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Intra- and extraluminal sarcoplasmic reticulum membrane regulatory sites for Ca^{2+}-induced Ca^{2+} release

Thomas E. Nelson and Kenneth E. Nelson

Department of Anesthesiology, University of Texas Health Science Center, 6431 Fannin, MSB 5.020, Houston, TX 77030, USA

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A heavy skeletal muscle sarcoplasmic reticulum (SR) fraction was actively loaded stepwise with calcium until Ca^{2+}-induced Ca^{2+} release occurred. The total Ca^{2+} load, T1, at which release occurred is postulated to be regulated by an intraluminal, low-affinity receptor. After obtaining T1, the critical concentration of Ca^{2+} required extraluminally (T2) was determined. T1 averaged 58.6 ± S.D., 6.9 nmol Ca^{2+}/mg SR and T2 averaged 2.14 ± S.D., 0.24 μM. Both T1 and T2 were increased by Mg^{2+} and decreased by caffeine. Ruthenium red increased T2 more than T1 while ryanodine had no effect on T1 but markedly increased T2. The results suggest that two Ca^{2+} regulatory sites may be functional for Ca^{2+}-induced Ca^{2+} release from SR.

Sarcoplasmic reticulum; Ca^{2+} regulation; Ca^{2+} release; Skeletal muscle; Ca^{2+} channel

1. INTRODUCTION

The functional role of Ca^{2+}-induced Ca^{2+} release in physiological excitation-contraction (E-C) coupling is controversial [1–3]. As recently discussed by Ikemoto et al. [4], the drug-induced contracture sensitivity of intact skeletal muscle is retained in certain elements of isolated sarcoplasmic reticulum (SR) membrane vesicles. It has been suggested that the Ca^{2+} release channel has 2 regulatory sites, one cytoplasmic and the other intraluminal [4]. In the present study, we utilize a Ca^{2+}-induced Ca^{2+} release model of actively Ca^{2+}-loaded SR vesicles to provide new evidence for intra- and extraluminal regulatory sites for the Ca^{2+} release channel.

2. MATERIALS AND METHODS

Heavy SR vesicles (8–12 000 × *g* fraction) were prepared from biopsied pig longissmus dorsi muscle. The protocol for pig muscle biopsies was approved by our Institutional Animal Welfare Committee. Ca^{2+}-induced Ca^{2+} release was measured spectrophotometrically using arsenazo III as a Ca^{2+} dye indicator. The heavy SR was added to a concentration of 1 mg/ml in a 30°C thermostated cuvette containing 1 mM MgATP, 20 mM histidine (pH 6.8), 150 mM KCl, 5 mM NaN_3, 16 μM arsenazo III, 10 mM phosphocreatine, 5 μg/ml creatinephosphokinase; and the final cuvette volume was 1.0 ml. Two different Ca^{2+} thresholds were determined for Ca^{2+}-induced Ca^{2+} release. The first threshold, T1 was determined by serial additions of 10 nmol Ca^{2+}. These additions of 10 nmol Ca^{2+} were repeated at 30 s intervals until Ca^{2+} release from the SR was observed (Fig. 1). The total amount of Ca^{2+} added to the cuvette when Ca^{2+} release first occurred is reported as T1, nmol Ca^{2+}/mg protein. In Fig. 1, 7 additions of 10 nmol Ca^{2+} each were made until Ca^{2+} release occurred. Thus, this T1 threshold is 70 nmol Ca^{2+}/mg SR protein.

Correspondence address: T.E. Nelson, Department of Anesthesiology, University of Texas Health Science Center, 6431 Fannin, MSB 5.020, Houston, TX 77030, USA

Following this, one more addition of 10 nmol Ca^{2+} was made to confirm repeatability of Ca^{2+}-induced Ca^{2+} release. A value for T2 was then determined by adding increasing concentrations of Ca^{2+} from 0.5 nmol, in 0.5 nmol increments until Ca^{2+} release occurred, and as illustrated in the example of Fig. 1, the T2 value is 2.0 μM. The effects of Mg^{2+}, caffeine, Ruthenium red and ryanodine on the values for T1 and T2 were determined by a 5 min preincubation with varying concentrations of each agent. Each variable was measured in duplicate on SR isolated from 3 different pigs. Analysis of variance and Newman-Keuls tests were used for statistical evaluation of the data.

3. RESULTS

The total intraluminal Ca^{2+} accumulated, T1, for Ca^{2+}-induced Ca^{2+} release averaged 58.6 ± 6.9 SD nmol Ca^{2+}/mg SR and average values were 50, 60 and 63 nmol Ca^{2+}/mg SR for the individual pig samples. After sufficient Ca^{2+} was accumulated for T1, the critical extraluminal Ca^{2+} concentration required to promote Ca^{2+} release, T2, averaged 2.14 ± 0.24 SD μM and average values were 2.0, 2.17 and 2.25 μM for the individual pig samples. After T1 and T2 were established for a given SR sample, repeated additions of T2 would result in reasonably consistent amounts of Ca^{2+} release, i.e. T2 did not change as more and more Ca^{2+} was actively loaded into the SR.

Increasing the amount of magnesium added to the SR from 0.25 to 1.5 mM resulted in increased amounts of Ca^{2+} required for Ca^{2+}-induced Ca^{2+} release. As a percent of control values, i.e. no additional Mg^{2+}, there was a proportionate increase in both T1 and T2 up to 160% and 175%, respectively. Except for T2 at 0.25 and 0.5 mM added magnesium (Fig. 2), the effect was to significantly ($P<0.05$) increase the threshold Ca^{2+} values for intra- and extraluminal Ca^{2+} necessary to open the Ca^{2+} channel. There was no

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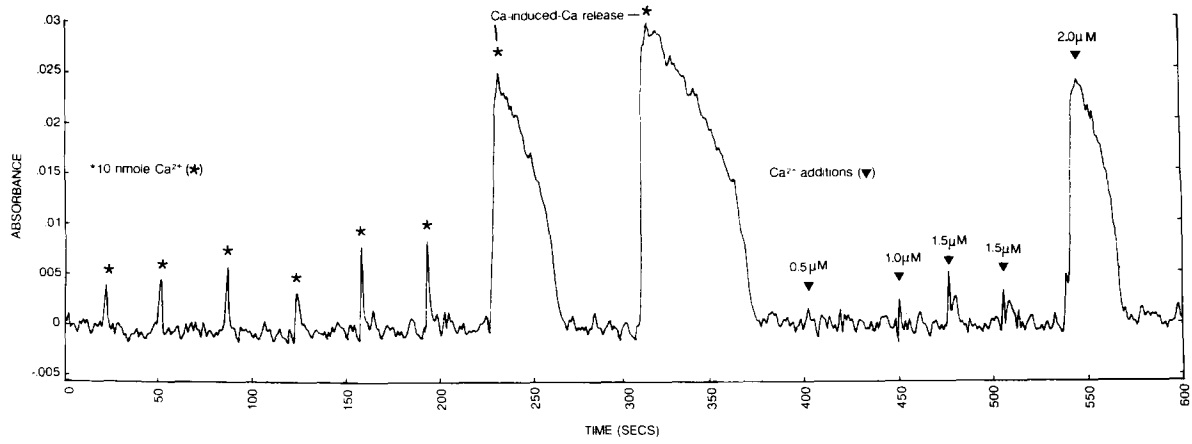

Fig. 1. Ca^{2+}-induced Ca^{2+} release thresholds. Eight additions of 10 nmol Ca^{2+} (*) resulted in Ca^{2+} release on the 7th and 8th additions. Threshold 1 (T1) is defined as $7 \times 10 = 70$ nmol Ca^{2+} per mg SR. Following this, increasing concentrations of Ca^{2+} were added (▼) to determine Threshold 2 (T2), the concentration of Ca^{2+} that produced Ca^{2+} channel opening. In this example, T2 = 2.0 μM. A 2nd addition of 1.5 μM Ca^{2+} was to show that T2 does not change within this time-span or Ca^{2+} load. See section 2 for experimental details.

statistically significant difference for the magnesium effect between T1 and T2. Caffeine from 0.1 to 1.0 mM lowered both T1 and T2 similarly, with 1.0 mM caffeine reducing each to about 60% of the control values (Fig. 2). Ryanodine had no effect on the intraluminal Ca^{2+} concentration required for Ca^{2+} release while it significantly ($P<0.01$) increased T2, the extraluminal Ca^{2+} level required (Fig. 3). At a ryanodine concentration of 0.3 μM, the extraluminal Ca^{2+} concentration required for Ca^{2+} release was approximately double that of the control value. Ruthenium red in concentrations ranging from 20 to 200 nM effectively increased both T1 and T2 but the effect on T2 was greater than that for T1 (Fig. 3). Increasing the concentration of SR protein in the cuvette from 0.5 to 1.5 mg/ml produced increases in T1 but was without effect on T2 (Fig. 4).

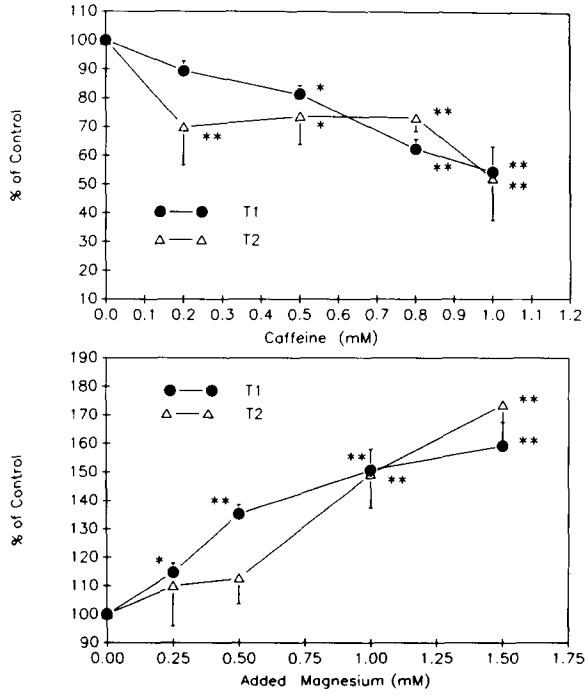

Fig. 2. Effects of caffeine and magnesium on T1 and T2 for Ca^{2+}-induced Ca^{2+} release. (Upper panel) Increasing caffeine from 0.1 to 1.0 mM reduced T1 and T2 to the same degree. (Lower panel) Increasing amount of Mg added from 0.25 to 1.5 mM increased T1 and T2 to the same extent. Asterisks indicate values significantly (* = $P<0.05$, ** = $P<0.01$) different from controls.

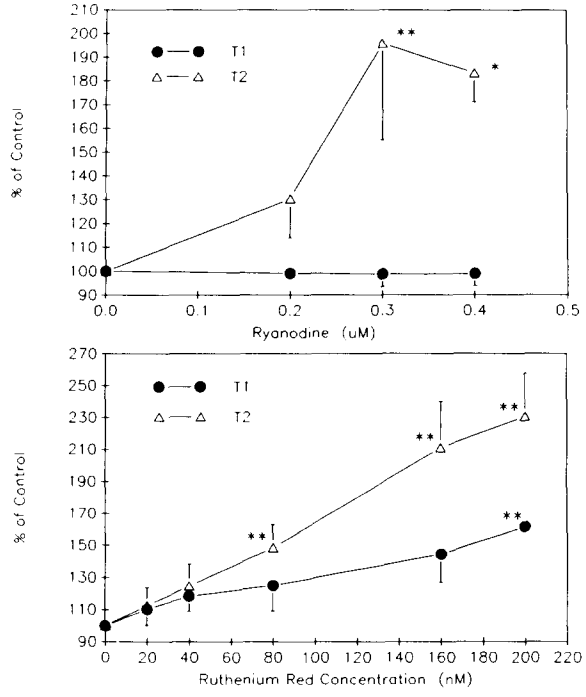

Fig. 3. Effects of ryanodine and Ruthenium red on T1 and T2 for Ca^{2+}-induced Ca^{2+} release. (Upper panel) Ryanodine (0.1–0.4 μM) increased T2 required for Ca^{2+} channel opening but had no effect on T1. (Lower panel) Ruthenium red (20–200 nM) increased T1 and T2 but the effect on T2 was greater. Asterisks indicate significant (*$P<0.05$, **$P<0.01$) difference from controls.

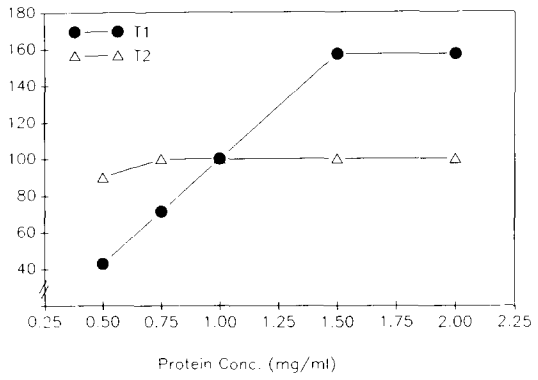

Fig. 4. Effect of SR protein concentration on T1 and T2. Increasing SR concentration from 0.5 to 2.0 mg/ml resulted in increase in T1 and no effect on T2.

4. DISCUSSION

The mechanism of E-C coupling in skeletal muscle remains a well-pursued mystery. Although our data provide little insight, it does provide new information supporting the existence of two calcium receptors involved in regulation of a Ca^{2+} channel in skeletal muscle SR. The experimental SR model used in this study in itself identifies two regulatory sites. First, the Ca^{2+}-induced Ca^{2+} release mechanism cannot occur until a critical load of Ca^{2+} is obtained within the SR, an observation previously noted by Endo [1,5]. One could assume that all Ca^{2+} binding sites, i.e. calsequestrin, etc., inside the SR must be saturated until an intraluminal pool of unbound Ca^{2+} accumulates and becomes available for release. It might be expected that this intraluminal pool of Ca^{2+} could vary and that amount of Ca^{2+} release would vary with the size of the pool available for release [4]. In the SR model used in this study that was not the case, because after obtaining the critical Ca^{2+} load for T1, serial additions of Ca^{2+} to produce Ca^{2+}-induced Ca^{2+} release did not alter the amount of Ca^{2+} released. As we previously reported [6], this suggests that inactivation of the Ca^{2+} channel is influenced by the extraluminal Ca^{2+} concentration and holds the amount of Ca^{2+} released constant, regardless of the Ca^{2+} preload. The fact that increasing SR protein produced an increase in T1 but not in T2 also supports the existence of two different sites.

Caffeine and magnesium had opposing effects on T1 and T2, caffeine lowered both while magnesium increased both. It is possible that either of these agents could be affecting only one of the putative receptors and in so doing alter indirectly the function of the other receptor but our data cannot distinguish such effects. In contrast, Ruthenium red and ryanodine effects appear to be more specific for the T2 receptor site. Ruthenium red sensitivity has been used as a marker for the Ca^{2+}-induced Ca^{2+} release channel [7] but its exact site of action is unknown. As a highly charged molecule, Ruthenium red access to SR intraluminal sites may be limited but an extraluminal target would be accessible. Ryanodine has been used as a specific marker for the ryanodine receptor protein, a Ca^{2+} channel that has been electrophysiologically investigated [8] and cloned for rabbit SR [9]. The fact that ryanodine only affected the extraluminal receptor, i.e. T2, is further support that 2 receptors may exist. We have previously demonstrated that ryanodine blocks rate and amount of Ca^{2+}-induced Ca^{2+} release [10]. It was also shown that increasing the concentration of trigger Ca^{2+}, i.e. T2 in the present study, reduced the inhibitory effects of ryanodine on Ca^{2+} release [10]. The fact that ryanodine increased the amount of T2 in this study is consistent with our previous findings and suggests that Ca^{2+} and ryanodine are acting on a common regulatory site. Based on our findings, we postulate the existence of two Ca^{2+} receptors for the regulation of Ca^{2+}-induced Ca^{2+} release from skeletal muscle SR. One receptor, T1 is intraluminal and because the Ca^{2+} concentration here is higher than in the cytoplasm, low affinity binding is predicted. The other receptor, T2, is extraluminal and high affinity in binding properties, requiring only micromolar concentrations of Ca^{2+} for activation. These putative receptors could represent two regulators for a single Ca^{2+} channel and a model constructed for the ryanodine receptor Ca^{2+} channel has predicted cytoplasmic binding sites for modulators of the Ca^{2+} release channel [9]. Alternatively, each of the putative receptors could be regulating two different Ca^{2+} release pathways in the SR membrane.

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