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Modulation of the Calmodulin-induced Inhibition of Sarcoplasmic Reticulum Calcium Release Channel (Ryanodine Receptor) by Sulfhydryl Oxidation in Single Channel Current Recordings and [^{3}H]Ryanodine Binding

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Abstract. The modulation of the calmodulin-induced inhibition of the calcium release channel (ryanodine receptor) by two sulfhydryl oxidizing compounds, 4-(chloromercuri)phenyl–sulfonic acid (4-CMPS) and 4,4′-dithiodipyridine (4,4′-DTDP) was determined by single channel current recordings with the purified and reconstituted calcium release channel from rabbit skeletal muscle sarcoplasmic reticulum (HSR) and [^{3}H]ryanodine binding to HSR vesicles. 0.1 μM CaM reduced the open probability (P_o) of the calcium release channel at maximally activating calcium concentrations (50–100 μM) from 0.502 ± 0.02 to 0.137 ± 0.022 (n = 28), with no effect on unitary conductance. 4-CMPS (10–40 μM) and 4,4′-DTDP (0.1–0.3 mM) induced a concentration dependent increase in P_o (> 0.9) and caused the appearance of longer open states. CaM shifted the activation of the calcium release channel by 4-CMPS or 4,4′-DTDP to higher concentrations in single channel recordings and [^{3}H]ryanodine binding. 40 μM 4-CMPS induced a near maximal (P_o > 0.9) and 0.3 mM 4,4′-DTDP a submaximal (P_o = 0.74) channel opening in the presence of CaM, which was reversed by the specific sulfhydryl reducing agent DTT. Neither 4-CMPS nor 4,4′-DTDP affected Ca-[^{125}I]calmodulin binding to HSR. 1 mM $MgCl_2$ reduced P_o from 0.53 to 0.075 and 20–40 μM 4-CMPS induced a near maximal channel activation (P_o > 0.9). These results demonstrate that the inhibitory effect of CaM or magnesium in a physiological concentration is diminished or abolished at high concentrations of 4-CMPS or 4,4′-DTDP through oxidation of activating sulfhydryls on cysteine residues of the calcium release channel.

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Key words: Skeletal muscle — Sarcoplasmic reticulum — Calcium release channel — Ryanodine receptor — Calmodulin — Sulfhydryl oxidation

Introduction

Calcium release in skeletal muscle occurs via the calcium release channel (ryanodine receptor) located in the terminal cysternae of the sarcoplasmic reticulum (Inui, Saito & Fleischer, 1987; Imagawa et al., 1987; Lai et al., 1988). The skeletal muscle calcium release channel (RyR-1) is a homotetramer with a molecular mass of 2,26 million-Da (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 immunophilin FK506-binding protein, phosphorylation by protein kinases, sulfhydryl oxidation by nitric oxide and various exogenous effectors (*see* reviews, Meissner, 1994; Coronado et al., 1994; Melzer, Hermann-Frank & Lüttgau, 1995; Sorrentino, 1995; Zuchi & Ronca-Testoni, 1997).

The 17-kDa calcium binding protein calmodulin consists of two lobes connected by a central alpha-helix, with two calcium binding domains in each lobe (Klee & Vanaman, 1982; Babu et al., 1985). Calmodulin has a dual effect on the calcium release channel. Calcium saturated calmodulin inhibited calcium release from sarcoplasmic reticulum vesicles (Meissner, 1986; Plank et al., 1988; Fuentes et al., 1994; Tripathy et al., 1995) and reduced the open probability of the calcium release channel (Smith, Rousseau & Meissner, 1989; Fuentes et al., 1994; Tripathy et al., 1995). Ca^{2+}-free calmodulin increased the release of calcium from sarcoplasmic reticulum vesicles and increased the open probability of the

calcium release channel (Tripathy et al., 1995, Burrati et al., 1995). The effect of calmodulin on the calcium release channel is modified by endogenous effectors, such as magnesium and ATP (Tripathy et al., 1995), by phosphorylation (Witcher et al., 1991; Suko et al., 1993) and exogenous ligands such as suramin or suramin analogues (Klinger et al., 1999).

The gating of the calcium release channel is markedly influenced by oxidation of sulfhydryls on cysteine residues of the calcium release channel (Abramson & Salama, 1989; Liu et al., 1994). Compounds, such as 4-(chloro-mercuri)phenyl-sulfonic acid (4-CMPS) (Oba, Koshita & van Helden, 1996; Suko & Hellmann, 1998; Suko, Drobny & Hellmann, 1999), thimerosal (Abramson et al., 1995; Marengo, Hidalgo & Bull, 1998) or 4,4′-dithiodipyridine (4,4′-DTDP; Nagura et al., 1988; Eager, Roden & Dulhanty, 1997; Marengo et al., 1998) activated the calcium release channel at low concentrations while high concentrations inhibited the channel. The aim of the present investigation was to study the modulation of the inhibitory effect of calmodulin on the skeletal muscle calcium release channel by sulfhydryl oxidizing agents. The organic mercurial 4-CMPS and 4,4′-dithiodipyridine were used as tools and selected as sulfhydryl oxidizing compounds. The results demonstrate that the channel-activating effect of oxidation of sulfhydryls on cysteine residues by high concentration of sulfhydryl oxidizing agents overrules the inhibitory effect of calmodulin (or magnesium in a physiological concentration) and causes a maximal or near maximal channel opening in the presence of calmodulin without affecting calcium-calmodulin binding.

Materials and Methods

Materials

4-(chloromercuri)phenyl-sulfonic acid (4-CMPS), 4,4′-dithiopyridine (4,4′-DTDP), Mops, Hepes, Tris, histidine, CsCl (ultra pure), ruthenium red, leupeptin, pepstatin, antipain, phenylmethylsulfonyl fluoride were purchased from Sigma-Aldrich GmbH (Vienna); [^{3}H]ryanodine and [^{125}]calmodulin from Dupont New England Nuclear (Boston, MA); ryanodine from Agrisystems International (Wind Gap); phosphatidylserine, phosphatidylethanolamine and phosphatidylcholine from Avanti Polar Lipids, (Alabaster, AI); Delrin bilayer chambers (CD22-200; CD13-200) from Warner Instrument (Hamden, NJ). Aprotinin was a generous gift from Bayer Austria AG (Vienna). 4,4′-DTDP was dissolved in methanol; all other reagents were dissolved in MilliQ deionized water.

Preparation of Sarcoplasmic Reticulum

Heavy sarcoplasmic reticulum vesicles (HSR) from rabbit skeletal muscle were prepared as described previously (Wyskovsky et al., 1990; Suko & 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, 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, 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 cysternae of sarcoplasmic reticulum vesicles was prepared as described previously (Suko & Hellmann, 1998), a slight modification of the preparation used previously (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 HSR/ml; 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 7.5–20% 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, 1 mM benzamidine for 14 hr at 2°C (Beckman SW28 rotor; 38 ml tubes). Fractions containing the ryanodine receptor (determined by SDS-PAGE) were pooled and dialysed for 24 hr in a medium containing 40 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, 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 2–4°C.

SDS-Polyacrylamide Gel Electrophoresis (SDS-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, 10% glycerol and boiled for 2 min. Gels were stained with 0.05% Coomassie Blue in 10% acetic acid. Molecular weight standards were run on two separate lanes of the same gel: Ferritin (440,000), thyreoglobulin (330,000), myosin (212,000). 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 following incorporation of purified calcium release channels (ryanodine receptors) into planar lipid bilayers, essentially as described previously (Suko & Hellmann, 1998). Planar lipid bilayers were formed from phosphatidylserine (10 mg/ml) and phosphatidylethanolamine (10 mg/ml) in decane (Avanti Polar Lipids, Alabaster, Al). The lipid solution was spread over a 200

μm diameter aperture in a delrin cup (Warner Instrument) 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, BRD) 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 50–100 μM $CaCl_2$ or 100 μM $CaCl_2$ plus 50 μM EGTA (free calcium 50 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 and stored on the hard disc of a McIntosh/PC. Single channel events were identified using TAC V2.5 software (Skalar Instruments, 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).

[^{3}H]Ryanodine Binding

[^{3}H]ryanodine binding was measured as described previously (Suko & Hellmann, 1998). Unless stated otherwise, controls and test samples were assayed in duplicate or triplicate for 60 min at 25–26°C in 0.2 ml solution containing 40 mM Mops/Tris (pH 7.0), 0.5 M KCl, 100 μM $CaCl_2$, 0.1 mg HSR, 0.5 μg/ml leupeptin, 1.4 μg/ml aprotinin, 1 μM pepstatin, 0.1 mM PMSF, 5 nM [^{3}H]ryanodine without or with calmodulin (0.1 nM–10 μM), 4-CMPS (0.01 μM–100 μM) or 4,4′-DTDP (0.01 μM–10 mM). In a few experiments the above control medium contained in addition 5 mM EGTA or 10 mM $MgCl_2$ or 10 μM ruthenium red. Nonspecific [^{3}H]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.

[^{125}]Calmodulin Binding

[^{125}]calmodulin binding to HSR was performed according to Tripathy et al. (1995). Assays were carried out in duplicate or triplicate for 60 min at 25–26°C in a solution containing 40 mM Mops/Tris (pH 7.0), 0.1 M KCl, 100 μM $CaCl_2$, 200 μg HSR, 0.5 μg/ml leupeptin, 1.4 μg/ml aprotinin, 1 μM pepstatin, 0.1 mM PMSF, 0.5–1 μM [^{125}]calmodulin without or with 4-CMPS (10–100 μM) or 4,4′-DTDP (0.5–1 mM) or suramin (0.5–1 mM). Nonspecific [^{125}]calmodulin binding was measured in the presence of 40–50 μM unlabeled calmodulin. Samples were filtered on Millipore cellulose filters (0.45 μM, type HA) presoaked in a solution containing 40 mM Mops/Tris (pH 7.0), 0.1 M KCl, 100 μM $CaCl_2$ and 1 mg/ml BSA and washed with 3×5 ml of the above solution containing 0.1 mg/ml BSA.

Protein Assay

Protein was measured by the Folin method and in the presence of detergents plus phosphatidylcholine, according to Kaplan & 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). Averaged results are presented as means ± SEM.

Abbreviations

HSR, heavy sarcoplasmic reticulum; CaM, calmodulin; Hepes, N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid; Mops, 3-(N-morpholino)-propanesulfonic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid; CHAPS, 3-[(cholamidopropyl) dimethylammonio]-1-propane sulfonate; DTT, 1,4-dithiothreitol; PMSF, phenylmethylsulfonyl fluoride; 4-CMPS, 4-(chloromercuri)phenyl-sulfonic acid; 4,4′-DTDP, 4,4′-dithiodipyridine; P_o, open probability.

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 50–100 μM free calcium in a 50–480 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. The slope conductance of single calcium release channels with Cs^+ as permeant ion was 511 ± 6 pS ($n = 16$) determined from nine different channel preparations used in the present study. Calmodulin (CaM) and the sulfhydryl oxydizing compounds 4-CMPS or 4,4′-DTDP were added to the *cis* chamber which corresponded to the cytosolic face of the calcium release channel.

An example of the inhibitory effect of CaM on the purified calcium release channel in the presence of 50 μM free Ca^{2+} is shown in Fig. 1. The inhibitory effect of CaM was usually seen 2–3 min after the addition of the calcium binding protein and was maximal within approximately 5–7 min. 0.1 μM CaM reduced the open probability from 0.640 to 0.147 (23% of the control; Fig. 1). With calcium as the sole activator of the calcium release channel (50–100 μM Ca^{2+}), 0.1 μM CaM caused a 3–5-fold reduction of the open probability of the calcium release channel in individual experiments. On average, in 28 single channel experiments 0.1 μM calmodulin reduced the open probability to 27% of the controls (control: $P_o = 0.502 \pm 0.020$; CaM: $P_o = 0.137 \pm 0.022$; means ± SEM, $n = 28$). 0.3 μM CaM, added sequentially, gave a similar inhibition of the open probability (28%; $n = 9$). The CaM-induced reduction of P_o in channels subsequently activated by the sulfhydryl oxidizing compounds 4-CMPS or 4,4′-DTDP is shown in Tables 1 and 2. Means of current amplitudes, mean open and closed channel duration and the distribution of the open and closed lifetimes of controls and of the same channels inhibited by CaM and reactivated by 4-CMPS or 4,4′-DTDP are given in Table 3 and Table 4. CaM

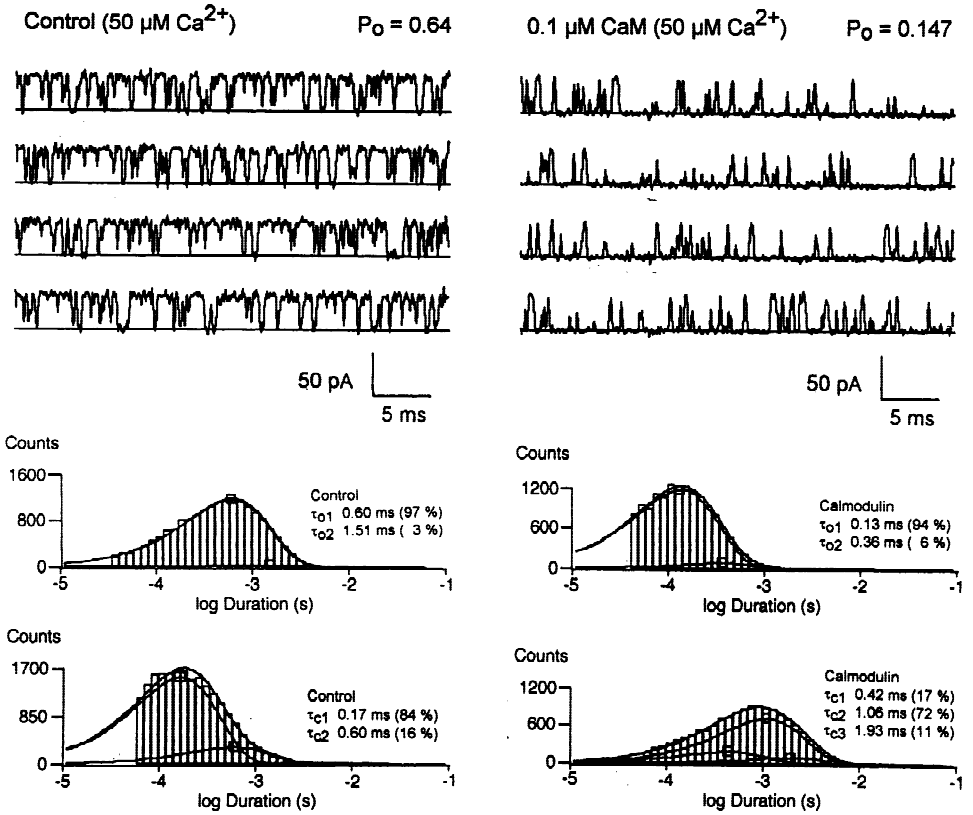


Fig. 1. Inhibition of a single purified skeletal muscle calcium-release channel by 0.1 μM calmodulin (CaM). Single channel currents, shown as upward deflections, were recorded at +20 mV holding potential with 480 mM/50 mM CsCl (*cis/trans*). The baselines are indicated by the solid lines. 50 μM Ca^{2+} and 0.1 μM CaM was added to the *cis* side. Control (left panel), $P_o = 0.64$, and test records (CaM, right panel), $P_o = 0.147$, are from the same channel. Channel open probabilities (P_o), cumulative mean open and closed channel time constants (τ_o, τ_c) and values of the percent of the channel represented by a time constant for purified calcium release channels were calculated from 38,000 events (control) and 30,000 events (CaM). Calibration bars represent 50 pA and 5 msec.

caused a reduction of the frequency of channel opening (Fig. 1), a significant reduction of the mean channel open duration (T_o) and a significant increase of the mean channel closed duration (T_c), a significant decrease of the open lifetimes (τ_o) and significantly prolonged closed lifetimes (τ_c). In controls the open and closed lifetimes were best fitted by the sum of two exponentials; in the presence of CaM the best fit of the closed lifetimes was obtained by the sum of three exponentials with a shift to longer closed times (Fig. 1; Table 3). Almost identical values for T_o, T_c, τ_o and τ_c in controls and in the presence of CaM from 10 analyzed calcium release channels are shown in Table 4. Prolonged closed time constants of the calcium release channel in the presence of CaM and calcium as channel activator, with no effect on the open time constants, have been reported (Fuentes et al., 1994; Tripathy et al., 1995), but the mean open times where reduced by CaM in channels activated by Ca^{2+} and ATP (Tripathy et al., 1995).

An example of the concentration dependence of the activation of a purified calcium release channel by 4-CMPS, carried out in the absence of calmodulin, is shown in Fig. 2. In three experiments the mean open probability of purified calcium release channels increased from 0.50 to 0.77, 0.90 and 0.93 upon sequential addition of 10, 20 and 40 μM 4-CMPS (Table 1*A; n* = 3). With 10 μM 4-CMPS P_o fluctuated between higher and lower activity states. The activation of the calcium release channel by 20 μM or 40 μM 4-CMPS was near maximal and not significantly different. The 4-CMPS-induced activation of the calcium release channel was identical to the previously reported increase in the open probability by 20 μM 4-CMPS (or 4-CMB) measured at 20 μM Ca^{2+} and 0 mV holding potential (control: $P_o = 0.43 \pm 0.05$; 4-CMPS: $P_o = 0.88 \pm 0.01$; means ± SEM, $n = 7$; Suko & Hellmann, 1998). 40 μM 4-CMPS induced a low activity state within 3–8 min (P_o = 0.01–0.02; Fig. 2*E*). A further addition of 7 μM ruthenium red closed the channel (Fig. 2*F*). The markedly prolonged open times in the presence of 4-CMPS with a shift to $\tau_o 2$ and $\tau_o 3$ (Suko & Hellmann, 1998) are illustrated in Fig. 2*G*. With 100 μM 4-CMPS channels were maximally

Table 1. Activation of single purified skeletal muscle calcium-release channels by 4-CMPS at 50–100 μM activating Ca^{2+} in the absence and presence of calmodulin and reversibility by DTT

	Open probability (P_o)	n
(A) Control	0.50 ± 0.08	3
10 μM 4-CMPS	0.77 ± 0.04[b]	3
20 μM 4-CMPS	0.90 ± 0.03	3
40 μM 4-CMPS	0.93 ± 0.02	3
(B) Control	0.54 ± 0.02[a]	7
0.1/0.3 μM CaM	0.17 ± 0.02[a]	7
10 μM 4-CMPS	0.53 ± 0.04[b]	7
20 μM 4-CMPS	0.81 ± 0.06	6
40 μM 4-CMPS	0.94 ± 0.01	4
40 μM 4-CMPS + 4 mM DTT	0.24 ± 0.02	3

Single channel currents were recorded at +20 mV holding potential with 480 mM/50 mM CsCl (*cis/trans*) in the presence of 50–100 μM Ca^{2+} (*cis*). Control and test records are from the same channel. (*A*) Activation of single channel current by 10, 20 and 40 μM 4-CMPS added sequentially to the *cis* side. (*B*) Inhibition of the open probability by 0.1 μM CaM ($n = 4$) or 0.3 μM CaM ($n = 3$) and reactivation by 10, 20 and 40 μM 4-CMPS added sequentially to the *cis* side. Reversibility of the 4-CMPS-induced channel activation by 4 mM DTT. Channel open probabilities (P_o) were calculated from 30,000 to 50,000 events. Values are means ± SEM for the number of experiments (n) given in parenthesis. Significantly different: [a]control (*B*) *vs.* CaM (*B*), $P < 10^{-7}$; [b]10 μM 4-CMPS (*A*) *vs.* 0.1/0.3 μM CaM + 10 μM 4-CMPS (*B*), $P < 0.01$.

Table 2. Activation of single purified skeletal muscle calcium-release channels by 4,4′-DTDP at 50–100 μM activating Ca^{2+} in the absence and presence of calmodulin

	Open probability (P_o)	n
(A) Control	0.44 ± 0.03	9
0.1 mM 4,4′-DTDP	0.81 ± 0.04[b]	6
0.3 mM 4,4′-DTDP	0.91 ± 0.01[c]	4
(B) Control	0.51 ± 0.02[a]	10
0.1 μM CaM	0.15 ± 0.05[a]	10
0.1 mM 4,4′-DTDP	0.45 ± 0.07[b]	6
0.3 mM 4,4′-DTDP	0.74 ± 0.04[c]	6

Single channel currents were recorded at 0 mV or +20 mV holding potential with 480 mM/50 mM CsCl (*cis/trans*) in the presence of 50–100 μM Ca^{2+} (*cis*). Control and test records are from the same channel. (*A*) Activation of single channel current (0 mV) by 0.1 mM and/or 0.3 mM 4,4′-DTDP added to the *cis* side (sequentially: $n = 6$; only 0.3 mM: $n = 3$). (*B*) Inhibition of the open probability (20 mV) by 0.1 μM calmodulin and activation by 0.1 mM and/or 0.3 mM 4,4′-DTDP added to the *cis* side (sequentially: $n = 2$. Channel open probabilities (P_o) were calculated from 30,000 to 50,000 events. Values are means ± SEM for the number of experiments given in parenthesis. Significantly different: [a]control (*B*) *vs.* CaM (*B*) $P < 10^{-7}$; [b]0.1 mM 4,4′-DTDP (*A*) *vs.* 0.1 μM CaM + 0.1 mM 4,4′DTDP (*B*), $P < 0.001$; [c]0.3 mM DTDP (*A*) *vs.* 0.1 μM CaM + 0.3 mM 4,4′-DTDP (*B*), $P = 0.025$.

activated within 30–60 sec followed by a more rapid transition to a low activity state as described previously (Suko & Hellmann, 1998).

An example of a CaM inhibited purified calcium release channel subsequently activated by sulfhydryl oxidation with 4-CMPS is shown in Fig. 3. Channel open probability was reduced by addition of 0.1 μM CaM to the *cis* side (Fig. 3*A* and *B*) and sequential addition of 10, 20 and 40 μM 4-CMPS added to the *cis* side activated the calcium release channel concentration-dependently (Fig. 3*C–E*). The calmodulin-induced inhibition of the calcium release channel was reversed by 4-CMPS, but the activation of the calcium release channel was shifted to higher concentrations of the sulfhydryl oxidizing compound. Statistical analysis of seven experiments with 4-CMPS showed that 0.1–0.3 μM CaM reduced the open probability from 0.54 to 0.17 and 10 μM 4-CMPS increased the open probability to 0.53, i.e., the P_o level observed in the absence of CaM; 20 μM 4-CMPS increased P_o to 0.81 and 40 μM 4-CMPS induced a nearly maximal activation of the calcium release channel ($P_o = 0.94$), (Table 1*B*). The open probability in the presence of calmodulin plus 10 μM 4-CMPS was significantly less than in the absence of calmodulin. The open probability of channels activated by 20 μM 4-CMPS in the presence of CaM was less than in the absence of CaM, however the difference was not statistically significant (Tables 1 and 3). The increase in the open probability by 20 and 40 μM 4-CMPS was associated with a marked increase of the mean channel open duration and a decrease in the mean channel closed duration (Table 3). The open lifetimes (τ_o) of the 4-CMPS activated channel were increased and the closed lifetimes (τ_c) were reduced; the former were best fitted by the sum of three exponentials and showed a clear shift to longer open times ($\tau_o1 = 0.51$ msec (30%), $\tau_o2 = 2.07$ msec (59%), $\tau_o3 = 6.91$ msec (11%); 0.1 μM CaM + 40 μM 4-CMPS, Table 3), similarily as in the absence of CaM.

Experiments showing the activating effect of 4-CMPS starting with a low P_o have been carried out with 10 μM activating calcium to show the increase in P_o under these conditions. When calcium release channels were activated by addition of 10 μM total $CaCl_2$ the open probability was 0.079 ± 0.031 and increased to 0.475 ± 0.095 following addition of 10 μM 4-CMPS to the *cis* bath, which represents an about sixfold increase in P_o; 20–40 μM 4-CMPS induced a near maximal activation of the calcium release channel ($P_o = 0.870 \pm 0.035$; means ± SEM, $n = 4$). The specific thiol-reducing agent DTT inhibited the 4-CMPS-induced activation of the calcium release channel as shown previously (Suko & Hellmann, 1998). Similarily, the channel activation by 4-CMPS in the presence of inhibitory CaM concentrations was almost completely reversed by DDT. In three experiments 40 μM 4-CMPS increased the open probability in the

Table 3. Mean open probability, mean current amplitude and mean open and closed lifetimes of controls, 0.1 μM CaM and 0.1 μM CaM plus 10–40 μM 4-CMPS

	Control		0.1–0.3 μM CaM		0.1–0.3 μM CaM + 10 μM 4-CMPS		0.1–0.3 μM CaM + 20 μM 4-CMPS		0.1–0.3 μM CaM + 40 μM 4-CMPS	
	$n = 7$		$n = 7$		$n = 7$		$n = 6$		$n = 4$	
	Means ± SEM	Area (%)	Means ± SEM	Area (%)	Means ± SEM	Area (%)	Means ± SEM	Area (%)	Means ± SEM	Area (%)
P_o	0.54 ± 0.02		0.168 ± 0.019		0.53 ± 0.04		0.81 ± 0.06		0.94 ± 0.01	
Amplitude (pA)	31.2 ± 0.7		30.6 ± 0.6		31.3 ± 0.9		30.9 ± 0.8		31.9 ± 1.5	
Mean T_o (msec)	0.42 ± 0.02		0.19 ± 0.01[a]		0.41 ± 0.05		0.81 ± 0.15		1.34 ± 0.41	
Mean T_c (msec)	0.36 ± 0.01		1.00 ± 0.08[a]		0.44 ± 0.07		0.21 ± 0.05		0.10 ± 0.03	
τ_o1 (msec)	0.32 ± 0.02	(80)	0.14 ± 0.01[a]	(94)	0.25 ± 0.03	(76)	0.23 ± 0.03	(47)	0.51 ± 0.17	(30)
τ_o2 (msec)	0.68 ± 0.05	(20)	0.49 ± 0.03[b]	(6)	0.79 ± 0.08	(24)	1.31 ± 0.27	(40)	2.07 ± 0.28	(59)
τ_o3 (msec)	—		—		—		3.31 ± 0.05	(13)	6.91 ± 0.90	(11)
τ_c1 (msec)	0.29 ± 0.02	(90)	0.38 ± 0.05	(20)	0.23 ± 0.02	(76)	0.13 ± 0.02	(71)	0.09 ± 0.003	(99)
τ_c2 (msec)	0.72 ± 0.08	(10)	0.98 ± 0.08[c]	(61)	0.68 ± 0.13	(24)	0.29 ± 0.10	(29)	—	
τ_c3 (msec)	—		2.70 ± 0.47	(11)			—		—	

Channel open probabilities (P_o), mean current amplitude (pA), mean channel open (T_o) and closed (T_c) duration (msec), cumulative mean open and closed channel time constants (τ_c) and values of the percent of the channel represented by a time constant for purified calcium release channels activated by 50–100 μM *cis* Ca^{2+} in controls, in the presence of 0.1/0.3 μM CaM or 0.1/0.3 μM CaM plus 10–40 μM 4-CMPS. Calmodulin (CaM) and 4-CMPS were added to the *cis* chamber. Calcium current was recorded at +20 mV voltage holding potential with 480 mM/50 mM CsCl (*cis/trans*). Values are means ± SEM from seven channels included in the analysis. Significantly different from control values: [a] $P < 0.00001$; [b] $P = 0.003$; [c] $P = 0.05$.

presence of 0.1–0.3 μM CaM to 0.92 and addition of 4 mM DTT to the *cis* side reduced P_o to 0.24 (Table 1*B*), i.e., close to the open probability observed in the presence of CaM. This DTT effect confirms that the 4-CMPS-induced activation was due to a modulation of sulfhydryls of the calcium release channel protein. The open probability of calcium release channels in the absence of 4-CMPS was not significantly changed by 2–8 mM DTT (Suko et al., 1999).

In control experiments with 4,4′-DTDP (Fig. 4), carried out in the absence of calmodulin, 0.1 mM 4,4′-DTDP increased the open probability of the purified calcium release channel by about 70% and 0.3 mM 4,4′-DTDP caused a near maximal channel activation ($P_o > 0.9$; Fig. 4). 0.1 mM 4,4′-DTDP caused a transition to a low activity state followed by a complete channel closure within 5–7 min and 0.3 mM 4,4′-DTDP within about 5 min after addition of the compound. To assure the determination of a maximal channel activation at the low 4,4′-DTDP concentration (0.1 mM) the activity of the calcium release channel was in most experiments monitored until channel inhibition occurred (six of nine experiments; Table 2*A*). The effect of the high 4,4′-DTDP concentration (0.3 mM) was in one experiment determined by sequential addition and in three of four experiments with separate channels (Table 2*A*). A further addition of 7 μM ruthenium red 2 min after 0.3 mM 4,4′-DTDP closed the channel (Fig. 4*E*). Statistical analysis of the effects of 4,4′-DTDP on the purified calcium release channel showed that 0.1 mM 4,4′-DTDP increased the open probability from 0.44 to 0.81; 0.3 mM 4,4′-DTDP induced a near maximal channel activation ($P_o > 0.9$; Table 2*A*). The markedly prolonged open times in the presence of 0.3 mM 4,4′-DTDP with a shift to τ_o2 and τ_o3, similarily as observed with 4-CMPS, are illustrated in Fig. 4F.

An example of the effect of 4,4′-DTDP on the CaM inhibited purified calcium release channel is shown in Fig. 5. Channel open probability was reduced by addition of 0.1 μM CaM to the cis side (Fig. 5*A* and *B*) and addition of 0.1 and 0.3 mM 4,4′-DTDP to the *cis* side activated the calcium release channel concentration-dependently (Fig. 5*C* and *D*). Statistical analysis of ten experiments showed that 0.1 μM CaM reduced the open probability from 0.51 (control) to 0.15; 0.1 mM and/or 0.3 mM 4,4′-DTDP increased the open probability to 0.45 and 0.74 (Table 2*B*). These results show that 0.1 mM 4,4′-DTDP increased the open probability close to the control level observed in the absence of calmodulin. 0.3 mM 4,4′-DTDP in the presence of calmodulin increased P_o considerably above the control level observed in the absence of calmodulin, but a maximal channel opening was not observed. The activating effect of 4,4′-DTDP (0.1 and 0.3 mM) on the open probability in the presence of calmodulin was significantly less than the channel activation in the absence of calmodulin (Table

Table 4. Mean open probability, mean current amplitude and mean open and closed lifetimes of controls, 0.1 μM CaM and 0.1 mM and/or 0.3 mM 4,4′-DTDP

	Control		0.1 μM CaM		0.1 μM CaM + 0.1 mM 4,4′-DTDP		0.1 μM CaM + 0.3 mM 4,4′-DTDP	
	$n = 10$		$n = 10$		$n = 6$		$n = 6$	
	Means ± SEM	Area (%)	Means ± SEM	Area (%)	Means ± SEM	Area (%)	Means ± SEM	Area (%)
P_o	0.51 ± 0.03		0.15 ± 0.02		0.45 ± 0.07		0.74 ± 0.04	
Amplitude (pA)	30.7 ± 0.8		30.7 ± 0.7		30.9 ± 1.1		28.5 ± 1.6	
Mean T_o (msec)	0.41 ± 0.03		0.18 ± 0.01		0.31 ± 0.03		0.82 ± 0.11	
Mean T_c (msec)	0.39 ± 0.03		1.13 ± 0.14		0.45 ± 0.11		0.23 ± 0.03	
τ_o1 (msec)	0.31 ± 0.05	(85)	0.13 ± 0.01	(96)	0.23 ± 0.02	(80)	0.31 ± 0.04	(42)
τ_o2 (msec)	0.70 ± 0.12	(15)	0.48 ± 0.03	(4)	0.61 ± 0.07	(20)	0.99 ± 0.17	(56)
τ_o3 (msec)	—		—		—		4.99 ± 0.82	(3)
τ_c1 (msec)	0.28 ± 0.04	(84)	0.38 ± 0.04	(30)	0.25 ± 0.03	(89)	0.15 ± 0.02	(88)
τ_c2 (msec)	0.74 ± 0.06	(16)	1.03 ± 0.09	(60)	0.93 ± 0.42	(12)	0.65 ± 0.07	(12)
τ_c3 (msec)	—		2.96 ± 0.45	(10)	—		—	

Channel probabilities (P_o), mean current amplitude (pA), mean channel open (T_o) and closed (T_c) duration (msec), cumulative mean open and closed channel time constants (τ_o, τ_c) and values of the percent of the channel represented by a time constant for purified calcium release channel activated by 50–100 μM *cis* Ca^{2+} in controls, in the presence of 0.1 μM CaM or 0.1 μM CaM plus 0.1 mM and/or 0.3 mM 4,4′-DTDP. Calmodulin (CaM) and 4,4′-DTDP were added to the *cis* chamber. Calcium current was recorded at +20 mV voltage holding potential with 480 mM/50 mM CsCl (*cis/trans*). Values are means ± SEM from ten channels included in the analysis. 0.1 mM and 0.3 mM 4,4′-DTDP was added sequentially in two experiments; in 8 experiments channel activation was induced by either 0.1 mM ($n = 4$) or 0.3 mM ($n = 4$) 4,4′-DTDP; for explanation *see* text.

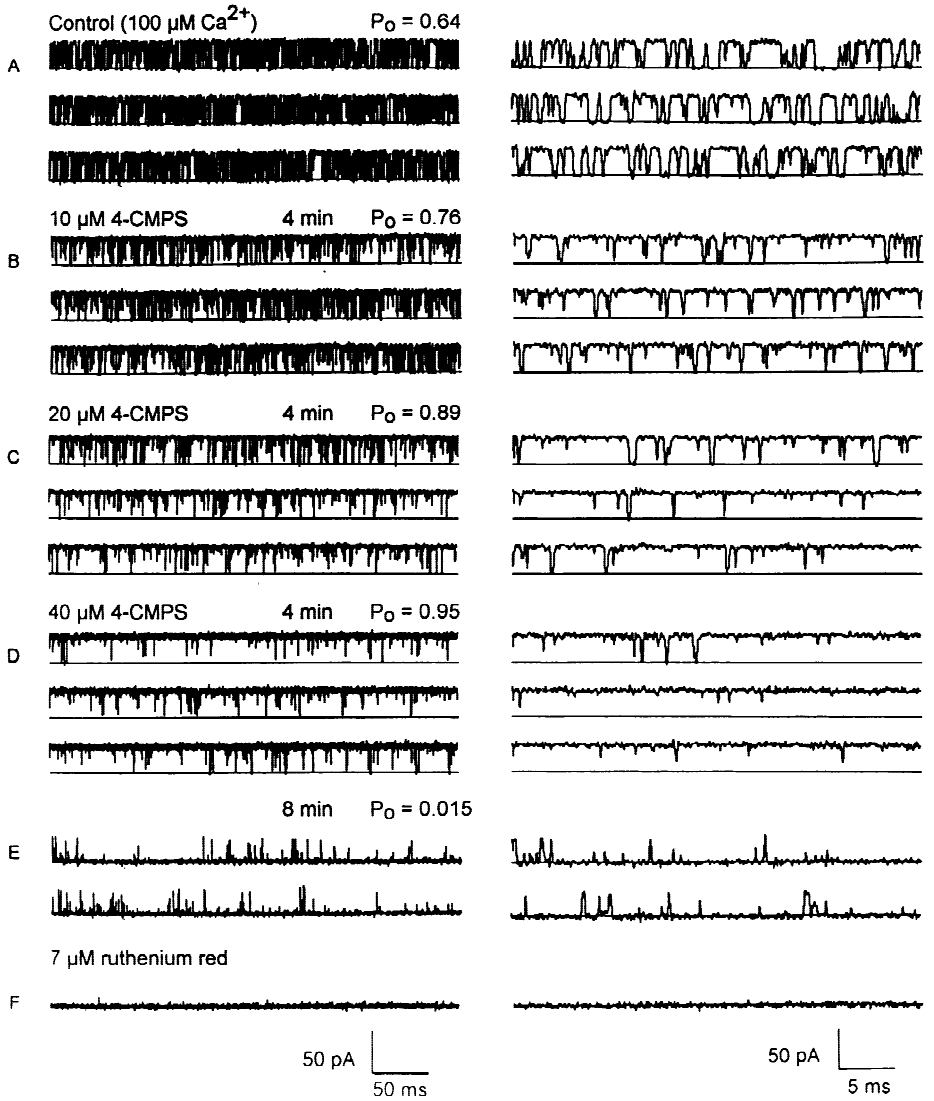
2*A* and *B*). The increase in the open probability by 0.3 mM 4,4′-DTDP in the presence of CaM was associated with a marked increase of the mean channel open duration (T_o), and a decrease in the mean channel closed duration (T_c); the open lifetimes (τ_o) of the 4,4′-DTDP activated channel, were increased and the closed lifetimes (τ_c) were reduced (Table 4). The former were best fitted by the sum of three exponentials and showed a shift to longer open times ($\tau_o1 = 0.31$ msec (42%), $\tau_o2 = 0.99$ msec (56%), $\tau_o3 = 4.99$ msec (3%), Table 4), similarily as observed with 4-CMPS.

When calcium release channels were activated by addition of 40 μM Ca^{2+} the open probability was 0.537 ± 0.037 and decreased to 0.075 ± 0.021 following addition of 1 mM $MgCl_2$ to the *cis* bath; subsequent addition of 10 μM 4-CMPS increased P_o to 0.506 ± 0.101 (means ± SEM, $n = 6$), which represents an about 6.5-fold increase in P_o. 20–40 μM 4-CMPS abolished the inhibitory effect of 1 mM magnesium ($P_o = 0.91 \pm 0.03$; means ± SEM, $n = 4$). The effect of 4-CMPS in the presence of higher magnesium concentrations was not tested.

[^{3}H]Ryanodine Binding

Specific high affinity [^{3}H]ryanodine binding to HSR vesicles (corrected for unspecific [^{3}H]ryanodine binding obtained in the presence of an excess of unlabeled ryanodine) was carried out in 60 min assays at 25°C in the presence of 40 mM Mops/Tris (pH 7.0), 0.5 M KCl, 100 μM activating Ca^{2+} and a [^{3}H]ryanodine concentration of 5 nM.

The concentration dependence of inhibition of [^{3}H]ryanodine binding by CaM was determined in three independent experiments with different HSR preparations with 5 nM [^{3}H]ryanodine, 100 μM Ca^{2+} and 0.5 M KCl. Calcium-calmodulin reduced high affinity [^{3}H]ryanodine binding half-maximally at 15.8 ± 2.2 nM (means ± SEM, $n = 3$). The inhibition of [^{3}H]ryanodine binding by CaM showed a positive cooperative effect with a Hill coefficient of 1.44 ± 0.01 (means ± SEM, $n = 3$). CaM was not capable to induce a complete inhibition of [^{3}H]ryanodine binding. In the calmodulin concentration dependence experiments the maximum reduction of high affinity [^{3}H]ryanodine binding was 23.8 ± 2.1% of the controls at 10 μM CaM and 24.8 ± 0.8% in the presence of 0.5 μM CaM (means ± SEM; $n = 3$). In the presence of 0.5 μM CaM, the concentration which has been selected for experiments with sulfhydryl oxidizing compounds (4-CMPS and 4,4′-DTDP), specific [^{3}H]ryanodine binding was reduced from 0.49 to 0.13 (4-CMPS group) and from 0.47 to 0.11 (4,4′-DTDP group), i.e., close to 25% of the controls (Table 5). In contrast to CaM, zero free Ca^{2+} or 10 mM Mg^{2+} or 5 μM ruthenium red induced a complete inhibition of specific [^{3}H]ryano-



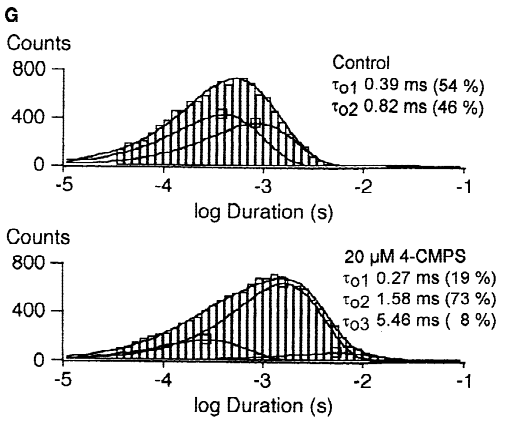


Fig. 2. Activation and inhibition of a single purified skeletal muscle calcium-release channel by 10–40 μM 4-CMPS. Single channel currents, shown as upward deflections, were recorded at +20 mV holding potential with 480 mM/50 mM CsCl (*cis/trans*). The baselines are indicated by the solid lines. Control and test records (*A–F*) are from the same channel. 4-CMPS was added sequentially to the *cis* side. Left panel: 400 msec recordings. Right panel: the first 40 msec of each 400 msec recording. (*A*) Control: 100 μM Ca^{2+}, P_o = 0.64. (*B*) Activation of the channel by 10 μM 4-CMPS, P_o = 0.76. (*C*) Activation of the channel by 20 μM 4-CMPS, P_o = 0.89. (*D*) Activation of the channel by 40 μM 4-CMPS, P_o = 0.95. (*E*) Inhibition of the channel by 40 μM 4-CMPS (8 min after addition), P_o = 0.015. (*F*) Channel closure by 7 μM ruthenium red. (*G*) Cumulative mean open channel time constants (τ_o) for control and 20 μM 4-CMPS and values of the percent of the channel represented by a time constant. Channel open probabilities (P_o) and τ_o were calculated from 23,000 events (control), 31,000 events (10 μM 4-CMPS), 25,000 events (20 μM 4-CMPS), 5,000–7,000 events (40 μM 4-CMPS), respectively. Calibration bars represent 50 pA and 50 msec or 5 msec.

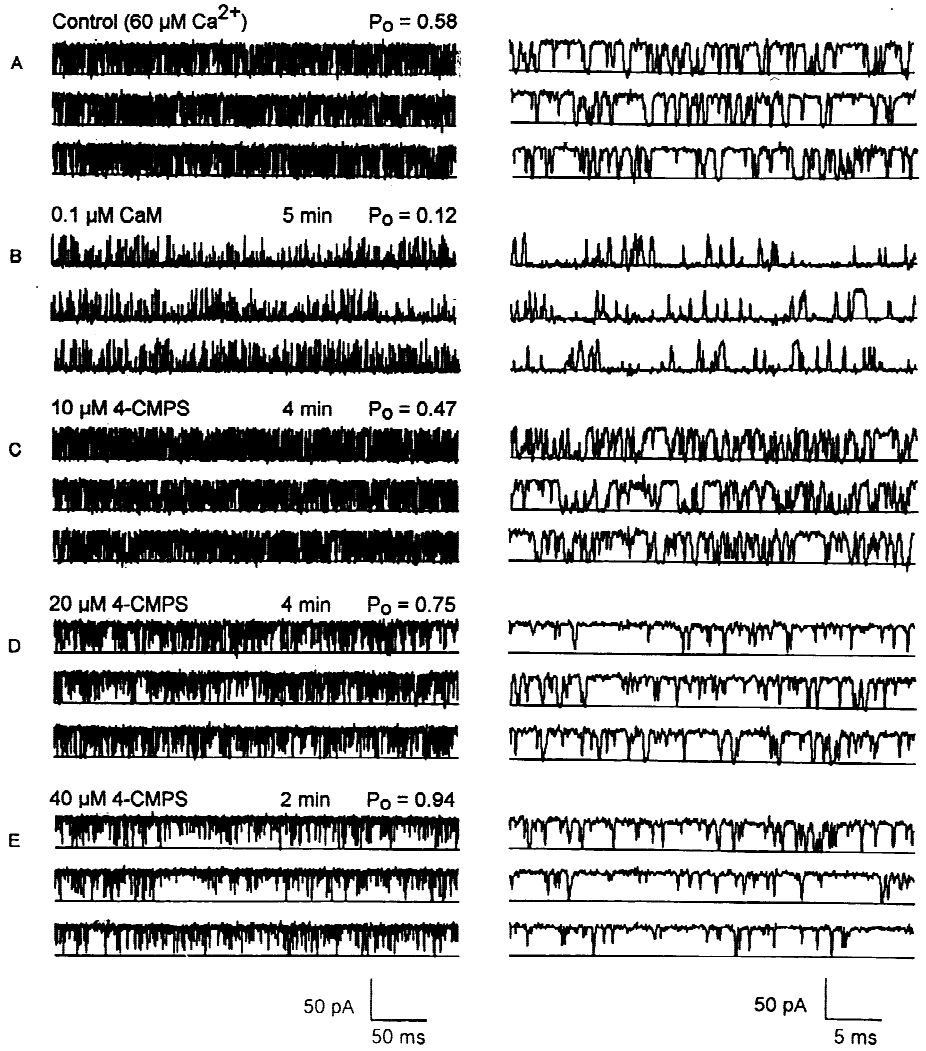
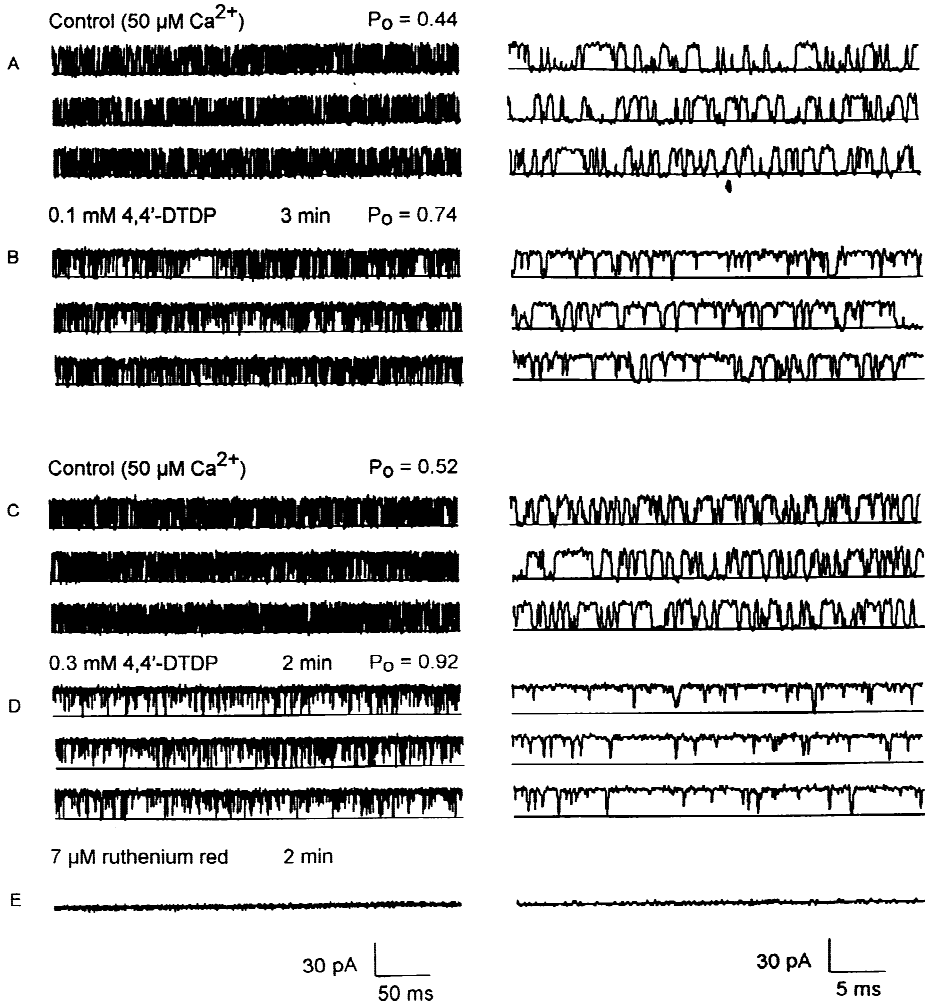


Fig. 3. Inhibition of a single purified skeletal muscle calcium-release channel by 0.1 μM CaM and reactivation by 10–40 μM 4-CMPS. Single channel currents, shown as upward deflections, were recorded at +20 mV holding potential with 480 mM/50 mM CsCl (*cis/trans*). The baselines are indicated by the solid lines. Control and test records (*A–E*) are from the same channel. CaM and 4-CMPS were added sequentially to the *cis* side. Left panel: 400 msec recordings. Right panel: the first 40 msec of each 400 msec recording. (*A*) Control: 60 μM Ca^{2+}, $P_o = 0.58$. (*B*) Inhibition of the channel by 0.1 μM CaM, $P_o = 0.12$. (*C*) Activation of the channel by 10 μM 4-CMPS, $P_o = 0.47$. (*D*) Activation of the channel by 20 μM 4-CMPS, $P_o = 0.75$. (*E*) Activation of the channel by 40 μM 4-CMPS, $P_o = 0.94$. Channel open probabilities (P_o) were calculated from 40,000 events (control), 36,000 events (CaM), 60,000 events (10, 20 μM 4-CMPS), and 9,000 events (40 μM 4-CMPS), respectively. Calibration bars represent 50 pA and 50 msec or 5 msec.

dine binding, i.e., binding was reduced to values for unspecific [^{3}H]ryanodine binding obtained in the presence of excess cold ryanodine.

The effects of 4-CMPS in controls (absence of CaM) were similar as reported previously under slightly different assay conditions (37°C; 10 nM [^{3}H]ryanodine, 1 M NaCl). 4-CMPS induced a dose-dependent increase in [^{3}H]ryanodine binding at low concentrations and reduced [^{3}H]ryanodine binding at high concentrations (Fig. 6). The activation of [^{3}H]ryanodine binding at low concentrations of 4-CMPS (3 μM and 10 μM) was significantly inhibited in the presence of CaM (Table 5). Activation of [^{3}H]ryanodine binding in the presence of CaM was shifted to higher 4-CMPS concentrations. Half-maximum activation of [^{3}H]ryanodine binding was significantly increased from 10.9 ± 0.6 μM 4-CMPS (Hill coefficient: 1.8 ± 0.1) in the absence of CaM to 15.1 ± 0.9 μM 4-CMPS in the presence of 0.5 μM CaM (Hill coefficient: 4.9 ± 0.3), ($n = 3$; means ± SEM); the maximum amount of [^{3}H]ryanodine bound was not significantly changed. Half-maximum inhibition of [^{3}H]ryanodine binding by 4-CMPS in the absence or presence of CaM was not significantly different. 4,4′-DTDP had a similar activating and inhibitory effect on



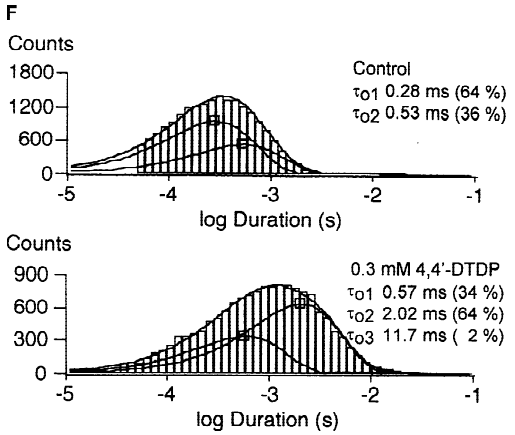


Fig. 4. Activation of a single purified skeletal muscle calcium-release channel by 0.1–0.3 mM 4.4′-DTDP. Single channel currents, shown as upward deflections, were recorded at 0 mV holding potential with 480 mM/50 mM CsCl (*cis/trans*). 4,4′-DTDP was added to the *cis* side. The baselines are indicated by the solid lines. Control and test records for 0.1 mM 4,4′-DTDP (*A* and *B*) and 0.3 mM 4,4′-DTDP (*C–E*) are from separate channels. Left panel: 400 msec recordings. Right panel: the first 40 msec of each 400 msec recording. (*A*) Control: 50 μM Ca^{2+}, P_o = 0.44. (*B*) Activation of the channel by 0.1 mM 4,4′-DTDP, P_o = 0.74. (*C*) Control: 50 μM Ca^{2+}, P_o = 0.52. (*D*) Activation of the channel by 0.3 mM 4,4′-DTDP, P_o = 0.92. (*E*) Channel closure by 7 μM ruthenium red. (*F*) Cumulative mean open channel time constants (τ_o) for control and 0.3 mM 4,4′-DTDP and values of the percent of the channel represented by a time constant. Channel open probabilities (P_o) and τ_o were calculated from 26,000 (control in *A*), 40,100 events (control in *C*), 19,000 (0.1 mM 4,4′-DTDP) and 30,000 events (4,4′-DTDP), respectively. Calibration bars represent 30 pA and 50 msec or 5 msec.

[^{3}H]ryanodine binding as observed with 4-CMPS (Table 5, Fig. 7), but activation and inhibition of [^{3}H]ryanodine binding by 4,4′-DTDP occured over a greater concentration range. Inhibition of [^{3}]ryanodine binding with 1 mM 4,4′-DTDP in the 60 min assays was much less than observed with 0.1 mM 4-CMPS (Figs. 6 and 7). Similar to the effect of 4-CMPS, the activation of [^{3}H]ryanodine binding at low concentrations of 4,4′-DTDP (3 and 10 μM) was significantly inhibited in the presence of CaM (Fig. 7, Table 5). Activation of [^{3}H]ryanodine binding

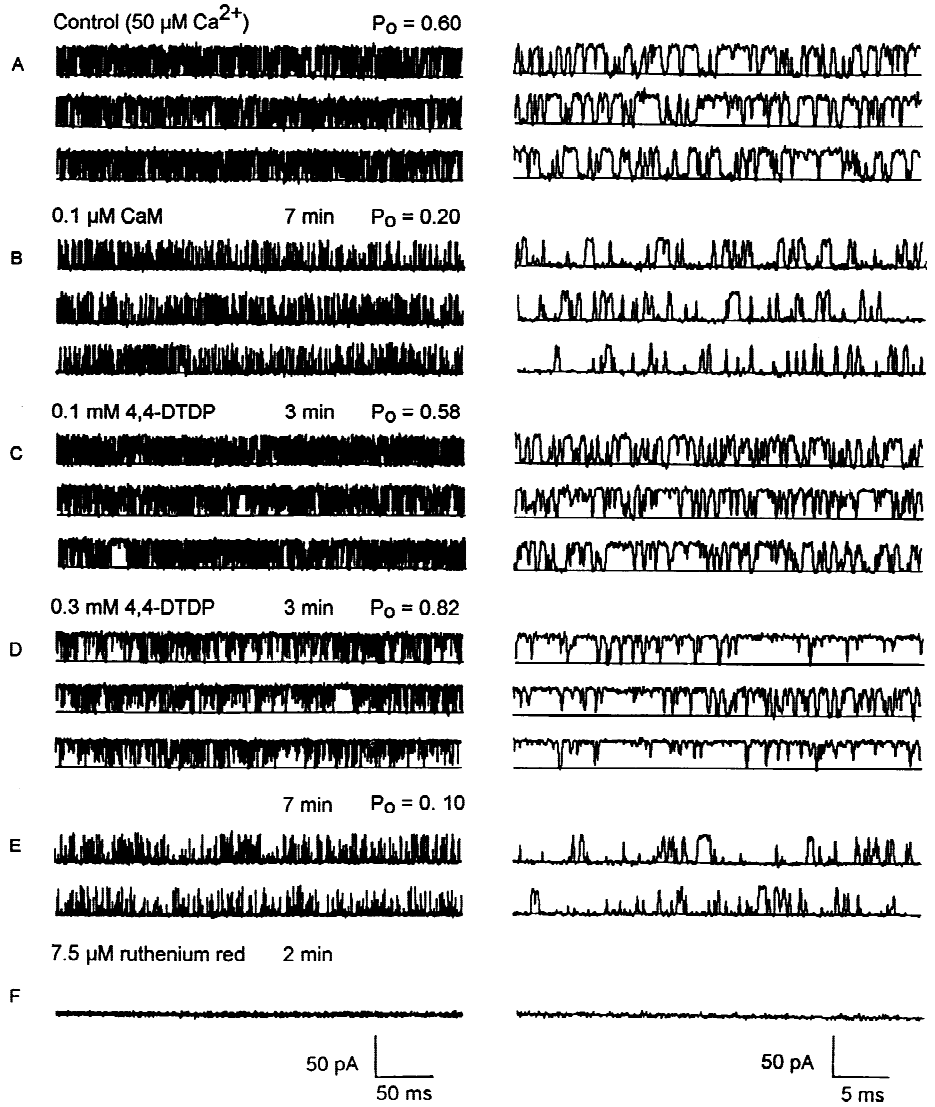


Fig. 5. Inhibition of a single purified skeletal muscle calcium-release channel by 0.1 μM CaM and reactivation by 0.1–0.3 mM 4,4′-DTDP. Single channel currents, shown as upward deflections, were recorded at +20 mV holding potential with 480 mM/50 mM CsCl (*cis/trans*). The baselines are indicated by the solid lines. Control and test records (*A–F*) are from the same channel. CaM and 4,4′-DTDP were added sequentially to the *cis* side. Left panel: 400 msec recording. Right panel: the first 40 msec of each 400 msec recording. (*A*) Control: 50 μM Ca^{2+}, P_o = 0.60. (*B*) Inhibition of the channel by 0.1 μM CaM, P_o = 0.20. (*C*) Activation of the channel by 0.1 mM 4,4′-DTDP, P_o = 0.58. (*D*) Activation of the channel by 0.3 mM 4,4′-DTDP, P_o = 0.82. (*E*) Inhibition of the channel by 0.3 mM 4,4′-DTDP (7 min after addition), P_o = 0.10. (*F*) Channel closure by 7.5 μM ruthenium red. Channel open probabilities (P_o) were calculated from 32,000 events (control), 26,000 events (CaM) and 34,000–40,000 events (4,4′-DTDP), respectively. Calibration bars represent 50 pA and 50 msec or 5 msec.

by 4,4′-DTDP in the presence of 0.5 μM CaM was shifted to higher 4,4′-DTDP concentrations. Half-maximum activation occurred at 9.8 ± 1.2 μM 4,4′-DTDP in the absence of CaM and at 22.6 ± 3.0 μM 4,4′-DTDP in the presence of 0.5 μM calmodulin (n = 3; means +/− SEM; Fig. 7). The maximum [^{3}H]ryanodine binding was not significantly changed in the three 4,4′-DTDP concentration-dependence experiments; it was lower at 100 μM DTDP in the presence of CaM compared to controls in seven independent experiments, but the difference was statistically not significant at a 5% level (P = 0.056), (Table 5). Half-maximum inhibition of [^{3}H]ryanodine binding by 4,4′-DTDP was not affected by CaM.

The 4-CMPS- or 4,4′-DTDP-induced activation of specific [^{3}H]ryanodine binding in the absence or presence of CaM was completely reversed by DTT (Figs. 8 and 9; Suko & Hellmann, 1998), demonstrating that the effects observed with both compounds were due to oxidation of sulfhydryls of the calcium release channel.

Table 5. Inhibition of 4-CMPS- or 4,4′-DTDP-induced stimulation of [^{3}H]ryanodine binding to HSR by CaM

	Drug [μM]	[^{3}H]ryanodine binding (pmol/mg HSR)		[^{3}H]ryanodine binding (pmol/mg HSR)	
		−CaM	*n*	+ CaM (0.5 μM)	(*n*)
Control	—	0.49 ± 0.03	(10)	0.13 ± 0.01[a]	(10)
4-CMPS	3	1.18 ± 0.21	(9)	0.40 ± 0.09[b]	(9)
	10	2.06 ± 0.22	(10)	0.94 ± 0.10[c]	(10)
	20	3.46 ± 0.22	(5)	3.12 ± 0.24	(5)
Control	—	0.47 ± 0.01	(8)	0.11 ± 0.01[a]	(8)
4,4′-DTDP	3	1.31 ± 0.11	(7)	0.47 ± 0.08[b]	(7)
	10	1.69 ± 0.11	(8)	0.87 ± 0.12[c]	(8)
	100	2.31 ± 0.16	(7)	1.86 ± 0.07	(7)

Specific [^{3}H]ryanodine binding to sarcoplasmic reticulum vesicles (HSR) was performed for 60 min at 25° C in a medium containing 40 mM MOPS/Tris (pH 7.0), 100 μM $CaCl_2$, 500 mM KCL, 5 nM [^{3}H]ryanodine, 0.1 mM PSMF, 0.5 μg/ml leupeptin, 1.4 μg/ml aprotinin, 1 μM pepstatin, 100 μg HSR/0.2 ml volume in the presence or absence of 0.5 μM CaM, and 3, 10 and 20 μM 4-CMPS or 3, 10 and 100 μM 4,4′-DTDP. Values are means ± SEM for the number of experiments (*n*) given in parenthesis. Significant differences between [^{3}H]ryanodine binding in the absence and presence of CaM: [a] $P < 10^{-8}$; [b] $P < 0.004$; [c] $P < 0.0004$.

[^{125}I]Calmodulin Binding

Calmodulin binding to HSR was performed with 0.5–1 μM calcium-[^{125}I]calmodulin in the absence and presence of 4-CMPS, 4,4′-DTDP and suramin in two different HSR preparations. 10–100 μM 4-CMPS and 0.5–1 mM 4,4′-DTDP had no significant effect on calcium-[^{125}I]calmodulin binding to HSR, while 0.5–1 mM suramin, which was used as a positive control, caused an almost complete inhibition of calcium-[^{125}I]calmodulin binding (Table 6).

Discussion

The present study demonstrates that the inhibitory effect of the calcium binding protein calmodulin on the sarcoplasmic reticulum calcium release channel depends on the state of oxidation of sulfhydryls of the channel. Two sulfhydryl oxidizing agents, the organic mercurial compound 4-CMPS and 4,4′-dithiodipyridine have been used as tools to study the effect calmodulin on the calcium release channel under conditions of sulfhydryl oxidation. Both agents were selected because of their well known action and the reversibility of channel activation by the specific sulfhydryl reducing agent DTT. 4-CMPS forms a mecaptide complex (mercuri-thiol bonds) with cysteines (Riordan & Vallee, 1972) of the calcium release channel subunits (Oba, Ischikawa & Yamaguchi, 1986; Suko & Hellmann, 1998); 4,4′-DTDP affects sulfhydryls of the calcium release channel by the formation of mixed disulfides (Eager et al., 1997).

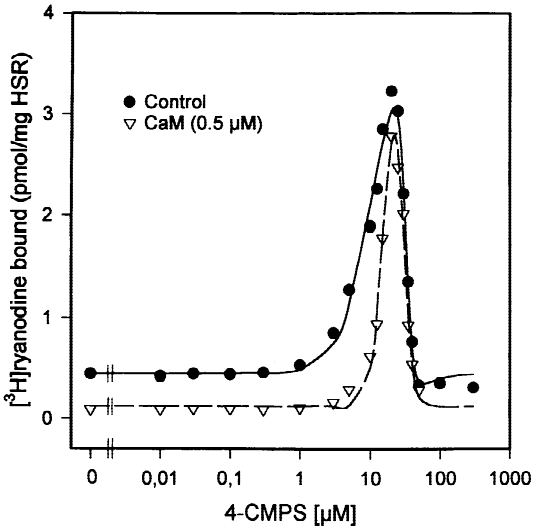


Fig. 6. Concentration dependence of activation and inhibition of specific [^{3}H]ryanodine binding to HSR by 4-CMPS in the absence or presence of 0.5 μM calmodulin. Specific [^{3}H]ryanodine binding to sarcoplasmic reticulum vesicles (HSR) was performed for 60 min at 25°C in a medium containing 40 mM MOPS/Tris (pH 7.0), 100 μM $CaCl_2$, 0.5 M KCl, 5 nM [^{3}H]ryanodine, 0.1 mM PSMF, 0.5 μg/ml leupeptin, 1.4 μg/ml aprotinin, 1 μM pepstatin, 0.5 μM CaM and 100 μg HSR/0.2 ml in the presence of the indicated concentrations of 4-CMPS. The solid and dotted lines represent a fit of the data according to the sum of two Hill equations (Suko et al., 1998). The calculated half maximum activation of [^{3}H]ryanodine binding increased significantly from 10.9 ± 0.6 μM (control) to 15.2 ± 0.9 μM 4-CMPS ($P = 0.017$) in the presence of CaM; the half maximum inhibition was not significantly changed (control: 33.9 ± 2.5 μM 4-CMPS; CaM: 32.2 ± 2.0 μM 4-CMPS; the Hill coefficient for activation by 4-CMPS increased significantly from 1.8 ± 0.1 to 4.9 ± 0.3 ($P < 0.001$) in the presence of CaM (means ± SEM; $n = 3$). The maximum [^{3}H]ryanodine binding was not significantly changed.

The low concentration of CaM (0.1–0.5 μM) used in the single channel experiments and [^{3}H]ryanodine binding excludes a significant reduction of the channel activating calcium concentration (50–100 μM) on binding of calcium to the two high affinity calcium binding sites on the C-terminal half and to the two low affinity calcium binding sites on the N-terminal half of CaM and formation of the inhibitory calcium-CaM complex (Klee & Vanaman, 1982; Suko et al., 1986).

Calcium-CaM is not capable of inducing a complete inhibition of the calcium release from HSR (Meissner, 1986; Plank et al., 1988; Fuentes et al., 1994) or complete inhibition of the calcium release channel or channel closure (Smith et al., 1989; Fuentes et al., 1994; Tripathy et al., 1995). The 3–4-fold reduction in P_o and [^{3}H]ryanodine binding observed in the present study is in agreement with the reported data on the maximum inhibition

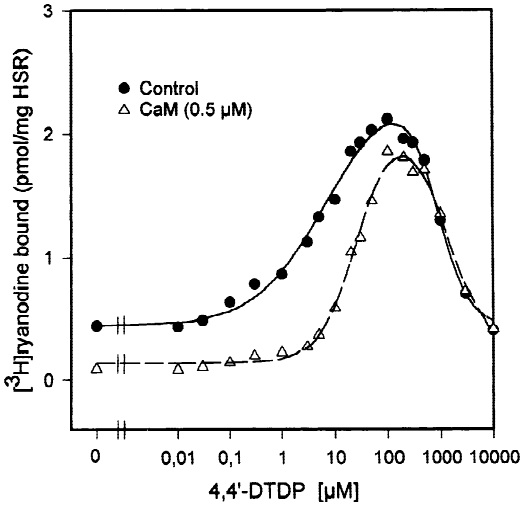


Fig. 7. Concentration dependence of activation and inhibition of [^{3}H]ryanodine binding to HSR by 4,4′-DTDP in the absence or presence of 0.5 µM calmodulin. Specific [^{3}H]ryanodine binding to sarcoplasmic reticulum vesicles (HSR) was performed for 60 min at 25°C in a medium containing 40 mM MOPS/Tris (pH 7.0), 100 µM $CaCl_2$, 0.5 M KCl, 5 nM [^{3}H]ryanodine, 0.1 mM PSMF, 0.5 µg/ml leupeptin, 1.4 µg/ml aprotinin, 1 µM pepstatin, 0.5 µM CaM and 100 µg HSR/0.2 ml. The solid line and the dotted line represent a fit of the data according to the sum of two Hill equations. The calculated half maximum activation of [^{3}H]ryanodine binding increased significantly ($P = 0.017$) from in 9.8 ± 1.2 µM to 22.6 ± 3.0 µM 4,4′-DTDP in the presence of 0.5 µM CaM; the half maximum inhibition was not significantly changed (control: 729 ± 69.5 µM 4,4′-DTDP; CaM: 1287 ± 234 µM 4,4′-DTDP; the Hill coefficient for activation by 4,4′-DTDP was 0.58 ± 0.02 in controls and increased significantly to 1.35 ± 0.1 in the presence of CaM ($P = 0.002$), (means ± SEM; $n = 3$). The maximum [^{3}H]ryanodine binding was not significantly changed.

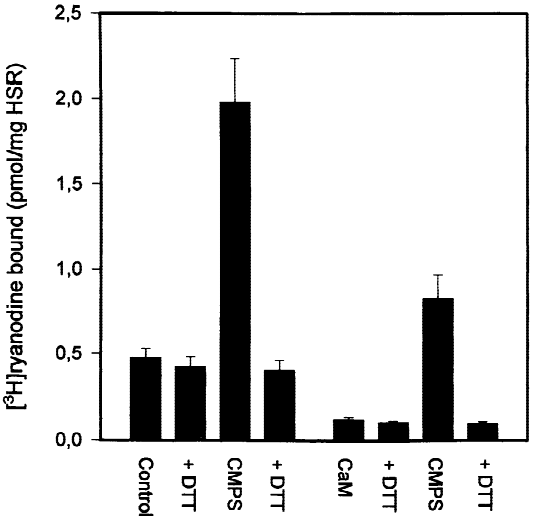


Fig. 8. Inhibition of 4-CMPS-induced stimulation of [^{3}H]ryanodine binding to HSR by DTT in the absence or presence of 0.5 µM calmodulin. Specific [^{3}H]ryanodine binding to sarcoplasmic reticulum vesicles (HSR) was performed for 60 min at 25°C in a medium containing 40 mM MOPS/Tris (pH 7.0), 100 µM $CaCl_2$, 0.5 M KCl, 5 nM [^{3}H]ryanodine, 0.1 mM PSMF, 0.5 µg/ml leupeptin, 1.4 µg/ml aprotinin, 1 µM pepstatin, 100 µg HSR/0.2 ml in the presence or absence of 0.5 µM CaM, 10 µM 4-CMPS and/or 2 mM DTT. Values are means ± SEM of five independent experiments with different HSR preparations. Significantly different: control *vs.* 4-CMPS ($P < 0.001$); control *vs.* CaM ($P < 0.001$); 4-CMPS *vs.* CaM + 4-CMPS ($P = 0.004$); control *vs.* DTT or *vs.* 4-CMPS + DTT, CaM *vs.* CaM + DTT or *vs.* CaM + 4-CMPS + DTT were not significantly different.

of the calcium release channel in single channel recordings or [^{3}H]ryanodine binding by the calcium-CaM complex (Fuentes et al., 1994; Tripathy et al., 1995; O'Driscoll et al., 1996). The effects of CaM on the open and closed time distribution (Tables 3 and 4) were similar to those seen on reduction of the free calcium concentration (Smith et al., 1988) or in the presence of inhibitory concentrations of magnesium.

Oxidation of sulfhydryls of cysteine residues of the calcium release channel by 4-CMPS or 4,4′-DTDP had a profound and opposite effect on channel gating as observed with calmodulin (Tables 1 and 2). The results show (*a*) In the presence of maximally activating calcium concentrations and 0.1 µM CaM the concentration dependence of channel activation by 4-CMPS or 4,4′-DTTP was shifted to the right in single channel current recordings (Figs. 3 and 5, Tables 3 and 4) and [^{3}H]ryanodine binding to HSR (Figs. 6 and 7), demonstrating that the channel activity (P_o) was lower in the presence of calmodulin at low concentrations of sulfhydryl oxidizing compounds as compared to controls. High concentrations of 4-CMPS abolished the inhibitory effect of CaM on the channel open probability, i.e., a maximal channel activation was obtained by 40 µM 4-CMPS ($P_o > 0.9$), with 0.3 mM 4,4′-DTDP (larger concentrations have not been tested) the activation was submaximal (Tables 1 and 2). However, calmodulin did not affect the sulfhydryl oxidation-induced inhibition of the calcium release channel by 4-CMPS or 4,4′-DTDP in single channel current recordings or in [^{3}H]ryanodine binding (Figs. 6 and 7). Activating and inhibitory sulfhydryls are most probably on separate cysteine(s) of the calcium release channel (Eager et al., 1997; Suko & Hellmann, 1998).

(b) The greater percentage increase in P_o by 4-CMPS or 4,′4-DTDP in the presence of calmodulin than in controls at maximally activating Ca^{2+} (Tables 1 and 2), requires some comment. The relative greater increase with CaM is due to the fact that the percentage increase in channel activity is greater when the activation is started from a lower P_o. This is demonstrated in single channel experiments with 4-CMPS, carried out with a low activating Ca^{2+} (10 µM) or in the presence 40 µM Ca^{2+} plus 1 mM magnesium (P_o about 0.07–0.08); under these conditions the percentage increase in P_o by 10 µM 4-CMPS (about sixfold) was greater than at maximally activating calcium concentrations in the absence or presence of calmodulin (Table 1).

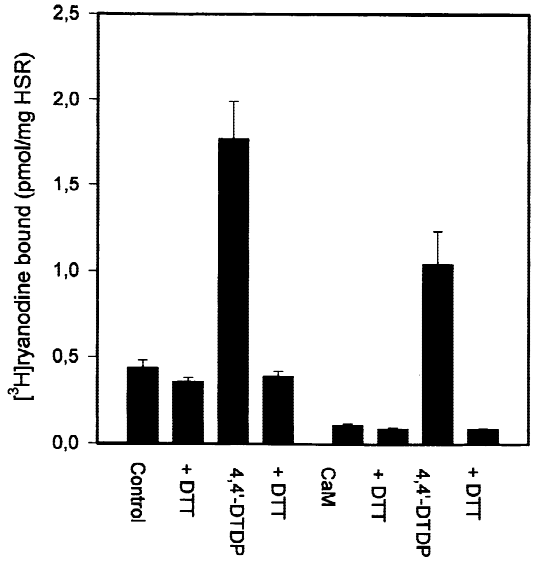


Fig. 9. Inhibition of 4,4′-DTDP-induced stimulation of [^{3}H]ryanodine binding to HSR by DTT in the absence or presence of 0.5 μM calmodulin. Specific [^{3}H]ryanodine binding to sarcoplasmic reticulum vesicles (HSR) was performed for 60 min at 25°C in a medium containing 40 mM MOPS/Tris (pH 7.0), 100 μM $CaCl_2$, 0.5 M KCl, 5 nM [^{3}H]ryanodine, 0.1 mM PSMF, 0.5 μg/ml leupeptin, 1.4 μg/ml aprotinin, 1 μM pepstatin, 100 μg HSR/0.2 ml in the presence or absence of 0.5 μM CaM, 10 μM 4,4′-DTDP and/or 2 mM DTT. Values are means ± SEM of four independent experiments with different HSR preparations. Significantly different: control *vs.* 4,4′-DTDP ($P = 0.001$); control *vs.* CaM ($P < 0.001$); 4,4′-DTDP *vs.* CaM + 4,4′-DTDP ($P = 0.05$); control *vs.* DTT or *vs.* 4,4′-DTDP + DTT, CaM *vs.* CaM + DTT or *vs.* CaM + 4,4′-DTDP + DTT were not significantly different.

Table 6. [^{125}]calmodulin binding to HSR

		[^{125}]calmodulin binding (pmol/mg HSR)	(*n*)
Control	—	27.9 ± 3.5	(4)
4-CMPS	10 μM	26.4 ± 3.6	(4)
	20 μM	26.0 ± 4.0	(4)
	40 μM	25.8 ± 4.9	(4)
	100 μM	25.5 ± 4.6	(4)
4,4′-DTDP	0.5–1 mM	29.8 ± 3.3	(4)
Suramin	0.5–1 mM	1.4 ± 0.7	(4)

Calcium-[^{125}]calmodulin binding to HSR was performed as described in Materials and Methods. Values are means ± SEM for the number of experiments given in parenthesis.

The above results suggest that activation of the calcium release channel by sulfhydryl oxidation and inhibition by calmodulin (or magnesium) are additive. Activation of the calcium release channel by sulfhydryl oxidation might add onto the calmodulin-induced inhibition resulting in less channel activity compared to controls. An additive effect of inhibition of magnesium and activation of the sheep cardiac calcium release channel by 4,4-DTDP has been shown by Eager & Dulhunty (1998). Inhibition of binding of CaM (calcium-free) and Ca-CaM to HSR by the sulfhydryl oxidizing and crosslinking compound diamide, and inhibition of the binding of CaM, but not of Ca-CaM to HSR by NEM was reported recently by Zhang et al. (1999). Calcium-[^{125}I]calmodulin binding to HSR was neither influenced by 4-CMPS nor 4,4′-DTDP (Table 6). These results exclude a displacement of calmodulin from the calcium release channel following oxidation of sulfhydryls in calmodulin binding regions or by conformational alterations of channel subunits that could possibly cause the dissociation of bound calmodulin. It is noted that neither oxidation of channel activating or channel inhibiting sulfhydryls affected Ca-[^{125}I]calmodulin binding to HSR. Suramin, which was used as reference agent, markedly reduced [^{125}I]calmodulin binding to HSR. It has previously been shown, that suramin displaced the ryanodine receptor bound to a calmodulin sepharose, thus demonstrating that suramin affects calmodulin binding to the calcium release channel (Klinger et al., 1999). Up to nine calmodulin binding sites have been predicted from the primary sequence of the ryanodine receptor (Takeshima et al., 1989; Zorzato et al., 1990). Six (Chen et al., 1994) or three (Menegazzi et al., 1994) calmodulin binding sites per ryanodine receptor subunit have been detected by CaM overlays to ryanodine receptor fusion proteins, which differed in their calcium-dependence.

Magnesium is a more potent inhibitor of the calcium release channel than calmodulin and high magnesium concentrations cause a complete inhibition of the calcium release channel (Smith et al., 1988; Meissner, 1994; Coronado et al., 1994; Eager & Dulhunty, 1998). The inhibitory effect of a physiological concentration of magnesium (about 1 mM Mg^{2+}), which reduced the open probability to about 14% of the controls, was abolished following sulfhydryl oxidation of the calcium release channel by 20–40 μM 4-CMPS.

Inhibitors such as calcium-CaM or magnesium may play a role under physiological and pathophysiological conditions associated with sulfhydryl oxidation of the calcium release channel. Nitric oxide, which is produced in skeletal muscle and cardiac muscle (Kobzik et al., 1994) activated the calcium release channel in skeletal muscle and cardiac muscle (Stoyanovsky et al., 1996; Aghadasi, Reid & Hamilton, 1997; Xu et al., 1998; Suko et al., 1999) by oxidation/nitrosylation of cysteine residues and may play a physiological role in the regulation of the gating of the calcium release channel. Activation of the calcium release channel by reactive oxygen species (Abramson et al., 1995; Oba et al., 1998), which contribute to calcium release from sarcoplasmic reticulum and muscle fatigue, could be partly counteracted by a calmodulin-induced inhibition.

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