Azumolene Inhibits a Component of Store-operated Calcium Entry Coupled to the Skeletal Muscle Ryanodine Receptor

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Dantrolene reduces the elevated myoplasmic Ca\(^{2+}\) generated during malignant hyperthermia, a pharmacogenetic crisis triggered by volatile anesthetics. Although specific binding of dantrolene to the type 1 ryanodine receptor (RyR1), the Ca\(^{2+}\) release channel of skeletal muscle sarcoplasmic reticulum, has been demonstrated, there is little evidence for direct dantrolene inhibition of RyR1 channel function. Recent studies suggest store-operated Ca\(^{2+}\) entry (SOCE) contributes to skeletal muscle function, but the effect of dantrolene on this pathway has not been examined. Here we show that azumolene, an equipotent dantrolene analog, inhibits a component of SOCE coupled to activation of RyR1 by caffeine and ryanodine, whereas the SOCE component induced by thapsigargin is not affected. Our data suggest that azumolene distinguishes between two mechanisms of cellular signaling to SOCE in skeletal muscle, one that is coupled to and one independent from RyR1.

Malignant hyperthermia (MH) is a potentially fatal pharmacogenetic syndrome in which exposure to volatile anesthetics triggers uncontrolled elevation of myoplasmic Ca\(^{2+}\) concentrations (\([\text{Ca}^{2+}]_i\)), skeletal muscle hypercontracture, and hypermetabolism, resulting in a dramatic rise in body temperature (1, 2). Mutations in the type 1 ryanodine receptor (RyR1), the major Ca\(^{2+}\) release channel in skeletal muscle, are linked to MH susceptibility in pigs in an autosomal recessive manner (3–5). In humans, MH is transmitted as an autosomal dominant trait by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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2 The abbreviations used are: MH, malignant hyperthermia; BSS, balanced salt solution; \([\text{Ca}^{2+}]_i\), myoplasmic Ca\(^{2+}\) concentrations; \([\text{Ca}^{2+}]_o\), extracellular Ca\(^{2+}\) concentrations; CHO, Chinese hamster ovary; CR, caffeine and ryanodine; FCCP, 3,3’-dithiopropionimidate; [Ca\(^{2+}\)]\(_{ER}\), endoplasmic reticulum; FDB, flexor digitorum brevis; RyR1, type 1/skeletal muscle ryanodine receptor; SOC, store-operated Ca\(^{2+}\) channel; SOCE, store-operated Ca\(^{2+}\) entry; Sr, sarcoplasmic reticulum; t-tubule, transverse tubule; BES, 2-(bis(2-hydroxyethyl)amino)ethanesulfonic acid.

Both partial inhibition of Ca\(^{2+}\) release from isolated sarcoplasmic reticulum (SR) (11, 15), and partial suppression of the elemental Ca\(^{2+}\) spark signals in adult muscle fibers (16). However, functional studies have been unable to unequivocally demonstrate direct inhibition of the RyR1 Ca\(^{2+}\) channel activity by dantrolene (12, 17).

Sustained opening of RyR1 Ca\(^{2+}\) channels leads to reduction of the Ca\(^{2+}\) store within the SR lumen, a signal that activates store-operated Ca\(^{2+}\) entry (SOCE) in skeletal muscle (18, 19). Recent evidence from heterologous expression systems supports a role for the amino-terminal cytoplasmic foot structure of RyR1 in coupling to channels possibly involved in SOCE (20). Additionally, elevated Ca\(^{2+}\) entry through cell surface Ca\(^{2+}\) channels with pharmacology similar to SOCE has been linked to the elevation of \([\text{Ca}^{2+}]_i\), in muscular dystrophy (21). The contribution of SOCE to MH and the potential effect of dantrolene on SOCE have not been examined. In this study, we test the hypothesis that azumolene can influence SOCE function. Using Ca\(^{2+}\)-sensitive fluorescence measurements of RyR1-dependent intracellular Ca\(^{2+}\) transients and extracellular Ca\(^{2+}\) entry via SOCE, we show that azumolene inhibits SOCE both in skeletal muscle fibers and in cultured cells expressing RyR1. Our results reveal two modes of SOCE activation. One mode is RyR1-dependent and can be inhibited by the action of azumolene; the other is RyR1-independent and is insensitive to azumolene.

**EXPERIMENTAL PROCEDURES**

*Cell Culture—C1148 cells, a Chinese hamster ovary (CHO) cell line stably expressing RyR1, were maintained in Ham’s F-12...*
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medium supplemented with 10% fetal bovine serum, 1% penicillin and streptomycin, and 0.5 mg/ml G-418 (22). Culture of C2C12 myogenic cells was described previously (23). Myotubes derived from C2C12 were used in experiments at day 5 of differentiation.

Intracellular Ca\(^{2+}\) Measurement—C1148 cells were loaded with 10 \(\mu\)M Fura-2 AM (Invitrogen) for 45 min at 37 °C and allowed to de-esterify for 15 min at 25 °C. Cells were then harvested and resuspended in balanced salt solution (BSS) containing (in mM) the following: 140 NaCl, 2.8 KCl, 2 CaCl\(_2\), 2 MgCl\(_2\), 10 HEPES, pH 7.2. 2.5 \(\times\) 10\(^5\) cells were transferred into the cuvette system of a PTI spectrofluorometer (Photon Technology International, Princeton, NJ), and the changes in \([\text{Ca}^{2+}]_o\) were measured as changes in the ratio of Fura-2 fluorescence at excitation wavelength of 350 nm (\(F_{350}\)) and 380 nm (\(F_{380}\)), following exposure to various concentrations of caffeine and ryanodine (C/R). For measurement in 0.5 mM EGTA, cells were centrifuged and resuspended in BSS without CaCL\(_2\), and 0.5 mM EGTA was added immediately before recordings. Measurement of Ca\(^{2+}\) in individual C2C12 myotubes was performed as described before (18). All experiments were conducted at 25 ± 2 °C.

SOCE Assay: Mn\(^{2+}\) Quenching of Fura-2—Mn\(^{2+}\) is known to be able to permeate into cells via store-operated Ca\(^{2+}\) channels (SOC), but it is impervious to surface membrane extrusion processes or SR uptake by Ca\(^{2+}\) pumps. Hence, Mn\(^{2+}\) fluorescence quenching represents a measurement of unidirectional Ca\(^{2+}\) flux into cells via SOC (18, 19). Briefly, to measure the Mn\(^{2+}\) influx rate through the SOC machinery, thapsigargin (TG), or C/R, was applied to C1148 cells or C2C12 myotubes to induce SR Ca\(^{2+}\) depletion in 0 mM extracellular Ca\(^{2+}\) ([Ca\(^{2+}\)]\(_o\)). The decay of Fura-2 fluorescence upon Mn\(^{2+}\) addition was expressed as percent decrease in Fura-2 fluorescence per unit time (the initial fluorescence is set to be equal to 100%). For all measurements of SOCE by Mn\(^{2+}\) quenching, the maximally quenched fluorescence signal was established at the end of the experiment by lysing the cells with 1% Triton and was set equal to 0% fluorescence.

Dissociation of Individual Flexor Digitorum Brevis (FDB) Fibers and Measurement of SOCE—FDB fibers were enzymatically dissociated from 2- to 4-month-old C57Bl6/J male mice, following the procedure described in our previous study (24). For experiments performed at room temperature (25 ± 2 °C), individual muscle fibers were plated onto either uncoated (for TG treatment) or a silicon-coated ΔTC3 dish (for C/R treatment) and loaded with 10 \(\mu\)M Fura-2 AM at room temperature for 1 h. FDB fibers were then fastened by silicon drops at both ends to avoid contraction induced by C/R (25). For experiments performed at 35 °C, FDB fibers were plated on uncoated ΔT4 dishes, and the temperature was controlled by a thermal controller (Biopotech Inc., Butler, PA). To prevent motion artifact in fibers associated with intracellular Ca\(^{2+}\) release, 20 \(\mu\)M N-benzyl-p-toluene sulfonamide (Sigma), a specific myosin II inhibitor (26), was applied.

Spatial and Temporal Resolution of SOCE in Skinned Muscle Preparation—The detailed procedure for the application of this methodology in SOCE measurement has been described before (27). Briefly, single muscle fibers were dissected from extensor digitorum longus muscle of C57Bl6/J mice and cultured for 72 h. For SOCE measurements, fibers were mechanically skinned in the presence of Rhod-5N potassium salt (Invitrogen) to trap the dye conjugated with Ca\(^{2+}\) into transverse tubules (T-tubule). For SR Ca\(^{2+}\) content assessment, the fiber was mechanically skinned in the absence of Rhod-5 and Ca\(^{2+}\) and then incubated with an intracellular-like solution (in mM, 140 potassium glutamate, 6.5 MgCl\(_2\), 6 creatine phosphatase, 0.5 CaCl\(_2\), 20 2-bromoethanesulfonate/BES-KOH) containing 0.2 mM EGTA and 20 \(\mu\)M Rhod-5N AM (Invitrogen) for 1 h at room temperature to load the SR, followed by extensive washes and incubation for an additional 30 min to allow the complete de-esterification of the dye. Treatment with 5 \(\mu\)M p-trifluromethoxy carbonyl cyanide phenylhydrazone (FCCP) (Sigma) effectively eliminated the fluorescence signal from mitochondria. A Bio-Rad 2100 confocal microscope (Zeiss, Thornwood, NY) was used to resolve the spatial and temporal distribution of Rhod-5N inside the T-tubules or SR compartment as described in Zhao et al. (27), with the following exception. The fiber was perfused with SR loading solution plus 20 \(\mu\)M azumolene or 0.1% Me\(_2\)SO for 2 min. After that, the fiber was exposed to an SR-depleting solution (in mM, 100 potassium glutamate, 16 sodium glutamate, 20 EGTA-KOH, 5 1,2-bis(o-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid, 0.07 MgCl\(_2\), 0.25 ATP, 1 creatine phosphatase, 10 BES-KOH) with C/R (30 mM/5 \(\mu\)M), in the presence of azumolene or Me\(_2\)SO carrier for 1000 s. These experiments were repeated six times, and mean values of 10 regions of interest per fiber were analyzed. Rhod-5N intensity was normalized to the maximal loading intensity, prior to the onset of SR Ca\(^{2+}\) depletion. The above experiments were conducted at 25 ± 2 °C.

Statistics—Values are mean ± S.E. Significance was determined by Student's t test or one-way analysis of variance. A value of \(p < 0.05\) was used as criterion for statistical significance.

RESULTS

Azumolene Inhibits Extracellular Ca\(^{2+}\) Entry in CHO Cells Stably Transfected with RyR1—C1148 is a cell line derived from CHO cells that are stably transfected with RyR1 (22). These cells contain functional RyR1 channel on the endoplasmic reticulum (ER) membrane, in addition to the presence of an endogenous SOCE pathway. Thus, cell population assays (e.g. 2.5 \(\times\) 10\(^5\) cells) using a cuvette system can be applied to evaluate RyR1-mediated changes in intracellular Ca\(^{2+}\) signaling. As shown in Fig. 1, exposure of these cells to C/R leads to release of Ca\(^{2+}\) from the ER. C/R treatment results in complete depletion of the ER Ca\(^{2+}\) store, since it has been demonstrated previously that no further Ca\(^{2+}\) release is observed with subsequent addition of TG or ionomycin (28). When the bath solution contains 2 mM Ca\(^{2+}\), the C/R-induced Ca\(^{2+}\) transient displays an initial peak followed by a sustained tail component. The peak is somewhat attenuated, and the sustained Ca\(^{2+}\) elevation is absent when the bath solution contains BSS without Ca\(^{2+}\) and 0.5 mM Ca\(^{2+}\).
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EGTA, which results in a nominally 0 mM [Ca^{2+}]_{i}. This indicates that extracellular Ca^{2+} entry contributes to the development of both the peak and the sustained component of the C/R-induced Ca^{2+} transient, although quite significantly to the latter. The sustained Ca^{2+} elevation observed in 2 munits [Ca^{2+}]_{i} could be substantially inhibited by 20 mM 2-aminodiphenyl borate, an inhibitor of SOCE (29) (Fig. 1A). Taken together, these results suggest that an endogenous SOCE pathway likely mediates the entry of extracellular Ca^{2+} following depletion of the C/R-dependent ER Ca^{2+} store in C1148 cells.

To test the effects of azumolene on the RyR1-dependent Ca^{2+} signaling, azumolene was added to C1148 cells from a Me_{2}SO stock solution. In the presence of 20 mM azumolene, the peak amplitude of C/R-induced Ca^{2+} transients (in 2 mM [Ca^{2+}]_{i}) was significantly reduced from F_{350}/F_{380} = 0.37 ± 0.06 (in Me_{2}SO carrier) to 0.20 ± 0.03 (plus azumolene). This is consistent with the previously reported partial inhibitory effect of dantrolene on RyR1-dependent Ca^{2+} transients in cells (17). Significantly, the sustained post-peak elevation of Ca^{2+}, which our results suggest is because of SOCE, was significantly reduced by the presence of azumolene.

Note that the addition of azumolene leads to apparent instantaneous elevation of the Fura-2 signal, as shown in Fig. 1A, inset. This is because of the intrinsic autofluorescence of azumolene. The autofluorescence of azumolene displays a sharp peak at an excitation wavelength of 348 nm, and thus likely contributes to the apparent elevation of F_{350}/F_{380} Fura-2 signal shown in Fig. 1A. To correct for the autofluorescence of azumolene, we subtracted the component of F_{350} Fura-2 fluorescence associated with azumolene addition in the calculation of F_{350}/F_{380}. As shown in Fig. 1B, the sustained component of Ca^{2+} transients was completely inhibited by azumolene. This observation led us to hypothesize that azumolene may suppress extracellular Ca^{2+} entry through SOCE.

We next used Mn^{2+} quenching of Fura-2 fluorescence to test whether azumolene directly affects SOCE in C1148 cells. In this assay, Mn^{2+} is supplied to the extracellular solution, and its entry through SOCE quenches the intracellular Ca^{2+}-dependent Fura-2 fluorescence (30–32). Measurement of the decrease in Fura-2 fluorescence at an excitation wavelength of 360 nm, the Ca^{2+}-independent isosbestic point of Fura-2, provides an assessment of SOCE function. We found that preincubation of C1148 cells with 20 mM azumolene significantly reduced the rate of Mn^{2+} entry following depletion of the ER Ca^{2+} store with C/R (Fig. 1, C and D). These results directly demonstrate that azumolene is capable of inhibiting SOCE induced by C/R in CHO cells expressing RyR1.

Azumolene Inhibits SOCE in C2C12 Myotubes—The effect of azumolene on SOCE was further evaluated in myotubes derived from the C2C12 mouse myogenic cell line. Differentiated C2C12 myotubes were treated with C/R for 5 min in the absence of extracellular Ca^{2+} to allow for complete depletion of the SR Ca^{2+} stores (18). As shown in Fig. 2A, individual myotubes treated with Me_{2}SO (vehicle control) exhibited steep quenching of Fura-2 fluorescence because of Mn^{2+} influx. This defines the maximal C/R-triggered activation of SOCE measured in our system. C2C12 myotubes exposed to 20 mM azumolene prior to C/R stimulation exhibited an ~70% reduction of SOCE compared with that of vehicle-treated control cells (Fig. 2, B and D). The peak amplitude of C/R-induced Ca^{2+}
release in the absence of extracellular Ca\(^{2+}\) in C2C12 myotubes was not altered by azumolene (Fig. 2, A and B), with \(F_{360}/F_{390} = 0.32 \pm 0.03\) in Me\(_2\)SO control versus 0.28 \(\pm\) 0.03 in azumolene-treated myotubes, a change that was not statistically significant \((n = 8–11, p = 0.38)\). Thus, this decrease in SOCE in azumolene-treated C2C12 myotubes does not appear to result from an inhibition of SR Ca\(^{2+}\) release by azumolene.

Interestingly, changing the order of drug treatment in the experimental protocol produces a significantly different result (Fig. 2C). Here, incubation of C2C12 myotubes with azumolene after C/R-initiated SR Ca\(^{2+}\) depletion resulted in loss of the inhibitory effect of the drug on SOCE (Fig. 2D, also compare Fig. 2, C and A). Me\(_2\)SO carrier alone had no effect on the slope of Mn\(^{2+}\) quenching, regardless of the order of application. Because a significant decrease in the slope of Mn\(^{2+}\) quenching of Fura-2 fluorescence occurred only when azumolene was added prior to C/R stimulation, and not after, the effect of azumolene on SOCE in C2C12 myotubes may depend on the conformation of the RyR1 channel at the time of incubation with azumolene, if azumolene acts by binding to RyR1.

Dose-dependent Effects of Azumolene on SOCE in C2C12 Myotubes—Our previous study showed that SOCE in fetal skeletal muscle can be activated in a graded manner by the reduction of SR Ca\(^{2+}\) store (25). To determine the effects of azumolene on the graded activation of SOCE in C2C12 myotubes, the quenching of Fura-2 by Mn\(^{2+}\) was monitored from the beginning of C/R-initiated SR Ca\(^{2+}\) release. As shown in Fig. 3A, myotubes pretreated with Me\(_2\)SO exhibited a sigmoidal Mn\(^{2+}\) quench curve, reflecting the graded activation of SOCE that follows reduction of the SR Ca\(^{2+}\) store. Prior incubation of myotubes with 10 \(\mu\)M azumolene for 2 min led to significant delay in activation of SOCE and an altered slope of the Mn\(^{2+}\) quench curve. To quantify the graded activation of SOCE, the first-order derivative of changes in \(F_{360}\), \(dF_{360}/dt\), was determined (Fig. 3B). This analysis led to the definition of the following two kinetic parameters of SOCE in skeletal muscles: \(m_{\text{max}}\), the peak slope of Mn\(^{2+}\) quenching, reflecting the maximum degree of SOCE activation; and \(\Delta r\),

FIGURE 2. Inhibition of C/R-activated SOCE by azumolene in C2C12 myotubes. A, individual C2C12 myotubes were treated with 0.1% Me\(_2\)SO (DMSO) control for 2 min and C/R for 5 min, and 0.5 mM Mn\(^{2+}\) was then perfused onto the myotube for 7 min. The decrease in fluorescence at 390 nm \(F_{390}\) reflects an elevation of \([Ca^{2+}]_i\), and the concurrent decrease in both \(F_{360}\) and \(F_{390}\) reflects the quenching of fluorescence by Mn\(^{2+}\) entry. Horizontal dashed line represents the basal Mn\(^{2+}\) entry rate, whereas the oblique dashed line represents the Mn\(^{2+}\) entry activated by SR store depletion. B, the \(F_{360}\) and \(F_{390}\) traces in C2C12 cells preincubated with 20 \(\mu\)M azumolene for 2 min before SR Ca\(^{2+}\) depletion. Addition of Mn\(^{2+}\) does not induce quenching of Fura-2 fluorescence. C, the \(F_{360}\) and \(F_{390}\) traces in C2C12 cells incubated with 20 \(\mu\)M azumolene for 6 min after SR Ca\(^{2+}\) depletion prior to initiating measurement of SOCE by Mn\(^{2+}\) fluorescence quenching. D, average data for initial rates (slopes) of Mn\(^{2+}\) quenching; open bar is for Me\(_2\)SO group, and hatched bar is for azumolene treatment group. Only the slope of the Mn\(^{2+}\) quench curve of the azumolene pretreatment group (azumolene before) was significantly suppressed relative to control \((n = 8)\). Experiments were performed at room temperature \((25 \pm 2 \, ^\circ\text{C})\). *, \(p < 0.05\).
the delay time to reach $m_{\text{max}}$ from the onset of Ca$^{2+}$ release from SR after addition of C/R. Using this analysis, we conducted systematic studies to resolve the dose-dependent effect of azumolene on $m_{\text{max}}$ and $\Delta \tau$ of SOCE in C2C12 myotubes. As shown in Fig. 3, C and D, the steepest range of azumolene effect on $\Delta \tau$ and $m_{\text{max}}$ was observed between concentrations of 0.1 to 20 $\mu$M, a clinically relevant concentration range. Note that there appears to be a biphasic effect of azumolene on SOCE, e.g. a high affinity effect with an apparent $K_d$ close to 2 $\mu$M, and a low affinity one that does not saturate under our experimental conditions.

Azumolene Does Not Affect TG-induced Activation of SOCE
SR Ca$^{2+}$ store in skeletal muscle can be depleted using other methods besides activation of RyR1 by C/R. TG, an inhibitor of the Ca$^{2+}$-ATPase on SR, has been classically used to passively deplete the SR Ca$^{2+}$ store to activate SOCE (19). As shown in Fig. 4A, treatment of C2C12 myotubes with 10 $\mu$M TG for 5 min in a bath solution containing 0 [Ca$^{2+}$]$_o$ led to depletion of SR Ca$^{2+}$ stores that is not affected by azumolene treatment ($p > 0.05$). The $F_{360}/F_{390}$ equaled 0.27 ± 0.02 in Me$_2$SO control versus 0.24 ± 0.02 in the azumolene-treated group. This SR Ca$^{2+}$ depletion leads to maximum activation of SOCE, which is reflected in the steep Mn$^{2+}$ quenching of Fura-2 fluorescence. In contrast to the results shown in Fig. 2A, we found that the TG-induced activation of SOCE was not affected by azumolene, e.g. the slope of Mn$^{2+}$ quenching of Fura-2 fluorescence did not change significantly with the addition of azumolene (Fig. 4A).

The ability of azumolene to discriminate between C/R- and TG-induced SR Ca$^{2+}$ depletion suggests the existence of at least two pathways of SOCE activation in skeletal muscle, one RyR1-dependent and the other RyR1-independent. To further test this hypothesis, we performed the following studies. First, we tested whether there are additive effects of TG and C/R on SOCE activation in C2C12 myotubes. As shown in Fig. 4B, the slope of Mn$^{2+}$ quenching induced by prior exposure to C/R did not increase following subsequent addition of TG, suggesting that TG does not induce additional activation of SOCE. The lack of additive effects of TG- and C/R-induced activation of SOCE in C2C12 myotubes was further demonstrated in Fig. 4C, where addition of C/R to a C2C12 myotube that was previously treated with TG also did not elicit additional activation of SOCE. Second, we tested whether C/R and TG mediation of SOCE involve interacting or parallel pathways in C2C12 myotubes by determining the effect of order of addition of the two sets of drugs on the parameters of SOCE and the effect of azumolene on these. As shown in Fig. 4B, the inhibitory effect of azumolene on C/R-induced SOCE in C2C12 myotubes could be completely overcome by subsequent treatment with TG.

**FIGURE 3.** Dose-dependent effects of azumolene on graded SOCE in C2C12 myotubes. A, simultaneous application of Mn$^{2+}$ and C/R after pre-treatment with either 0.1% Me$_2$SO (gray) or 20 $\mu$M azumolene (black). Fura-2 fluorescence is quenched in a sigmoidal manner in Me$_2$SO (DMSO)-treated myotubes, whereas azumolene reduces the Mn$^{2+}$ quenching slope and delays SOCE activation. B, plot of $dF_{360}/dt$ derived from the control trace in A. The lowest point on the y-axis was defined as $m_{\text{max}}$, the maximal slope of SOCE, and the value of this point on the x-axis (time) relative to the initiation of Ca$^{2+}$ release from SR ($t = 0$) was defined as $\Delta \tau$; the duration from initiation of SR Ca$^{2+}$ release to the point where $m_{\text{max}}$ is reached. Dose-dependent changes in $\Delta \tau$ (C) and $m_{\text{max}}$ (D), as a function of azumolene concentration, were averaged from six separate experiments. Experiments were performed at room temperature (25 ± 2°C). *, $p < 0.05$. **
Azumolene does not affect TG-triggered SOCE in C2C12 myotubes. A, traces represent changes in F_360 from C2C12 myotubes following passive depletion of SR Ca\(^{2+}\) stores induced by 10 \(\mu\)M TG for Me\(_2\)SO (DMSO) control (upper trace) or 20 \(\mu\)M azumolene (lower trace). Horizontal dashed lines represent the basal Mn\(^{2+}\) entry rate, whereas oblique dashed lines represent the Mn\(^{2+}\) entry activated by SR store depletion. B, Me\(_2\)SO or azumolene was added to C2C12 myotubes before TG-induced SR Ca\(^{2+}\) depletion. Cells were treated with caffeine/ryanodine for 5 min, followed by Mn\(^{2+}\) addition to the perfusate for 4 min, and then TG plus Mn\(^{2+}\) was added to the perfusate. C, identical to B, with the order of caffeine/ryanodine and TG addition reversed. D, average data for the slope of F_360 derived from A to C. The two sequential slopes after each of the two treatment regimens in B and C were designated as S1 (boxed) and S2 (short dashed line), respectively (\(n = 10\) for each group tested). *, \(p < 0.05\). Experiments were performed at room temperature (25 ± 2 °C).
This result suggests that TG can maximally activate SOCE despite azumolene inhibition of RyR1-mediated SOCE, indicating the two pathways to SOCE are parallel and access the same store of SR Ca\(^{2+}\). Indeed, in the converse experiment, azumolene does not inhibit TG-induced SOCE, and subsequent addition of C/R does not appear to affect the degree of SOCE activation (Fig. 4C), supporting the argument for parallel pathways to SOCE activation.

The data from multiple measurements summarized in Fig. 4D substantiate our conclusions. Although TG and C/R may share a final common target that leads to activation of SOCE, only the C/R-induced SOCE pathway is significantly affected by azumolene.

**Differential Effects of Azumolene on SOCE Activation in FDB Muscle Fibers**—To complement our cell culture-based measurements of azumolene effects on SOCE, we tested azumolene in enzymatically dissociated FDB fibers from mice. For these measurements, we used 20 mM/5 μM C/R or 20 μM TG to deplete the SR Ca\(^{2+}\) store for maximum activation of SOCE. To prevent motion artifacts, we employed two techniques. First, the silicon-grease method was used to immobilize the individual FDB fibers onto the culture dish (25). Second, N-benzyl-p-toluene sulfonamide, a specific myosin II inhibitor with minimum alteration of Ca\(^{2+}\) signaling (26), was used to prevent muscle contraction associated with intracellular Ca\(^{2+}\) release. Similar to our results with C2C12 myotubes, we found that 20 μM azumolene could significantly inhibit C/R-activated SOCE (Fig. 5A) but not the TG-induced SOCE (Fig. 5B).

The above analyses of the effect of azumolene on SOCE in FDB muscle fibers were all performed at room temperature (25 ± 2 °C) (Fig. 5C). Because previous studies have suggested that the effect of dantrolene on RyR-mediated Ca\(^{2+}\) release may display temperature dependence (33), we performed a
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Azumolene Uncouples Graded Activation of SOCE from SR Ca2+ Release in Adult Skeletal Muscle Fibers—To investigate the relationship between SR store depletion and SOCE activation, we monitored SOCE and SR Ca2+ release by C/R in mechanically skinned skeletal muscle fibers using a recently adapted confocal microscopy methodology (27). The membrane-impermeant salt of Rhod-5N can be trapped inside the sealed T-tubule compartments of skinned skeletal muscle fibers (Fig. 6A). Upon initiation of Ca2+ release from SR with C/R, activation of SOCE allows the flow of Ca2+ ions from the sealed T-tubule compartment to the cytoplasm, resulting in decreased Rhod-5N fluorescence. In the presence of azumolene, the rate and extent of decrease in Rhod-5N fluorescence are significantly reduced compared with the MeSO control group. At the end of the exposure to the SR Ca2+ depletion solution (1000 s), Rhod-5N fluorescence intensity equaled 0.40 ± 0.03 in the MeSO control group, whereas in the azumolene-treated fibers this value was 0.68 ± 0.02 (p < 0.05). This demonstrated that SOCE was reduced by azumolene.

In parallel experiments, we determined the effect of azumolene on C/R-induced Ca2+ release by loading Rhod-5N AM, the membrane-permeable form of the dye, into the SR of skinned muscle fibers, rather than the T-tubule system (Fig. 6B). To eliminate potentially confounding changes resulting from a mitochondrial Rhod-5N Ca2+ signal, the mitochondrial electron transport inhibitor, 5 μM FCCP, was added to the skinned muscle fiber prior to induction of SR Ca2+ release by C/R and was present throughout the series of experiments at 35 °C. As shown in Fig. 5D, azumolene affected SOCE in these experiments in a manner virtually identical to those observed at 25 °C; the drug inhibited C/R-induced SOCE without significantly affecting TG-induced SOCE. Similar to our results in the C2C2 myotube, the SR Ca2+ release in response to both C/R (0.47 ± 0.06 in control group versus 0.46 ± 0.06 in azumolene group, p > 0.05) and TG (0.48 ± 0.05 in control group versus 0.46 ± 0.09 in azumolene group, p > 0.05) was unaltered by azumolene.

Ca2+ depletion process. In the presence of FCCP, C/R induces SR Ca2+ depletion that is demonstrated by a time-dependent decrease in SR Rhod-5N fluorescence. Fibers pretreated with azumolene display a slightly slower rate of fluorescence decrease (0.63 ± 0.01), but no significant difference in final fluorescence was found when compared with the MeSO control (0.56 ± 0.01, p = 0.26). This result suggests that the inhibitory effect of azumolene on SOCE does not result from its direct inhibition of the SR Ca2+ release process.
The correlation between changes in SR Ca\(^{2+}\) content and SOCE activation is illustrated in Fig. 6C, where the normalized Rhod-5N fluorescence intensity of the T-tubule compartment is plotted against the intensity in the SR compartment over a time interval of 500 s following addition of C/R. During this period, close coupling between changes in SR Ca\(^{2+}\) release and SOCE activation are observed under control conditions. In the presence of azumolene, however, this close coupling is disrupted, as reflected by the shallower correlation between SR Ca\(^{2+}\) release and SOCE activation.

**DISCUSSION**

Elucidating the cellular mechanism(s) of dantrolene action on skeletal muscle Ca\(^{2+}\) signaling is of great interest from both physiological and pathophysiological points of view. Because MH syndromes are linked to mutations in the RyR1 channel in various vertebrates, as well as in humans (2, 4, 34, 35), and because dantrolene binds to a specific site on RyR1 (36), previous studies have focused on the role of dantrolene in modulating RyR1 channel activity. Functional studies demonstrate only partial inhibition of Ca\(^{2+}\) release from isolated SR membrane vesicles (11, 12, 15) and partial suppression of the elemental Ca\(^{2+}\) spark signals in adult muscle fibers (16). As a muscle relaxant, dantrolene can suppress the elevation of [Ca\(^{2+}\)]\(_{c}\) in intact muscle fibers, yet conclusive evidence for direct inhibition of RyR1 channel activity by dantrolene is lacking. Our data suggest that a significant portion of the action of dantrolene and related compounds in the therapy of MH may stem from their inhibition of RyR1-coupled SOCE.

Further, we demonstrate that azumolene disrupts the tight Ca\(^{2+}\) release-coupled graded activation of SOCE that is normally seen in adult mouse skeletal muscle fibers without substantially inhibiting SR Ca\(^{2+}\) release. Second, we show that azumolene can substantially inhibit the C/R-triggered SOCE in heterologous cells expressing RyR1, in cultured C2C12 myotubes, and in adult mouse skeletal muscle fibers, suggesting that this effect is dependent on RyR1. Third, we found that although TG-induced depletion of SR Ca\(^{2+}\) stores leads to maximal rates of SOCE, this process was not susceptible to azumolene inhibition, indicating that azumolene does not inhibit all signals that can lead to the stimulation of SOCE. Fourth, we show that substantial inhibition of RyR1-coupled SOCE by azumolene occurs only when cells are treated with this drug prior to C/R-induced RyR1 activation and SR Ca\(^{2+}\) depletion. Because C/R treatment produces prolonged RyR1 channel opening, the inability of azumolene to inhibit SOCE when added after C/R treatment is consistent with previously published in vitro studies demonstrating that dantrolene interacts preferentially with the closed state of RyR1 (36, 37). Therefore, we hypothesize that the inhibitory effect of azumolene and, by extension, of dantrolene on SOCE results from drug binding to the closed state of RyR1. The discordance between the ability of azumolene to inhibit SOCE versus SR Ca\(^{2+}\) release in FDB muscle fibers suggests that Ca\(^{2+}\) itself is not the direct signal from RyR1 that stimulates SOCE. Furthermore, because azumolene does not inhibit TG-induced SOCE, azumolene cannot be acting at the level of the SOCE machinery itself. It therefore follows that azumolene is likely uncoupling the efficiency of a Ca\(^{2+}\)-dependent RyR1 signal coupled directly or indirectly to the SOCE machinery and represents a novel hypothesis for the mechanism of action of this drug.

Because under control conditions there is no additivity between saturating effects of C/R- and TG-induced Ca\(^{2+}\) release in their effects on SOCE, it is likely that the two systems for activating SOCE result from competition for the same intracellular Ca\(^{2+}\) store in the cells examined here. Even when RyR1-coupled SOCE is inhibited by azumolene, TG is still able to activate SOCE at nearly the same rate as if azumolene is absent. Taken together, our data suggest that at least two different mechanisms, either through RyR1 or ER/SR Ca\(^{2+}\)-ATPase, are capable of activating the SOCE machinery in mammalian skeletal muscle, which is consistent with previous studies (20, 38).

Recent studies from Pessah and co-workers (39, 40) have demonstrated a process of excitation-coupled Ca\(^{2+}\) entry (ECCE) experimentally distinct from SOCE in cultured myotubes. ECCE is not sensitive to Ca\(^{2+}\) store depletion but is activated by membrane depolarization and is sensitive to RyR1 conformation and mutations (39, 40). Furthermore, they have presented preliminary evidence that dantrolene also affects ECCE, but not TG-induced SOCE, thereby discriminating between the two processes (41). It is possible that the integral membrane machinery and/or the attendant signaling mechanisms underlying these pathways of extracellular Ca\(^{2+}\) entry (i.e. SOCE and ECCE) may be similar, if not identical, because both involve coupling to RyR1 and azumolene/dantrolene sensitivity.

Because both dantrolene and azumolene are therapeutic in the treatment of MH, the novel mechanism of drug action described here leads us to suggest that an elevated RyR1-coupled signal to SOCE may contribute appreciably to the pathophysiology of MH, i.e. MH is as much a syndrome of exaggerated Ca\(^{2+}\) entry as it is of exaggerated Ca\(^{2+}\) release. By extension then, the therapeutic activity of dantrolene in MH may result from its ability to inhibit exaggerated Ca\(^{2+}\) influx, rather than from its ability to inhibit SR Ca\(^{2+}\) efflux. Further defining the role of dantrolene and azumolene in modulating various Ca\(^{2+}\)-dependent aspects of muscle physiology should improve our knowledge of the machinery responsible for cellular Ca\(^{2+}\) homeostasis. This may provide novel therapeutic targets for various human disorders linked to dysfunctional Ca\(^{2+}\) signaling involving susceptible RyR isoforms and SOCE mechanisms.

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