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Aging Impairs Regulation of Ryanodine Receptors from EDL but not Soleus Muscles

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Abstract

Introduction—Because impaired excitation-contraction coupling and reduced sarcoplasmic reticulum Ca^{2+} release may contribute to the age-associated decline in skeletal muscle strength, we investigated the effect of aging on RyR1 regulation by physiological channel ligands.

Methods— $[^3\text{H}]$ Ryanodine binding to membranes from 8 and 26 month old Fischer 344 extensor digitorum longus (EDL) and soleus muscles was used to investigate the effects of age on RyR1 modulation by Ca^{2+} and calmodulin (CaM).

Results—Aging reduced maximal Ca^{2+} -stimulated binding to EDL membranes. In $0.3 \mu\text{M}$ Ca^{2+} , age reduced and CaM increased binding to EDL membranes. In $300 \mu\text{M}$ Ca^{2+} , CaM reduced binding but the age effect was not significant. Aging did not affect Ca^{2+} or CaM regulation of soleus RyR1.

Discussion—In aged fast-twitch muscle, impaired RyR1 Ca^{2+} regulation may contribute to lower SR Ca^{2+} release and reduced muscle function.

Keywords

Excitation-contraction coupling; aging; ryanodine receptor; sarcoplasmic reticulum; calmodulin; calcium; skeletal muscle

Introduction

Aging is associated with a decrease in skeletal muscle mass and contractile force, particularly in type II fibers ^{1, 2}. Although the loss of mass contributes to decreased force production, incomplete contractile activation¹, possibly due to excitation-contraction coupling (ECC) dysfunction, might contribute.

ECC begins with the spread of the action potential over the sarcolemma and into the depths of the transverse-tubules. Depolarization of the transverse-tubules induces a conformational change in the dihydropyridine receptors which opens physically coupled sarcoplasmic

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reticulum (SR) Ca^{2+} release channels/ryanodine receptors (RyR1) and initiates SR Ca^{2+} efflux. The reduced SR Ca^{2+} release in aged muscle in response to either physiological³ or pharmacological^{4,5} triggers suggests aged RyRs respond inadequately to regulatory signals.

In vitro, RyR1 exhibits a biphasic Ca^{2+} dependence, opening in submicromolar Ca^{2+} and closing in micromolar Ca^{2+} . Numerous endogenous effectors modulate RyR1 Ca^{2+} dependence⁶. Here we focus on the Ca^{2+} -binding protein calmodulin (CaM). In submicromolar Ca^{2+} (near resting intracellular Ca^{2+}), Ca^{2+} -free CaM (apo-CaM) enhances RyR1 opening. In micromolar Ca^{2+} , as occurs in the vicinity of RyR1 during Ca^{2+} release, CaM binds Ca^{2+} and promotes channel closing⁷. Here we asked whether aging alters RyR response to Ca^{2+} and CaM.

Materials and Methods

Animals

Eight month (young; survival rate >90%⁸) and 26 month (old; survival rate ~25%⁸) old male Fischer 344 rats were obtained from the National Institute on Aging aged rodent colony maintained by Harlan Sprague Dawley, Inc.

Isolation of muscle membranes

Animals were euthanized by CO_2 inhalation in accordance with the Public Health Services Policy on Humane Care and Use of Laboratory Animals and with the approval of the Institutional Care and Use Committee of the Georgia Institute of Technology. *Soleus* (82% type I, 18% type II MHC⁹) and *extensor digitorum longus* (EDL; 4% type I, 96% type II MHC⁹) muscles of 6 animals were pooled for each membrane preparation. In three preparations, an animal was omitted due to tumor or overt disease. Muscles were homogenized in ice-cold 100 mM NaCl, 5 mM Tris pH 6.8 with protease inhibitors and centrifuged at 2,600 g for 30 min at 4° C. Filtered supernatant was centrifuged for at 15,000 g for 30 min at 4° C. Pellets were suspended 300 mM sucrose, 100 mM KCl, 5 mM Tris pH 6.8 and stored at -80° C.

[³H]Ryanodine binding

Ryanodine binds open RyRs with high affinity and specificity, providing an indirect indicator of channel activity¹⁰. The Ca^{2+} , apo-CaM and Ca^{2+} -CaM dependence of [³H]ryanodine binding was performed as previously described¹¹ at pH 7.0 and the free Ca^{2+} concentration indicated in the figures¹². Each assay run consisted of a randomly chosen, unblinded young sample paired with similarly chosen old sample. Each sample was assayed in duplicate and averaged. The number of samples assayed is reported in the figure legends. Unequal numbers of observations were due to limiting amounts of sample.

Calmodulin expression and purification

Rat CaM cDNA in a pET-7 vector was expressed in *E. coli* and purified as previously described¹¹.

Analysis and statistics

Ca^{2+} dependence of ryanodine binding was fit with equation 1 that assumes a high affinity Ca^{2+} -binding activation site and a lower affinity Ca^{2+} -binding inhibition site.

$$\% \text{ Ryanodine Bound} = B_o \left(\frac{[\text{Ca}^{2+}]}{[\text{Ca}^{2+}] + \text{EC}_{50}} \right) \left(1 - \frac{[\text{Ca}^{2+}]}{[\text{Ca}^{2+}] + \text{IC}_{50}} \right) \quad \text{Equation 1}$$

where B_o is the maximal Ca^{2+} -activated binding, EC_{50} is the Ca^{2+} concentration required to achieve one-half B_o and IC_{50} is the Ca^{2+} concentration required to half-inhibit $[\text{H}^3]$ ryanodine binding. Data are presented as mean $\pm$ standard error. Binding was analyzed using repeated measures ANOVA or Student's t-test, as appropriate. Assumptions of normality and equality of variances were tested. If either was violated, comparisons were made using a Mann-Whitney Rank Sum Test. Effect size was calculated as partial eta squared (η^2). Curve fitting and statistical analysis utilized SigmaPlot v.13.0 and SPSS v.22. The level of significance was set *a priori* at $p < 0.05$.

Results

Ca^{2+} dependence of $[\text{H}^3]$ ryanodine binding

Ca^{2+} -dependence of $[\text{H}^3]$ ryanodine binding to membranes from young and old soleus and EDL muscles was compared (Fig 1). Repeated measures ANOVA was performed with within factor Ca^{2+} concentration and between factor AGE. For the EDL Ca^{2+} dependence (Fig 1A) Mauchley's test of sphericity was significant ($p < 0.001$), therefore a Huynh-Feldt correction was applied. There was a significant AGE- Ca^{2+} interaction ($p = 0.008$) and significant main effects for AGE ($p = 0.034$) and Ca^{2+} ($p < 0.001$). Partial η^2 for the main effects of AGE, Ca^{2+} and the AGE- Ca^{2+} interaction were 0.38, 0.94 and 0.34 respectively. Post hoc tests with Bonferroni correction indicate aging significantly reduced binding at 10, 30 and 100 μM Ca^{2+} . This is consistent with age significantly reducing B_o , maximal Ca^{2+} -activated $[\text{H}^3]$ ryanodine binding but not altering the EC_{50} or IC_{50} (Table 1).

Repeated measures ANOVA on the soleus Ca^{2+} dependence (Fig 1B) indicated a significant main effect of Ca^{2+} ($p < 0.001$) but no effect of AGE ($p = 0.695$). This is consistent with no significant difference in B_o , EC_{50} , and IC_{50} (Table 1).

CaM modulation of $[\text{H}^3]$ ryanodine binding

CaM regulation of $[\text{H}^3]$ ryanodine binding was examined in buffers containing either 0.3 μM or 300 μM Ca^{2+} (Fig 2 & Supplemental Table 1). A three-way repeated measures ANOVA was conducted for the EDL with between factor AGE and within factors Ca^{2+} and CaM. ANOVA revealed a significant Ca^{2+} -CaM interaction ($p < .001$). Therefore, Ca^{2+} conditions were analyzed separately using two-way ANOVAs with between factor AGE and within factor CaM. Two-way CaM-AGE interactions for both Ca^{2+} concentrations were non-significant. Both 0.3 and 300 μM Ca^{2+} showed a significant main effect for CaM ($p < 0.001$ and $p = 0.007$ respectively). Pairwise multiple comparisons with Bonferroni corrections

indicate in 0.3 μM Ca^{2+} , 100 nM CaM was significantly different from 0 and 30 nM CaM. AGE was significantly different for 0.3 μM Ca^{2+} ($p=0.030$) but not for 300 μM Ca^{2+} ($p=0.056$). Partial η^2 for AGE was 0.315 and 0.252 in 0.3 and 300 μM Ca^{2+} respectively.

Due to potential violations of normality and equality of variances in at least one group in the soleus data, a non-parametric approach was used. Mann-Whitney U revealed no significant effect of age on [^3H]ryanodine binding either in the absence or presence of CaM at either Ca^{2+} concentration.

Animal and muscle characteristics

Body and muscle weights are presented in Supplemental Table 2. EDL and soleus muscles of old animals weighed approximately 20 and 26% less, respectively, than their young counterparts. Elderly animals exhibited significant skeletal muscle atrophy and cardiac hypertrophy.

Discussion

Consistent with previous work, we found atrophy^{1, 2, 13} and altered RyR responsiveness¹⁴⁻¹⁶ in aged skeletal muscle. Although the atrophy was similar in EDL and soleus, only RyRs from aged EDL differed from their young counterparts. RyRs from these muscles had a reduced maximal Ca^{2+} activation that may contribute to depressed SR Ca^{2+} release in aged skeletal muscle.

Russ et al^{4, 5} reported reduced 4-chloro-m-cresol induced Ca^{2+} release from SR of aged fast but not slow rat muscle. Although lower SR Ca^{2+} load in aged muscle may contribute to reduced release, the current work indicates a role for altered RyR activation.

Damiani et al¹⁶ also examined regulation of RyRs from old animals. In contrast to the current work, they reported an increased Ca^{2+} sensitivity of activation but no change in the extent of activation. The disparate results may be due to different strains of rats (Wister), ages (~50% mortality) and muscles (tibialis anterior).

The role of RyR Ca^{2+} activation *in vivo* is unclear^{17, 18} but Ca^{2+} release has been suggested to induce additional RyR opening. However, drastically reducing RyR1 Ca^{2+} activation had little effect on depolarization induced Ca^{2+} release¹⁸. Alternatively, Ca^{2+} may contribute to the termination of release¹⁹. Despite the uncertain role of RyR Ca^{2+} activation *in vivo*, interventions that modify *in vitro* RyR Ca^{2+} activation generally have similar effects on voltage activation of skeletal muscle²⁰⁻²³. Thus, the decreased Ca^{2+} activation of aged EDL RyR1 may contribute to reduced depolarization-induced Ca^{2+} release in senescent muscle^{24, 25}.

In our CaM experiments, consistent with reduced Ca^{2+} activation, we found reduced ryanodine binding in aged EDL at low Ca^{2+} . Age did not affect EDL or soleus RyR1 regulation by CaM in either high or low Ca^{2+} . Although, we must be cautious when interpreting our negative results due to the low statistical power, we are in agreement with Damiani et al¹⁶ who also noted no effect of aging on RyR1 Ca^{2+} -CaM inhibition. They did not examine CaM activation at low Ca^{2+} . Although aged RyRs may respond normally to

CaM, CaM is subject to modification and oxidized CaM has been isolated from tissues of old animals ²⁶. We previously reported that CaM oxidation reduced the efficacy of RyR1 regulation regardless of Ca²⁺ concentration ¹¹.

These results are consistent with a greater impact of aging on fast-twitch muscles. In humans, muscle atrophy is attributed to a reduced fiber number and size, particularly of type II fibers ². At the single fiber level atrophy in the rat EDL is greater than in the soleus ²⁷. ECC also appears to be differentially affected, with the EDL exhibiting a greater increase in resting Ca²⁺ ²⁷, a greater decrease in RyR content ³ and impaired RyR function (Figs 1 & 2).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

Apo-CaM	calcium-free calmodulin
CaM	calmodulin
Ca²⁺-CaM	calcium-bound calmodulin
ECC	excitation contraction coupling
E. coli	Escherichia coli
EDL	extensor digitorum longus
RyR1	skeletal muscle isoform of the ryanodine receptor

SR sarcoplasmic reticulum

Tris tris(hydroxymethyl)aminomethane

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Figure 1. Aging decreased the extent of Ca^{2+} -activated [^3H]ryanodine binding to membranes derived from aged extensor digitorum longus (EDL) but not soleus muscles
 [^3H]Ryanodine binding to membranes derived from 8 (○) and 26 (●) month old EDL (A) and soleus (B) muscles. Symbols are the mean $\pm$ SEM of 6 EDL and 4 Soleus experiments performed in duplicate. Lines represent fits of equation 1. *Significantly different ($p < 0.05$) from 8 month old at the indicated Ca^{2+} concentration.

Figure 2. Effects of aging on apo-CaM activation and Ca^{2+} -CaM inhibition of [3H]ryanodine binding to membranes from extensor digitorum longus (EDL) and soleus muscles
 [3H]Ryanodine binding to membranes derived from 8 (open bars) and 26 (filled bars) month old EDL (A & B) and soleus (C & D) muscles was performed in assay buffers with 0.3 μM (A & C) or 300 μM (B & D) free Ca^{2+} and the indicated concentration of CaM. Bars are the mean $\pm$ SEM. Number of observations is given in parentheses. *100 nM CaM significantly different ($p < 0.05$) from No CaM and 30 nM.

Table 1Effects of Age on the Ca^{2+} Dependence of [^3H]Ryanodine Binding.

Muscle/Age (n)	B_o (%)	EC_{50} (μM)	IC_{50} (μM)
EDL 8 month (6)	55.6 $\pm$ 3.8	3.2 $\pm$ 0.8	259.0 $\pm$ 37.0
EDL 26 month (6)	36.9 $\pm$ 2.8	2.0 $\pm$ 0.1	241.9 $\pm$ 23.7
P value	0.0015	0.132 ^{<i>I</i>}	0.327
Partial η^2	0.604	0.148	0.015
Soleus 8 month (4)	71.5 $\pm$ 12.3	4.0 $\pm$ 0.5	237.0 $\pm$ 41.0
Soleus 26 month (4)	84.9 $\pm$ 10.7	6.2 $\pm$ 2.1	314.6 $\pm$ 245.1
P value	0.291	0.443	0.327
Partial η^2	0.102	0.153	0.015

^{*I*} Failed normality test, comparison made using Mann-Whitney Rank Sum test.

EDL = extensor digitorum longus