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Enhanced Excitation-Coupled Calcium Entry (ECCE) in Myotubes Expressing Malignant Hyperthermia Mutation R163C is Attenuated by Dantrolene.

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Running Title: Dantrolene inhibits normal and MH susceptible ECCE

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ABBREVIATIONS:
BLM, bilayer lipid membrane; EC, Excitation-contraction; ECCE, excitation coupled calcium entry; EGTA, ethylene glycol-bis(2-aminoethylether)-N,N,N',N'-tetraacetic acid; FDB, flexor digitorum brevis; FKBP12, FK506 binding protein 12 kilodalton; HEPES, (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid); MH, malignant hyperthermia; *P*o, open probability; RyR1, ryanodine receptor type 1; SR, sarcoplasmic reticulum; SOCE, store operated calcium entry; τ_c, mean closed-dwell time; τ_o, mean open-dwell time; TG, thapsigargin.

Abstract

Dantrolene is the drug of choice for the treatment of malignant hyperthermia (MH) and is also useful for treatment of spasticity or muscle spasms associated with several clinical conditions. The current study examines the mechanisms of dantrolene's action on skeletal muscle and shows that one of dantrolene's mechanisms of action is to block excitation coupled calcium entry (ECCE) in both adult mouse flexor digitorum brevis fibers and primary myotubes. A second important new finding is that myotubes isolated from mice heterozygous and homozygous for the RyR1 R163C MH susceptibility mutation show significantly enhanced ECCE rates that could be restored to those measured in wild type (WT) cells after exposure to clinical concentrations of dantrolene. We propose that this gain of ECCE function is an important etiological component of MH susceptibility and possibly contributes to the fulminant MH episode. The inhibitory potency of dantrolene on ECCE found in wild type and MH susceptible muscle is consistent with the drug's clinical potency for reversing the MH syndrome, and is incomplete as predicted by its efficacy as a muscle relaxant.

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Dantrolene is a hydantoin derivative (1-[[[5-(4-Nitrophenyl)-2-furanyl]methylene]-imino]-2,4-imidazolidinedione) imino]-2,4-imidazolidinedione) that is currently the drug of choice for the treatment of the pharmacogenic syndrome malignant hyperthermia (MH) in humans (Gronert, 2004; Krause et al., 2004) and animals (Bjurström, 1995; Klont et al., 1994; Nelson, 1991; Roberts et al., 2001). MH is associated with acute increase in intracellular Ca^{2+} (Reulbach et al., 2007) resulting from exposure to volatile anesthetics and/or depolarizing muscle blockers (Ali et al., 2003; Gronert, 2004; Krause et al., 2004). Heat stress has also been clearly shown to trigger fulminant MH in susceptible mice (Chelu et al., 2006; Yang et al., 2006), although its etiological role in human MH and MH in other species is debated (Robinson et al., 2003). Dantrolene is also clinically useful for treatment of spasticity or muscle spasms associated with several clinical conditions including spinal cord injuries, stroke, multiple sclerosis, and cerebral palsy (Chou et al., 2004; Dressler and Benecke, 2005; Saulino and Jacobs, 2006; Verrotti et al., 2006). Dantrolene has been used successfully to mitigate symptoms arising from neuroleptic malignant syndrome (NMS) (Lappa et al., 2002; Strawn et al., 2007), but meta-analysis of 271 case reports has indicated an overall higher mortality rate when dantrolene monotherapy is used to treat NMS (Reulbach et al., 2007). The muscle relaxant properties of dantrolene have been closely correlated with its ability to both reduce basal resting free Ca^{2+} (Lopez et al., 1987) and evoked increases in Ca^{2+} in the myoplasm, although the exact mechanisms by which dantrolene interrupts the MH episode and promotes skeletal muscle relaxation are complex (Krause et al., 2004).

Although dantrolene shows selectivity as a skeletal muscle relaxant, with little or no effect on smooth or cardiac muscle, it has relatively low apparent potency. In humans, blood concentrations of 2.8-4.2 mg/L (~9-13 μM) are needed to depress the response of a

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single muscle twitch by 70%–75% (Flewellen et al., 1983) . Recent simulations of dantrolene's pharmacokinetic profile indicate that the European recommendations for treating MH result in 24 h plasma concentrations of 14 –18 mg/L (45-57 μM), whereas MHAUS guidelines result in plasma concentrations ranging from 7–23 mg/L (22.5-73μM) (Podranski et al., 2005).

The plasma concentrations of dantrolene needed to successfully treat clinical MH (Podranski et al., 2005), and those required to attenuation contractile force (1-10μM) (Krause et al., 2004), suggest that this drug may influence multiple homeostatic mechanisms that affect not only Ca^{2+} release from sarcoplasmic reticulum (SR) but also Ca^{2+} entry into the muscle cell. Whether all the effects of dantrolene on attenuating myoplasmic Ca^{2+} are mediated through a common pathway is not clear. The prevailing data support a view that ryanodine receptor type 1 (RyR1) is a direct and selective molecular target of dantrolene's muscle-relaxant action. RyR1 is the calcium release channel of sarcoplasmic reticulum that is essential for normal excitation-contraction (EC) coupling in skeletal muscle. [^{3}H]Dantrolene was shown to bind with high affinity (K_D=277 nM) to SR membrane preparations enriched in RyR1 and its binding site appears distinct from the site that recognizes the alkaloid ryanodine (Palnitkar et al., 1997; Parness and Palnitkar, 1995). Results from affinity labeling studies with [^{3}H]azido-dantrolene (Palnitkar et al., 1999; Paul-Pletzer et al., 2001) and site-directed antibodies (Paul-Pletzer et al., 2002) identified a potential dantrolene binding site in the N-terminal region bounded by AA 590-609. This region of RyR1 was used previously as a peptide to activate RyR1 and was defined by the authors as *domain peptide 1* (DP1) (El-Hayek et al., 1999). One mechanism proposed for dantrolene's inhibitory activity is through the stabilization of the interactions of two RyR1 domains AA 590-609 and AA 2442–2477. The second domain had also been

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used as a peptide to activate RyR1 and had been defined as DP4 (Yamamoto et al., 2000). Dantrolene has been shown to inhibit the ability of DP4 to enhance the binding of [^{3}H]ryanodine to SR membranes containing RyR1 (Kobayashi et al., 2005). It has been proposed that dantrolene stabilizes DP1-DP4 interactions in a manner that prevents the "unzipping" of these domains; a process that has been theorized to be necessary for transitioning from the closed to the open channel state; and thereby stabilizing the closed state of RyR1 channels (Kobayashi et al., 2005; Yamamoto et al., 2000; Yamamoto and Ikemoto, 2002a; Yamamoto and Ikemoto, 2002b). MH mutations within DP1 or DP4 had been suggested to act by destabilizing the closed channel state and contribute to MH susceptibility (Murayama et al., 2007). However no direct evidence has been presented to confirm either of these theories.

Attempts to demonstrate that the mechanism of dantrolene's action in both normal and MH muscle are solely the result of a direct and selective inhibition of RyR1 channels remains controversial. Fruen and coworkers demonstrated that dantrolene can inhibit both SR Ca^{2+} release and [^{3}H]ryanodine binding to SR vesicles isolated from both normal and MH swine skeletal muscle under special experimental conditions that require the presence of AMP-PCP (a non-hydrolyzable ATP analog) and calmodulin in a buffer containing the organic anion methylproprionate to substitute for chloride (Fruen et al., 1997; Zhao et al., 2001). However Szentesi and coworkers failed to inhibit the gating activity of RyR1 channels incorporated into bilayer lipid membranes (BLM) in the presence or absence of ATP (Szentesi et al., 2001). Dantrolene and its analog azumolene have been shown to reduce the resting myoplasmic free Ca^{2+} concentration ($[Ca^{2+}]_{rest}$) when it is administered to humans and animals *in vivo* when RyR1 channel activity is very low (Lopez et al., 1985; Lopez et al., 1987; Lopez et al., 2000; Lopez et al., 1992; Lopez et al., 1990). In addition,

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azumolene has been shown to inhibit a specialized form of store operated Ca^{2+} entry (SOCE) that is activated as a consequence of constraining an RyR1 open conformation produced by the presence of micromolar ryanodine and millimolar caffeine (Zhao et al., 2006). Whether these effects are the consequence of dantrolene modification of RyR1 conformation and Ca^{2+} channel gating was not demonstrated. Therefore it appears that dantrolene may have more than one mechanism through which it can influence Ca^{2+} fluxes.

In the present study, we demonstrate for the first time that one of dantrolene's mechanisms of action is to block excitation coupled calcium entry (ECCE) in both adult mouse flexor digitorum brevis (FDB) fibers and primary myotubes. Dantrolene's ability to block ECCE is independent of either its ability to inhibit RyR1 channel activity reconstituted in BLM or activate Ca^{2+} release triggered by EC coupling. The inhibitory potency of dantrolene on ECCE is consistent with the drug's clinical potency for reversing the MH syndrome, and is incomplete as would be predicted by dantrolene's efficacy as a muscle relaxant. A second important new finding is that myotubes isolated from heterozygous and homozygous mice possessing MH missense mutation R163C-RyR1 show significantly enhanced ECCE rates that could be restored to those measured in wild type (WT) cells after exposure to clinical concentrations of dantrolene. We propose that this gain of ECCE function is an important etiological component of MH susceptibility and possibly contributes to the MH episode. We identify inhibition of ECCE as novel mechanism for dantrolene's clinical efficacy as a muscle relaxant.

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Methods

Preparation of primary myotubes

Primary skeletal myoblast lines were isolated from 1- to 2-day old C57/B6 wild type (WT) mice and from newborn mice heterozygous (HET) and homozygous (HOM) for point mutation R163C-RyR1 MH (Yang et al., 2006) as previously described (Cherednichenko at al., 2004; Hurne et al., 2005; Rando and Blau, 1997). The myoblasts were expanded in 10 cm cell-culture treated Corning dishes coated with collagen (Calbiochem, Richmond, CA) and were plated onto 96-well µ-clear plates (Greiner) coated with MATRIGEL (Becton Dickinson) for Ca^{2+} imaging studies. Upon reaching ~80% confluence, growth factors were withdrawn and the cells allowed to differentiate into myotubes over a period of 3 days.

Preparation of FDB fibers

Flexor digitorum brevis (FDB) muscles were dissected from adult mice (C57/B6) and single intact FDB myofibers were enzymatically isolated as previously described (Brown et al., 2007). Following isolation, FDB's were plated on ECM (Sigma) coated glass bottom imaging dishes (Matek) and maintained in DMEM (GIBCO) supplemented with 0.2% BSA and 100 mM gentamicin (Sigma). Fibers were kept overnight in a 5% CO_2 incubator and experiments were conducted within 12-24 hours of plating. Dishes of myofibers were randomized to control treatment (0.1% DMSO), ryanodine (Calbiochem) treatment (250µM, ~ 1 hour), or ryanodine treatment followed by a 10 min application of dantrolene (10µM).

Ca^{2+} and Mn^{2+} imaging

Differentiated primary myotubes were loaded with 5 µM Fura-2-AM to measure the rate of dye quench by Mn^{2+} entry, or Fluo-4-AM to measure Ca^{2+} transients (Molecular Probes, Eugene, OR) at 37°C for 20 min in imaging buffer (in mM; 125 NaCl, 5 KCl, 2

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$CaCl_2$, 1.2 $MgSO_4$, 6 dextrose, and 25 HEPES, pH 7.4 supplemented with 0.05% bovine serum albumin). The cells were then washed 3 times with imaging buffer and transferred to the stage of a Nikon Diaphot inverted microscope and illuminated at the isosbestic wavelength for fura-2 or 494 nm for fluo-4 with a Delta Ram excitation source (Photon Technology International; PTI, Lawrenceville, NJ). Fluorescence emission at 510nm was captured from regions of interest within each myotube from 3-10 individual cells at 5 frames per second using an Olympus 40x oil na 1.3 objective, IC-300 ICCD camera and digitized and analyzed with ImageMaster software (PTI). When used, electrical field stimuli were applied using two platinum electrodes fixed to opposite sides of the well and connected to an AMPI Master 8 stimulator set at 3V, 25ms bipolar pulse duration, over a range of frequencies (0.05-20 Hz; ~40 sec pulse train duration). Alternatively, higher frequency stimuli were applied to the cells at 30 Hz for 1.5 sec (7V; 1ms bipolar pulse duration) as described above and fluo-4 fluorescence emission from individual cells was measured at 100Hz using photometry (PTI, model 614). In some experiments, KCl was focally applied for 2 sec to depolarize the cells using Multivalve Perfusion System (Automate Scientific Inc., Oakland, CA). When K^+ (40mM) was applied the concentration of Na^+ was lowered accordingly to preserve osmolarity in the external medium.

Mn^{2+} influx into myotubes was measured as previously described, or with minor modification (Clementi et al., 1992; Fessenden et al., 2000). Final concentrations of 500 µM $MnCl_2$ and 1.2 mM Mg^{2+} were added to a nominally Ca^{2+}-free (~7µM free Ca^{2+}) external solution to measure Mn^{2+} entry rates in response to depolarization. Independent experiments were performed with the external solution containing 300 µM Ca^{2+} to determine if competition between Mn^{2+} and Ca^{2+} affected the rates of Mn^{2+} quench

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attributed to ECCE. Cells were stimulated by electrical field or with focal application of K^+ or as described above.

Mn^{2+} influx into FDB's was performed using dishes of adhered FDBs loaded with Fura-2 AM (5μM; 20 min) in normal mouse Ringer (NR) containing (in mM; 146 NaCl, 4.7 KCl, 0.6 $MgSO_4$, 1.6 $NaCO_3$, 0.13 NaH_2PO_4, 7.8 glucose, 20 HEPES and 1.8 $CaCl_2$). Fura-2 fluorescence was imaged on an inverted microscope (Olympus IX-50, 40x H_2O objective 1.15 na) equipped for fluorescence excitation (Lambda DG-4) and coupled to a CCD based imaging system (CoolSnap HQ CCD camera) all controlled by IP Lab 4.0 (BD Biosciences). During the study the myofibers were excited at the isosbestic wavelength of fura-2 (360 ±7nm) (Klein et al., 1988), and images of emission intensity (510nm) sampled for 50 ms at a rate of 1Hz for 3-5 min. During the imaging period, the myofibers were continuously superfused (~ 2 ml/min) first with normal ringer (NR) followed by perfusion with manganese containing ringer (Mn-R) in which Ca^{2+} (1.8 mM) was replaced with equimolar [Mn^{2+}]). Following a ~ 1 min application of Mn-R, fibers were superfused with Mn-R ringer containing 40mM K^+ (adjusted for ionic balance by lowering Na^+ in the buffer).

The rate of and extent of changes in Ca^{2+} measured with fluo-4 and the quench rate of fura-2 fluorescence measured in both myotube and myofiber preparations were determined by linear least-squares regression (OriginLab 7.5) and expressed as Means±SEM. Statistical analysis was performed in SigmaStat (v. 3.1; Jandel) using one-way ANOVA and Tukey's post-hoc to establish significance ($p<0.05$).

Recording and analysis of RyR1 single channel activity in bilayer lipid membranes

Preparation of SR Membranes and purified RyR1. Junctional SR membranes enriched in RyR1 were prepared from skeletal muscle of New Zealand White rabbits and

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stored in 10% sucrose, 10mM HEPES, pH 7.4, at -80°C until needed for solubilization in CHAPS detergent as previously described (Pessah et al., 1986).

RyR1 was purified from the CHAPS-solubilized proteins by column chromatography through Sephacryl S-300 HR (Amersham Biosciences) and the RyR1 peak further purified on a 5-20% (W/V) linear sucrose gradient. The ~30S fraction containing enriched RyR1 was then concentrated on a HiTrap Heparin HP column (Amersham Biosciences) (Samso et al., 2005). Purity of RyR1 was assessed by SDS-PAGE and silver stain. For preparation of the RyR1–FKBP12 complex, purified RyR1 was supplemented with an eightfold molar excess of recombinant FKBP12 (Sigma-Aldrich) immediately before channel reconstitution experiments.

Measurement and analysis of purified RyR1 single channel reconstituted in planar lipid bilayer. Bilayers were composed of phosphatidylethanolamine: phosphatidylserine: phosphatidylcholine (5:3:2 w/w, Avanti Polar Lipids, Inc., AL) dissolved in decane at a final concentration of 30mg/ml across a 200µm aperture on a polysulfone cup (Warner Instrument Corp., CT). The bilayer partitioned two chambers (*cis* and *trans*) with buffer solution (in mM) 500 CsCl, defined free Ca^{2+} (buffered by certain EGTA calculated according to the software Bound and Determined) and 20 HEPES-Tris (pH 7.4) on *cis*, 500 or 50 CsCl and 20 HEPES-Tris (pH 7.4) on *trans*. The addition of protein was made to the *cis* solution that was held at the virtual ground, whereas the *trans* solution was connected to the head stage input of an amplifier (Bilayer Clamp BC 525C, Warner Instrument, CT). BLM-TC Planar Lipid Bilayer Thermocycler (Warner Instruments) was used to control the recording temperature throughout the experiment at a setting of either 25^{o}C or 35^{o}C. After supplementation of purified RyR1 with a molar excess of FKPB12, single channels were reconstituted by introducing the FKBP12-RyR1 protein preparation in the *cis* chamber.

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Single channel gating was monitored and recorded at a holding potential of -40mV (applied to the *trans* side). The sidedness (cytosolic) of the channel was verified by the positive response to addition of micromolar Ca^{2+} once the channel was reconstituted. Additionally the response of most channels to 2μM ryanodine or ruthenium red was tested at the end of the experiment. The amplified current signals, filtered at 1 kHz (Low-Pass Bessel Filter 8 Pole, Warner Instrument) were digitized and acquired at a sampling rate of 10 kHz (Digidata 1320A, Axon-Molecular Devices, Union City, CA). All the recordings were made for at least 2 to 10 min under each defined experimental condition. The channel open probability (P_o), mean open-, and mean closed-dwell times (τ_o and τ_c) were obtained by using Clampfit, pClamp software 9.0 without further filtration (Axon-Molecular Devices, Union City, CA).

Reagents

Dantrolene sodium, calmodulin, and FKBP12 were obtained from Sigma-Aldrich. Fura-2 AM and Fluo-4 AM were obtained from Molecular Probes. Dantrolene was dissolved in dried DMSO (HPLC grade). All other reagents were of the highest purity commercially available.

Results and Discussion

Dantrolene suppresses electrically evoked Ca^{2+} transients but does not directly inhibit RyR1 in BLM

We assessed if dantrolene (10μM) could depress the amplitude of Ca^{2+} transients evoked in intact wild type skeletal myotubes loaded with fluo-4 in response to repetitive 30Hz electrical pulse trains lasting 1.5 sec (1 ms bipolar pulse duration). Under these conditions the rising phase of the Ca^{2+} transient reached a plateau within 500 ms and the

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maximum amplitude persisted until termination of the stimuli (Fig. 1A&B). Responses from individual myotubes were obtained before and 10 min after the application of dantrolene (10μM) into the external bath. Figure 1 (panels A&B) showed that the maximum amplitudes of the triggered Ca^{2+} transients were depressed by 18±5% within 10 min of dantrolene application ($p<0.05$), and that this effect was completely reversed within 10 min of wash-out with the same external medium lacking the drug (Fig. 1 A&C). Dantrolene under these experimental conditions did not significantly ($p>0.1$) influence either the initial rate of rise (Fig. 1 B) or the rate of decay of the Ca^{2+} transients upon termination of the pulse train. The decay rates, which reflect cessation of SR Ca^{2+} release and the re-sequestration of myoplasmic Ca^{2+}, were not significantly different before (295±35 counts/ms) and 10min after (277±33 counts/ms) exposure to dantrolene ($p>0.1$). The fact that relaxation of the Ca^{2+} transients upon termination of the pulse trains was unaffected indicates that dantrolene did not impair SR Ca^{2+} uptake. Although subtle, the observed reduction in the Ca^{2+} transient amplitude in the presence of dantrolene was a consistent observation and reflects a reduction in the rapid release of SR Ca^{2+} triggered by EC coupling in skeletal myotubes. These data are consistent with dantrolene's proposed mechanisms of direct attenuation of RyR1 activity (Fruen et al., 1997; Paul-Pletzer et al., 2001; Paul-Pletzer et al., 2002). However, given dantrolene's relatively low (micromolar) potency for attenuating Ca^{2+} transient amplitude and contractility, its pharmacological activity could be the result of additional yet undiscovered mechanisms and these were further investigated.

To directly assess the possible inhibition of RyR1 activation by dantrolene, we therefore reconstituted purified RyR1/FKBP12 complexes in bilayer lipid membrane (BLM) and directly studied the effects of dantrolene on single channel gating kinetics. Figure 2A

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shows the high molecular band of solubilized RyR1 protomer purified by column chromatography and visualized by SDS-PAGE and silver stain. Purified RyR1 was incubated with recombinant FKBP12 before reconstitution in BLM and recording channel gating activity. The first trace of Figure 2B shows the activity of a representative RyR1 channel in the presence of CaM (2 μM), ATP (2 mM), and Ca^{2+} (10μM) on the cytoplasmic (*cis*) side at 25^{o}C. Addition of 10 μM dantrolene to the *cis* chamber did not change gating parameters including channel open probability (Po) or open and closed dwell time constants (τ_o and τ_c, respectively) over a 10 min recording period (trace 2 shows gating parameters for >50 s of continuous recording). Dantrolene was then increased to 20μM in the *cis* solution of the BLM chamber at which time addition of 10μM dantrolene was made to the *trans* (lumenal) side, but the drug failed to affect changes in channel gating parameters over an additional 10 min of recording (Fig 2B, third trace). RyR1 channel activity was also studied under conditions where the BLM solutions where equilibrated at 35^{o}C to test the possible temperature dependence of dantrolene inhibition of RyR1 (Fruen et al., 1997). The fourth and fifth traces in Figure 2B showed that, under these conditions, addition of CaM (1 μM) to the *cis* chamber rapidly enhanced channel Po as a result of prolonging τ_{o1} and shortening τ_c (traces 4 and 5). Subsequent addition of 10 and 20 μM dantrolene to the *cis* chamber failed to diminish channel gating parameters over the 20 min of recording (traces 6 and 7), although the channel remained responsive to addition of ryanodine and ruthenium red to the *cis* chamber (Fig 2B, bottom traces).

Collectively the data presented in Figures 1 and 2B suggest that dantrolene is unlikely to directly interact with the minimal functional unit of the channel complex (i.e., the FKBP12-RyR1-CaM complex) to attenuate the gating activity of the Ca^{2+} release channel, even when measurements are made at physiological temperatures for mammalian skeletal

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muscle (*e.g.,* 35^{o}C). These results are consistent with and extend the findings of Szentesi and coworkers who failed to identify detectable inhibition of ATP-activated RyR1 channels reconstituted in BLM (Szentesi et al., 2001). Recently Zhang and coworkers reported that although dantrolene's structural analog azumolene depressed spark frequency in permeabilized frog skeletal muscle fibers, it did not significantly alter the properties of individual spark events (Zhang, 2005). Compounds known to directly bind to RyR1, or affect channel activity through their influences on protein-protein interactions known to occur within the triadic complex, have been shown to alter the intensity and spatial spread of individual sparks in addition to their frequency in a manner consistent with their influence on single RyR1 channel kinetics (Gonzalez, 2001). Since we failed to demonstrate a direct action of dantrolene on the functioning FKBP12-RyR1-CaM channel complex, we therefore examined the possible influence of dantrolene on two different forms of Ca^{2+} entry, SOCE and ECCE, as possible mechanisms contributing to dantrolene's pharmacological activity as a muscle relaxant.

Dantrolene does not suppress SOCE elicited by SR store depletion

To test dantrolene's possible inhibition of SOCE induced by SR store depletion, fluo-4 loaded skeletal myotubes were exposed to thapsigargin (200 nM) to block the SR/ER Ca^{2+} ATPase (SERCA) for 10 min in nominal Ca^{2+} free external medium. Under these extreme conditions of chronic SR Ca^{2+} store depletion, most cells (>90%) failed to respond to electrical stimulation or activation by caffeine (i.e., their Ca^{2+} stores were fully depleted), and the cells that did not respond were chosen for further analysis. When 2 mM Ca^{2+} was added back to the external solution, a large Ca^{2+} transient was observed as a result of rapid depletion-activated Ca^{2+} entry, assumed to be mediated by activation of

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SOCE channels within the plasma membrane. In all cells rapid depletion-activated Ca^{2+} entry was observed. Neither the rate nor the magnitude of the Ca^{2+} transient attributed to SOCE was influenced by the presence of 10 μM dantrolene (Fig. 3).

Dantrolene suppresses ECCE elicited by electrical pulse trains or KCl depolarization

Since dantrolene failed to have measurable effect on the gating activity of purified reconstituted FKBP12-RyR1-CaM channels or on SR depletion-activated SOCE, we next tested whether or not dantrolene may inhibit ECCE. Using the Mn^{2+} quench technique with fura-2 loaded WT myotubes, the quench of fura-2 fluorescence was measured before (Fig. 4 trace "Ctrl") and during electrical stimulation with 20 Hz electrical pulse trains lasting ~40 s in a nominally Ca^{2+} free external buffer containing 500 μM Mn^{2+}. Under these assay conditions, electrical stimulation of the cells resulted in rapid enhancement of the rate of Mn^{2+} quench of fura-2 that we attribute to ECCE (Fig. 4A). If cells were pre-incubated with 10 μM dantrolene for 10 min prior to applying the electrical pulse train, the initial rate of Mn^{2+} quench was reduced by 72±4% ($p<0.001$).

Ryanodine has been previously shown to transiently activate then persistently block RyR1 channel activity in a concentration and time-dependent manner (Pessah and Zimanyi, 1991). Pretreatment of RyR1 with ryanodine in intact myotubes has been shown to accentuate ECCE by slowing its inactivation/deactivation (Cherednichenko at al., 2004; Hurne et al., 2005). In the present study, we pre-treated myotubes with 500 μM ryanodine for 30 min. Under these conditions, the cells failed to respond to 20 mM caffeine (data not shown), indicating that ryanodine blocked RyR1 activity. Never the less, stimulation of the same cells with a 20Hz pulse train resulted in a large Ca^{2+} transient, which we attributed to the rapid entry of extracellular Ca^{2+} into the cells *via* ECCE (Fig 5A). Dantrolene inhibited ECCE in ryanodine-pretreated myotubes in a dose dependent manner with an IC_{50} of 4.2

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±1.9 μM but failed to completely inhibit ECCE, reaching a maximum inhibition of 69% of the control rate between 10 and 50 μM dantrolene (Fig. 5A, inset). Dantrolene also inhibited the rate of Mn^{2+} quench triggered by electrical pulse trains delivered to cells not pretreated with ryanodine in an external medium containing 500 μM Mn^{2+} and nominally free of Ca^{2+} with a similar dose-response characteristic as ryanodine pre-treated cells tested for ECCE (Fig. 5B *vs.* 5A, respectively).

ECCE in FDB fibers is inhibited by dantrolene

The existence of ECCE in adult skeletal muscle fibers has not been directly investigated previously. We therefore performed experiments with adult FDB skeletal muscle fibers (FDBs) dissected from WT adult mice as described in Methods. Figure 6A shows representative traces of Mn^{2+} quench before and after sequential addition of ryanodine (Ry) to block RyR1 and 40 mM K^+ external potassium to depolarize the fiber. This protocol permitted direct assessment of ECCE in FDBs. As previously observed in myotubes, FDBs exposed to ryanodine in this manner, failed to respond to 20mM caffeine, indicating complete block of RyR1-mediated Ca^{2+} release (data not shown). Upon addition of 40 mM external K^+, the rate of Mn^{2+} quench increased an average of >6.5- fold (n= 23; $p<0.001$; Fig 6B, bar labeled Ry). Importantly ECCE triggered by addition of 40 mM K^+ to the external medium is also clearly observable in the absence of ryanodine pre-treatment (Fig 6A, trace “Ctrl”), reflected as a 25% increase in the rate of Mn^{2+} entry (n=8; p=0.03; Fig 6C, bar labeled Ctrl) in response to depolarization. Ryanodine pre-treated FDBs were exposed to dantrolene (10 μM) 10 min prior to testing for ECCE. As would be predicted from our results with myotubes, dantrolene reduced the rate of Mn^{2+}-mediated fura-2 quench by 62% compared to cells exposed to ryanodine alone (n=19; $p<0.001$; Fig 6B, bar labeled Ry+Dan). Collectively these data show that ECCE is present in both naïve skeletal

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myotubes and adult fibers of WT mice. Importantly ECCE can be attenuated by clinically relevant concentrations of dantrolene in fibers pre-treated with ryanodine. Initial results from a limited number of FDB fibers not pre-treated with ryanodine also indicates a tendency for dantrolene to reduce the rate of Mn^{2+} quench elicited by K^+ depolarization (Figure 6A and C), although the variance associated with these measurements necessitates a larger number of fibers be tested to obtain sufficient statistical power.

Enhanced ECCE in myotubes expressing R163C-RyR1 is restored by dantrolene

To determine whether or not dantrolene's effects on ECCE were relevant to its ability to prevent or treat MH, myotubes prepared from WT, HET, and HOM R163C-RyR1 mice were tested for their rates of Mn^{2+} quench elicited by 20 Hz electrical pulses described above and the effects of dantrolene on these quench rates. In these experiments the extracellular Mn^{2+} was set at 500 μM, but instead of the nominally Ca^{2+}-free external solution used in experiments shown in Fig. 4 and Fig. 5B, external Ca^{2+} was set to 300 μM Ca^{2+}. Under these experimental conditions (500 μM Mn^{2+} + 300 μM Ca^{2+}), the Mn^{2+} quench rates triggered by 20 Hz pulse trains were approximately 50% of those measured in nominally Ca^{2+}-free solutions. Figure 7 shows that the rate of Mn^{2+} quench elicited by this stimulus protocol was enhanced 1.8- and 2-fold for HET and HOM R163C-RyR1 myotubes compared to WT ($p<0.0001$). When the myotubes were pretreated with 10 μM dantrolene 10 min prior to measuring quench rates using solvent as a control, it had profound effects on ECCE similar to what we observed in WT myotubes. Figure 8 shows that dantrolene reduced ECCE rates (measured as the rate of Mn^{2+} entry elicited by a 20Hz electrical train, ~40 sec in duration) to 60-70% of the respective control rate irrespective of genotype. In this regard, dantrolene was able to lower ECCE rates in HET and HOM myotubes to below those measured in WT myotubes in the absence of

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dantrolene (Fig. 8), but was not able to lower them to the rate of dantrolene treated WT cells, suggesting that additional mechanisms might also be important in the actions of dantrolene.

ECCE was first identified in skeletal myotubes were it was found essential for sustaining the amplitude of the Ca^{2+} transient in response to prolonged electrical pulse trains or KCl depolarization in a manner independent of SR Ca^{2+} store depletion (Cherednichenko at al., 2004; Hurne et al., 2005). Here we provide the first evidence that ECCE is also operant in mature adult skeletal muscle fibers (i.e., FDBs) and implicates a potential physiological role for ECCE in adult skeletal muscle. A defining characteristic of ECCE seen in both FDBs and myotubes is that the magnitude of ECCE (defined by the rate of Mn^{2+} quench triggered by addition of K^{+} to the external medium) is greatly enhanced when measured after blocking RyR1 with micromolar ryanodine; a concentration sufficient to eliminate responses to caffeine.

It is well established that exposure of mice to ryanodine and its derivatives triggers hyper-contraction of skeletal muscle with rapid onset that is invariably lethal (Waterhouse et al., 1987). To date skeletal muscle hyper-contraction elicited by ryanodine has been interpreted as being solely the result of stabilizing a persistent open state of RyR1 (Meissner, 2002). However, ryanodine has been shown to stabilize multiple RyR1 channel conductance states, and predominating among them is a highly stable fully closed state (Bidasee et al., 2003; Buck et al., 1997; Zimanyi et al., 1992). The novel observation reported here is that the ryanodine-modified RyR1 state in which ryanodine completely locks the RyR1 channel in a non-conducting conformation greatly enhances ECCE in myotubes and adult fibers, suggesting a potentially important role for Ca^{2+} entry in the etiology of ryanodine-induced hyper-contraction. In support of this hypothesis the actions

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of ryanodine have been documented to be dependent on the activity of the EC coupling elicited by transverse (T) tubule depolarization (Hillyard and Procita, 1959; Procita, 1956; Procita, 1958). Moreover evidence for the role of ECCE in normal use-dependent physiology and pathophysiology of adult skeletal muscles may have been described in earlier studies, but underappreciated. For example the rate of relaxation of contractures after prolonged applications of extracellular K^+ was faster in the absence of extracellular Ca^{2+} than in its presence (Caputo and Gimenez, 1967), whereas brief depolarization in Ca^{2+} free solutions was not different than the response in Ca^{2+} replete external medium (Caputo and Gimenez, 1967; Grabowski et al., 1972). More recently, age-related denervation of fast skeletal muscle was found in senescent mice, and muscle fibers isolated from them were found to show dependence on extracellular Ca^{2+} to maintain tetanic force (Payne et al., 2007).

Results from the present study also identify ECCE as a target of dantrolene's pharmacological actions in both FDBs and myotubes prepared from WT mice. The potency (IC_{50}= 4 μM) and limited maximum efficacy (60-70% diminution) of dantrolene toward attenuating ECCE is consistent with its pharmacological activity as a muscle relaxant. The efficacy of 10 μM dantrolene towards attenuating ECCE is similar in naïve and ryanodine-exposed preparations in both models, suggesting that a common mechanism is involved. Collectively these results confirm that myotubes represent a predictive and useful model of adult fibers in which to study physiological, pathophysiological and pharmacologic processes associated with ECCE. Dantrolene's inhibitory activity could be measured in both native and ryanodine-treated myotubes by monitoring either Mn^{2+} (Figs. 4 & 5B) or Ca^{2+} (Fig. 5A) entry. In this regard, Mn^{2+} and Ca^{2+} appear to compete for a common ECCE influx pathway since the rate of Mn^{2+} quench of fura-2 is reduced by approximately 2-fold

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when the Ca^{2+} concentration in the external buffer is raised from nominally free Ca^{2+} (Fig. 4) to one that contains 300 μM Ca^{2+} (Fig. 7 and 8). As a skeletal muscle relaxant dantrolene is active in the dose range of 1-10 μM and it produces a maximum of 60-70% attenuation of contractile force (Gronert, 2004; Krause et al., 2004).

Measurements of RyR1 channel gating kinetics in the BLM preparation have previously failed to support (Szentesi et al., 2001) the biochemical evidence that is the basis for the hypothesis that the muscle relaxant properties of dantrolene result from a direct interaction with RyR1 domains that stabilize the closed conformation of the channel (Kobayashi et al., 2005; Palnitkar et al., 1999; Palnitkar et al., 1997; Paul-Pletzer et al., 2001; Paul-Pletzer et al., 2002). Several prominent methodological differences between studies of single channels reconstituted in BLM and previous biochemical and biophysical studies could account for the discrepancy. For example Fruen and colleagues demonstrated the importance of CaM, ATP and temperature in order to measure dantrolene's inhibition of [^{3}H]ryanodine binding to skeletal muscle membranes and enhanced SR Ca^{2+} mobilization from SR vesicles elicited by addition of caffeine (Fruen et al., 1997; Zhao et al., 2001). However, despite the fact that the conditions used in our BLM studies closely mimicked those used by Fruen and coworkers, they still do not support a direct mechanism by which dantrolene promotes channel closure at 35^{o}C in the presence of CaM and ATP. Moreover, the RyR1 channels remained responsive to ryanodine even in the presence of dantrolene. While the present results do not refute the presence of [^{3}H]dantrolene or [^{3}H]azido-dantrolene binding sites on RyR1, they do not support the interpretation that occupation of these sites suppresses SR Ca^{2+} release from a given Ca^{2+} release unit, nor that it interferes with the gating activity of RyR1 channels. Our results do support the concept that dantrolene binding to the RyR1 complex could weaken its

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functional coupling to ECCE in native or ryanodine-modified states without affecting its inherent channel activity. It is important to note that attenuation of ECCE in intact skeletal muscle cells represents the first evidence of a pharmacologically relevant mechanism by which dantrolene suppresses sarcolemmal Ca^{2+} entry in response to a physiologically relevant trigger (*i.e.,* membrane depolarization). By contrast, its proposed inhibition of a specific type of SOCE could only be demonstrated when stores were depleted by caffeine in combination with a ryanodine modified RyR1 (Zhao et al., 2006).

We have previously shown that point mutations C4958S and C4961S changes in the conformation of these Ca^{2+} channels to a persistent inactive state that can in turn influence ECCE inactivation/deactivation (Hurne et al., 2005). Similar influences on ECCE were reported in ryanodine pre-treated myotubes (Cherednichenko et al., 2004). In the present paper, we demonstrate enhanced ECCE in R163C HET and HOM myotubes that could be reduced by 10μM dantrolene to a rate similar to that seen in naïve WT cells. It remains to be seen if enhanced rates of ECCE in MH myotubes could represent a risk factor for triggering clinical MH with halogenated general anesthetics and if triggering agents further exacerbate the rate of ECCE in MH muscles to that observed with ryanodine-modified channels. A role for ECCE in MH susceptibility, clinical MH and possible other disorders of skeletal muscle deserves further attention.

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Footnotes

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Figure Legends

Figure 1. Dantrolene depresses Ca^{2+} transient amplitude in skeletal myotubes. **A)** Representative EC coupling response from WT myotubes triggered by repetitive pulse trains of 30 Hz (1ms pulse duration) before (Ctrl), 10 min after perfusion of 10 μM dantrolene (Dan), and 10 min after initiating wash-out of the drug (Wash). The records shown were taken from continuous measurements of a single myotubes. **(B)** Expanded time scale showing the initial rate of intracellular Ca^{2+} rise attributed to SR Ca^{2+} release during the first 100 ms of EC coupling elicited by a 30Hz pulse train before (Ctrl) and 10 min after (Dan) application of dantrolene (Dan=10 μM). The initial rate of Ca^{2+} rise was limited by the affinity of Ca^{2+} binding to Fluo-4. **C)** Mean and S.E. of normalized Ca^{2+} transient amplitude in n=15 cells before (Ctrl), and 10 min after application of 10 μM dantrolene (Dan), and after 10-min of wash-out period (Wash). Measurements of intracellular Ca^{2+} were acquired at 100Hz using photometry of individual myotubes loaded with fluo-4 as described in Methods.

Figure 2. Dantrolene does not attenuate gating properties of reconstituted RyR1 channels at either 25°C or 35°C. (A) SDS-PAGE gel showing typical purity of RyR1 preparation used in BLM reconstitutions. **(B)** Recording and analysis of purified RyR1-FKBP12 single channel activity were made as described in detail in the Methods. The specific compositions of the cytoplasmic (*cis*) and lumenal (*trans*) solutions are indicated above each representative trace. Upper three traces show representative segments of recordings obtained from a channel at 25°C lasting >20 min after addition of dantrolene to the *cis/trans* solutions. This entire experiment was performed on two independent bilayer

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experiments. The remaining traces (traces 4-8) were obtained from an independent BLM experiment that incorporated two channels equilibrated in *cis/trans* buffers at 35^{o}C. Results from this experimental protocol were replicated in n=4 independent experiments with the same results. Open probability (Po) and mean open/closed dwell time values (τ_o/τ_c) were calculated using Clampfit 9.0 and are denoted above each representative trace. For the two-channel's mean open time values, only level one (τ_{o1}) was presented, whereas Po represents the open probability of the two channels. The dashed line with "c" indicates the zero current level.

Figure 3. Dantrolene does not interfere with SOCE. A) Store-operated Ca^{2+} entry (SOCE) was measured under extreme conditions of chronic store depletion. Fluo-4 loaded myotubes were challenged with 200 nM thapsigargin (TG) an irreversible blocker of SR Ca^{2+} pump in the presence (Dan) or absence (Ctrl) of 10 μM dantrolene. At the end of TG treatment ($\geq$10 min; depletion phase not shown), ~90% of the cells failed to respond to electrical stimulation and caffeine (not shown). After depletion, the external solution was changed to one containing 2 mM Ca^{2+} and the rate of SOCE monitored. **B)** Summarized data of experiments shown in panel A for n = 24 (Ctrl) and n = 25 treated (Dan).

Figure 4. Dantrolene inhibits Mn^{2+} entry triggered by electrical stimulation of primary myotubes. A) Primary WT myotubes exhibited enhanced Mn^{2+} entry (500 μM in a nominally Ca^{2+}-free external solution) in response to a 20 Hz train of electrical pulses (ES) monitored by the rate of quench of fura-2 fluorescence excited at the isosbestic wavelength (trace "Ctrl"). Pretreatment of myotubes for 10 min with 10 μM dantrolene

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(Dan) reduced the initial rate of Mn^{2+} entry by 72%. **B)** Summarized data of experiments shown in panel A for n = 18 Ctrl and n = 24 Dan cells. *p<0.001

Figure 5. Dantrolene inhibits ECCE and Mn^{2+} entry in a dose-dependent manner.

A) Pre-treatment of WT myotubes with 500 μM ryanodine for 30 min locked RyR1 in an inactive conformation unresponsive to caffeine (not shown). Despite the lack of any response to caffeine in cells loaded with fluo-4, depolarization of ryanodine-treated myotubes triggered a large extracellular Ca^{2+} entry (ECCE) that persisted for the duration of electrical stimulation (ES) indicated by the representative trace labeled "0". Dantrolene (2–50 μM) inhibited ECCE in ryanodine-treated WT cells in a dose-dependent manner with IC_{50} of 4.2 μM (**Inset**). **B)** Mn^{2+} entry rate in fura-2 loaded WT myotubes (not blocked with ryanodine) was also inhibited dose dependently by dantrolene. n = 12 cells in panel A and n = 12-20 cells in panel B.

Figure 6. Adult skeletal muscle fibers exhibit ECCE that is inhibited by dantrolene.

A) WT adult FDB myofibers were assayed with the Mn^{2+} quench assay for the presence of ECCE. Fura-2 loaded myofibers were excited at 360 nm and emission was collected at 510 nM. Bath perfusion of normal mouse Ringer was followed by perfusion with Mn^{2+} containing Ringer (bar labeled Mn^{2+}), then Mn^{2+} Ringer containing 40 mM K^+ (bar labeled KCl). Dishes of myofibers were randomized to control treatment (no drug; Ctrl), dantrolene treatment (+Dan), ryanodine treatment (250μM, ~ 1 hour; Ry), or ryanodine treatment followed by a 10 minute application of Dantrolene (10μM; Ry+Dan). **B**) Summarized data from 23 and 19 fibers for Ry, and Ry+Dan treatments, respectively. **C**) Summarized data from 9 fibers for Ctrl and Dan.

The rates of Mn^{2+} quench of fura-2 during depolarization with K^+ were normalized to the quench rate before depolarization of each fiber. ANOVA revealed that mean rates were significantly enhanced after depolarization compared to before depolarization for each treatment group (*= $p<0.05$).

Figure 7. Myotubes expressing R163C-RyR1 have an exaggerated ECCE.

A) Rates of Mn^{2+} entry were measured in fura-2 loaded myotubes prepared from WT mice and mice heterozygous (HET) or homozygous (HOM) for MH susceptibility mutation R163C-RyR1. The rates of Mn^{2+} quench were measured in an external buffer containing 500 μM Mn^{2+} + 300 μM Ca^{2+} (bar labeled Mn^{2+}) before and after delivery of a 20 Hz electrical pulse train (ES). **B)** Summarized data of mean rate of Mn^{2+} quench for n = 50 WT, n = 69 HET, and n = 69 HOM myotubes. Rates of electrically evoked Mn^{2+} entry for WT was significantly lower than either HET or HOM (*$p<0.0001$).

Figure 8. Dantrolene attenuates ECCE in WT, HET and HOM R163C-RyR1 expressing myotubes. Mytotubes were challenged with electrical pulse trains and monitored for Mn^{2+} entry as described in Fig 7. Another group of cells were treated with 10 μM dantrolene for 10 min. Data represents the mean rate calculated from responses of n = 20 (WT), 31 (HET), and 43 (HOM) cells. *$p<0.001$ compared to corresponding non-treated genotype.

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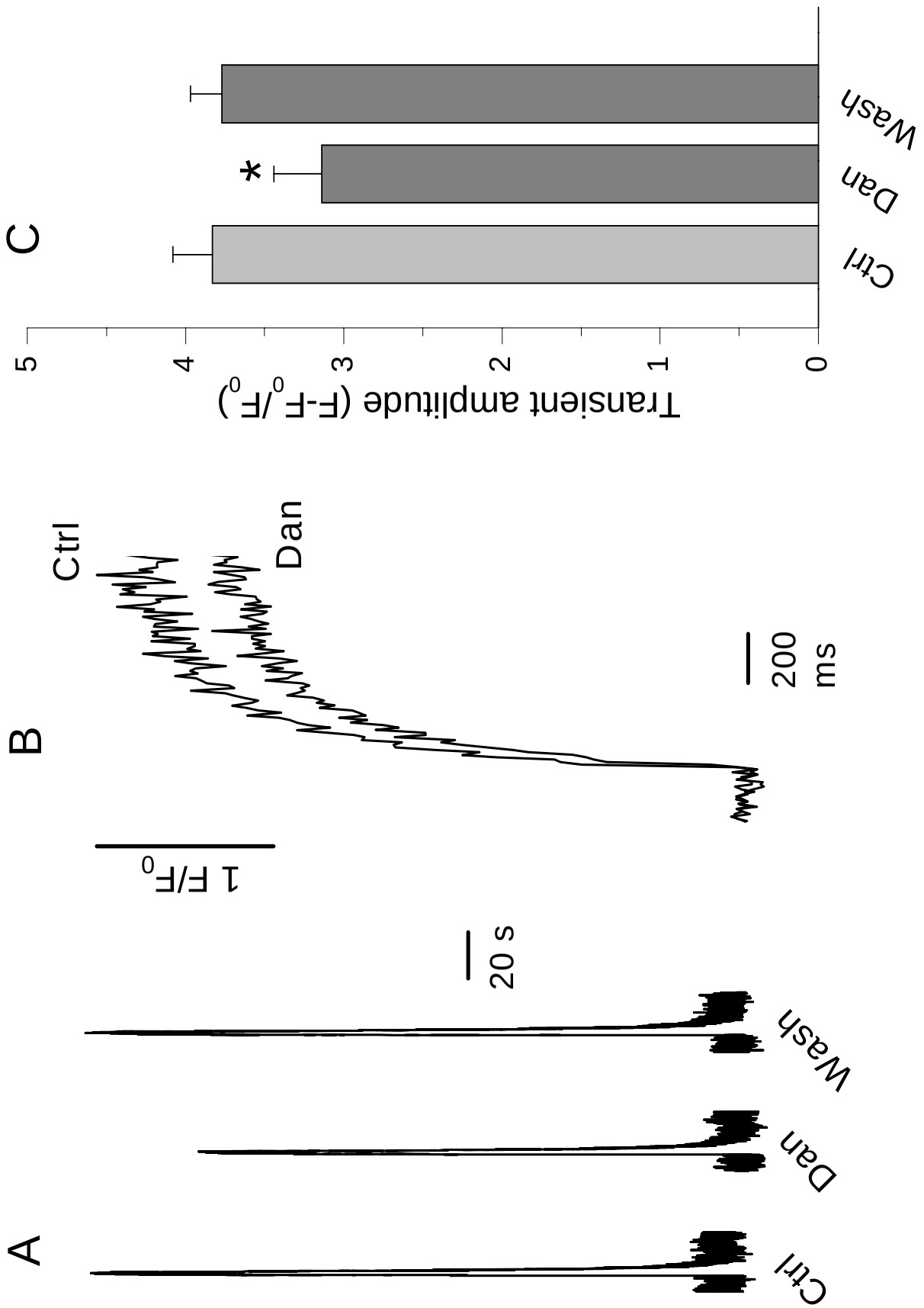

Figure 1

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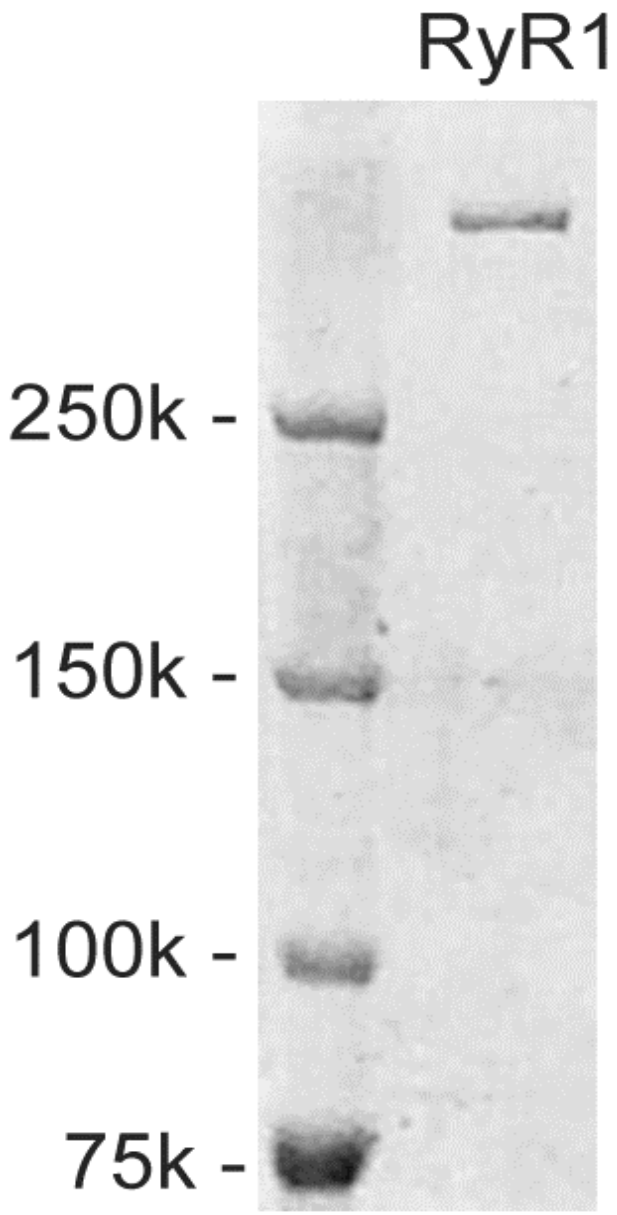

Figure 2A

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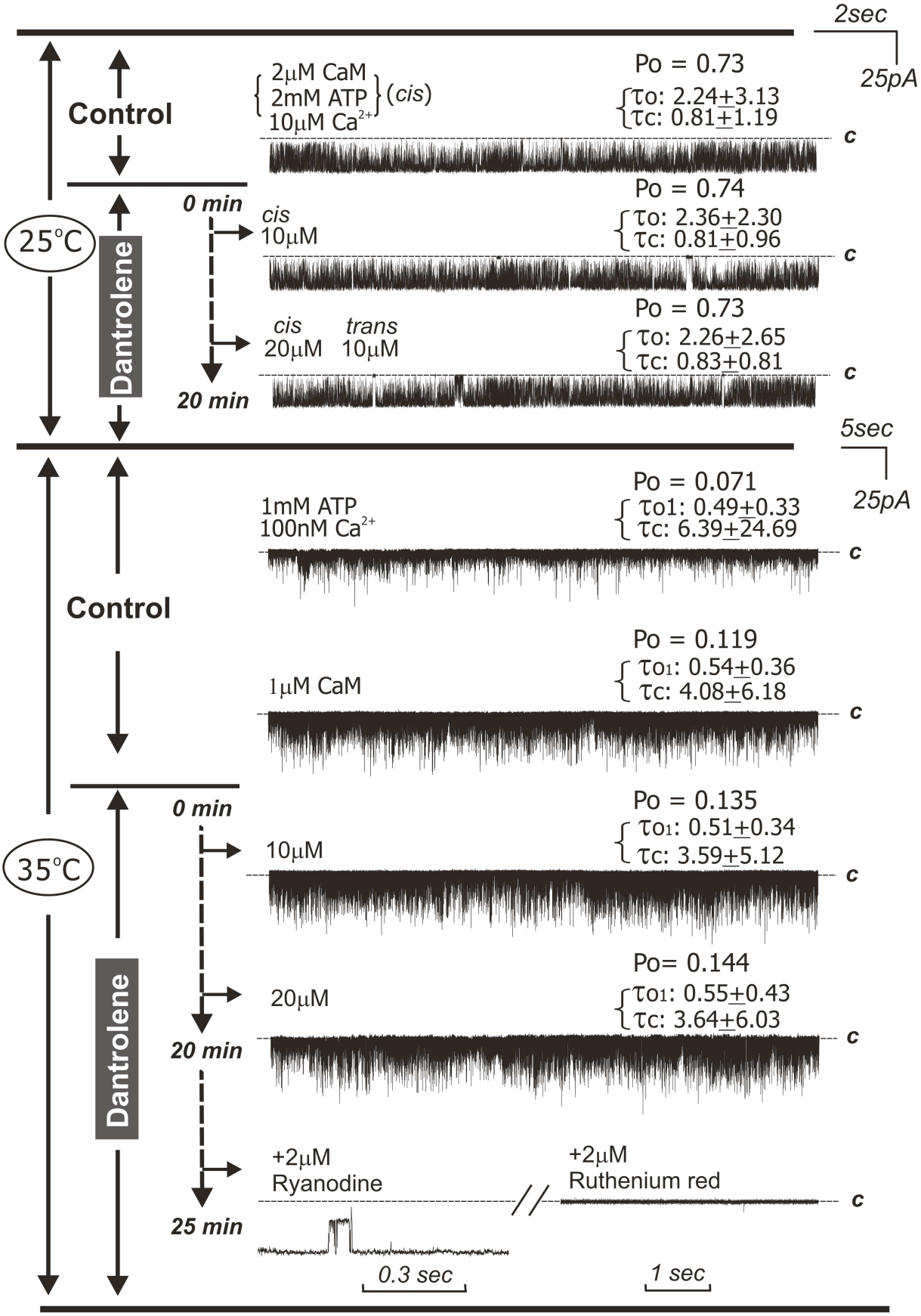
Figure 2B



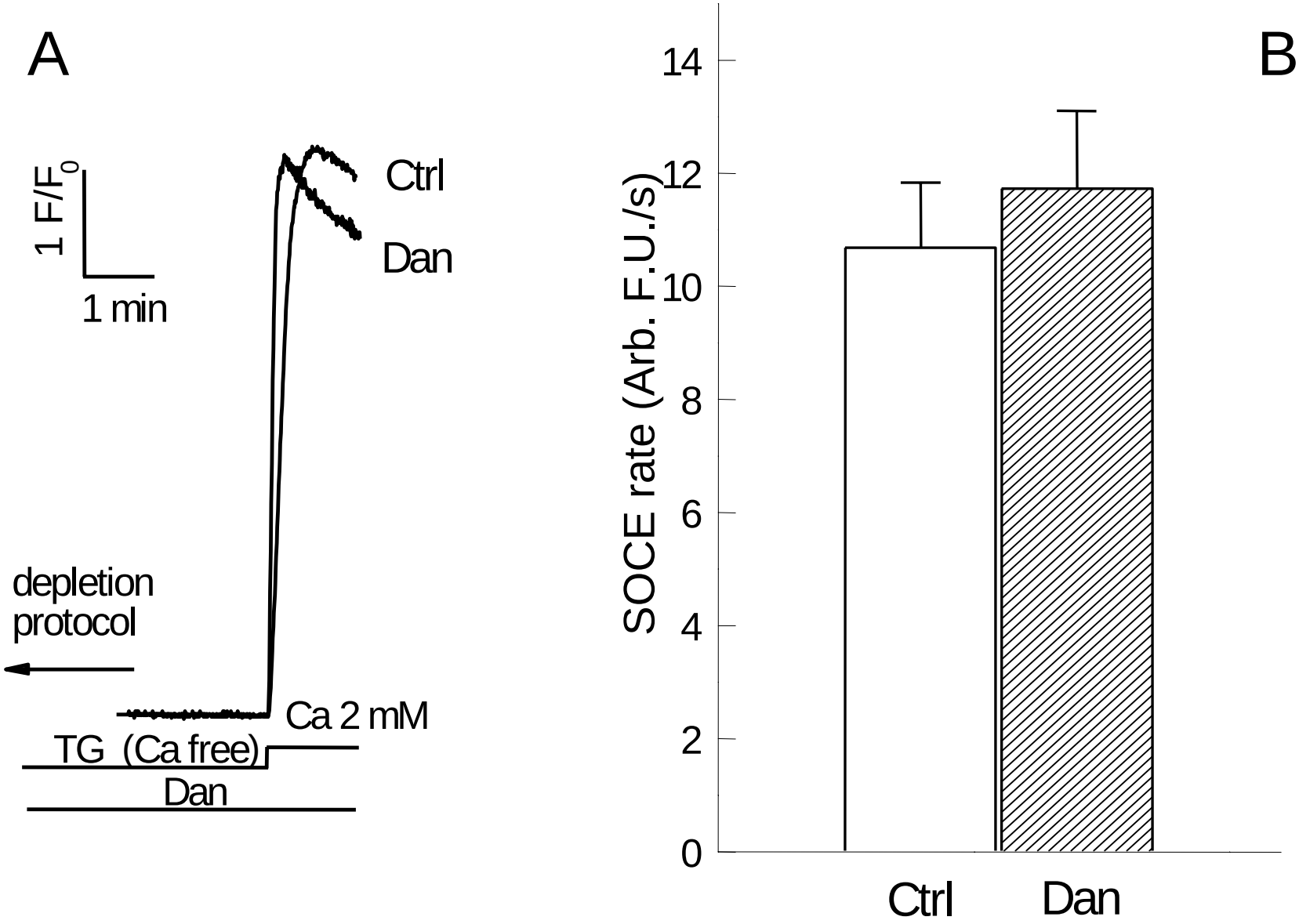


Figure 3

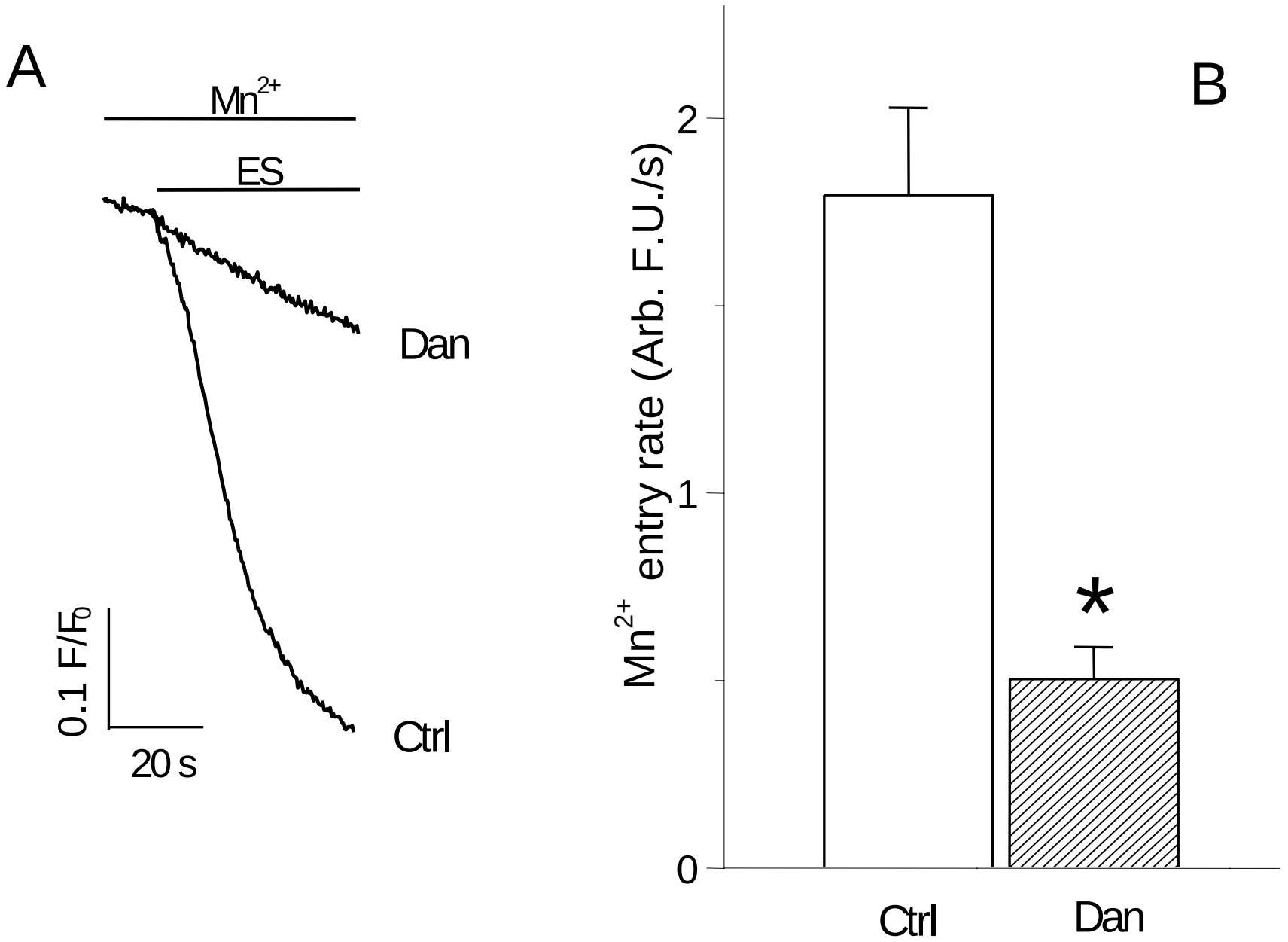


Figure 4

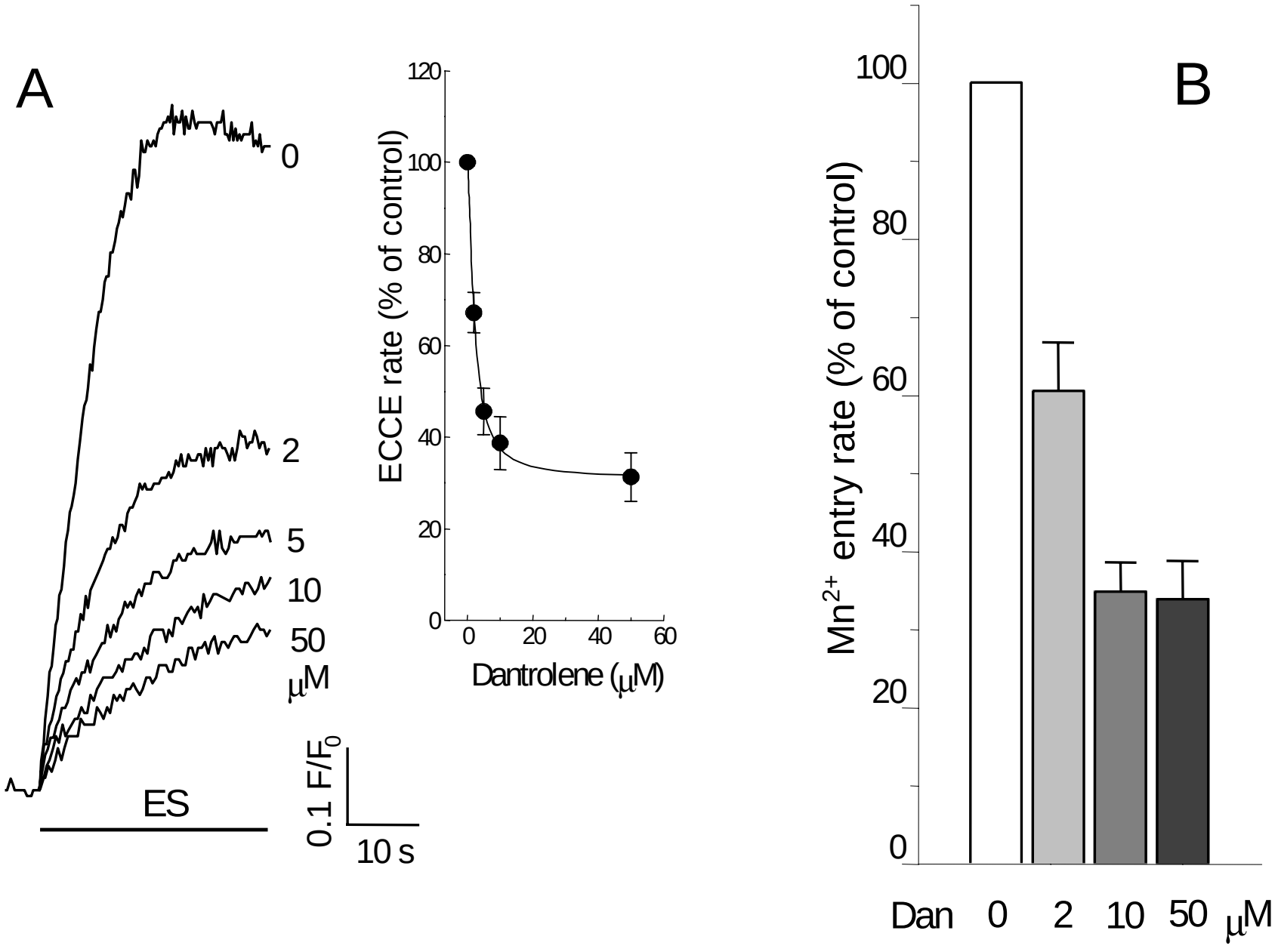


Figure 5

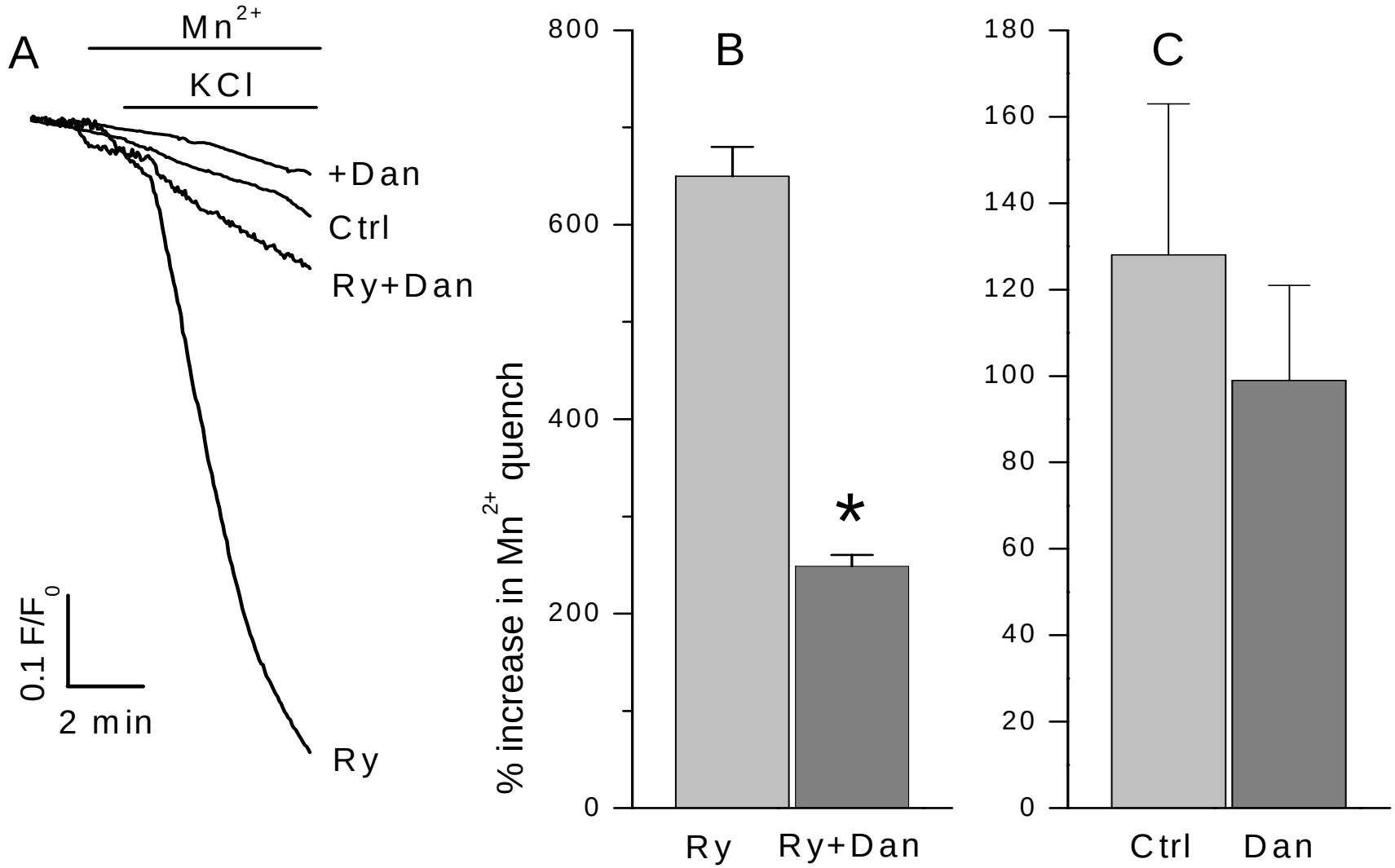


Figure 6

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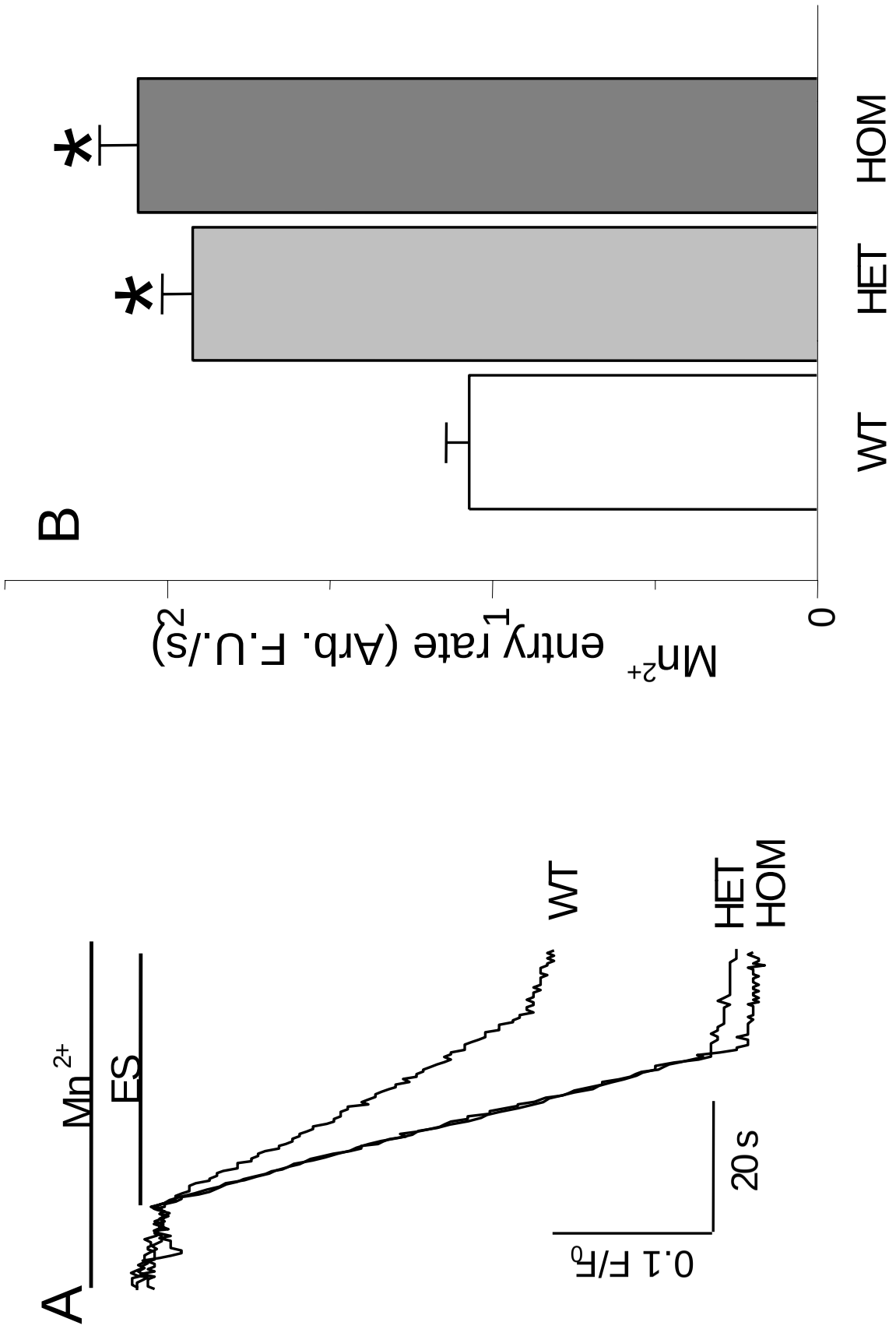

Figure 7

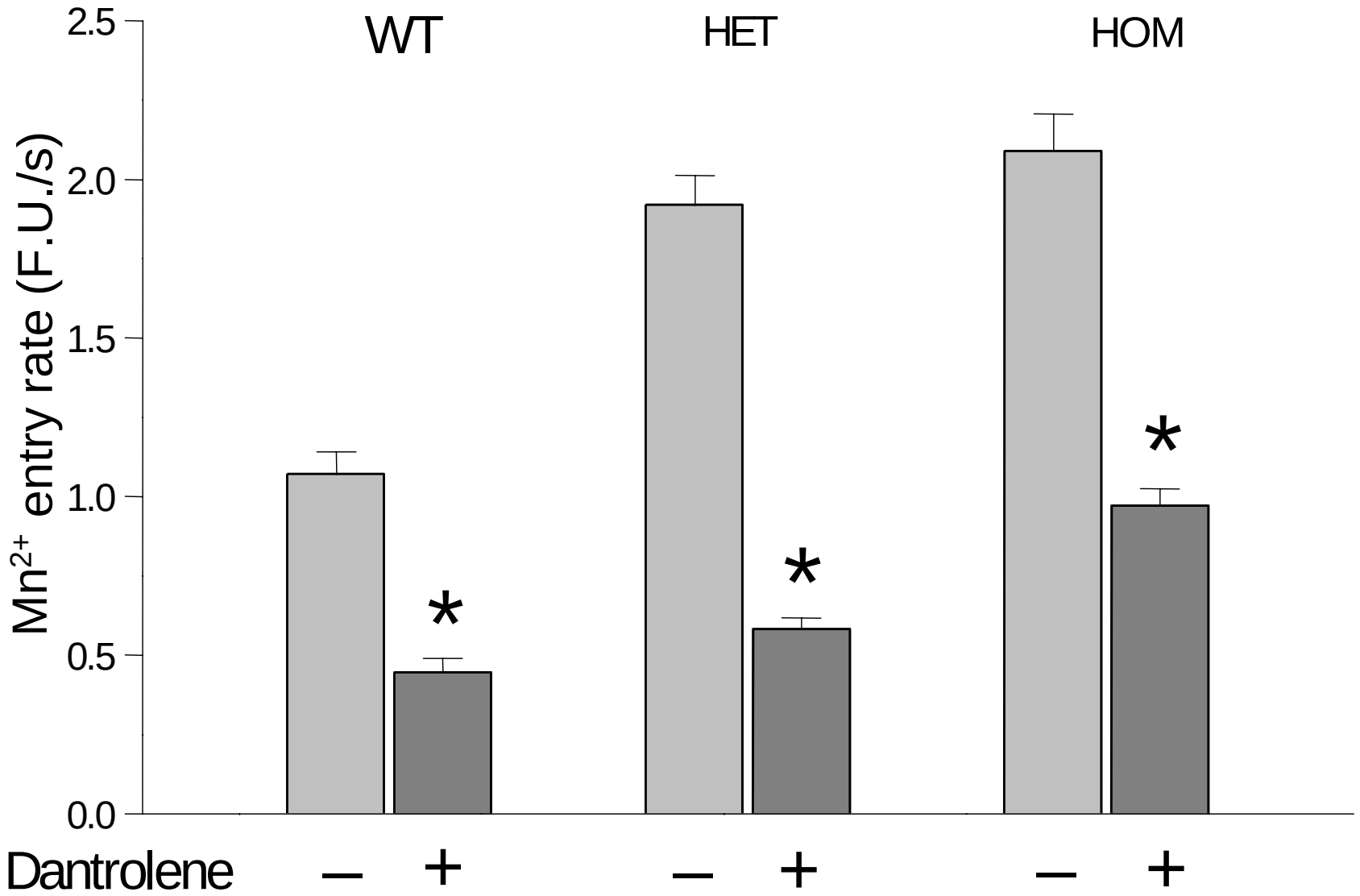

Figure 8