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Inhibition of calcium release from skeletal muscle sarcoplasmic reticulum by calmodulin

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The effect of calmodulin on calcium release from heavy sarcoplasmic reticulum isolated from rabbit skeletal muscle was investigated with actively and passively calcium loaded sarcoplasmic reticulum vesicles and measured either spectrophotometrically with arsenazo III or by Millipore filtration technique. The transient calcium-, caffeine- and AMP-induced calcium release from actively loaded sarcoplasmic reticulum vesicles was reduced to 29%, 51% and 59% of the respective control value by 1 μM exogenous calmodulin. Stopped-flow measurements demonstrate that calmodulin reduces the apparent rate of caffeine-induced calcium release from actively loaded sarcoplasmic reticulum. The rate of calcium uptake measured in the presence of ruthenium red, which blocks the calcium release channel, was not affected by calmodulin or calmodulin-dependent phosphorylation of sarcoplasmic reticulum vesicles with ATP[S]. The rate of the calcium-, caffeine- and AMP-induced calcium release from passively loaded sarcoplasmic reticulum vesicles was reduced 1.4–2.0-fold by 1 μM exogenous calmodulin, i.e. the half-time of release was maximally increased by a factor of two, whilst calmodulin-dependent phosphorylation of a 57 kDa protein with ATP[S] had no effect. The data indicate that calmodulin itself regulates the calcium release channel of sarcoplasmic reticulum.

Introduction

Calcium release from sarcoplasmic reticulum and the ATP-driven calcium uptake by the calcium transport ATPase of sarcoplasmic reticulum membranes control muscle contraction and relaxation [1–4]. Calcium-induced calcium release from sarcoplasmic reticulum via a calcium channel has been observed in skinned fibres of skeletal [5–7] and cardiac [8,9] muscle and isolated sarcoplasmic reticulum vesicles by calcium flux measurements and spectrophotometric methods [10–18], as well as by single channel measurements with sarcoplasmic reticulum vesicles fused into lipid bilayer [19]. The so-called 'calcium-gated calcium channel' or 'calcium release channel' is enriched in heavy sarcoplasmic reticulum and located in sarcoplasmic reticulum derived from the terminal cysternae [11,20,21].

It was shown recently by Meissner [22] that calmodulin inhibits calcium-induced calcium release and calcium release by the ATP analog $p[CH_2]ppA$ from skeletal muscle sarcoplasmic reticulum vesicles. On the other hand, skeletal muscle sarcoplasmic reticulum contains an endogenous

Abbreviations: $p[CH_2]ppA$, adenosine 5′-[β,γ-methylene]triphosphate; ATP[S], adenosine 5′-[γ-thio]triphosphate ([S]pppA).

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calmodulin-dependent protein kinase which phosphorylates several proteins [23,24]. Since calmodulin-dependent phosphorylation did not influence calcium uptake [23,24], (in contrast to the increase in the rate of calcium uptake induced in cardiac sarcoplasmic reticulum [25–30]), it was suggested by MacLennan et al. [31] on grounds of indirect experimental evidence that calmodulin-dependent phosphorylation plays a role in calcium release from skeletal muscle sarcoplasmic reticulum due to an inhibitory effect on the calcium release channel. Furthermore, Kim and Ikemoto [32] obtained a good correlation between inhibition of the rate of calcium-induced calcium release and calmodulin-dependent phosphorylation of a 60 kDa protein.

In the present study we have investigated the effect of exogenous calmodulin on the calcium-, caffeine- and AMP-induced calcium release from actively and passively loaded heavy sarcoplasmic reticulum vesicles. ATP[S], which does not energize calcium uptake, was used with passively loaded sarcoplasmic reticulum vesicles to determine whether calmodulin itself or calmodulin-dependent phosphorylation of sarcoplasmic reticulum proteins affects the calcium release channel.

Materials and Methods

Reagents

Arsenazo III, ATP, Mops, caffeine, ruthenium red were purchased from Sigma Chemical Co. (St. Louis); phospho*enol*pyruvate, pyruvate kinase, ATP[S] from Boehringer (Mannheim); $^{45}CaCl_2$ from Amersham International (Amersham); [^{35}S]ATP[S] from New England Nuclear (Boston).

Preparation of sarcoplasmic reticulum and calmodulin

Heavy sarcoplasmic reticulum vesicles from rabbit skeletal muscle were prepared according to Yamamoto and Kasai [10]. Briefly, white back and leg muscle (fast twitch muscle) were put through a meat grinder and homogenized in a Waring blender for 3 min in a medium containing 5 mM histidine buffer (pH 7.0), 5 mM NaN_3, 100 mM NaCl, and centrifuged for 30 min at $4000 \times g$. The supernatant was filtered through cheese cloth and centrifuged for 30 min at $10000 \times g$. The pellet was resuspended in 5 mM histidine buffer (pH 7.0), 5 mM NaN_3, 0.6 M KCl and centrifuged for 30 min at $100000 \times g$. The pellet was washed once in the same medium, except that the concentration of KCl was 0.1 M, centrifuged again for 30 min at $100000 \times g$, resuspended in 5 mM histidine buffer (pH 7.0), 5 mM NaN_3, 0.1 M KCl and stored at $-70\,^{\circ}C$.

Calmodulin from bovine brain (obtained from the slaughter-house) was prepared by Phenyl-Sepharose affinity chromatography according to Gopalakrishna and Anderson [33], as described previously [34].

Analyses

Calcium release from actively loaded sarcoplasmic reticulum. Calcium uptake was carried out in a 1×1 cm cuvette in a medium containing 20–50 mM Mops-Tris (pH 7.0), 5 mM NaN_3, 100–150 mM KCl, 15–100 μM arsenazo III, 100–180 μM added $CaCl_2$, 4 mM phospho*enol*pyruvate, 40 μg/ml pyruvate kinase, 0.5 mM $MgCl_2$, 0.1 mM ATP and 2 mg/ml sarcoplasmic reticulum; total volume 2.0 ml. Calcium uptake was started by addition of ATP via injection by a Hamilton syringe under vigorous stirring. Calcium release was started by injection of the calcium, caffeine or AMP into the cuvette by a Hamilton syringe (in a volume ranging from 1% to maximally 5% of that of the uptake medium), when calcium uptake had reached a plateau value (approximately 4–8 min after ATP addition, depending on the amount of added calcium). All incubations were performed at $25\,^{\circ}C$, pH 7.0. Alterations in absorption were monitored by a dual-wavelength spectrophotometer (Sigma ZWS II) at wavelength pairs of 675 nm and 685 nm or 650 nm and 690 nm [35].

The contaminating calcium in the medium with or without sarcoplasmic reticulum was determined by titration in the presence of arsenazo III, calibrated by addition of known amounts of calcium, plus chelation of calcium by EGTA. Contaminating calcium amounted to 5–6 μM in the complete medium without protein and to about 15 μM in the presence of 1 mg/ml sarcoplasmic reticulum. The total contaminating calcium in the calcium uptake medium with 2 mg/ml sarcoplasmic reticulum was therefore about 25 μM.

Rates of calcium release were determined by stopped-flow with the Sigma ZWS II spectrophotometer equipped with a stopped-flow apparatus (with a 2 × 2 mm cuvette and a 10 cm quartz rod transmitting the light to the photomultiplier) at wavelength pairs of 650 nm and 690 nm. Syringes I and II: 20 mM Mops-Tris (pH 7.0), 5 mM NaN_3, 150 mM KCl, 40 μM arsenazo III, 4 mM phospho*enol*pyruvate, 40 μg/ml pyruvate kinase, 0.1 mM $MgCl_2$; Syringe I contained in addition 140–160 μM added $CaCl_2$, 0.5 mM ATP, 2 mg/ml sarcoplasmic reticulum; Syringe II contained in addition 40 μM calcium or 5 mM caffeine or 1–4 mM AMP. The contents of syringes I and II were mixed in the ratio 1 : . Calcium release was started 3 min after starting the uptake with ATP. Dual-wavelength traces were displayed on a digital storage oscilloscope (Tektronix model 468) and stored by a COmmodore CBM 8032 computer on diskettes or displayed by a plotter (Digiplot, model WX 4675, Watanabe Instruments Corp.).

Calcium release from passively loaded sarcoplasmic reticulum. Freshly prepared heavy sarcoplasmic reticulum vesicles were preincubated for 24–48 h on ice in medium containing 50 mM Mops – Tris (pH 7.0), 5 mM NaN_3, 150 mM KCl, 4 mM $CaCl_2$ or 4 mM $^{45}CaCl_2$, 20 mg/ml sarcoplasmic reticulum [36]. Calcium release was started by injection of the preloaded sarcoplasmic reticulum by a Hamilton syringe under vigorous stirring into a 1 × 1 cm cuvette containing 50 mM Mops, 5 mM NaN_3, 150 mM KCl, 0.5–1.5 mM $MgCl_2$ and 40 μM arsenazo III, 30 μM $CaCl_2$, 0.15 mg/ml sarcoplasmic reticulum (final concentration; dilution 1 : 133) or 100 μM arsenazo III, 50 μM $CaCl_2$, 0.25 mg/ml sarcoplasmic reticulum (final concentration; dilution 1 : 80). The dual-wavelength traces were digitized by an analog-digital converter and stored on diskettes by a Commodore CBM 8032 computer.

Calcium-induced calcium release from sarcoplasmic reticulum vesicles passively loaded with $^{45}CaCl_2$ was determined by a filtration technique. Calcium release was started by dilution of the preloaded vesicles in a medium containing 50 mM Mops (pH 7.0), 5 mM NaN_3, 150 mM KCl, 0.5 mM $MgCl_2$, 0.1 mg/ml sarcoplasmic reticulum and 20 μM $^{45}CaCl_2$ (1 : 200 dilution) or 0.5 mM $^{45}CaCl_2$ plus 0.5 mM EGTA. Calcium-independent calcium release was measured in the above medium with 20 μM calcium (contaminant) plus 1 mM EGTA; $T = 25\,^{\circ}C$. The reaction was stopped by addition of a solution containing 50 mM Mops (pH 7.0), 5 mM NaN_3, 150 mM KCl, 10 mM $MgCl_2$ and 20 μM ruthenium red [16] and filtered through a cellulose filter of 0.3 μm pore size (Schleicher & Schüll; membrane filters Type BA84) and washed twice with 3 ml of the same medium. The radioactivity on the filter was measured in a liquid scintillation counter in 2 ml ethylglycol and 6 ml Atomlight (New England Nuclear, Boston), as described previously [27,36].

Calmodulin-dependent phosphorylation of heavy sarcoplasmic vesicles from skeletal muscle [23,24,31] by [^{35}S]ATP[S] and autoradiography of SDS gels was carried out as previously described for cardiac sarcoplasmic reticulum [37].

Protein was measured by the Folin method [38] standardized against bovine serum albumin.

Calculations. The theoretical curves were calculated by fitting one or two exponential functions to the experimental data using an iterative, non-linear least-squares procedure as described elsewhere [39].

Results

Typical experiments on calcium uptake by heavy sarcoplasmic reticulum vesicles and calcium-, caffeine- and AMP-induced calcium release from the actively loaded sarcoplasmic reticulum, measured spectrophotometrically with arsenazo III, are shown in Fig. 1. The increase in the calcium uptake signal over the amount of added calcium is due to the uptake of added and contaminating calcium (about 20–25 μM in the presence of 2 mg/ml sarcoplasmic reticulum). The calcium release induced by calcium, caffeine or AMP was transient and the released calcium was taken up again into the sarcoplasmic reticulum vesicles by the calcium pump [3,4]. The total amount of calcium released by calcium, caffeine or AMP, estimated semi-quantitatively from the height of the release signal, (corrected for uptake during the release), was 20.2 nmol/mg, 18.4 nmol/mg and 28.6 nmol/mg, respectively (Table I), representing 23.3%, 22.2% and 31.8% of the calcium load. Since the signal after addition of 20

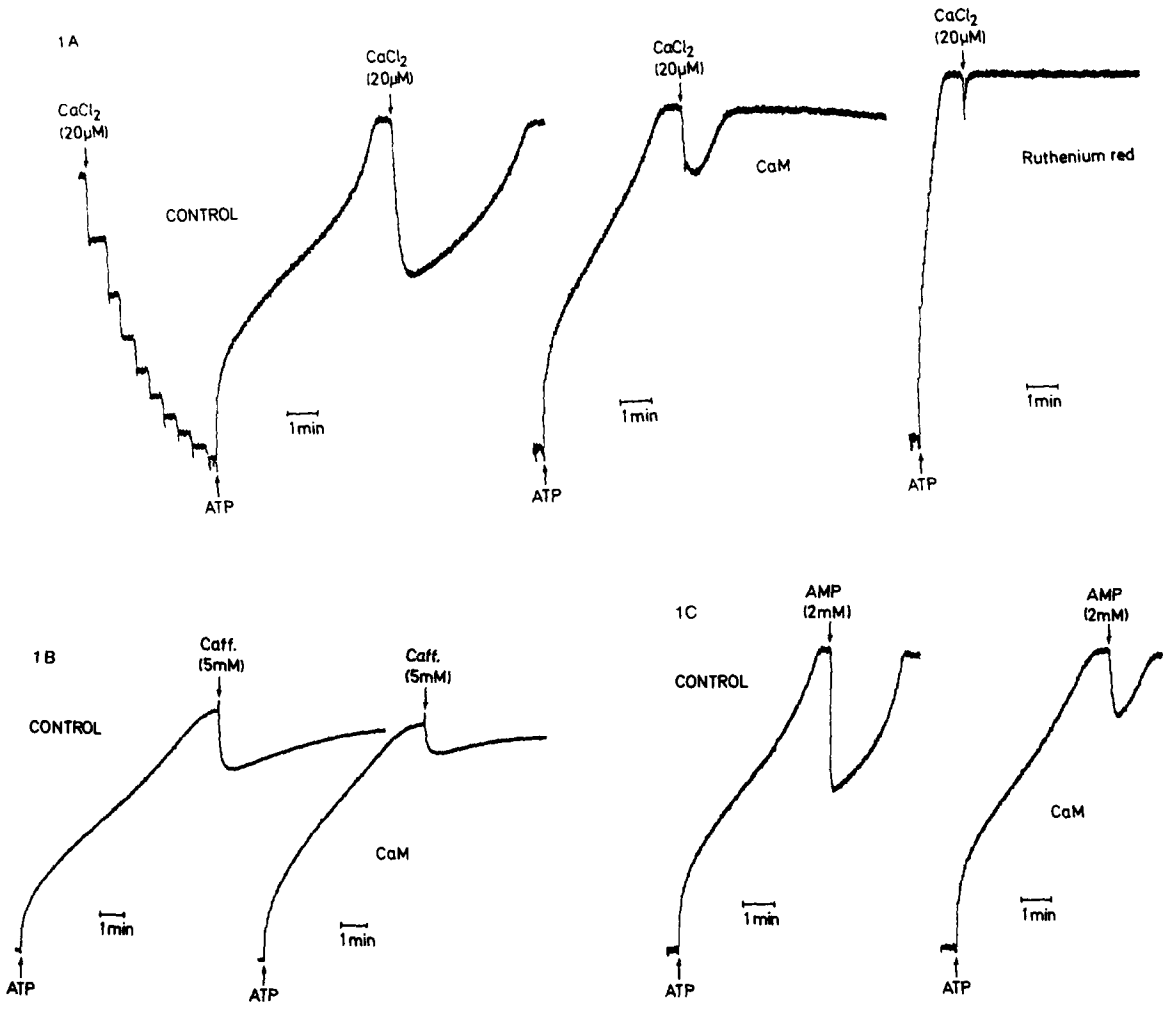


Fig. 1. Inhibition of calcium-, caffeine- and AMP-induced calcium release from actively loaded heavy sarcoplasmic reticulum by calmodulin. Uptake medium: 20 mM Mops, 150 mM KCl, 5 mM NaN_3, 4 mM phospho*enol*pyruvate, 40 μg/ml pyruvate kinase, 0.5 mM $MgCl_2$, 100 μM ATP, 2 mg/ml sarcoplasmic reticulum, 160–180 μM added calcium, 15 μM arsenazo III. Calcium- (A). caffeine- (B) and AMP-induced calcium release (C) without and with 1 μM calmodulin. Dual-wavelength spectrophotometric traces; wavelength pairs 650 nm and 690 nm. A calibration curve, made for each trace by addition of $CaCl_2$ in 20 μM steps, is shown only in (A).

μM calcium in the presence of ruthenium red, which blocks the calcium-induced calcium release, was about 70% of the signal in the calibration curve, carried out in each experiment in the absence of ATP, it was assumed that approximately 30% of the released calcium is taken up during the release phase. Calcium release in terms of percent of the applied load, was not much altered at calcium loads varying from 70 to 110 nmol/mg sarcoplasmic reticulum protein.

Calcium-induced calcium release was inhibited by 1 μM calmodulin, but less than by ruthenium red, both added before start of the calcium uptake with ATP (Fig. 1A). Calcium release from actively loaded isolated sarcoplasmic reticulum vesicles triggered off by caffeine (5 mM) or AMP (2 mM) was similarly inhibited by 1 μM calmodulin (Figs. 1B, 1C). Inhibition of calcium release during the uptake phase was clearly observed in the presence of ruthenium red (with a low ATP concentration

TABLE I

INHIBITION OF CALCIUM-, CAFFEINE- AND AMP-INDUCED CALCIUM RELEASE FROM ACTIVELY LOADED HEAVY SARCOPLASMIC RETICULUM BY CALMODULIN

Conditions are given in Fig. 1; values are means ± S.E.; n = number of determinations; CaM, calmodulin.

	n	Calcium released (nmol/mg SR)	Inhibition (% of control)
Calcium-induced release			
Control	8	20.18 ± 1.60	
CaM (1 μM)	8	5.86 ± 0.70	71
Caffeine-induced release			
Control	10	18.37 ± 0.95	
CaM (1 μM)	10	9.54 ± 1.02	49
AMP-induced release			
Control	5	28.61 ± 2.18	
CaM (1 μM)	5	16.90 ± 2.03	41

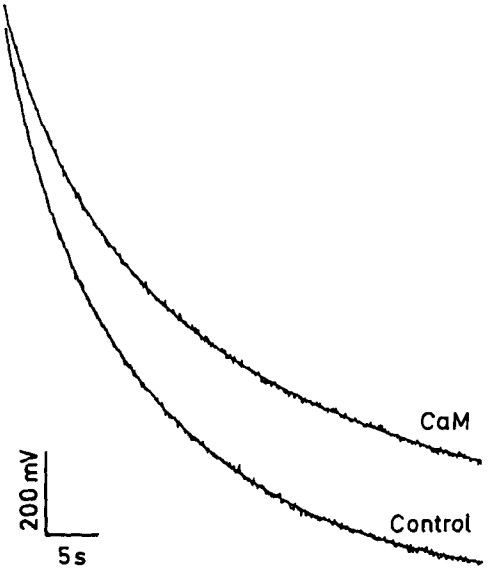


Fig. 2. Inhibition of the rate of caffeine-induced calcium release from actively loaded heavy sarcoplasmic reticulum by calmodulin (CaM) measured by stopped-flow. Syringe 1: 20 mM Mops, 150 mM KCl, 5 mM NaN_3, 4 mM phospho*enol*-pyruvate, 40 μg/ml pyruvate kinase, 0.1 mM ATP, 0.5 mM $MgCl_2$, 40 μM arsenazo III, 160 μM $CaCl_2$, 2 mg/ml sarcoplasmic reticulum, ±5 μM calmodulin. Syringe 2: 20 mM Mops, 150 mM KCl, 5 mM NaN_3, 4 mM phospho*enol*pyruvate, 40 μg/ml pyruvate kinase, 40 μM arsenazo III, 10 mM caffeine. Two-exponential fit.

and 80–100 μM calcium per mg sarcoplasmic reticulum protein), i.e. the time required for the uptake of added and contaminating calcium was markedly shorter than in the absence of ruthenium red (Fig. 1A). Inhibition of the calcium release by 1 μM calmodulin, semi-quantitatively estimated from the height of the release signal, was 71%, 49% and 41% for the calcium, caffeine- and AMP-induced release, respectively (Table I).

Stopped-flow measurements of the caffeine-induced calcium release from actively loaded heavy sarcoplasmic reticulum vesicles showed a small, but significant reduction in the apparent rate of calcium release by calmodulin (Fig. 2). The calcium release by caffeine was biphasic in the presence or absence of calmodulin. The apparent rate constants, calculated from a two-exponential fit of the release signal, decreased for both phases in the presence of exogenous calmodulin, i.e. the apparent half-time of the release increased 1.25–1.50 fold (control: $t_{1/2}(1)$ 3.09 ± 0.13 s; $t_{1/2}(2)$ 12.89 ± 0.40 s; calmodulin: $t_{1/2}(1)$ 3.88 ± 0.11 s; $t_{1/2}(2)$ 19.14 ± 1.20 s; means ± S.E.; $n = 6$ or 7). The calculated maximum calcium release of the phases was about equal in the presence or absence of calmodulin. On the other hand, a reduction in the rate of AMP-induced calcium release by calmodulin, measured under similar conditions at AMP concentrations ranging from 0.5 to 2 mM was obtained only in some preparations; the effect was not reproducible in all tested sarcoplasmic reticulum preparations; the reason for this inconsistency remains unclear.

The rate of calcium uptake, measured in the presence of ruthenium red in order to block the calcium-activated calcium release, was not increased by preincubation of heavy sarcoplasmic reticulum vesicles with 2–5 μM calmodulin and 20 to 40 μM calcium in the absence of ATP for 1–10 min, indicating that calmodulin itself does not affect the calcium pump directly. Since the half-time for the uptake reaction was rather short (about 1 s; Table II), maximum phosphorylation might not have been achieved within such a short time period, hence, the experiments are not conclusive as to whether or not calmodulin-dependent

TABLE II

RATE OF CALCIUM UPTAKE BY HEAVY SARCOPLASMIC RETICULUM IN THE PRESENCE OF RUTHENIUM RED WITH AND WITHOUT CALMODULIN OR CALMODULIN-DEPENDENT PHOSPHORYLATION

Conditions are given in Fig. 3; values are means ± S.E.; n = number of determinations; CaM, calmodulin.

	n	$t_{1/2}$ (s)
Control	5	1.06 ± 0.11
CaM	5	1.18 ± 0.13
Control + ATP[S]	5	1.13 ± 0.33
CaM + ATP[S]	5	1.15 ± 0.10

phosphorylation might influence the rate of calcium uptake. Prephosphorylation of heavy sarcoplasmic reticulum vesicles was, therefore, carried out with ATP[S] plus calmodulin in the presence of calcium for 10 min, followed by

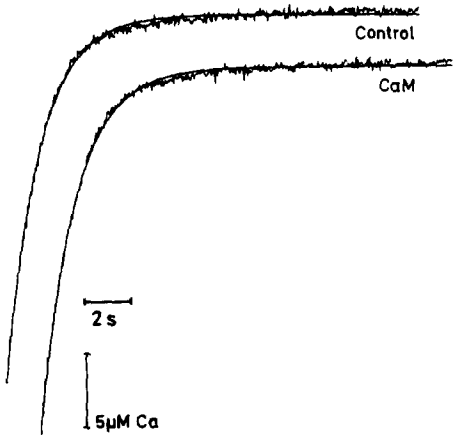


Fig. 3. Rate of calcium uptake of heavy sarcoplasmic reticulum vesicles in the presence of ruthenium red after calmodulin-dependent phosphorylation with ATP[S]. Preincubation of 20 mg/ml sarcoplasmic reticulum in 5 mM histidine buffer (pH 7.0), 100 mM KCl, 5 mM NaN_3, 200 μM $CaCl_2$, 1 mM $MgCl_2$, 0.5 mM ATP[S] with and without 10 μM calmodulin (CaM) for 10 min (T = 25 °C) followed by dilution 1 : 10 in the medium of Syringe 1 : 20 mM Mops, 150 mM KCl, 5 mM NaN_3, 4 mM phospho*enol*pyruvate, 40 μg/ml pyruvate kinase, 20 μM ruthenium red, 0.5 mM $MgCl_2$, 40 μM arsenazo III, 20 μM $CaCl_2$, 2 mg/ml sarcoplasmic reticulum. Syringe 2: 20 mM Mops, 150 mM KCl, 5 mM NaN_3, 4 mM phospho*enol*-pyruvate, 40 μg/ml pyruvate kinase, 20 μM ruthenium red, 40 μM arsenazo III, 100 μM ATP. Mono-exponential fit.

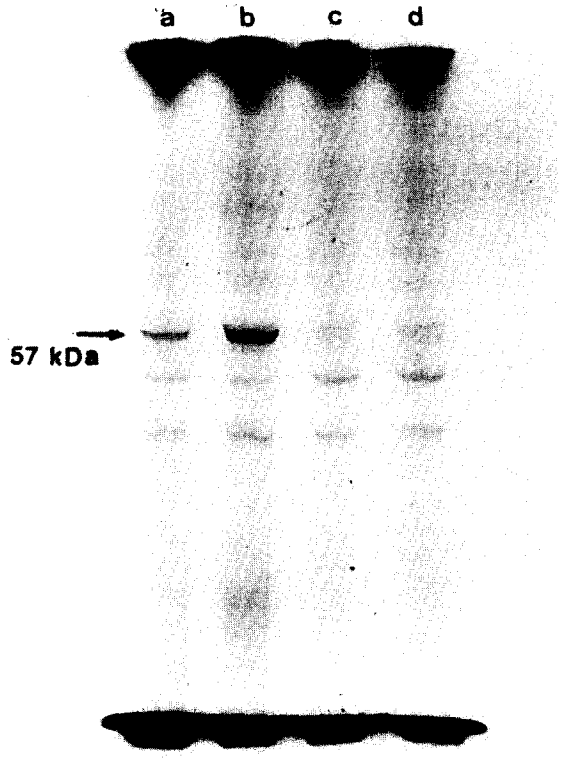
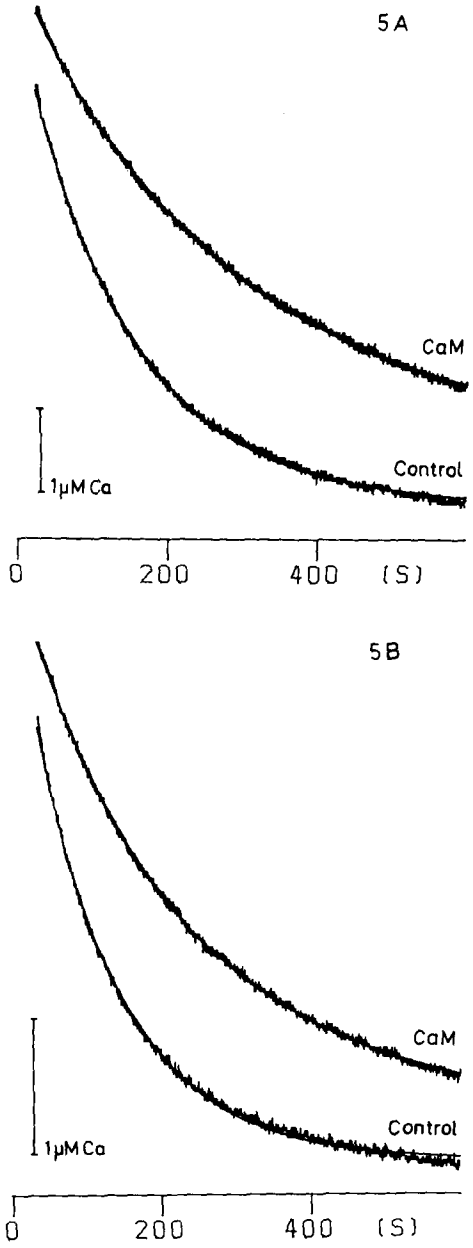


Fig. 4. Autoradiogram of an SDS-polyacrylamide gel electrophoretogram of calmodulin-dependently phosphorylated heavy sarcoplasmic reticulum with [^{35}S]ATP[S]. Phosphorylation was carried out for 10 min (25 °C, pH 7.0); lane a: 50 mM Mops, 100 mM KCl, 5 mM NaN_3, 1 mM $MgCl_2$, 0.25 mM [^{35}S]ATP[S], 0.2 mM $CaCl_2$, 20 mg/ml sarcoplasmic reticulum; lane b: plus 10 μM calmodulin; lane c: zero added $CaCl_2$ plus 2 mM EGTA, zero calmodulin; lane d: zero added $CaCl_2$ plus 2 mM EGTA, 10 μM calmodulin (Refs. 26 and 37).

sarcoplasmic reticulum dilution 1 : 10 and mixing with ATP. Again no increase was observed in the rate of calcium uptake, indicating that calmodulin-dependent phosphorylation does not affect the calcium uptake process (Fig. 3; Table II). Autoradiography of SDS gels of phosphorylated sarcoplasmic reticulum vesicles with either [^{32}P]ATP or [^{35}S]ATP[S] revealed a calmodulin-dependent phosphate incorporation into a 57 kDa protein (Fig. 4).

The effect of calmodulin on calcium-, caffeine- and AMP-induced calcium release from passively loaded sarcoplasmic reticulum (carried out in the presence of 4 mM calcium, 20 mg/ml sarco-

plasmic reticulum for 24 on ice) was measured spectrophotometrically by diluting the vesicles in the release medium without (Fig. 5A) or with 5 mM caffeine (Fig. 5B) or 1.5 mM AMP (Fig. 5C). The magnesium concentration was kept at 0.5 mM in the calcium-induced release and at 1.0 mM in the caffeine- and 1.5 mM in AMP-induced release in order to slow the rate of calcium release to an extent easily measurable under the experimental conditions. The apparent rate constants, calculated from a mono-exponential fit of the release signals decreased significantly in the three types of release in the presence of 1 μM exogenous calmodulin (Table III), i.e. the apparent half-times were increased 1.4–2-fold. Inhibition of the rate of calcium release by 0.1 μM calmodulin, measured



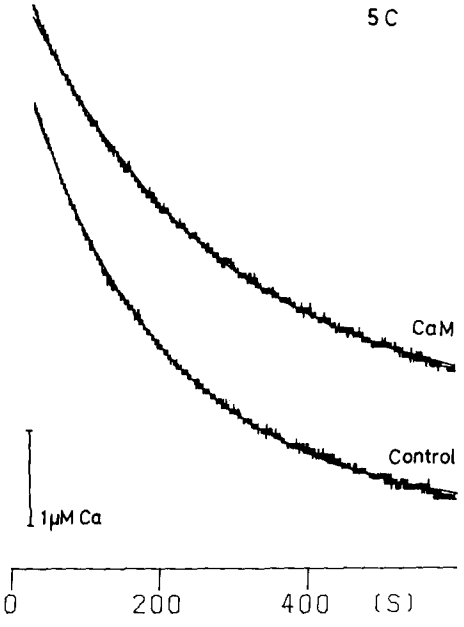


Fig. 5. Inhibition of calcium-, caffeine- and AMP-induced calcium release from passively loaded heavy sarcoplasmic reticulum by calmodulin. Preload of sarcoplasmic reticulum: 50 mM Mops, 150 mM KCl, 5 mM NaN_3, 4 mM $CaCl_2$, 20 mg/ml sarcoplasmic reticulum (24–48 h on ice). The protein was diluted 1:133 in 50 mM Mops, 150 mM KCl, 5 mM NaN_3, 0.5 mM $MgCl_2$, 40 μM arsenazo III, 30 μM $CaCl_2$, 0.15 mg/ml sarcoplasmic reticulum without or with 1 μM calmodulin (final concentration). (A) Calcium-induced release. (B) Caffeine-induced release. Conditions as in (A), except 1 mM $MgCl_2$, 5 mM caffeine. (C) AMP-induced release. Conditions as in (A), except 1.5 mM $MgCl_2$, 0.5 mM AMP. Dual-wavelength spectrophotometric traces; wavelength pairs 650 nm and 690 nm. Mono-exponential fit. The maximum calcium release ranged between 21.8 nmol/mg and 32.6 nmol/mg sarcoplasmic reticulum protein. Rate parameters are given in Table III.

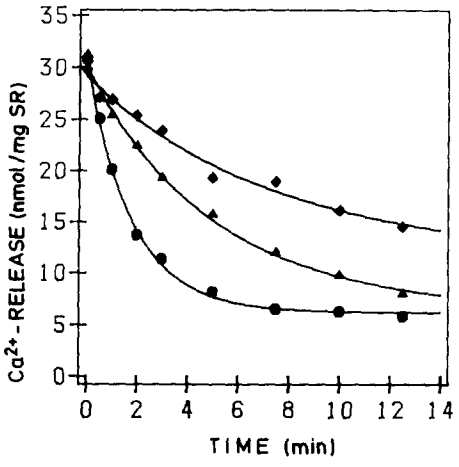


Fig. 6. Inhibition of calcium-induced calcium release from passively loaded sarcoplasmic reticulum vesicles by calmodulin measured with $^{45}CaCl_2$. Preload of sarcoplasmic reticulum: 50 mM Mops, 150 mM KCl, 5 mM NaN_3, 4 mM $^{45}CaCl_2$, 20 mg/ml sarcoplasmic reticulum (24–48 h on ice). The protein was diluted 1:200 in 50 mM Mops, 150 mM KCl, 5 mM NaN_3, 0.5 mM $MgCl_2$, 20 μM $CaCl_2$, 0.1 mg/ml sarcoplasmic reticulum (●), plus 1 μM calmodulin (▲); plus 20 μM ruthenium red, 10 mM $MgCl_2$ (◆). Mono-exponential fit.

in calcium-induced release only, was inhibited to the same degree as with 1 μM calmodulin. The maximum calcium release varied between 21 and 32 nmol/mg sarcoplasmic reticulum protein, in agreement with the data obtained with $^{45}CaCl_2$.

TABLE III

INHIBITION OF CALCIUM-, CAFFEINE- AND AMP-INDUCED CALCIUM RELEASE FROM PASSIVELY LOADED HEAVY SARCOPLASMIC RETICULUM BY CALMODULIN MEASURED SPECTROPHOTOMETRICALLY

Conditions are given in Fig. 5; values are means ± S.E.; n = number of determinations; CaM, calmodulin.

	n	$t_{1/2}$ (s)
Calcium-induced release		
Control	8	109.74 ± 4.57
CaM (1 μM)	6	209.18 ± 10.71
(0.1 μM)	5	207.31 ± 6.83
Control (ATP[S])	4	93.48 ± 7.70
CaM (1 μM + ATP[S])	6	154.88 ± 7.40
Caffeine-induced release		
Control	4	80.34 ± 1.73
CaM (1 μM)	5	159.22 ± 5.14
AMP-induced release		
Control	3	131.48 ± 2.27
CaM (1 μM)	3	188.11 ± 14.16

TABLE IV

INHIBITION OF CALCIUM-INDUCED CALCIUM RELEASE FROM PASSIVELY LOADED HEAVY SARCOPLASMIC RETICULUM BY CALMODULIN MEASURED WITH $^{45}CaCl_2$

Values are means ± S.E.; n = number of determinations. Conditions are given in Materials and Methods; SR, sarcoplasmic reticulum; CaM, calmodulin.

	n	Ca^{2+} released (nmol/mg SR)	$t_{1/2}$ (s)
Calcium-induced release			
Control	6	25.8 ± 1.0	92 ± 11
CaM (1 μM)	6	27.4 ± 1.7	189 ± 32
Ruthenium red (20 μM)	6	19.9 ± 1.3	332 ± 61
EGTA	4	17.5 ± 1.6	266 ± 53

The total average calcium load obtained in four different sarcoplasmic reticulum preparations by preloading with 4 mM $^{45}CaCl_2$ for 24 h or 48 h on ice, measured by filtration technique, was 32.2 ± 0.56 nmol/mg and 33.5 ± 1.64 nmol/mg sarcoplasmic reticulum, respectively (mean ± S.E.) and about 85% of calcium load was released. The release was monophasic and the rate of calcium-induced calcium release was maximal at 10–20 μM Ca^{2+}. The apparent rate of release in the presence of ruthenium red was almost identical to the rate obtained in the absence of calcium, indicating a complete block of the calcium-induced calcium release (Table IV).

Inhibition of the rate of calcium-induced calcium release by 1 μM calmodulin from sarcoplasmic reticulum vesicles passively preloaded with $^{45}CaCl_2$, measured in the presence of 0.5 mM $MgCl_2$ and either 20 μM total $^{45}CaCl_2$ (Fig. 6) or 0.5 mM $^{45}CaCl_2$ plus 0.5 mM EGTA (free Ca^{2+} 10–20 μM) was similar to that observed spectrophotometrically, i.e. the mean apparent half-time increased about 2-fold (Table IV).

Discussion

Calcium release through the calcium-gated channel of heavy sarcoplasmic reticulum is activated by caffeine [6,11,14,15,40–42] and adenine nucleotides [12,15–18,22,43] and inhibited

by magnesium [11,16,18,41–43], ruthenium red [10,11,22,42] and local anaesthetics such as procaine or tetracaine [11,14,41]. Inhibition by calmodulin of the calcium-induced calcium release and calcium release by the ATP analog p[CH_2]ppA has been reported quite recently by Meissner [22].

In the present investigation the effect of calmodulin on the calcium release channel was studied using calcium-, AMP- and caffeine-induced release from actively and passively loaded heavy sarcoplasmic reticulum vesicles. AMP, as adenine nucleotide, is an effector of the release channel [12,17] and caffeine mimics the calcium-induced release by increasing the apparent calcium affinity on the receptor site of the calcium channel [43].

Inhibition of all three types of calcium release by calmodulin was obtained with actively and passively calcium loaded sarcoplasmic reticulum, but evidence for inhibition of calcium release from actively loaded sarcoplasmic reticulum is indirect. Since the rate of calcium uptake was neither activated by calmodulin nor calmodulin-dependent phosphorylation, when the release channel was blocked by ruthenium red, strongly indicates that indeed the calcium release was affected by calmodulin. An inhibition of the apparent rate of the caffeine-induced clacium release could be demonstrated.

The data on calcium release from passively loaded sarcoplasmic reticulum vesicles are more straight forward, because in the absence of ATP there is no interference by re-uptake of released calcium by the calcium pump or by calmodulin-dependent phosphorylation of the calcium channel and/or a regulatory site of the channel. The results demonstrate that the calcium-calmodulin complex itself affects the calcium release channel, resulting in an about 2-fold reduction in the rate of calcium release measured spectrophotometrically or by Millipore filtration with $^{45}CaCl_2$. A complete blockade of calcium release, as in the case of ruthenium red, was not achieved even at high calmodulin concentrations.

No effect of calmodulin-dependent phosphorylation of sarcoplasmic reticulum proteins by the calmodulin-dependent protein kinase of skeletal muscle sarcoplasmic reticulum [23,24] was observed on the rate of calcium release. Phosphorylation of heavy sarcoplasmic reticulum fractions with [^{35}S]ATP[S] following passive loading of sarcoplasmic reticulum vesicles, resulted in calcium-, calmodulin-dependent phosphorylation of a 57 kDa protein of the sarcoplasmic reticulum, as evidenced by autoradiography of sarcoplasmic reticulum SDS gels, but did not increase the degree of inhibition of the rate of calcium release beyond that obtained with calmodulin in the absence of ATP[S]. Since ATP[S] is not a substrate for the calcium transport ATPase, it appears a very suitable tool to obtain stable phosphorylation for release experiments without influencing the calcium pump. The above results do not support an influence of calmodulin-dependent phosphorylation on the calcium release channel of skeletal muscle sarcoplasmic reticulum. On the other hand, it cannot be ruled out that a regulatory unit of the calcium release channel, modifies calcium release via a calmodulin-dependent phosphorylation process, was removed during preparation of the sarcoplasmic reticulum.

On the basis of the incomplete inhibition of calcium release a predominantly regulatory role of calmodulin on the calcium channel of sarcoplasmic reticulum was postulated by Meissner [22] which is supported by the present data. Our results contrast to the findings of Palade [44], of a negligible effect of calmodulin on thymol-induced calcium release (about 10% inhibition of release at 0.6 μM calmodulin) and even less in the case of the caffeine-induced calcium release, which were interpreted as indicating that calmodulin does not have a regulatory function on the sarcoplasmic reticulum calcium release channel.

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