Effects of azumolene on Ca\(^{2+}\) sparks in skeletal muscle fibers *

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Running title: Azumolene decreases activation of spontaneous Ca\textsuperscript{2+} sparks

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**ABBREVIATIONS:** MH, malignant hyperthermia; SR, sarcoplasmic reticulum; RyR, Ryanodine receptor; DHPR, dihydropyridine receptor; E-C coupling, excitation-contraction coupling; CICR, calcium induced calcium release; RT, rise time; FDHM, full duration at half-max; FWHM, full width at half-max.

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Abstract

Azumolene is an analog of dantrolene, the only approved medicine for treatment of malignant hyperthermia (MH). The pharmacological mechanism of these drugs is to inhibit skeletal muscle sarcoplasmic reticulum (SR) Ca\(^{2+}\) release by modulating the activity of the SR ryanodine receptor (RyR) Ca\(^{2+}\) release channel. To investigate the effects of azumolene on SR Ca\(^{2+}\) channel gating within skeletal muscle fibers, we monitored Ca\(^{2+}\) sparks in permeabilized frog skeletal muscle fibers. Application of 0.0001~10 \(\mu\)M azumolene suppressed the frequency of spontaneous Ca\(^{2+}\) sparks in a dose-dependent manner (EC\(_{50}=0.25\mu\)M, Hill coefficient =1.44), but did not cause systematic dose-dependent effects on the properties of the Ca\(^{2+}\) sparks. These results suggest that azumolene decreases the likelihood of Ca\(^{2+}\) release channel openings that initiate Ca\(^{2+}\) sparks, thereby decreasing spark frequency, but has little effect on aggregate Ca\(^{2+}\) channel open times during a spark. To assess azumolene inhibition of RyRs activated in a manner analogous to those activated during an MH episode, we applied DP4, a synthetic peptide corresponding to a central region of RyR1 (Leu2442 to Pro2477), which mimics an MH modification. Azumolene also decreased Ca\(^{2+}\) spark frequency in a dose-dependent manner without altering spark properties in the DP4 MH model. We conclude that azumolene suppresses the opening rate but not the open time of RyR Ca\(^{2+}\) release channels within skeletal fibers.
Introduction

Malignant hyperthermia (MH) is a pharmacogenetic disorder of skeletal muscle in which the excitation-contraction (E-C) coupling processes are disrupted, leading to uncontrolled Ca^{2+} release from sarcoplasmic reticulum (SR) in response to volatile anesthetics or depolarizing muscle relaxants (Denborough, 1996). Dantrolene is a postsynaptic muscle relaxant and the only approved effective treatment of MH. Early investigations have shown that dantrolene shifts the excitation threshold to more positive voltages (Hainaut and Desmedt, 1974) and reduces contractile force (Flewellen et al., 1983) in both normal and MH skeletal muscles, providing a lifesaving treatment for the deadly MH episode. The molecular basis of the action of dantrolene is generally presumed to involve either direct or indirect inhibitory effects on the ryanodine receptor (RyR). Mammalian RyRs have three tissue-specific isoforms: RyR1, which is mainly expressed in skeletal muscle; RyR2, which is mainly expressed in cardiac muscle; and RyR3, which is more widely expressed, including neuronal tissues (Sutko and Airey, 1996). The effect of dantrolene appears to be tissue-specific. Dantrolene inhibits calcium efflux from both the normal and MH-susceptible SR isolated from skeletal muscle but has no effects on cardiac muscle SR (Fruen et al., 1997; Chamberlain et al., 1984). The contractility of cardiac muscle is not affected by dantrolene while the contractility of skeletal muscle is remarkably reduced (Fratea et al., 1997). Dantrolene, and its more water-soluble analog azumolene, also inhibit [^3H] ryanodine binding to skeletal SR membrane fractions but have no effects on [^3H] ryanodine binding to cardiac
SR vesicles (Freren et al., 1997). [3H] azidodantrolene, a pharmacologically active photoaffinity analog of dantrolene, photo cross-links to the N-terminal fragment of RyR1, which plays a significant role of the regulation of channel function (Paul-Pletzer et al., 2002; Paul-Pletzer et al., 2001). Thus, dantrolene may directly target to RyR1 and modulate the activity of RyR1, thereby reducing the Ca^{2+} release in skeletal muscle during an MH episode. Dantrolene’s effect on RyR3 is not as extensively studied as for RyR1 and RyR2, but it is clear that dantrolene and azumolene also inhibit the activity of RyR3. Dantrolene and azumolene inhibit Ca^{2+} release via RyR3 in neuronal cells (Wei et al., 1996; Pelletier et al., 1999; Mattson et al., 2000). Heterologously expressed RyR3 isoform was also significantly inhibited by dantrolene and azumolene, and this inhibition was comparable with the dantrolene inhibition of RyR1 (Zhao et al., 2001).

In frog skeletal muscle Ca^{2+} sparks underlie the global Ca^{2+} transient during depolarization (Tsugorka et al., 1995; Klein et al., 1996). Ca^{2+} sparks are brief, highly localized elevations of myoplasmic [Ca^{2+}]_{free} that can be visualized by fluorescent Ca^{2+} indicators (Cheng et al., 1993). These events provide a means to evaluate the opening and closing properties of Ca^{2+} release channels (the RyR) or small groups of channels in functional muscle fibers. In the present study, we sought to identify the effect of azumolene, a more water-soluble analog of dantrolene, on the opening and closing properties of RyR Ca^{2+} release channels in permeabilized frog skeletal muscle fibers. Using this approach it is possible to determine whether the inhibition of Ca^{2+} release by azumolene in muscle fibers is due to changes in Ca^{2+} spark frequency (i.e., channel opening rate), changes in Ca^{2+} spark spatio-temporal properties (i.e., channel open time and/or conductance), or both. Permeabilized muscle fibers maintain most of the
macromolecular interactions present in intact muscle fibers, and provide a convenient means to apply a range of conditions, reagents, peptides and proteins to assess their effects on RyR activity (for review, see Schneider and Ward, 2002). The sparks observed in permeabilized fibers are ‘spontaneous’ calcium release events, and their spatio-temporal properties reflect the influence of activating and inhibiting agents in the absence of voltage sensor activity (Klein et al., 1996). Here we find that azumolene can completely inhibit the frequency of spontaneous Ca$^{2+}$ sparks in a dose-dependent manner, with little alteration in the spatio-temporal properties of the Ca$^{2+}$ sparks. Furthermore, using a synthetic peptide segment of the central domain of RyR1 from Leu2442 to Pro2477 (DP4) to mimic an MH episode (Yamatomo et al., 2000), we show that the increased spontaneous Ca$^{2+}$ spark frequency in the presence of DP4 is also greatly reduced by azumolene in a dose-dependent manner. Our data suggest that azumolene acts to stabilize the closed state of the RyR Ca$^{2+}$ release channel and thereby inhibits the initiation of Ca$^{2+}$ sparks, but has no effects on the termination of the Ca$^{2+}$ release underlying the Ca$^{2+}$ sparks.

Methods

Frogs (*Rana pipiens*) were first placed into a crushed ice-water slurry for 30 min followed by rapid decapitation and spinal cord destruction according to protocols approved by the University of Maryland Institutional Animal Care and Use Committee. The ileofibularis muscle was removed and pinned in a dissecting chamber containing
Ringer’s solution (in mM): 115 NaCl, 2.5 KCl, 1.8 CaCl2, 1.0 MgCl2, 10 HEPES (pH 7.0). Small fiber segments (~5 mm) were manually dissected in relaxing solution containing (in mM): 120 K-glutamate, 2 MgCl2, 0.1 EGTA, 5 Na-Tris-maleate (pH 7.0), and mounted in an experimental chamber, stretched to 3.6±0.4 µm per sarcomere. The fiber was bathed in a relaxing solution containing 0.01% saponin and 1.0mM EGTA for 30-40 seconds for chemical permeabilization, allowing solution equilibration into the myoplasm. Immediately following the permeabilization procedure, the fiber was bathed in internal solution containing (in mM): 80 K-glutamate, 5 Na2ATP, 4.79 MgCl2 (0.42 [Mg2+]free), 20 tris-maleate, 0.1 EGTA, 20 Na2 creatine phosphate, 5 glucose, 0.05 Fluo-3 (pentapotassium salt) (Molecular Probes, Eugene, OR), pH 7.0 supplemented with 8% Dextran (41kD). The [Ca2+]free (100nM) and the [Mg2+]free in our internal solution were calculated using WinMaxC 2.5 (Patton et al, 2004). After control data collection, the bathing solution was changed to an experimental internal solution containing 0.2% DMSO plus azumolene sodium (1-(((5-(4-bromophenyl)-2-oxazolyl)methylene)amino)-2,4-imidazolidinedione, gift from Dr. Jerome Parness, UMDNJ-Robert Wood Johnson Medical School, Piscataway, NJ) at a final concentration of 0-10µM. The fiber was allowed to equilibrate for 10 minutes before another period of data collection. To control for effects of solution change, “sham” fibers were exposed to azumolene free DMSO (0.2%) in internal solution. To minimize muscle-to-muscle variation, a group of sham fibers ([azumolene]=0) was obtained from the same muscles as used for each azumolene concentration.

For experiments mimicking an MH episode, the fiber was first bathed in internal solution with the [Mg2+]free increased to 1.2mM (6.73 mM total MgCl2) to decrease the
frequency of Ca\(^{2+}\) sparks. After data collection in the absence of DP4, the bathing solution was changed to the internal solution containing 150\(\mu\)M DP4 (a synthetic domain peptides of RyR1 from Leu2442 to Pro2477, a gift from Dr. Noriaki Ikemoto, Harvard Medical School, Boston, MA) plus 0.2% DMSO. The fibers were then allowed to equilibrate for 10 minutes before the second data collection (=“control”). The bathing solution was then changed to internal solution containing DP4 (150\(\mu\)M), 0.2% DMSO and 0.04-5\(\mu\)M azumolene (“experimental”) or 150 \(\mu\)M DP4, 0.2% DMSO and no azumolene (“sham”).

Ca\(^{2+}\) sparks in fibers were monitored on an inverted microscope (Olympus IX-70 with a X60, 1.4NA oil-immersion objective). The line-scan images were recorded with a laser scanning confocal system (Bio-Rad MRC 600, 488nm excitation) operated in linescan (\(x-t\)) mode, with the scan line parallel to the fiber axis (2ms per line, 768 pixels per line, 0.18 \(\mu\)m per pixel, 512 lines per image, total line scan image duration 1.024 s). The scan-line was 138\(\mu\)m long parallel to the fiber’s long axis. To avoid laser damage to the fiber, the linescan was repeated for five images at one location and then moved 0.9\(\mu\)m perpendicular to the fiber’s long axis between runs. Multiple successive runs of images were recorded in each condition. Line-scan images were computer-processed to identify and record spark locations using a detection algorithm as previously described (Cheng et al., 1999; Shtifman et al., 2000).

Images were corrected for PMT offset and converted to \(\Delta F\) images by subtraction of resting fluorescence (\(F\)) along the scan line averaged in time, excluding the contribution of potential Ca\(^{2+}\) spark regions of interest (ROIs). \(\Delta F\) images were then normalized pixel by pixel by \(F\) and smoothed 3x3 to get the \(\Delta F/F\) images. Spatial and
temporal profiles were extracted from each ROI as previously described (Lacampagne et al., 1999). Events with a \( \Delta F/F < 0.4 \) were excluded from data analysis post-hoc.

The frequency of occurrence of Ca\(^{2+} \) sparks (number of events per sarcomere per second) was calculated from the number of sparks per image divided by the number of sarcomeres along the line and by the image duration (1.024s). Spark frequency was determined in each fiber for control and either sham or experimental conditions. Because of the variability in the starting Ca\(^{2+} \) spark frequency amongst fibers, the frequency in a given fiber under experimental or sham conditions was normalized to the mean of the control Ca\(^{2+} \) spark frequencies for the same group of experimental or sham fibers at each azumolene concentration. Ca\(^{2+} \) spark frequency results are reported as means ± SEM of these normalized frequencies from N fibers divided by the mean normalized frequency for the shams at the same azumolene concentration. Analysis if variance (ANOVA) was used as statistical analysis for comparison of means, with a significance level of \( P < 0.05 \).

The spatio-temporal properties of Ca\(^{2+} \) sparks, such as amplitude, rise time, full duration at half-max (FDHM), full width at half-max (FWHM) and spark mass, were not normally distributed; therefore, a non-parametric ANOVA was performed (Dunn’s) to compare spark properties under different experimental conditions. All statistical analysis was performed with SigmaStat. Non-linear curve fitting was performed in SigmaPlot (Jandel).

**Results**
Azumolene decreases spontaneous Ca$^{2+}$ spark frequency

To investigate the effects of azumolene on localized Ca$^{2+}$ release permeabilized, cut frog skeletal muscle fibers were first incubated in an internal solution (control), then either in an internal solution either with 0.2% DMSO (sham) or in an internal solution containing the appropriate concentration of azumolene and 0.2% DMSO (experimental). The [Mg$^{2+}$]$_{free}$ in the internal solution was 0.42 mM. At this low Mg$^{2+}$ concentration the spontaneous Ca$^{2+}$ spark frequency would be relatively high (Lacampagne et al., 1998), thereby facilitating the acquisition of sufficient numbers of Ca$^{2+}$ sparks for statistical analysis subsequent to an inhibition of spark frequency in the presence of azumolene.

Fig. 1 shows representative line-scan fluorescence ($\Delta F/F$) images of permeabilized frog muscle fibers in control (A and C) and after addition of either 0.2% DMSO (B, sham) or 1µM azumolene with 0.2% DMSO (D) to the bathing solution. Distance along the fiber ($x$) is represented vertically and time ($t$) is represented horizontally to give the $x$ versus $t$ image in each panel. Each localized increase in [Ca$^{2+}$] (Ca$^{2+}$ spark) is characterized by a brief and localized increase in fluorescence (Klein et al., 1996; Schneider and Klein, 1996). When added to the permeabilized muscle fibers, 1 µM azumolene appeared to modulate SR Ca$^{2+}$ release by producing a large decrease in the frequency of Ca$^{2+}$ sparks.

Concentration dependent effects of azumolene in muscle fibers
We next evaluated the relationship between the decrease in Ca\(^{2+}\) spark frequency and the concentration of applied azumolene. Figure 2 shows that azumolene concentrations from 10\(^{-4}\) µM to 10 µM elicited a nonlinear, dose-dependent decrease in Ca\(^{2+}\) spark frequency. The solid line was obtained by fitting the data to the Hill equation:

\[
f = f_{\text{min}} + \frac{(f_{\text{max}} - f_{\text{min}})}{1 + ([AZ]/K)^b}
\]

where \(f\) is the event frequency normalized to the average control event frequency in the same groups of fibers divided by the mean normalized frequency in the corresponding sham; \(b\) is the Hill coefficient and \(K\) is the concentration of azumolene that elicits a 50% decrease in Ca\(^{2+}\) spark frequency (EC50). Since spark frequencies at each azumolene concentration were expressed relative to the mean frequency in the corresponding sham fibers, \(f_{\text{max}}\) was constrained to 1. Fitting of the data to equation 1 provided an \(f_{\text{min}}\) value of 0.00±0.15, indicating full suppression of all spark activity at saturating azumolene concentrations. 50% inhibition occurred at an EC50 of 0.25±0.12µM azumolene. A Hill coefficient of 1.44±0.99 suggests a single binding site for azumolene on RyR.

Nelson et al. (1996) have previously reported the presence of two concentration-dependent effects of dantrolene on isolated RyR1 channels reconstituted into the planar lipid bilayers. They found that low (nM) concentrations of dantrolene activated RyR1, causing a three to five-fold increase in \(P_{\text{open}}\) and open state dwell time, whereas higher (µM) concentrations decreased \(P_{\text{open}}\). Here we applied very low concentrations of azumolene, from 0.1 to 10nM, but did not observe any clear increase in Ca\(^{2+}\) spark frequency.
The effects of azumolene on Ca\(^{2+}\) spark spatio-temporal properties

To determine whether the decrease in Ca\(^{2+}\) spark frequency in the presence of azumolene was associated with changes in the spatio-temporal properties of individual Ca\(^{2+}\) release events we analyzed the spatio-temporal properties of the detected Ca\(^{2+}\) sparks. Fig 3 shows box plots of the azumolene concentration dependence of the distribution of values for the spatio-temporal properties of Ca\(^{2+}\) release events (\(\Delta F/F \geq 0.4\)) after addition of 0.2% DMSO (sham; [azumolene] = 0 \(\mu\)M), or after the addition of azumolene (0.0001- 1 \(\mu\)M). Although at 1\(\mu\)M azumolene the frequency of Ca\(^{2+}\) spark occurrence was considerably inhibited (Fig. 2) the number of Ca\(^{2+}\) spark events was still practical for analysis of their spatio-temporal properties. At 10 \(\mu\)M azumolene the number of events was too small for analysis of spark properties. Fig 3 shows that there were no systematic concentration dependent effects of azumolene on the temporal properties (rise time and FDHM), amplitude or spatial spread (FWHM) of the Ca\(^{2+}\) sparks.

To determine whether there was an azumolene dependent change in the amount of Ca\(^{2+}\) released from SR by the individual sparks, spark mass at time of spark peak was calculated for each spark using the equation (Hollingworth et al., 2001):

\[
\text{Mass}=\text{Amplitude} \times 1.206 \times \text{FWHM}^3
\]  

Fig. 4 shows that azumolene (0.0001 – 1 \(\mu\)M) caused no systematic concentration dependent change in spark mass. Overall, the predominant effect of azumolene in
permeabilized frog skeletal muscle was to greatly decrease the frequency of spontaneous 
Ca$^{2+}$ sparks while causing no systematic changes in spark properties.

Azumolene inhibits the increased Ca$^{2+}$ spark frequency in a DP4 induced model of MH

DP4 is a 36 amino acid synthetic peptide corresponding to the central domain of 
RyR1, from Leu2442 to Pro2477. Most MH mutations are found in this domain or in an 
N-terminal domain. These two domains are putative regulatory domains that are thought 
to intricately interact with each other and to be involved in the regulation of channel 
gating by stabilizing the closed channel of the channel (Ikemoto and Yamatomo, 2002). 
Point mutations within these two domains produce functional modifications that lead to 
destabilization and enhanced activation of RyRs and Ca$^{2+}$ release from SR. Since 
addition of DP4 mimics the MH/CCD modification (Yamatomo et al., 2000), we used 
DP4 to generate an MH model to determine whether azumolene can prevent the increased 
channel activity during an MH episode. Previous studies have shown that exogenous 
DP4 peptide significantly increases the spontaneous Ca$^{2+}$ spark frequency in 
permeabilized frog skeletal muscle fibers while the spatio-temporal properties of Ca$^{2+}$ 
sparks remained essentially unchanged (Shtifman et al., 2002). The activation effect of 
DP4 on spark frequency is specific in that a single amino acid substitution (Arg for Cys$^{17}$ 
within DP4), which mimics the in vivo mutation of Arg$^{2458}$-to-Cys$^{2458}$ in MH, abolishes 
the activating effects of DP4 (Shtifman et al., 2002). Here we applied 150µM DP4 in an 
internal solution having a free Mg$^{2+}$ concentration of 1.2mM. At this Mg$^{2+}$ concentration 
the spontaneous spark frequency is relatively low (Lacampagne et al, 1998; Shiftman et
al, 2002) so that the activating effect of DP4 on spark frequency would be moderate and apparent. We observed an 11-fold increase in spark frequency after 10 minutes exposure to a DP4-containing solution (Fig. 5a). Changing the 150 μM DP4 internal solution to another sample of the internal solution containing the same concentration of DP4 and waiting for an additional 10 minutes did not further change Ca^{2+} spark frequency (Fig. 5a). Some groups of fibers were incubated in internal solution containing 150μM DP4 for 10 minutes prior to data collection. These fibers were subsequently exposed to internal solution containing DP4 (150μM) plus azumolene at different concentrations, and again monitored for Ca^{2+} spark activity. We found that azumolene caused a dose dependent inhibition of Ca^{2+} spark frequency compared with fibers treated with DP4 alone (Fig. 5b). The fit of the data in Fig. 5b to the Hill equation (Eqn 1; f_{max}=1) gave full inhibition, with an EC50 of 2.35±4.02 and a Hill coefficient of 1.73±2.72, reminiscent of that observed for azumolene alone, but with an approximately 10-fold lower apparent affinity (EC50=0.25±0.12 without DP4, Fig. 2).

We next assessed whether the decrease in Ca^{2+} spark frequency in this DP4-MH model was associated with any changes in spatio-temporal properties of individual Ca^{2+} release events. Fig 6 shows the concentration dependence of the distribution of values for the spatio-temporal properties of Ca^{2+} release events (ΔF/F ≥ 0.4) after addition of 150μM DP4 alone to mimic the MH episode (sham; Fig 6, [azumolene] = 0) or after the addition of azumolene (0.04 - 5 μM) in the presence of 150μM DP4. The spatio-temporal properties exhibited no systematic variation as a function of azumolene concentration. Thus, although azumolene greatly decreased the frequency of Ca^{2+} sparks, presumably reflecting a decrease in the rate of opening of the RyR channels that initiate the sparks in
the MH-mimic model, the overall duration of channel opening and the amount of Ca$^{2+}$ released in a spark did not appear to be significantly changed by azumolene.

Discussion

This study describes the effects of azumolene on local SR Ca$^{2+}$ release events (Ca$^{2+}$ sparks) detected by laser scanning confocal microscopy in permeabilized, cut frog skeletal muscle fibers. Azumolene and dantrolene are similarly effective RyR inhibitors (Fruen et al., 1997), with both drugs targeting a common site on RyR1 at an N-terminal regulatory domain including amino acids 590-609 (Paul-Pletzer et al., 2002). Azumolene and dantrolene exhibit similar potency in the treatment and prevention of MH episodes due to administration of halothane or succinylcholine to MH susceptible swine (Dershwitz and Sreter, 1990). Here we use azumolene to study Ca$^{2+}$ sparks because azumolene is less fluorescent than dantrolene, and thus causes less interference with measurements using the fluorescent Ca$^{2+}$ indicator Fluo-3. Our results demonstrate that azumolene decreases the frequency of occurrence of Ca$^{2+}$ sparks in permeabilized fibers in a concentration-dependent manner, while the spatio-temporal properties of individual Ca$^{2+}$ sparks are essentially unchanged. These findings suggest that azumolene decreases the probability that a RyR channel or channels will open and initiate a Ca$^{2+}$ spark, i.e. azumolene decreases trigger events in the calcium release units. In contrast, azumolene must have minimal effects on the overall RyR channel open time and conductance during the Ca$^{2+}$ spark since spark properties were basically unchanged by azumolene. It is important to note that membrane permeabilization and the resulting fiber depolarization
may interfere with the interaction between the DHPR and RyR. Therefore, the data presented here may be best explained by azumolene targeting directly to the RyR, without interaction with the DHPR.

Previous studies have demonstrated that dantrolene decreases the sensitivity of isolated RyR1 and RyR3 to activation by Ca\(^{2+}\) in that it shifts the Ca\(^{2+}\) dependence of ryanodine binding to higher Ca\(^{2+}\) levels (Fruen et al., 1997, Zhao et al., 2001). The results here are also consistent with azumolene shifting Ca\(^{2+}\) sensitivity to CICR (calcium induced calcium release). In our experiments the global Ca\(^{2+}\) concentration is relatively low inside a muscle fiber equilibrated with our internal solution (~100nM), well under the Ca\(^{2+}\) concentration for full activation by CICR. Under these conditions, azumolene could decrease the rate of spontaneous openings of RyR channels by CICR, resulting in the observed decrease in the frequency of Ca\(^{2+}\) sparks. In contrast, the properties of the Ca\(^{2+}\) sparks that do occur may not be changed appreciably because the local Ca\(^{2+}\) concentration within the calcium release unit during a spark is high after opening of an RyR Ca\(^{2+}\) channel. Ca\(^{2+}\) activation of neighboring channels inside this cluster could then be maximal both with and without azumolene at this high local Ca\(^{2+}\) concentration, giving a similar local Ca\(^{2+}\) release event in the presence or absence of azumolene. This interpretation requires that the elevated local Ca\(^{2+}\) within a release unit can open azumolene-bound channels. Alternatively, azumolene would have to fully dissociate from the RyRs in the release unit within a time frame much shorter than the few millisecond rise time of a Ca\(^{2+}\) spark. However, either of these requirements would appear to be contrary to the ability of azumolene (and dantrolene) to suppress the Ca\(^{2+}\) regenerative aspects of an MH episode.
An alternative interpretation that does not rely on the ability of elevated Ca\textsuperscript{2+} levels to activate azumolene-bound channels during a spark could be based on having only a small number of channels (e.g., 2-4; Shtifman et al., 2000) underlying the generation of a Ca\textsuperscript{2+} spark. In this case, occupancy of a single channel in this small release unit could effectively eliminate that unit from generating a detectable spark. Thus frequency would be decreased as azumolene concentration was increased, but any events that did occur would be generated by release units without any bound azumolene and would thus exhibit normal spark properties.

There are two RyR isoforms in frog skeletal muscle, RyR\textalpha and RyR\textbeta, which are homologues of mammalian RyR1 and RyR3, respectively (Murayama and Ogawa, 2002). These two isoforms of RyR are expressed at about the same level in frog skeletal muscle. Dantrolene was found to decrease Ca\textsuperscript{2+} release from intracellular stores in central neurons (Wei et al., 1996; Pelletier et al., 1999; Mattson et al., 2000), which express predominantly RyR3. Heterologously expressed RyR3 was also significantly inhibited by dantrolene and azumolene, and this inhibition was comparable to the dantrolene inhibition of RyR1 (Zhao et al., 2001). In our studies, azumolene (10\mu M) completely inhibited Ca\textsuperscript{2+} sparks. Thus, our results are consistent with the notion that azumolene (or dantrolene) may have an inhibitory effect on both RyR1 and RyR3 isoforms.

T. E. Nelson et al. (1996) found that dantrolene and azumolene at nanomolar concentrations increased the open-state probability and open dwell time of single RyR1 channels from SR vesicles incorporated into lipid bilayer membranes. In contrast, at higher dantrolene concentration (5 \mu M) they observed a decrease in the open probability...
due to a decrease in the channel open time as well as a decrease in single channel conductance. In the present study low concentrations of azumolene, from 0.1 to 10 nM, did not markedly increase spark frequency, whereas at higher concentrations a decrease in spark frequency, which should correspond to an increase in channel closed time, was observed. There was no systematic alteration in the spatio-temporal properties of Ca\textsuperscript{2+} sparks. These differing results may reflect the different experimental preparations or different RyR isoform composition in frog skeletal muscle. T. E. Nelson’s experiments used crude RyR1 from mammalian SR. Though dantrolene in micromolar concentrations inhibits the activity of RyR3 (Zhao et al., 2001), and inhibits calcium efflux from the intracellular stores of neurons (T.J. Nelson et al., 1999), it has not been determined whether dantrolene or azumolene at nanomomolar concentrations would activate RyR3. However, evidence for such activation was not detected in our experiments. In other studies, using purified RyR in lipid bilayers, no effects of dantrolene were observed on purified RyR1 channels, which was interpreted as showing that the dantrolene effect on muscle fibers may be on an accessory protein (Szentesi et al., 2001). Finally, using SR vesicles in patch-clamped bilayers, dantrolene (50 µM) was found to decrease the bursts of Ca\textsuperscript{2+} channel activity (Suarez-Isla et al., 1986), consistent with our observation of decreased spark frequency.

DP4 is a synthetic peptide corresponding to a region of the central domain of RyR1 from Leu2442 to Pro2477. Most MH mutations are found in this domain or within an N-terminal domain. According to a ‘domain switch’ model proposed by Ikemoto, these two domains are putative regulatory domains that intricately interact with each other and are involved in the regulation of channel gating (Ikemoto and Yamamoto,
2002). In the resting or non-activated state, the N-terminal and central domains make contact with each other at several undetermined sub-domains, forming the ‘zipped’ configuration that promotes the closed state of RyR. Stimulation via E-C coupling or application of chemical agents weakens these inter-domain contacts, thereby lowering the energy barrier for RyR opening (Ikemoto and Yamatomo, 2002; Kobayashi et al., 2004). For MH mutants in either of these two domains, the domain switch is weakened, making the RyR hypersensitive to RyR agonists. DP4 is thought to weaken this inter-domain interaction, producing an MH-like activation/sensitization effect on the channel (El-Hayek et al., 1999; Yamamoto and Ikemoto, 2002; Shtifman et al., 2002; Kobayashi et al., 2004). Here we applied 150 µM DP4 to permeabilized fibers to mimic an MH episode and have shown that azumolene inhibited the Ca^{2+} spark frequency in a dose-dependent manner in the presence of DP4, whereas the Ca^{2+} spark properties were not changed. Compared to the inhibitory effect in normal fibers, an approximately 10-fold higher concentration of azumolene was needed to get the same inhibitory effect in the presence of DP4.

The cartoon in figure 7 integrates our current results with the inter-domain interaction model for single RyR channel gating. Azumolene (or dantrolene) might preferentially bind to the closed state of RyR (top of Fig. 7), promoting the domain-domain interaction and thereby stabilizing the closed configuration of RyR (Kobayashi et al., 2005). This stabilization would also be effective in the presence of DP4, which mimics an MH episode, if azumolene preferentially binds to the closed state of the channel (Fig. 7). The basis for the observed lack of effect of azumolene on the properties...
of the sparks that do occur would then depend on the number of interacting channels within the Ca\textsuperscript{2+} release unit and the nature of their interaction (see above).

In summary, we have investigated the effects of azumolene on local Ca\textsuperscript{2+} release in permeabilized frog skeletal muscle fibers and in a frog MH model generated by application of DP4. We found that azumolene significantly decreased the frequency of local Ca\textsuperscript{2+} release, presumably by reducing the RyR Ca\textsuperscript{2+} channel opening rate. Azumolene also decreased the frequency of local Ca\textsuperscript{2+} release events in an MH episode mimicked by DP4, which interferes with a domain-domain interaction within the RyR. This effect may reflect the mechanism of azumolene (or dantrolene) action during an MH episode, a stabilization of the closed state of RyR.
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References:


Footnotes:

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Legends for figures:

Figure 1. Effects of azumolene on spontaneous Ca\(^{2+}\) spark occurrence in permeabilized frog skeletal muscle fibers. Representative linescan images under control for DMSO (A), DMSO (0.2%, B), control for azumolene (C) and azumolene (1µM) + DMSO (0.2%), (D) conditions. Line scan images are presented for permeabilized frog skeletal muscle fibers bathed in internal solution containing the Ca\(^{2+}\) indicator Fluo-3. Images show the ∆F/F fluorescence resulting from line scan of a 138-µm length of fiber for 1.024s. 1µM azumolene decreased the Ca\(^{2+}\) spark events dramatically, while 0.2% DMSO sham solution had no apparent effect on spark frequency.

Figure 2. Azumolene decreases the calcium spark frequency in a dose-dependent manner. Ca\(^{2+}\) spark frequency in each fiber in the presence of the indicated azumolene was normalized to the average control frequency for that group of fibers prior to azumolene or sham solution change. Ca\(^{2+}\) spark frequency results are reported as means ± SEM of these normalized frequencies from N fibers divided by the mean normalized frequency for the shams at the same azumolene concentration. The line was obtained by fitting the data to the Hill equation (Eqn.1; \(f_{\text{max}}=1.00, f_{\text{min}}\geq 0.00\)), resulting in an EC50 of 0.25±0.12µM, a Hill coefficient of \(=1.44\pm0.99\), and \(f_{\text{min}}\) of 0.00±0.15sarc\(^{-1}\)s\(^{-1}\). \(R^2=0.93\).

Figure 3. Effects of azumolene on the distribution of individual spark spatio-temporal properties. Box plots indicate the 10, 25, 50 (i.e. median), 75 and 90 percentile values of the distribution (*, \(p<0.05\) compared to its matched sham). The boxes at 0 concentration
represent the properties of groups of shams, each matched to the azumolene concentration denoted by the same letter. The solid lines were obtained by fitting the median values of spark properties to the Hill equation (Eqn. 1; \( f \) now representing the indicated spark property). No systematic concentration dependent effects of azumolene were found on these spatio-temporal properties. The calcium spark numbers are: 778 for sham and 1101 for test at 0.0001 \( \mu \text{M} \) azumolene; 2349 for sham and 1258 for test at 0.001 \( \mu \text{M} \) azumolene; 2536 for sham and 1469 for test at 0.1 \( \mu \text{M} \) azumolene; 2488 for sham and 1466 for test at 0.01 \( \mu \text{M} \) azumolene; 4056 for sham and 2636 for test at 0.3 \( \mu \text{M} \) azumolene; 1622 for sham, and 453 for test at 1 \( \mu \text{M} \) azumolene.

Figure 4. Effects of azumolene on individual spark mass. Spark mass was calculated by equation 2. The boxes at 0 concentration represent the masses of shams matched to the azumolene concentration indicated by the same letter. The solid line was obtained by fitting the median values to the Hill equation (Eqn. 1; \( f = \text{spark mass} \)). There is no systematic concentration dependent effect of azumolene on spark mass. The numbers of sparks at each azumolene treatments are the same as in the previous figure. Box plots indicate the 10, 25, 50 (\( i.e. \) median), 75, 90 percentile values of the distribution (*, \( p<0.05 \) compared to its matched sham).

Figure 5. A. MH mimic episode generated by DP4. 150\( \mu \text{M} \) DP4 in an internal solution having a free Mg\( ^{2+} \) concentration of 1.2mM resulted in an 11-fold increase in spark frequency after 10 minutes exposure to a DP4-containing solution. The increased Ca\( ^{2+} \) spark frequency did not change further with time after changing the 150 \( \mu \text{M} \) DP4 internal
solution to another sample of the internal solution containing the same concentration of DP4 and waiting an additional 10 minutes. B. Azumolene decreases Ca$^{2+}$ spark frequency in a dose-dependent manner in the DP4 (150 µM) MH mimic system. The spark frequency of each fiber in the presence of the indicated DP4 and azumolene was normalized to the average frequency for that group of fibers in DP4 prior to azumolene or sham solution change. Ca$^{2+}$ spark frequency results are reported as means ± SEM of these normalized frequencies from N fibers divided by the mean normalized frequency for the shams at the same azumolene concentration. Fitting these data to equation 1 ($f_{\text{max}} = 1.00, f_{\text{min}} \geq 0.00$) resulted in an EC$_{50}$ of 2.35±4.02, Hill coefficient of 1.73±2.72, and $f_{\text{min}}$ of 0.00±1.14. $R^2=0.91$.

Figure 6. Effects of azumolene on individual spark spatio-temporal properties in the DP4-MH mimic model. Box plots indicate the 10, 25, 50 (i.e. median), 75, 90 percentile values of the distribution. The boxes at 0 concentration represent the properties of shams matched to the different azumolene concentration treatments. The solid lines were obtained by fitting the median values to the Hill equation (Eqn. 1) as in Fig. 3. No systematic concentration dependent effects of azumolene were found on these spatio-temporal properties. The calcium spark numbers are: 714 for sham ([DP4]=150 µM), 746 for DP4 (150 µM) + azumolene (0.04 µM); 341 for sham ([DP4]=150 µM), 332 for DP4 (150 µM) + azumolene (0.2 µM); 208 for sham ([DP4]=150 µM), 136 for DP4 (150 µM) + azumolene (2.24 µM); 207 for sham ([DP4]= 150µM), and 52 for DP4 (150µM) + azumolene (5 µM) treatment.
Fig 7. Cartoon model for azumolene modulation of the interactions between domains of RyR. Azumolene (AZ) binds to the N-terminal domain of closed RyR1 channels, promoting the stabilization of the closed ‘zipped’ state of the channel, even in the presence of DP4.
Figure 1

A. Control for DMSO

B. DMSO

C. Control for Azumolene

D. Azumolene

ΔF/F

20 μm

200 ms
Figure 2.
Figure 3

Rise Time

Temporal Half Duration

AMP

Spatial Half Width
Fig. 4

Spark Mass

[Azumolene] μM
Figure 5

A

![Bar graph showing Ca\textsuperscript{2+} Spark Frequency (sarc-1.s\textsuperscript{-1}) for Pre-DPA, DP4 first application, and DP4 second application.]

B

![Line graph showing Normalized Spark Frequency against [Azumolene] (\mu M).]

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Figure 6

- **Rise Time**
  - [Azumolene] μM vs. Rise Time (ms)
  - Data points and error bars for various [Azumolene] concentrations.

- **Temporal Half Duration**
  - [Azumolene] μM vs. FWHM (ms)
  - Data points and error bars for various [Azumolene] concentrations.

- **Spatial Half Width**
  - [Azumolene] μM vs. Amplitude (ΔF/F)
  - Data points and error bars for various [Azumolene] concentrations.

- **FWHM (μm)**
  - [Azumolene] μM vs. FWHM (μm)
  - Data points and error bars for various [Azumolene] concentrations.
Figure 7

[Diagram showing the interactions and calcium release process involving domains and calcium ions.]