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RyR1-mediated Ca^{2+} Leak and Ca^{2+} Entry Determine Resting Intracellular Ca^{2+} in Skeletal Myotubes*

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The control of resting free Ca^{2+} in skeletal muscle is thought to be a balance of channels, pumps, and exchangers in both the sarcolemma and sarcoplasmic reticulum. We explored these mechanisms using pharmacologic and molecular perturbations of genetically engineered (dyspedic) muscle cells that constitutively lack expression of the skeletal muscle sarcoplasmic reticulum Ca^{2+} release channels, RyR1 and RyR3. We demonstrate here that expression of RyR1 is responsible for more than half of total resting Ca^{2+} concentration ($[Ca^{2+}]_{rest}$) measured in wild type cells. The elevated $[Ca^{2+}]_{rest}$ in RyR1-expressing cells is not a result of active gating of the RyR1 channel but instead is accounted for by the RyR1 ryanodine-insensitive Ca^{2+} leak conformation. In addition, we demonstrate that basal sarcolemmal Ca^{2+} influx is also governed by RyR1 expression and contributes in the regulation of $[Ca^{2+}]_{rest}$ in skeletal myotubes.

In skeletal muscle active Ca^{2+} efflux from the sarcoplasmic reticulum (SR)[2] occurs fundamentally through RyR1 via an orthograde signal from DHPR. In the absence of stimuli the open probability of RyR1 is very low, and $[Ca^{2+}]_{rest}$ is maintained near 100 nM in frog (1), mammalian (2), and human skeletal muscle (3) and in mammalian skeletal myotubes (4). This stems from the fact that in the absence of depolarization, the DHPR appears to suppress spontaneous RyR1 activity (5, 6) as evidenced higher RyR1 activity in *mdg* myotubes that lack expression of the α_{1s}-DHPR (7, 8).

In addition to the "classical" release pathway mediated by RyR1 activation, at rest there is ample evidence for the existence of a second less defined SR Ca^{2+} efflux pathway that has been referred to as ryanodine (Ry)-insensitive "Ca^{2+} leak" (4, 9, 10). This Ca^{2+} leak can be broadly defined as a passive efflux of Ca^{2+} from the SR under resting or quiescent conditions. Part if not all of the Ry-insensitive Ca^{2+} leak pathway has been proposed to represent a conformation of RyR1 with a low conductance that is constitutively open ($P_O \sim 1$) and represents a distinct conformation from that of actively gated RyR1 channels involved in excitation contraction coupling (4, 10). It has been shown that Ry-insensitive Ca^{2+} leaks may contribute significantly to SR Ca^{2+} loading capacity and that they may have a significant contribution to regulation of $[Ca^{2+}]_{rest}$ in skeletal muscle. If this is correct, then RyR1 leak may have relevance in physiological and pathological regulation of muscle Ca^{2+} homeostasis.

Macrocyclic bastadins isolated from the marine sponge *Lanthella basta* are novel modulators of RyR1. Bastadin 5 (B5) has been shown to prolong dramatically both open and closed time constants of single RyR1 channels reconstituted in bilayer lipid membranes without changing their unitary conductance or overall open probability (11). Importantly, under conditions where RyR1 channels are pharmacologically blocked (with micromolar ryanodine or ruthenium red) both B5 and its related congener bastadin 10 have been shown to increase significantly the Ca^{2+} loading capacity in SR vesicles and increase the capacity of SR membranes to bind [^{3}H]Ry ~4-fold (B_{max}) (10).

We hypothesized that expression of RyR1 in RyR-null ($_{Null}$RyR) myotubes would increase $[Ca^{2+}]_{rest}$ and that this increase would be secondary to passive Ca^{2+} efflux from SR stores mediated by Ry-insensitive Ca^{2+} leak. As expected, expression of RyR1 in $_{Null}$RyR myotubes increased $[Ca^{2+}]_{rest}$ to concentrations typically found in wild type myotubes, and complete blockade of caffeine sensitive RyR1 Ca^{2+} release by ryanodine did not modify $[Ca^{2+}]_{rest}$ levels. When B5 was added to examine the contribution of RyR1 leaks toward the $[Ca^{2+}]_{rest}$, we found that Ry+B5 in combination reduced resting $[Ca^{2+}]_{rest}$ to essentially dyspedic levels in RyR1-expressing cells, but had no effect in $_{Null}$RyR cells. $[Ca^{2+}]_{rest}$ was further reduced when Ry+B5-pretreated $_{Null}$RyR and wild type ($_{Wt}$RyR) myotubes were exposed to low external Ca^{2+} solution. Similar results were obtained in primary myotubes generated from RyR1/3-null dyspedic mice and their wild type littermates. These results show that a fraction of RyR1 within the SR membrane exists in a Ry-insensitive conformation that mediates Ca^{2+} leak that determines $[Ca^{2+}]_{rest}$ levels in skeletal muscle. In addition, RyR1 expression also regulates basal sarcolemmal Ca^{2+} influx, which also contributes to $[Ca^{2+}]_{rest}$ in skeletal myotubes.

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[2] The abbreviations used are: SR, sarcoplasmic reticulum; DHPR, dihydropyridine receptor; Ry, ryanodine; $[Ca^{2+}]_{rest}$, resting Ca^{2+} concentration; B5, bastadin 5; $_{Null}$RyR, RyR-null; Wt, wild type; SERCA, SR Ca^{2+}-ATPase; PMCA, plasma membrane sarcolemmal Ca^{2+}-ATPase; NCX, Na^+-Ca^{2+} exchanger.

MATERIALS AND METHODS

Isolation of B5—B5 was extracted from lyophilized *Ianthella* basta sponge collected from Guam using methods described previously (11).

Cell Culture and Infection with RyR1 Herpes Simplex Virus Virions—1B5 cells that lack expression of RyR-1, RyR-2, and RyR-3 ($_{Null}$RyR) were cultured on Matrigel- (BD Bioscience) coated 10-cm dishes as described previously (4, 12, 13) and allowed to differentiate for 5 days. Grid plates containing differentiated myotubes were infected with helper-free herpes simplex virus type 1 virion particles containing $_{Wt}$RyR1 cDNA at a multiplicity of infection of 0.5 for 2 h, and then cultured for 48 h in differentiation media prior to being used experimentally (10, 14). Transduced cells were identified after making measurements of resting [Ca^{2+}] using immunofluorescence with Ab34C.

Primary myoblast cell lines were generated from the hindlimb and forelimb muscles of E18 RyR1/RyR3 double-null dyspedic mice and their Wt littermates (15, 16). Myoblasts were differentiated into myotubes by withdrawal of growth factors as described previously (14).

Ca^{2+}-selective Microelectrodes—Single- and Double-barreled Ca^{2+}-selective microelectrodes were prepared using thin walled borosilicate glass capillaries (WPI 2B150F-4, and WPI PB-150F-4 Sarasota, FL) as described previously (4). They were back-filled first with the neutral carrier ETH 129 (Fluka, Ronkontioma, NY) and then with pCa7 solution. Each Ca^{2+}-selective microelectrode was individually calibrated as described previously (1), and only those with a linear relationship between pCa3 and pCa7(Nernstian response, 29.5 mV/pCa unit) and at least 25 mV between pCa7 and pCa 8 were used experimentally.

To better mimic intracellular ionic conditions, all calibration solutions were supplemented with 1 mM Mg^{2+}. After making measurements of resting [Ca^{2+}], all electrodes were then recalibrated, and if the two calibration curves did not agree within 3 mV, the data from that microelectrode were discarded. Before starting the studies, we determined by direct calibration that the calcium sensitivity of the Ca^{2+} microelectrodes was not affected by any of the drugs used in the present study.

Microelectrode recordings were performed as described previously (1, 4). The potential from the 3 M KCl microelectrode (V_m) was subtracted electronically from potential of the Ca^{2+} electrode (VCa_E), to produce a differential Ca^{2+}-specific potential (VCa) that represents the $[Ca^{2+}]_{rest}$. V_m and VCa were filtered (30–50 KHz) to improve the signal-to-noise ratio and stored in a computer for further analysis.

Mn^{2+} Quench—Primary $_{Null}$RyR and $_{Wt}$RyR myotubes were loaded with 5 μM fura-2/AM to measure the rate of dye quench by Mn^{2+} entry (Molecular Probes, Eugene, OR) at 36 °C for 20 min in imaging buffer, pH 7.4. The myotubes were then washed three times with imaging buffer and transferred to the stage of a Nikon TE2000 inverted microscope and illuminated at the isosbestic wavelength for fura-2 (360 nm). Fluorescence emission was captured from regions of interest within each myotube from 3–10 individual cells at 5 frames/s using an Olympus 40 × oil 1.3 NA objective. Mn^{2+} influx into myotubes was measured as described previously with minor modification (17, 18). Final concentrations of 500 μM $MnCl_2$ and 1.2 mM Mg^{2+} were added to a nominally Ca^{2+}-free (~7 μM free Ca^{2+}).

SR Ca^{2+} Loading Content Determination—Relative SR Ca^{2+} content levels of primary $_{Null}$RyR and $_{Wt}$RyR myotubes were estimated from the magnitude of the Ca^{2+} release induced by 5 μM ionomycin in cells loaded with 5 μM fluo-5N/AM for 20 min at 37 °C. Cells were incubated in Ca^{2+}-free solution to avoid Ca^{2+} entry from the extracellular medium. Total SR calcium content was expressed as the area under the curve of the Ca^{2+} release transient.

Membrane Vesicle Preparation and Immunoblotting—Microsomal vesicles were prepared from cultured myotubes. Myotubes were homogenized in a Polytron cell disrupter in 5 mm imidazole, pH 7.4, 300 mm sucrose supplemented with protease inhibitor (Complete™; Roche Applied Science) and collected as described previously (19). Proteins were separated using SDS-PAGE (20) and transferred to polyvinylidene difluoride membranes. Expression of specific proteins was assessed by incubation of the membranes with poly- or monoclonal antibodies against; RyR1 (34C; Sigma-Aldrich), SR Ca^{2+}-ATPase 1 (SERCA-1) (ABR-Affinity BioReagents, Rockford, IL), Na^+-Ca^{2+} exchange 3 (NCX3) (a gift from Dr. Kenneth Philipson and 95209 Swant, Bellinzona, Switzerland), plasma membrane sarcolemmal Ca^{2+}-ATPase (PMCA) (sc-28765 Santa Cruz Biotechnology, Santa Cruz, CA), myosin (sc-20641 Santa Cruz Biotechnology), and glyceraldehyde-3-phosphate dehydrogenase (FL-335, Santa Cruz Biotechnology).

Solutions—Ionic composition of the mammalian Ringer solution was: 125 mM NaCl, 5 mM KCl, 1 mM $MgSO_4$, 25 mM HEPES, 6 mM glucose, 2 mM $CaCl_2$. Ryanodine (500 μM) and B5 (20 μM) solutions were prepared by adding these compounds to the desired concentration in normal mammalian Ringer solution. The low Ca^{2+} solution was prepared using the same protocol as the corresponding regular Ringer solution, but Ca^{2+} was omitted, and Mg^{2+} (2 mM) was added. Ca^{2+}-free solution was prepared by omitting Ca^{2+} and adding Mg^{2+} (2 mM) and EGTA (1 mM). All solutions were adjusted to pH 7.4. Solution exchange was realized by gentle aspiration of the media and application of the new media with a transfer pipette. Solution replacement was repeated several times to assure the complete exchange of media. Experiments were performed at 23 °C.

Drug Treatments—In the drug treatment studies, all cells were first incubated with 500 μM Ry for 45 min before $[Ca^{2+}]_{rest}$ measurements were made. In studies of the effects of B5, 20 μM B5 was added to the cells for 10 min prior to $[Ca^{2+}]_{rest}$ measurements. For studying the effect of Ry and B5 together, the myotubes were first incubated with Ry for 45 min followed by an additional 10-min incubation with both Ry and B5 before $[Ca^{2+}]_{rest}$ measurements were made. To explore the effect of low Ca^{2+} solution in cells treated with Ry and B5 together, the myotubes were first incubated with Ry 45 min followed by an additional 10-min incubation with both Ry and B5 and then were incubated in low Ca^{2+} in the presence of Ry and B5 for 2 min before $[Ca^{2+}]_{rest}$ measurements were made. We avoided

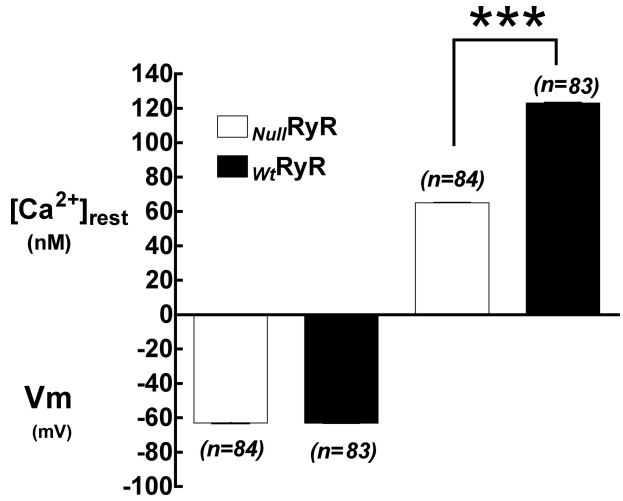

FIGURE 1. **Resting membrane potentials (*left*) and resting intracellular free Ca^{2+} concentrations (*right*) measured using double-barreled microelectrodes in RyR-null and Wt RyR1-expressing 1B5 myotubes.** Data are expressed as mean ± S.D., $n = 20$ cells/group.

making $[Ca^{2+}]_{rest}$ measurements after long incubations in low Ca^{2+} solution (more than 10 min) because despite the fact that 2 mM Mg^{2+} was added to the low Ca^{2+} solution all myotubes began to show a significant depolarization after this interval (>7 mV).

Statistics—All values are expressed as mean ± S.D. Paired and unpaired *t* tests were used to compare the $[Ca^{2+}]_{rest}$ in single myotubes and groups of myotubes before and after drug treatment(s); $p < 0.05$ was considered significant.

RESULTS

RyR1 Expression Significantly Increases $[Ca^{2+}]_{rest}$ in $_{Null}$RyR Myotubes—Resting membrane potentials and $[Ca^{2+}]_{rest}$ were measured in differentiated $_{Null}$RyR 1B5 myotubes and those transduced with $_{Wt}$RyR1 virions. The $[Ca^{2+}]_{rest}$ observed in myotubes expressing $_{Wt}$RyR1 was significantly higher than that observed in $_{Null}$RyR myotubes (123 ± 4.7 nM, $n = 83$ *versus* 65 ± 4 nM, $n = 84$, $p < 0.0001$) (Fig. 1). There were no differences in membrane potential of $_{Wt}$*Ry*R1-expressing myotubes compared with $_{Null}$RyR myotubes (63 ± 2.1 mV, $n = 84$ *versus* 63 ± 1.8 mV, $n = 83$, $p > 0.05$) (Fig. 1).

Ry Does Not Lower $[Ca^{2+}]_{rest}$—It is well established that low concentrations Ry can enhance the open probability of RyR1 and that high micromolar concentrations (such as the conditions used in the present study) fully block channel conductance (21–23) and prevent Ca^{2+} release induced by direct RyR agonists (*e.g.* caffeine, 4 CmC). Incubation of myotubes expressing $_{Wt}$RyR1 with 500 μM Ry completely abolished Ca^{2+} responses to the first and any subsequent exposure to caffeine (20 mM) (data not shown). Incubation of $_{Wt}$RyR1-expressing myotubes and $_{Null}$RyR myotubes in 500 μM Ry for 45 min did not modify $[Ca^{2+}]_{rest}$ in either group of cells. In $_{Null}$RyR myotubes$[Ca^{2+}]_{rest}$ was 65 ± 4.8 nM ($n = 30$) before and 63 ± 4 nM ($n = 30$) ($p > 0.05$) after Ry treatment, and in $_{Wt}$RyR1-expressing myotubes, $[Ca^{2+}]_i$ was 123 ± 3.6 nM ($n = 24$) before and 122 ± 3.2 nM ($n = 24$) ($p > 0.05$) after Ry incubation (Fig. 2*A*). There was no change in V_m in $_{Null}$RyR and $_{Wt}$RyR1myotubes after the treatment with Ry.

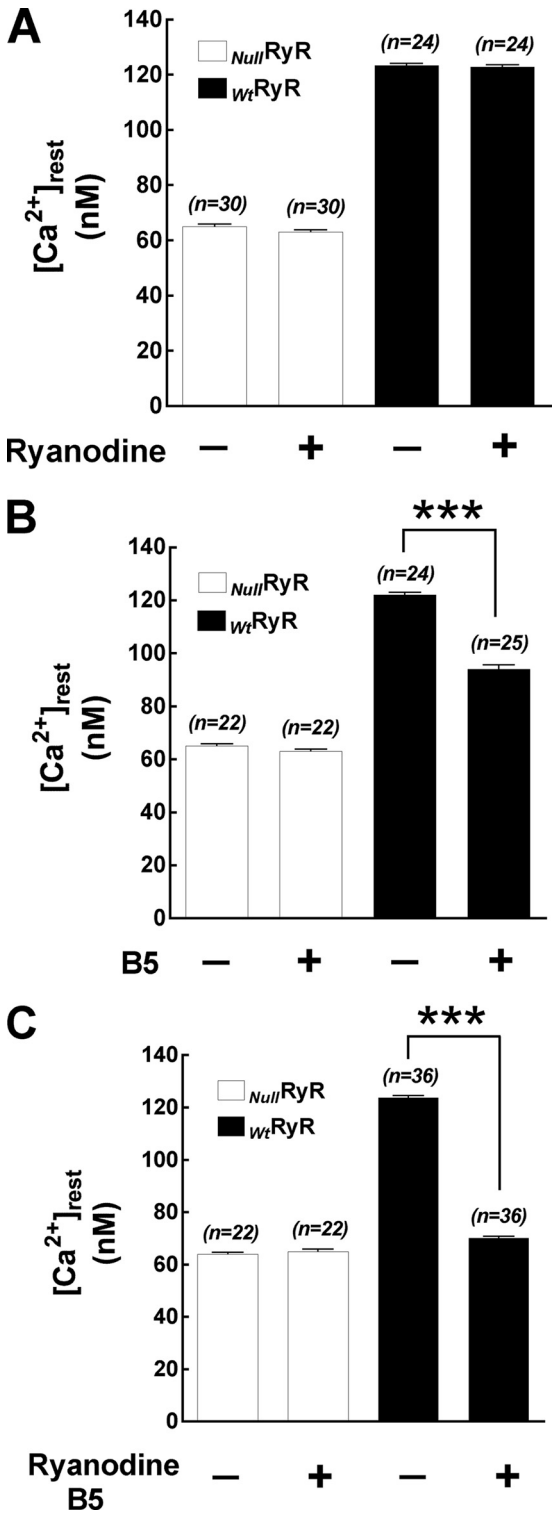

FIGURE 2. **Resting intracellular free Ca^{2+} concentrations measured in RyR-null and Wt RyR1-expressing 1B5 myotubes.** *A*, after treatment with 500 μM ryanodine. *B*, after treatment with 20 μM B5. *C*, after treatment with 500 μM ryanodine and 20 μM B5. Data are expressed as mean ± S.D., $n = 20$ cells/group.***, $p < 0.0001$.

B5 Reduces $[Ca^{2+}]_{rest}$ in Myotubes Expressing RyR1—Incubation of $_{Null}$RyR myotubes for 10 min with B5 did not affect the levels of $[Ca^{2+}]_{rest}$ (65 ± 4 nM, $n = 22$ before *versus* 63 ± 4 nM, $n = 22$, $p > 0.05$) (Fig. 2*B*). Interestingly, B5 alone diminished $[Ca^{2+}]_{rest}$ in $_{Wt}$RyR1-expressing myotubes by 25% in relation to

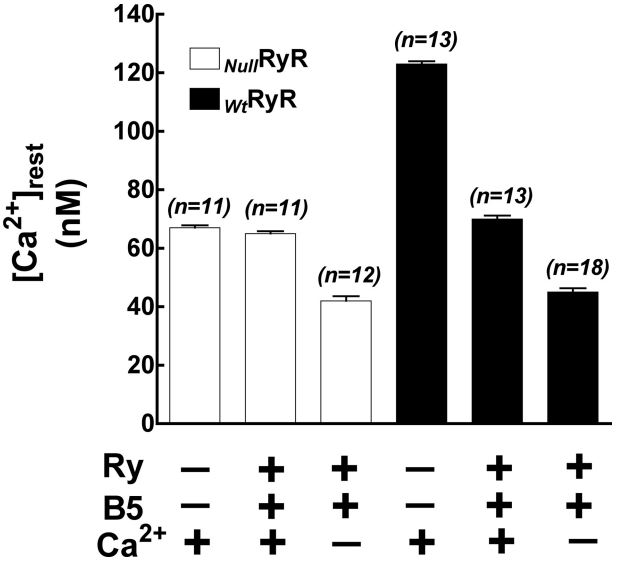

FIGURE 3. **Effects of removal of extracellular Ca^{2+} on intracellular free Ca^{2+} concentrations after pretreatment with ryanodine and B5.** Data are expressed as mean ± S.D., $n = 20$ cells/group.

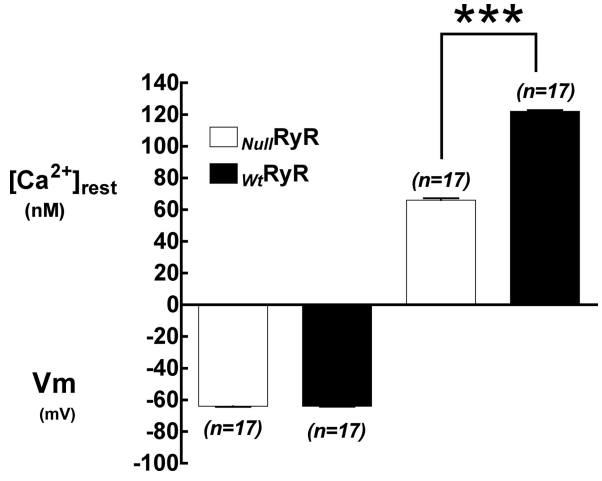

FIGURE 4. **Resting membrane potentials (*left*) and resting intracellular free Ca^{2+} concentrations (*right*) measured using double-barreled microelectrodes in Wt and RyR-null primary myotubes.** Data are expressed as mean ± S.D., $n = 20$ cells/group.

control, from 121 ± 5.3 nM ($n = 24$) to 94 ± 8.1 nM ($n = 25$) $p < 0.0001$ (Fig. 2*B*). There was no change in V_m in $_{Null}$RyR and $_{Wt}$RyR1 myotubes during the treatment with B5 from pretreatment values (data not shown).

Ry and B5 in Combination Restore $[Ca^{2+}]_{rest}$ to Dyspedic Levels in RyR1-expressing Cells—Incubation of $_{Null}$RyR myotubes with Ry+B5 had no effect on $[Ca^{2+}]_{rest}$ (64 ± 3 nM ($n = 22$) before and 65 ± 4.2 nM ($n = 22$), $p > 0.05$ after). However in $_{Wt}$RyR1-expressing myotubes, incubation with Ry+B5 significantly decreased $[Ca^{2+}]_{rest}$ in myotubes to a level not significantly different from that measured in $_{Null}$RyR myotubes, from 123 ± 4.8 nM ($n = 36$) to 70 ± 4.7 nM ($n = 44$, $p < 0.0001$) (Fig. 2*C*). As seen when treated with either agent alone, there was no change in V_m in $_{Null}$RyR and $_{Wt}$RyR1 myotubes during the incubation with Ry+B5 (data not shown).

Effect of Ry+B5 in low $[Ca^{2+}]_e$—To explore the contribution of extracellular Ca^{2+} to $[Ca^{2+}]_{rest}$ in skeletal myotubes, $_{Null}$RyR and $_{Wt}$RyR myotubes were incubated in Ry+B5 (see under "Materials and Methods"), and then the bath solution was then substituted with one containing low Ca^{2+} in the presence of Ry+B5. In $_{Null}$RyR myotubes, $[Ca^{2+}]_{rest}$ in normal Ringer was 67 ± 2.9 nM ($n = 11$), and after Ry+B5 incubation $[Ca^{2+}]_{rest}$ was 65 ± 2.6 nM ($n = 11$, $p > 0.05$) After substitution with the Ca^{2+} solution $[Ca^{2+}]_{rest}$ declined to 42 ± 5.6 nM ($n = 12$, $p < 0.0001$). Following the same experimental protocol in $_{Wt}$RyR-expressing myotubes resulted in $[Ca^{2+}]_{rest}$ values that shifted from 123 ± 3.4 nM ($n = 13$) in normal Ringer to 70 ± 4.2 nM ($n = 13$, $p < 0.001$) after Ry+B5 incubation, and to 45 ± 5.8 nM ($n = 18$, $p < 0.0001$) after incubation in low Ca^{2+} solution. The presence of Ry+B5 and low $[Ca^{2+}]_e$ resulted in $[Ca^{2+}]_{rest}$ in $_{Wt}$RyR and $_{Null}$RyR myotubes that were nearly identical (45 ± 5.8 nM *versus* 42 ± 5.6 nM, respectively) (Fig. 3).

$[Ca^{2+}]_{rest}$ in $_{Null}$RyR and $_{Wt}$RyR1 Primary Myotubes—Resting membrane potentials and $[Ca^{2+}]_{rest}$ were measured in differentiated dyspedic and wild type primary myotubes. Similar to the data for null and RyR1-transduced 1B5 myotubes,

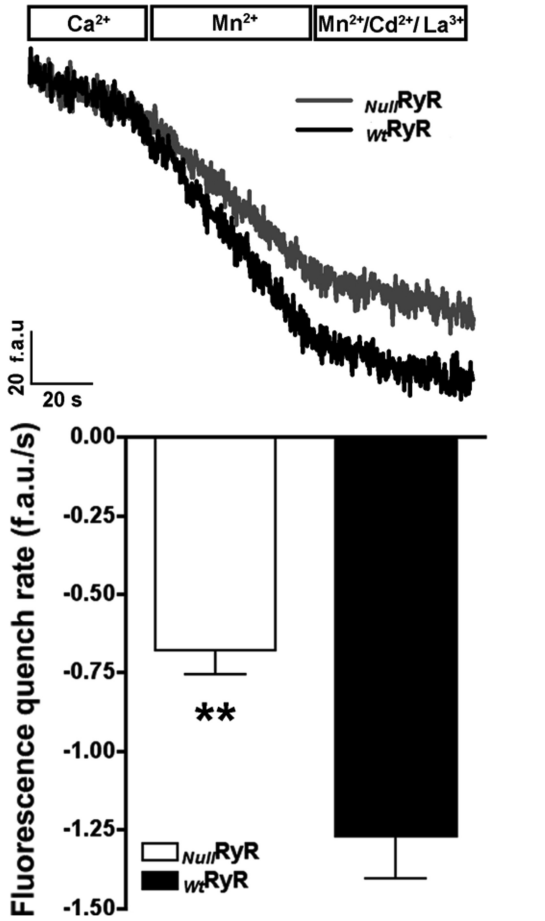

FIGURE 5. **Measurements of resting cation entry using Mn^{2+} quench in RyR-null and Wt primary myotubes.** *Upper*, fura-2 fluorescence raw traces from representative myotubes in the presence of extracellular Ca^{2+}, Mn^{2+} in the absence of Ca^{2+}, and Mn^{2+} in the absence of Ca^{2+} after the addition of extracellular Cd^{2+} and La^{3+}. *Lower*, comparison of the rate of Mn^{2+} quench between Wt and RyR-null primary myotubes. Data are shown as mean ± S.D., $n = 15$ cells/group. **, $p < 0.0001$.

$[Ca^{2+}]_{rest}$ observed in $_{Wt}$RyR1 primary myotubes was significantly higher than that observed in dyspedic myotubes (122 ± 3.6 nM, $n = 17$ *versus* 66 ± 5.2 nM, $n = 17$, $p < 0.001$) (Fig. 4)

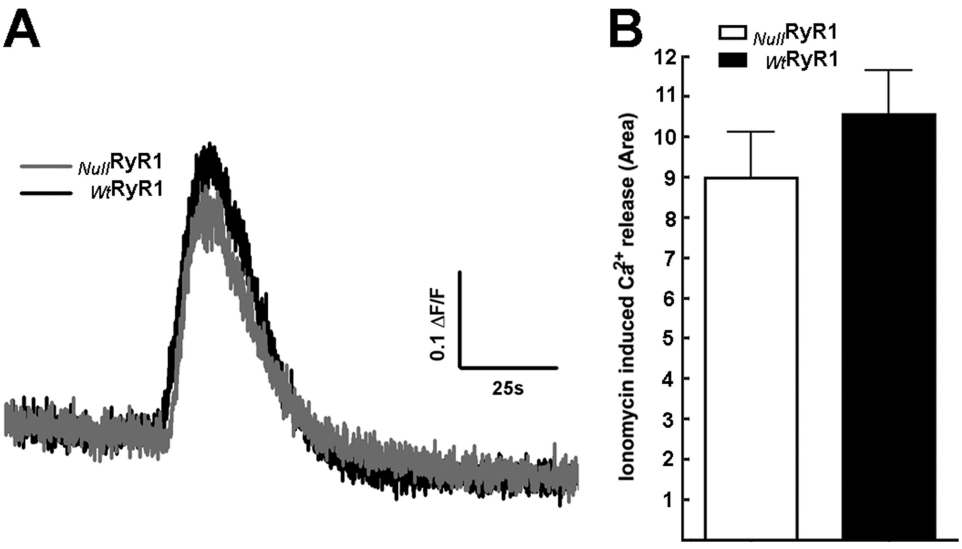

FIGURE 6. **Fluo-5N fluorescence signals after the addition of 5 μM inomycin to Wt and RyR-null primary myotubes in the presence of nominal free extracellular Ca^{2+} buffer.** *A*, representative curve of Wt and RyR-null responses. *B*, average area under the curve of the inomycin-induced Ca^{2+} release. Data are shown as mean ± S.D., $n = 20$ cells in each group. $p > 0.05$.

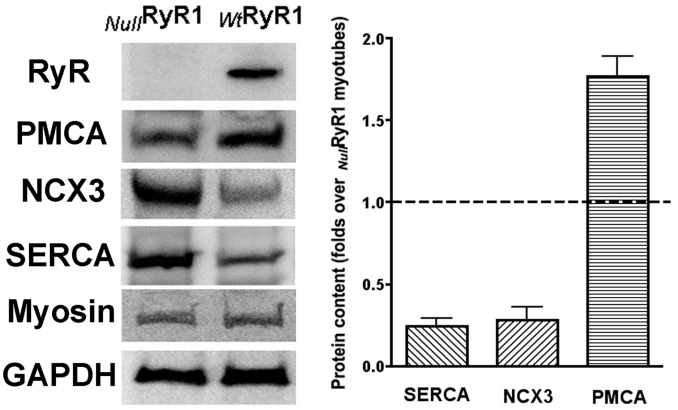

FIGURE 7. **Expression of Ca^{2+}-handling proteins in Wt and RyR-null primary myotubes.** *Left*, representative Western blots using antibodies directed against RyR1, PMCA, NCX3, SERCA1, myosin (to demonstrate similar differentiation state), and glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) as a loading control. *Right*, expression of RyR1 associated with a significant decrease in the expression of SERCA and NCX3 and a significant up-regulation of the expression of PMCA. Data are expressed as mean ± S.D., $n = 5$ Western blots/group.

with no difference in the resting membrane potential (64 ± 1.6 mV, $n = 17$ *versus* 64 ± 1.5 mV, $n = 17$, $p > 0.05$) between the two groups. In addition, these measurements were not different from the values seen in corresponding 1B5 cells.

Ca^{2+} Entry in $_{Null}$RyR and $_{Wt}$RyR1 Primary Myotubes—Using the Mn^{2+} quench technique, the quenching of fura-2 fluorescence was measured at its isosbestic point in primary $_{Null}$RyR and $_{Wt}$RyR myotubes at rest in a nominally Ca^{2+}-free external solution containing 500 μM Mn^{2+}. Under these assay conditions, the rate of Mn^{2+} quench of fura-2 signal can be attributed to Ca^{2+} entry (Fig. 5, *upper*). The rate of Mn^{2+} quench at rest was ~2-fold greater in $_{Wt}$RyR than in $_{Null}$RyR myotubes (1.26 *versus* 0.67 fluorescence (arbitrary units)$^{-s}$ respectively, $p < 0.0001$) (Fig. 5, *lower*). These data describe for the first time that resting Ca^{2+} entry is greater in $_{Wt}$RyR1 compared with $_{Null}$RyR myotubes.

SR Ca^{2+} Content—To quantify the level of SR Ca^{2+} content, we exposed $_{Null}$RyR and $_{Wt}$RyR1 primary myotubes loaded with Fluo-5N/AM and exposed to 5 μM ionomycin in Ca^{2+} free solution, to avoid Ca^{2+} entry from the extracellular medium. Fig. 6 shows that under these conditions the there is no significant difference in amplitude of the fluorescence signal (Fig. 6*A*) or the total Ca^{2+} released (*area* under the *curve*, Fig. 6*B*) from $_{Null}$RyR myotubes compared with $_{Wt}$RyR1 myotubes.

Ca^{2+} Handling Protein Expression—Western blot analysis for expression of RyR1, PMCA, NCX3, and SERCA performed on membranes isolated from $_{Null}$RyR and $_{Wt}$RyR1 primary myotubes is shown in Fig. 7 (representative blot, Fig. 7, *left*; densitometry analysis, Fig. 7, *right*). Expression of RyR1 was accompanied with increased expression of PMCA and a decreased expression of NCX3 and SERCA.

DISCUSSION

The purpose of this study was to examine whether the expression of RyR1 has any effect on $[Ca^{2+}]_{rest}$, the resting Ca^{2+} entry, and SR Ca^{2+} loading in skeletal muscle. Our study demonstrates that expression of $_{Wt}$RyR1 is associated with a significant increase in $[Ca^{2+}]_{rest}$, in resting Ca^{2+} entry, with no significant change in SR Ca^{2+} loading compared with $_{Null}$RyR myotubes. The fact that we observed the same results with RyR1-transduced 1B5 myotubes and in primary myotubes rules out the possibility that the observed difference in $[Ca^{2+}]_{rest}$ was related to overexpression of RyR1 when the differentiated myotubes were transduced with virion particles containing wild type RyR1 cDNA. A significant part of this elevation in $[Ca^{2+}]_{rest}$ appears to be related to the presence of RyR1 leaks because Ry+B5 was able to reduce $[Ca^{2+}]_{rest}$ to levels similar to those observed in $_{Null}$RyR myotubes. In addition, our results show clearly that extracellular Ca^{2+} also plays an important role in maintaining $[Ca^{2+}]_{rest}$ at physiological levels.

From these results it is clear that in addition to the control of well known intracellular Ca^{2+} regulatory mechanisms (PMCA, NCX, SERCA) on steady-state $[Ca^{2+}]_{rest}$ in skeletal muscle a significant portion of $[Ca^{2+}]_{rest}$ is also set by passive Ca^{2+} release, which appears to be the result of a fraction of $_{Wt}$RyR1s within SR that are in a Ry-insensitive Ca^{2+} leak conformation and by the Ca^{2+} influx via the sarcolemma that is modulated by the presence of RyR1. In fact, the combined effect of expression of RyR1 elevated the $[Ca^{2+}]_{rest}$ by ~2-fold. The precise mechanism underlying how RyR1 leaks and the expression of the RyR1 lead to a chronic elevation $[Ca^{2+}]_{rest}$ needs further study. If RyR1 leak exceeds Ca^{2+} uptake by the SR and extrusion by sarcolemmal mechanisms, a new dynamic equilibrium of Ca^{2+} mobilization must be established that allows the

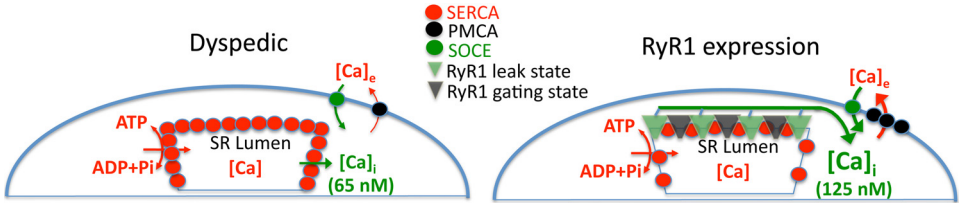

FIGURE 8. **Model showing the changes in expression of Ca^{2+}-handling proteins and the consequent changes in myoplasmic Ca^{2+} concentration, rate of resting Ca^{2+} entry, and Ca^{2+} removal.**

higher $[Ca^{2+}]_{rest}$. If the currently proposed mechanism that Ca^{2+}extrusion processes of the plasma membrane (PMCA, SERCA, and NCX) control $[Ca^{2+}]_{rest}$ they should be stimulated by elevations in resting $[Ca^{2+}]_i$, and in the steady state, such as those defined by our experimental conditions, these transport mechanisms should be sufficient to compensate for the increased Ca^{2+} leak/sarcolemmal Ca^{2+} entry, resulting in a return of $[Ca^{2+}]_{rest}$ toward that measured in $_{Null}$RyR myotubes. Because this does not happen, then the changes in $[Ca^{2+}]_{rest}$ observed with the expression of RyR1 must involve a modification of the set points of the activity of both SERCA and these sarcolemmal Ca^{2+} transport mechanisms and/or the amount of expression of such proteins at the SR and plasma membrane. In fact, it was found that expression of RyR1 was accompanied by an increase in the expression of PMCA and a decreased expression of NCX3 and SERCA, all of them linked to the regulation of intracellular $[Ca^{2+}]$. As modeled in Fig. 8, the decreased expression of SERCA, an increased expression of PMCA, elevated resting Ca^{2+} entry, and an elevated cytoplasmic Ca^{2+} are the costs for expressing RyR1 and maintaining SR stores at levels equal to that found in dyspedic cells. One explanation is that RyR1-expressing cells down-regulate SERCA as a compensatory adaptation to limit the consumption of ATP that would be needed to offset RyR1-mediated Ca^{2+} leak from SR. If the level of SERCA expression was maintained in the face of a sizable Ca^{2+} leak, futile cycling of Ca^{2+} between the SR lumen and the extracellular space would come at a great energy cost. Conversely, dyspedic cells express higher levels of SERCA because without the RyR1 Ca^{2+} leak there is a reduced energy cost. One intriguing discovery in the present study is that RyR1 expression appears to confer significant regulation of the density of SERCA protein found in SR membranes. These results are consistent with previous findings that indicated up-regulation of SERCA levels in skeletal muscle membranes isolated from dyspedic mice compared with those isolated from wild type (Fig. 6 in Ref. 24).

Second, the higher PMCA expression could help offset decreased rates of Ca^{2+} transient recovery (relaxation) in light of lower SERCA capacity by removing a larger fraction of released Ca^{2+} during excitation to the extracellular space. Why NCX protein is down-regulated and how it contributes to maintenance of resting Ca^{2+} are unclear.

B5, through its modulatory actions on the FKBP12·RyR1 complex, has been previously shown to increase SR Ca^{2+} loading capacity and concomitantly attenuate RyR1 Ca^{2+} leak. This property of the bastadins is the result of their ability to convert Ry-insensitive leak states (RyR1 leak) into ryanodine-sensitive channels (RyR1 Ca^{2+} channels) (10) and is demonstrated by their ability to increase B_{max} of Ry binding/mg of protein (10). Therefore, in the present study B5 was used to examine the relationship between Ry-sensitive and Ry-insensitive Ca^{2+} efflux pathways that coexist in the SR of $_{Wt}$RyR1-expressing myotubes. We found that B5 in combination with blocking concentrations of Ry decreased $[Ca^{2+}]_{rest}$ in $_{Wt}$RyR-expressing myotubes by 43% but had no effect in $_{Null}$RyR myotubes. These data are consistent with the hypothesis that bastadins can promote the conversion of RyR1 in the Ry-insensitive Ca^{2+} leak conformation into Ry-sensitive RyR1 channels. B5 alone, also reduced $[Ca^{2+}]_{rest}$ in $_{Wt}$RyR1-expressing myotubes but to a lesser degree (25%) compared with its effect in combination with Ry (43%) probably because the RyR1 leaks converted into gating channels do not have the same degree of negative control by the DHPR as normal gating channels.

Another interesting result is that the resting Ca^{2+} entry is greater in $_{Wt}$RyR than $_{Null}$RyR myotubes, suggesting that the magnitude of this entry is modulated by the presence of RyR1. The physiological role of this resting Ca^{2+} entry is poorly understood, but appears to be independent of resting membrane potential (myotubes polarized based on the Nernst equation for 23 °C) and/or the degree of SR depletion as we showed in Fig. 6, as has been postulated by Kurebayashi and Ogawa (25), since the experiments were conducted in unstimulated myotubes.

The existence of a RyR1-mediated Ca^{2+} leak pathway in the SR may have some implications for the pathophysiology of two well characterized disorders of skeletal muscle, malignant hyperthermia and central core disease. In muscle cells from the majority of patients with either disorder, there is a global elevation of $[Ca^{2+}]_{rest}$, which can be partially reversed by treating the muscle cells with B5 in combination with blocking concentrations of Ry (4). In summary, our results demonstrate that that expression of $_{Wt}$RyR1 is associated with an increase in $[Ca^{2+}]_{rest}$ and that in addition to traditionally proposed mechanisms involving SERCA, NCX, and PMCA, $[Ca^{2+}]_{rest}$ in skeletal muscle is determined in part by passive Ca^{2+} leak through $_{Wt}$RyR1 and increased basal sarcolemmal Ca^{2+} entry.

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