CALCIUM-INDUCED CALCIUM RELEASE AT TERMINAL CISTERNAE OF SKELETAL SARCOPLASMIC RETICULUM

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1. Introduction

The mechanism by which Ca^{2+} induces muscular contraction is now quite well understood [1–3]. The release mechanism from sarcoplasmic reticulum has remained unsolved [4–6]. Studies with skinned fibers and isolated sarcoplasmic reticulum vesicles have yielded two main hypotheses:

(i) The small amount of Ca^{2+} that crosses the sarcolemma during nerve stimulation releases Ca^{2+} from sarcoplasmic reticulum [7–9];

(ii) The action potential transmitted from the transverse tubules to the sarcoplasmic reticulum 'depolarizes' the membrane and permits efflux of Ca^{2+} [10–12].

Many observations still remain unexplained, including differences observed between cardiac and skeletal muscle [5].

Studies with isolated sarcoplasmic reticulum [9,13,14] have revealed a phenomenon of Ca^{2+}-induced Ca^{2+} release, but the parameters and kinetics of the process led to the conclusion supporting Endo's view [4] that Ca^{2+}-triggered Ca^{2+} release seems to play a role only in certain pharmacologically induced or pathological circumstances such as in the presence of caffeine or in malignant hyperthermia [14].

Here we present data on Ca^{2+}-induced Ca^{2+} release by terminal cisternae of sarcoplasmic reticulum at physiological $[Mg^{2+}]$. We propose that this process may serve as a trigger for a secondary membrane potential-dependent Ca^{2+} release from sarcoplasmic reticulum.

2. Materials and methods

2.1. *Fractionation of sarcoplasmic reticulum vesicles*

Sarcoplasmic reticulum vesicles were isolated from rabbit dorsal and hind leg muscles according to [15] with some essential modifications. Mature New Zealand white rabbits weighing at least 2 kg were decapitated, the muscles placed on ice and dissected. Muscles (50 g) were homogenized with 450 ml 0.25 M sucrose in a Waring blender for 60 s in 2 intervals of 30 s with 5 s between them. Enzyme grade sucrose filtered through 0.45 μm Millipore filter (HAWP 047) was used. Several batches of homogenates were combined and centrifuged at 9000 × *g* for 20 min. The supernatant was passed through 8 layers of cheese cloth and centrifuged in a Beckman type 19 rotor at 36 000 × *g* (av) for exactly 60 min. The pellet was suspended in 0.25 M sucrose and placed on top of a linear sucrose gradient between 0.85–1.6 M and centrifuged at 25 000 rev./min for 16 h in a Beckman SW 27 rotor. The light, white fraction between 0.9–1.05 M (LSR), the intermediate slightly yellowish fractions between 1.05–1.2 M (ISR), and the heavy, yellowish fractions between 1.2–1.4 M (HSR) were collected. The fractions above 0.85 M sucrose and the pellet at the bottom of the tube had low ATP-dependent Ca^{2+} uptake activity and were discarded. The fractions were diluted with an equal volume of 0.1 M KCl, 5 mM Tris- maleate (pH 6.5) and centrifuged at 130 000 × *g* for 60 min. The pellets were suspended in the same buffer at ~20 mg protein/ml and stored at 0°C.

2.2. *Calcium loading and release assay*

All fractions were equilibrated at 2 mg/ml prior to assay for 2 h at 0°C with 0.1 M K-glutamate (pH 6.9).

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Aliquots of 20 μl were loaded at 23°C with $^{45}Ca^{2+}$ by addition of 200 μl solution containing 0.1 M K-glutamate (pH 6.9), 2 mM ATP, 3 mM $MgSO_4$, and 10 μM $^{45}CaCl_2$. At 2 min 55 s, when almost all Ca^{2+} in the medium was taken up by sarcoplasmic reticulum vesicles, caffeine or $CaCl_2$ was added at the indicated concentrations. At 3 min 2 s an aliquot was passed through a Millipore filter (0.45 μm HAWP 025) and washed with 2 ml 0.1 M K-glutamate (pH 6.9). The Millipore filters were dried under an infrared lamp and counted in Liquiscint (National Diagnostics) in a Beckman scintillation counter. The standard error of the Ca^{2+} release measurements was <10%.

3. Results and discussion

3.1. *Caffeine-induced Ca^{2+} release is operative in terminal cisternae*

The release of Ca^{2+} induced by caffeine is most pronounced in the heavy fraction of sarcoplasmic reticulum (fig.1). These experiments were performed in the presence of 1.25 mM free Mg^{2+} and at low Ca^{2+} loading levels (~1/4 of maximum loading capacity in the absence of precipitating anions). It is known from skinned fiber experiments that calcium-induced Ca^{2+} release is dependent on the extent of filling of the sarcoplasmic reticulum with Ca^{2+} and also on the free [Mg^{2+}] in the medium. The threshold for Ca^{2+}-induced Ca^{2+} release should therefore be determined with the sarcoplasmic reticulum loaded to the physiological level and in the presence of physiological levels of free Mg^{2+} [4]. The heavy fraction of sarcoplasmic reticulum is mainly derived from terminal cisternae [15,16] which makes the junction with T-tubules. Although caffeine-induced Ca^{2+} release was also observed with other fractions of sarcoplasmic reticulum, much higher concentrations of caffeine were required. Even 10 mM caffeine did not give maximal responses whereas with HSR 0.5 mM caffeine was almost optimal. The caffeine-induced Ca^{2+} was only partially inhibited at 5 mM free Mg^{2+}.

3.2. *Ruthenium red inhibits caffeine-induced Ca^{2+} release from HSR*

Ruthenium red is known to inhibit Ca^{2+} uptake in mitochondria and at 20 μM also inhibited Ca^{2+} release induced by Ca^{2+} or caffeine in sarcoplasmic reticulum vesicles [14]. With HSR as little as 0.5 μM ruthenium red inhibited >50% of the Ca^{2+} release induced by 0.5 mM caffeine (fig.2). In the absence of caffeine, ruthenium red stimulated Ca^{2+} uptake of HSR prob-

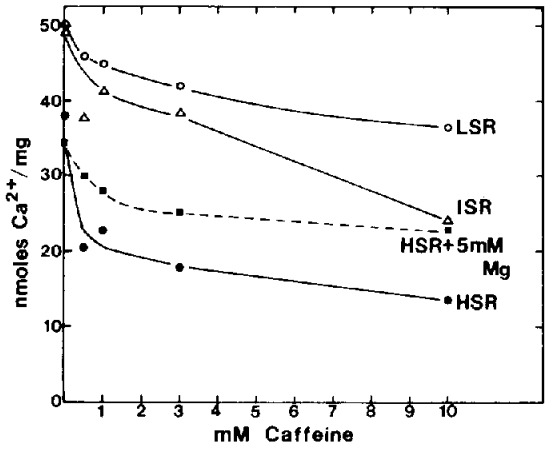

Fig.1. Caffeine-induced Ca^{2+} release from sarcoplasmic reticulum fractions. SR fractions were loaded with $^{45}Ca^{2+}$ as in section 2. At 2 min 55 s, 1–20 μl 100 mM caffeine was added to the reaction mixture and at 3 min 2 s, a 150 μl aliquot was passed through a Millipore filter and counted in section 2. As a control a 150 μl aliquot of the reaction mixture without caffeine was passed at 3 min through the filter: (○) LSR; (△) ISR; (●) HSR; (■) HSR + 5 mM free Mg^{2+}.

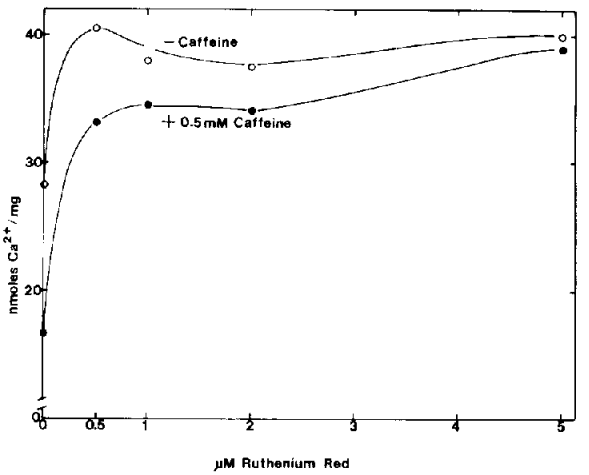

Fig.2. Inhibition of caffeine-induced Ca^{2+} release from heavy fraction of sarcoplasmic reticulum by ruthenium red. HSR was loaded with $^{45}Ca^{2+}$ as in section 2 in the absence and presence of indicated amounts of ruthenium red. At 2 min 55 s, 1 μl 100 mM caffeine was added to the reaction mixture as indicated. Assays were done as in section 2: (○) –caffeine; (●) +0.5 mM caffeine.

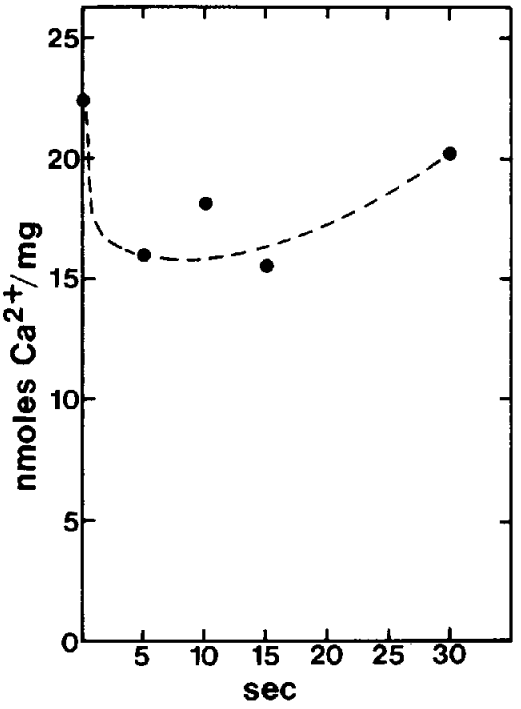

Fig.3. Time course of Ca^{2+} release induced by caffeine. HSR was loaded with $^{45}Ca^{2+}$ as in section 2. At 3 min, 1 μl 100 mM caffeine was added to the reaction mixture and at the indicated times 150 μl aliquots were passed through Millipore filter and assayed. As a control the same amount of reaction mixture was passed through the filter at 3 min without adding caffeine.

ably because of inhibition of Ca^{2+}-induced release, as described below.

3.3. *Time course of Ca^{2+} release induced by caffeine*

The time course of Ca^{2+} release upon addition of 0.5 mM caffeine was too fast to detect by the conventional filtration method. At 5 s, Ca^{2+} release was complete and at 30 s reuptake into HSR was observed (fig.3). It is likely that the Ca^{2+} pump was operative during Ca^{2+} release [17].

3.4. *Calcium-induced Ca^{2+} release at high [Mg^{2+}]*

Since the effect of caffeine on Ca^{2+} release from sarcoplasmic reticulum of muscle or skinned fibers is attributed to the stimulation of Ca^{2+}-induced Ca^{2+} release, the above results suggest that the Ca^{2+}-induced Ca^{2+} release is operative only in HSR and that it is inhibited by very low concentrations of ruthenium red. Calcium release from HSR was induced by $CaCl_2$ at <10 μM after loading calcium to a physiological (~25–30% of maximal) level (fig.4A). Since ATP-dependent Ca^{2+}/Ca^{2+} exchange of the Ca^{2+} pump may be operative and is not expected to be inhibited by ruthenium red, only ruthenium red-sensitive Ca^{2+} effluxes were considered to be Ca^{2+}-induced Ca^{2+} release. Even with LSR significant amounts of Ca^{2+} were released by addition of Ca^{2+} (fig.4B) but in contrast to the observation with HSR (fig.4A) this process was not inhibited by ruthenium red. The ruthenium red-insensitive release of $^{45}Ca^{2+}$ may well be due to an exchange via the ATP-driven pump which is known to be present in large amounts in LSR. It

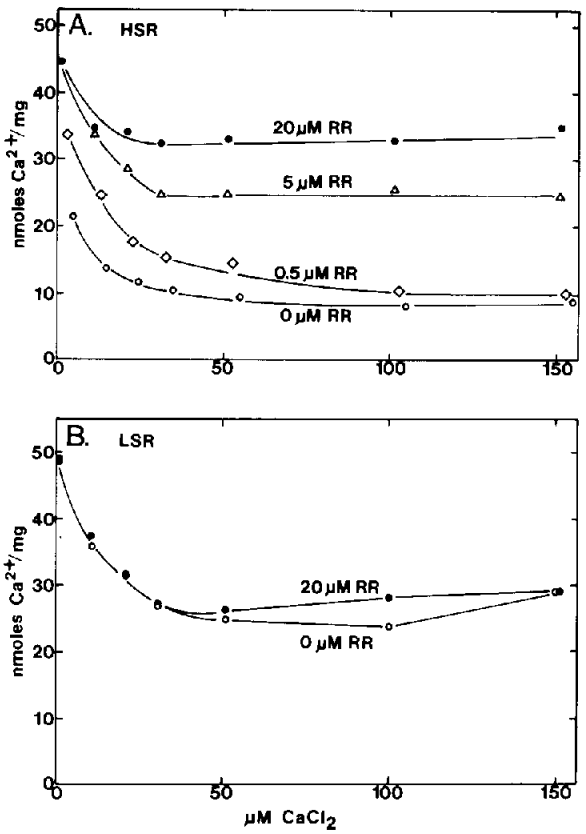

Fig.4. Ca^{2+}-induced Ca^{2+} release and its inhibition by ruthenium red. HSR (A) and LSR (B) were loaded with $^{45}Ca^{2+}$ as in section 2 in the absence (○) or presence of 0.5 (◇), 5 (△), 20 (●) μM ruthenium red. At 2 min 55 s when almost all the Ca^{2+} in the medium is taken up by the sarcoplasmic reticulum vesicles, 4 μl 0.5–10 mM $CaCl_2$ was added to the reaction mixture and at 3 min 2 s a 150 μl aliquot was passed through a Millipore filter and assayed as described. As a control the same amount of reaction mixture was passed at 3 min through the filter without adding $CaCl_2$: RR, ruthenium red.

should be emphasized that these experiments were done with free Mg^{2+} at 1.25 mM and at suboptimal levels of Ca^{2+} loading in order to counter the valid objections in [4]:

> 'A very high concentration of free calcium, higher than 3×10^{-4} M, is necessary to induce a net release of calcium by calcium under physiological conditions. It seems very unlikely that such a high concentration is attained even in the narrow space between the T-system and the sarcoplasmic reticulum by the calcium coming only from the T-system membrane.'

These data show a much greater sensitivity to Ca^{2+} in HSR than observed with skinned fibers. This allows us to reconsider Ca^{2+} as a transmitter in the excitation from the T-tubules to the terminal cisternae of sarcoplasmic reticulum.

One critical question is whether this Ca^{2+}-induced calcium release can supply sufficient Ca^{2+} required for contraction. Because of the fact that HSR isolated by this method represents only 9% of the total sarcoplasmic reticulum membrane, the amount of Ca^{2+} released is small compared to the total Ca^{2+} in sarcoplasmic reticulum. It seems likely that in vivo the organized structure of the sarcoplasmic reticulum can release more Ca^{2+} than observed with fragmented HSR. We would like to propose that the Ca^{2+}-induced Ca^{2+} release may give rise to a membrane potential change (inside negative) [18] which is propagated to the entire sarcoplasmic reticulum opening Ca^{2+} channels which supply sufficient Ca^{2+} for muscular contraction. Many reports on skinned fiber and fragmented sarcoplasmic reticulum suggest a mechanism of Cl^- or 'depolarization' induced Ca^{2+} release from sarcoplasmic reticulum of skeletal muscle [10–12,19–23]. This kind of Ca^{2+} release can be explained well by the proposed mechanism. Preliminary experiments showed that both caffeine- and Ca^{2+}-induced Ca^{2+} release from HSR were partially inhibited by dissipating the membrane potential with valinomycin and K^+. These findings suggest that the membrane potential-dependent Ca^{2+} release is operative in the HSR during the observed Ca^{2+}-induced Ca^{2+} release and are consistent with the above hypothesis.

Acknowledgement

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