Ca\(^{2+}\) leak through ryanodine receptor 1 regulates thermogenesis in resting skeletal muscle

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Mammals rely on nonshivering thermogenesis (NST) from skeletal muscle so that cold temperatures can be tolerated. NST results from activity of the sarcoplasmic reticulum (SR) Ca\(^{2+}\) pump in skeletal muscle, but the mechanisms that regulate this activity are unknown. Here, we develop a single-fiber assay to investigate the role of Ca\(^{2+}\) leak through ryanodine receptor 1 (RyR1) to generate heat at the SR Ca\(^{2+}\) pump in resting muscle. By inhibiting a subpopulation of RyRs in a single-fiber preparation via targeted delivery of ryanodine through transverse tubules, we achieve in-preparation isolation of RyR1 Ca\(^{2+}\) leak. This maneuver provided a critical increase in signal-to-noise of the SR-temperature-sensitive dye ER thermoyellow fluorescence signal from the fiber to allow detection of SR temperature changes as either RyR1 or SR Ca\(^{2+}\) pump activity was altered. We found that RyR1 Ca\(^{2+}\) leak raises cytosolic [Ca\(^{2+}\)] in the local vicinity of the SR Ca\(^{2+}\) pump to amplify thermogenesis. Furthermore, gene-dose-dependent increases in RyR1 leak in RYR1 mutant mice result in progressive rises in leak-dependent heat, consistent with raised local [Ca\(^{2+}\)] at the SR Ca\(^{2+}\) pump via RyR1 Ca\(^{2+}\) leak. We also show that basal RyR Ca\(^{2+}\) leak and the heat generated by the SR Ca\(^{2+}\) pump in the absence of RyR Ca\(^{2+}\) leak is greater in fibers from mice than from toads. The distinct function of RyRs and SR Ca\(^{2+}\) pump in endothermic mammals compared to ectothermic amphibians provides insights into the mechanisms by which mammalian skeletal muscle achieves thermogenesis at rest.

Skeletal muscle | thermogenesis | ryanodine receptor | SR Ca\(^{2+}\) pump | heat

Significance

The evolution of mammals to use skeletal muscle as a source of heat allowed them to spread to all parts of the globe. The generation of heat requires increased adenosine triphosphate (ATP) hydrolysis in the resting muscle in a regulated manner, but how this mechanism works is unknown. The results suggest that mammals increase their RyR1 Ca\(^{2+}\) leak rate to amplify a basal ATP turnover rate at the sarcoplasmic reticulum Ca\(^{2+}\) pump that is higher than that of lower vertebrates. Muscle-based thermogenesis allows regulation of body temperature that is essential for life in mammals and provides a potential pathway for manipulating body weight or temperature by altering metabolic rate.

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observed in mouse, indicating that the properties of RyR and SR Ca\(^{2+}\) pump in the mammalian skeletal muscle are critical for this tissue to become a heat generator.

**Results**

In this section, we demonstrate the local inhibition of RyR1s by ryanodine applied through a targeted delivery via the transverse tubular-system (t-system) lumen and the use of this preparation to determine the effect of different magnitudes of RyR1 Ca\(^{2+}\) leak or known [Ca\(^{2+}\)\(_{\text{cyto}}\)] on changes in the SR temperature. Our approach provides a reference condition, where RyR1 Ca\(^{2+}\) leak is locally blocked for the condition with functional RyR1s. This provides the signal-to-noise improvement required to resolve RyR1 Ca\(^{2+}\) leak-dependent heat generation within a single muscle fiber.

**RyR1 Ca\(^{2+}\) Leak Increases SR Temperature.** Single Tibialis Anterior (TA) muscle fibers from 12- to 16-wk-old mice were used in this study. Approximately 90% of the TA section used was glycolytic type IIB and X fibers (18–20). We isolated intact fibers under paraffin oil, and the sarcolemma of ~60% of the fiber length was mechanically removed (Fig. L4, step 1). The remaining intact fiber segment was exposed to an extracellular solution containing 50 μM ryanodine and 1 mM Fluo-5N using a 2-μL microcapillary tube (Fig. L4, step 2). A concentration of 50 μM ryanodine irreversibly locks the RyR1 in a closed state (21, 22) and so was preferred to the use of other RyR inhibitors such as tetracaine (13), which displays reversibility upon solution exchanging. The paraffin oil surrounding the fiber restricts diffusion of extracellular solution delivered from the microcapillary tube to a localized region of the fiber, where the applied solution enters the t-system lumen. After ~15 s, the external solution was removed, and the remaining sarcolemma of the fiber was peeled away (Fig. L4, step 3). A well-known feature of the mechanical skinning procedure is that the t-system membrane seals over at its former interface with the surface of the fiber, trapping the extracellular solution inside the t-system (23, 24); consequently, the localized region of the fiber exposed to the extracellular solution containing the inhibitor can be visualized by tracking the Fluo-5N signal across the length of the fiber (Fig. L4 A–C).

To test whether ryanodine-exposed RyR1s were blocked, we exposed the fibers to 3 mM caffeine to induce cytosolic Ca\(^{2+}\) waves, a signature of RyR1 activity (Fig. L8) (25–28). When added to the bathing solution, caffeine induced the appearance and propagation of Ca\(^{2+}\) waves. These waves occurred only across the section of the fiber without traces of Fluo-5N (Fig. 1 C–E and Movie S1). In contrast, Ca\(^{2+}\) waves propagated through fibers exposed to an external solution containing Fluo-5N and vehicle, indicating that the inhibition observed in Fig. 1C and Movie S1 is due to the presence of ryanodine. This result shows that the RyR1s of a restricted segment of a muscle fiber can be inhibited, allowing us to assess the contribution of the RyR1 in basal SR Ca\(^{2+}\) pump-dependent thermogenesis with an in-preparation reference section.

To measure the SR Ca\(^{2+}\) pump-mediated thermogenesis in resting muscle fibers, we loaded single fibers partially exposed to ryanodine and Fluo-5N (as described in Fig. L4) with the temperature-sensitive dye ER thermo yellow (ERTY) that localizes specifically to the SR (SI Appendix, Fig. 1A and B). ERTY fluorescence is inversely proportional to the SR temperature (29, 30). In contrast to the localized presence of Fluo-5N signal,
we observed a homogeneous ERTY signal across the long axis of the fiber (Fig. 2 A and B). Following the experimental protocol shown in Fig. 2C, we tested the contribution of a RyR1 Ca\textsuperscript{2+} leak on the SR Ca\textsuperscript{2+} pump-mediated heat generation in wild-type (WT; RYR1\textsuperscript{WT/WT}), heterozygous (RYR1\textsuperscript{WT/KI}), and homozygous (RYR1\textsuperscript{KI/KI}) mutant mouse muscle fibers carrying the RYR1 gain-of-function variant p.G2435R to exploit the gene-dose-dependent increase in RyR1 Ca\textsuperscript{2+} leak across the three genotypes (31). In addition to these genotypes, fibers from the amphibian R. marina expressing RYR\alpha and RYR\beta genes instead of RYR1 were tested to compare the role of RyR Ca\textsuperscript{2+} leak in SR Ca\textsuperscript{2+} pump-dependent thermogenesis between RyR1 and RyR\alpha/\beta in the ectothermic toad. From these experiments, the RyR-dependent and -independent SR Ca\textsuperscript{2+} pump...
heat generation were estimated by comparing ERTY-normalized fluorescence before and after SR Ca2+ pump inhibition with cyclopiazonic acid (CPA) (ERTY fluorescence was normalized to the intensity after SR Ca2+ pump inhibition). The changes in ERTY fluorescence intensity (ΔERTY, %) were transformed to changes in degrees Celsius (°C) by using the previously reported calibration (3.9%/°C) (29).

In the presence of RyR1 Ca2+ leak, the SR Ca2+ pump basal activity accounts for an increase in SR temperature of 1.6, 2.2, 2.8, and 0.4 °C in RyR1WT/WT, RyR1WT/KI, RyR1K1KI, and RyRαβ fibers, respectively (Fig. 2 D–G, black traces). Conversely, the basal SR temperature shift set by the SR Ca2+ pump ATP hydrolysis in the absence of RyR1 Ca2+ leak was 1.0, 0.9, 0.9, and 0.3 °C in RyR1WT/WT, RyR1WT/KI, RyR1K1KI, and RyRαβ, respectively (Fig. 2 D–G, green traces). In each mammalian genotype, the SR Ca2+ pump-dependent SR temperature in the presence of RyR1 Ca2+ leak was higher than in the absence of RyR1 Ca2+ leak (Fig. 1 H–J), showing that RyR1 Ca2+ leak in resting muscle fibers contributes to the basal thermogenic activity of the SR Ca2+ pump in mammalian muscle (Movie S3). In contrast, the SR Ca2+ pump-dependent SR resting temperature was not affected by RyRαβ leak in amphibian muscle (Fig. 2K).

From the results shown in Fig. 2 H–K, we calculated the RyR contribution to the SR Ca2+ pump-dependent SR temperature by subtracting the RyR-independent from the RyR-dependent Δ°C upon SR Ca2+ pump-inhibition values from the same fiber (Fig. 2C). Within the mammalian groups, the RyR1 contribution to the SR Ca2+ pump-dependent SR temperature progressively increased from the most stable to the leakiest RyR1s (RyR1WT/WT < RyR1WT/KI < RyR1K1KI) (Fig. 2L). In addition, RyR Ca2+ leak contribution in RyRαβ was lower than in RyR1WT/WT, suggesting that RyR1 Ca2+ leak provides a thermogenic source that skeletal muscle fibers with RyRαβ lack.

When the RyR1 Ca2+ leak was blocked (RyR1-independent Δ°C upon the SR Ca2+ pump inhibition), the three mammalian genotypes showed the same Δ°C change upon inhibition of the pump (Fig. 2M). In contrast, RyR-independent Δ°C change upon the SR Ca2+ pump inhibition was lower in RyRαβ (0.3 °C) compared to RyR1WT/WT (1 °C). Collectively, these results show that 1) in resting WT fibers, the SR Ca2+ pump basal activity shifts the SR temperature 1.6 °C, where 0.6 °C of that shift comes from the constitutive RyR1 Ca2+ leak; 2) increases in RyR1 Ca2+ leak lead to an increase in heat generation by the SR Ca2+ pump in resting skeletal muscle; and 3) in fibers with RyR α and β subtypes instead of RyR1, RyR Ca2+ leak is not a significant source of heat generation in resting skeletal muscle.

Next, we checked whether the increase in the SR Ca2+ pump heat generation exerted by RyR1 Ca2+ leak relied on cytosolic Ca2+ diffusion from the channel to the pump. To do this, we restricted the cytosolic Ca2+ diffusion by substituting the cytosolic Ca2+ buffer 0.1 mM ethylene glycol bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA) for 1 mM 2,2'-bis(o-aminophenoxy)ethane-N,N',N'-tetraacetic acid (BAPTA). In contrast to EGTA, BAPTA (1 mM) is a fast Ca2+ buffer that restricts the distance that a Ca2+ ion can travel freely before being chelated to 22.4 nm (13). To maximize the sensitivity of our assay, we tested the effect of CPA in the SR temperature of RyR1WT/WT and RyR1WT/KI fibers in the presence of BAPTA. These fibers were used in contrast to RyR1WT/WT or RyR1WT/KI fibers because of their higher contribution of RyR1 Ca2+ leak to thermogenesis (Fig. 2F).

Therefore, the RyR1WT/KI fibers should be the most sensitive to a restriction in cytoplasmic Ca2+ diffusion. Before testing the effect of cytoplasmic BAPTA on RyR1-dependent SR Ca2+ pump heat generation, we confirmed that the SR Ca2+ loading was comparable under the same [Ca2+]cyto (100 nM) in EGTA or BAPTA (SI Appendix, Fig. 2). When CPA was applied to RyR1WT/KI fibers partially exposed to ryanodine, the ERTY transients in sections with and without functional RyR1s were not different (P = 0.576; 4.053 ± 0.529% and 3.693 ± 0.442%, respectively) and were comparable to the values observed in ryanodine-exposed RyR1WT/KI fibers in 0.1 mM EGTA (Fig. 2 N and O). This shows that cytosolic Ca2+ diffusion from the RyR1 to the SR Ca2+ pump is required to increase the SR Ca2+ pump activity and heat generation.

Low [Ca2+]cyto Levels Regulate Heat Generation in Healthy, Resting Muscle. An increase in RyR1 Ca2+ leak has been proposed as a mechanism to maintain body temperature upon chronic cold exposure, where the increase in leak leads to greater SR Ca2+ pump activity to maintain the SR Ca2+ leak/uptake balance (6). Therefore, it is expected that mild changes in [Ca2+]cyto alter the activity of the pump in healthy muscle. To test this hypothesis, we coculated WT muscle fibers with ERTY and fluo-5N in the SR to track changes in SR temperature and [Ca2+]cyto, respectively, during exposure to increasing [Ca2+]cyto below the [Ca2+]cyto threshold for contraction (32) (Fig. 3). Increasing [Ca2+]cyto from 67 nM to 136 nM increased the [Ca2+]cyto, but no change in ERTY was observed. However, an increase to 165 nM [Ca2+]cyto caused a significant change in both [Ca2+]cyto (165 vs. 67 and 165 vs. 91 nM) and ERTY (165 vs. 67 and 165 vs. 91 nM), indicating that changes in heat generation. These results show that small changes in [Ca2+]cyto can produce a significant increase in SR Ca2+ pump thermogenic activity in WT muscle fibers.

RyR1 Ca2+ Leak Increases Local [Ca2+]cyto at SR Ca2+ Pump for Heat Generation. The rise in the SR Ca2+ pump-dependent thermogenesis generated by increases in RyR1 Ca2+ leak in mouse fibers (Fig. 2 D–F) and the reliance of the SR Ca2+ pump thermodogenic activity on Ca2+ diffusion from the channel to the pump (Fig. 2 N and O) suggest that RyR1 Ca2+ leak sets a local [Ca2+]cyto at the SR Ca2+ pump higher than the bulk [Ca2+]cyto set to 100 nM, RyR1 leak, set in our experiments to 100 nM. To calculate the local [Ca2+]cyto at the SR Ca2+ pump set by RyR1 Ca2+ leak, we generated a calibration curve of ERTY signal across a range of [Ca2+]cyto (500 to 50 nM) at the SR Ca2+ pump. For this calibration, RyR1 was blocked with 1 mM Tetracaine so that RyR1 Ca2+ leak did not influence [Ca2+]cyto. Fibers were initially bathed in 500 nM Ca2+ solution and progressively subjected to decreasing [Ca2+]cyto solutions (Fig. 4A). The average ERTY value from the last 30 s of each condition was calculated (Fig. 4B) and used to plot a curve of [Ca2+]cyto vs. ERTY (%) (Fig. 4C). Notably, the greatest changes in ERTY signal were achieved between 300 and 100 nM [Ca2+]cyto. Particularly, from 200 to 150 nM ERTY, fluorescence intensity increased from 104.984 ± 0.252 to 108.298 ± 0.313%, whereas from 150 to 100 nM, the intensity increased from 108.298 ± 0.319 to 113.163 ± 1.021%. To verify this result, an experiment was performed where the fiber was subjected to increasing concentrations of Ca2+ from 50 to 500 nM. A similar relationship between ERTY signal and [Ca2+]cyto was observed (SI Appendix, Fig. 3).

To calculate the contribution of RyR1 Ca2+ leak on the local [Ca2+]cyto at the SR Ca2+ pump, we used the previously obtained values of RyR1 contribution to ΔERTY (%) upon SR Ca2+ pump inhibition (Fig. 4D) in conjunction with the generated [Ca2+]cyto vs. ERTY (%) curve. Since the RyR leak contribution to ΔERTY (%) values were obtained from experiments performed at 100 nM [Ca2+]cyto, the ERTY % of fluorescence value at 100 nM Ca2+ within the calibration curve (113.162 ± 1.021%) was used as a reference value (Fig. 4E). We subtracted the values of RyR1 contribution to ΔERTY corresponding to 2.393 ± 0.456%, 5.025 ± 0.648%, and 7.747 ± 0.884% for RyR1WT/WT, RyR1WT/KI, and RyR1K1KI, respectively, to the percentage of fluorescence at 100 nM Ca2+ in the calibration curve. The obtained values were interpolated in the curve shown in Fig. 4C to estimate their corresponding values of [Ca2+]cyto. Using this calculation, with the bulk [Ca2+]cyto set to 100 nM, RyR1...
leakiness progressively increased the local \([\text{Ca}^{2+}]_{\text{cyto}}\) at the SR \(	ext{Ca}^{2+}\) pump to 125 ± 6, 157 ± 8, and 195 ± 14 nM \([\text{Ca}^{2+}]_{\text{cyto}}\) in RYR1\text{WT}/\text{WT}, RYR1\text{WT}/\text{KI}, and RYR1\text{KI}/\text{KI}, respectively (Fig. 4F). These results are consistent with RYR1 \text{Ca}^{2+}\) leak setting a local \([\text{Ca}^{2+}]_{\text{cyto}}\) at the SR \(	ext{Ca}^{2+}\) pump that drives its activity. 

Next, we used the estimates of local \([\text{Ca}^{2+}]_{\text{cyto}}\) at the SR \(	ext{Ca}^{2+}\) pump in the three RYRI genotypes to determine the SR \text{Ca}^{2+}\) pump ATP consumption rates and heat output using our previously established model (33). Fig. 5A shows the relationship of the SR \text{Ca}^{2+}\) pump ATP consumption rate (in \(\mu\text{M/s}\)) across the range of \([\text{Ca}^{2+}]_{\text{cyto}}\) from 50 to 500 nM. The ATP consumption rate increased with increasing RYRI leakiness across the genotypes (51.2 ± 5.3, 78.3 ± 8.5, and 126.8 ± 17.2 \(\mu\text{M/s}\) for RYR1\text{WT/WT}, RYR1\text{WT/KI}, and RYR1\text{KI/KI} fibers, respectively [Fig. 5B]).

In WT fibers, with the RYR1 blocked and the local \([\text{Ca}^{2+}]_{\text{cyto}}\) at the SR \text{Ca}^{2+}\) pump the same as the bulk \([\text{Ca}^{2+}]_{\text{cyto}}\) (100 nM), the estimated SR \text{Ca}^{2+}\) pump ATPase rate was 30.7 \(\mu\text{M/s}\). In contrast, with RYR1 \text{Ca}^{2+}\) leak present and local \([\text{Ca}^{2+}]_{\text{cyto}}\) at the SR \text{Ca}^{2+}\) pump raised to 125 nM \text{Ca}^{2+}, the SR \text{Ca}^{2+}\) pump ATPase rate was 51.2 \(\mu\text{M/s}\). This indicates that a 25% increase in \([\text{Ca}^{2+}]_{\text{cyto}}\) at the pump leads to a 66% increase in the SR \text{Ca}^{2+}\) pump activity (Fig. 5C). This nonlinear increase in the relationship between \([\text{Ca}^{2+}]_{\text{cyto}}\) and ATP consumption translates to an equivalent increase in the SR \text{Ca}^{2+}\) pump-dependent heat production in resting muscle. This suggests that the \text{Ca}^{2+}\) handling properties of the SR \text{Ca}^{2+}\) pump coupled with the endogenous cytosolic \text{Ca}^{2+}\) environment allow the pump to amplify the \text{Ca}^{2+}\) cycling-dependent heat production in response to RYR1 \text{Ca}^{2+}\) leak.

Finally, we used the estimated ATP consumption rate values to calculate the SR \text{Ca}^{2+}\) pump-dependent heat production at rest across the three genotypes. Assuming that in a steady state, ATP is regenerated through glucose oxidation (enthalpy change for this process is 2,802 kJ/mol at a stoichiometry of 38 ATP per glucose) at the same rate that it is consumed, the heat output is...
The evidence presented here shows that SR Ca\(^{2+}\) leak through the RyR1 directly affects the local \([\text{Ca}^{2+}]_{\text{cyto}}\) at the SR Ca\(^{2+}\) pump in mammalian resting skeletal muscle fibers, setting the thermogenic activity of the pump. We show that the SR Ca\(^{2+}\) pump contribution to SR temperature in resting skeletal muscle is different in mouse and toad skeletal muscle, examples of endotherms and ectotherms, respectively. The differences between the mouse and toad fibers at the SR was the heat generated by the isolated SR Ca\(^{2+}\) pump in the fiber and the additional contribution of Ca\(^{2+}\) via RyR leak to further amplify heat generation at the pump. In the RyR1 KI mice, it was not possible to resolve a difference in heat generated across the genotypes of the isolated SR Ca\(^{2+}\) pump in the fiber. This result indicates that RyR Ca\(^{2+}\) leak provides amplification of the heat generated by the pump by proportionally raising the local \([\text{Ca}^{2+}]\) at the pump in the resting fiber. Additionally, the lack of sarcolipin in the SR of TA fibers does not prevent the SR Ca\(^{2+}\) from generating significant heat, certainly in comparison to the toad, and the consistent heat generated by the isolated SR Ca\(^{2+}\) pump in the RyR1 KI fibers suggests that the composition of the SR is comparable between the genotypes.

To make measurements of the heat generated by the SR in resting muscle, we used a single-fiber approach, employing a targeted local inhibition of RyR1s with ryanodine delivered through the t-system. Ryanodine was chosen as the RyR inhibitor because of its high affinity for the target, which maintained binding to the RyR after the preparation was transferred from oil to a cytoplasmic bathing solution that did not contain ryanodine. The presence of a reference section in a single fiber where RyR1s were inhibited increased the signal-to-noise for detecting changes in RyR1 Ca\(^{2+}\) leak-dependent changes in SR temperature. Loading of the temperature-sensitive dye was not affected by local inhibition of RyR1s, and the presence of both conditions (± RyR1 Ca\(^{2+}\) leak) within the same biological sample allowed us to assess its contribution under the same dye-loading and acquisition settings.

RyR1 Ca\(^{2+}\) leak sets a standing Ca\(^{2+}\) gradient between the RyR1s and the SR Ca\(^{2+}\) pump, where the local \([\text{Ca}^{2+}]_{\text{cyto}}\) at the SR Ca\(^{2+}\) pump was maintained at a value greater than the bulk \([\text{Ca}^{2+}]_{\text{cyto}}\). This feature allows the Ca\(^{2+}\)-handling properties of the SR to set the basal use of ATP at the SR Ca\(^{2+}\) pump, setting the heat output. We were able to show that the magnitude of RyR1 Ca\(^{2+}\) leak is critical to heat generation by the SR by examining the SR Ca\(^{2+}\) pump-dependent heat generation of RyR1 KI mouse muscle fibers that show a gene-dose-dependent increase in RyR1 Ca\(^{2+}\) leak (31). In contrast, the ectothermic toad showed a smaller change in SR temperature. This is partially explained by the lower capacity of the toad fibers to leak Ca\(^{2+}\) through the RyR compared to mouse, where evidence suggests that Ca\(^{2+}\) leakage through RyRs in toad fibers is one order of magnitude lower than in mammals (34, 35).

We provide direct evidence that local \([\text{Ca}^{2+}]\) at the SR Ca\(^{2+}\) pump increases the pump basal activity and heat generation in healthy, resting mammalian skeletal muscle. Across a range of \([\text{Ca}^{2+}]_{\text{cyto}}\) at the resting muscle, the SR Ca\(^{2+}\) pump generated a nonlinear increase in SR temperature. This result indicates that thermogenic gain of the muscle is sensitive to small changes in RyR1 Ca\(^{2+}\) leak when the basal heat-generating capacity of the pump was relatively high, as in mouse muscle. The generation of a standing Ca\(^{2+}\) gradient set by the SR is consistent with raised \([\text{Ca}^{2+}]\) in the junctional space between the t-system and SR terminal cisternae, compared to the bulk cytoplasm, as shown previously by monitoring \([\text{Ca}^{2+}]_{\text{cyto}}\) levels in the presence and absence of RyR1 Ca\(^{2+}\) leak (13).

In the absence of RyR1 Ca\(^{2+}\) leak, the SR Ca\(^{2+}\) pump continued to generate heat in the fiber. The constant generation of heat indicates a basal ATP hydrolysis rate. Ca\(^{2+}\) slippage from the SR lumen to the cytoplasm through the pump, under a local build-up of adenosine diphosphate (36, 37), will generate a loss of SR Ca\(^{2+}\) that would require constant resequestering of Ca\(^{2+}\) to maintain the SR Ca\(^{2+}\) content. Importantly, the heat generated by the isolated SR Ca\(^{2+}\) pump provides a critical basal level that can be further amplified by RyR1 Ca\(^{2+}\) leak. The stark difference in heat generated by the isolated SR Ca\(^{2+}\) pump in mouse and toad fibers thus underscores the capacity of the resting muscle in these animals to act as a heat generator. The greater level of heat generated by the SR pump of the mouse than the toad isolated pump is likely set by differences in the SR Ca\(^{2+}\) pump modulation through regulatory proteins that interact with the pump and the lipidic composition where the SR Ca\(^{2+}\) pump is embedded, altering ATP hydrolysis–Ca\(^{2+}\) transport coupling ratios (5, 38). Furthermore, the fast-twitch fibers from

![Diagram A](image1.png)

**Fig. 5.** Local \([\text{Ca}^{2+}]_{\text{cyto}}\) at the SR Ca\(^{2+}\) pump sets the SR Ca\(^{2+}\) pump thermogenic activity. (A) Model of the SR Ca\(^{2+}\) pump ATP consumption rate across a range of 500 to 50 nM \([\text{Ca}^{2+}]_{\text{cyto}}\). (B) The SR Ca\(^{2+}\) pump activity (in μM/s) given the estimated local \([\text{Ca}^{2+}]_{\text{cyto}}\) at the pump across the three genotypes. One-way ANOVA with Tukey’s multiple comparisons revealed statistical significance across the genotypes (RYR1WT/WT vs. RYR1WT/KI, P < 0.0001; RYR1WT/KI vs. RYR1KI/KI, P = 0.0135). (C) Summary of local \([\text{Ca}^{2+}]_{\text{cyto}}\) at the SR Ca\(^{2+}\) pump in RYR1WT/WT in absence and presence of RyR1 Ca\(^{2+}\) leak (left y axis) (P < 0.001) compared to the SR Ca\(^{2+}\) pump activity at the given local \([\text{Ca}^{2+}]_{\text{cyto}}\) concentrations at the SR Ca\(^{2+}\) pump in both conditions (right y axis) (P < 0.01). (D) The SR Ca\(^{2+}\) pump-dependent heat production (in mW/g) across the three genotypes given the estimated the SR Ca\(^{2+}\) pump activity values shown in B. Results are mean ± SD. One-way ANOVA with Tukey’s multiple comparisons revealed statistical significance across the genotypes (RYR1WT/WT vs. RYR1WT/KI, P < 0.0001; RYR1WT/KI vs. RYR1KI/KI, P = 0.0135). *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001, ns, not significant.
TA used in this study do not contain sarcolipin (39), indicating that sarcolipin is not critical to the reduction in coupling efficiency of the SR Ca\(^{2+}\) pump. Across the muscle fibers in the body, the SR Ca\(^{2+}\) pump could be further regulated by sarcolipin, reactive oxygen species, phospholamban, heat shock proteins, and other regulators (5, 40), which vary in expression across fiber types (39) and possibly other factors.

From our calibration of the [Ca\(^{2+}\)]\(_{cyto}\) at the SR Ca\(^{2+}\) pump and the heat generated, we were able to show how leakier RyRs in fibers from the RYR1 KI mice provide more Ca\(^{2+}\) to the pump under the same bulk [Ca\(^{2+}\)]\(_{ISR}\) (Figs. 4 and 5). In the toad fibers, the low contribution of Ca\(^{2+}\) leak to heat generation at the pump may be, in part, due to a relatively low [Ca\(^{2+}\)]\(_{ISR}\) restricting the driving force for leak (41, 42). Additionally, the two sets of junctional membranous per sarcomere in mammalian fibers compared to one in amphibians (43) may increase the density of RyRs and SR Ca\(^{2+}\) pumps and decrease the diffusional distance for Ca\(^{2+}\) to travel in mice to increase the [Ca\(^{2+}\)] at the SR Ca\(^{2+}\) pump compared to toads.

Our direct demonstration that skeletal muscle RyR fibers can change their level of heat generation by changing RyR1 Ca\(^{2+}\) leak and setting a high basal level of heat generation solely through the SR Ca\(^{2+}\) pump provides key insights into how mammals can stay warm in a wide range of environments. Skeletal muscle is a large organ and it is highly metabolically efficient for handling the SR Ca\(^{2+}\), providing a source of heat generation at every sarcomere. The ability of mammals to adapt the Ca\(^{2+}\) handling apparatus of skeletal muscle under resting conditions for the purpose of generating volumes of heat that maintain body temperature (5) was a critical evolutionary step that allowed mammals to colonize all parts of the globe. Importantly, this evolutionary step involved altering the SR so that the pump became less efficient, consuming more energy at rest than ectothermic vertebrates, and, equally, the RyR1 provides an amplifier of the heat that can be generated at the pump to assist meeting the thermogenic need of the mammal. Furthermore, the sensitive method developed here to detect heat in a resting fiber with an in-preparation control for the RyR leak can be applied to any skeletal muscle fiber from vertebrates. For example, the contribution of the RyR leak and the isolated SR Ca\(^{2+}\) pump to SR heat generation can be assessed across fiber types and in models of aging and sarcopenia, exercise, and numerous other lifestyle and genetic factors. The results may form a basis for understanding changes in whole-body metabolism.

Methods

Muscle Preparation. All experimental methods using animals were approved by the Animal Ethics Committee at The University of Queensland. Male C57BL/6J mice were euthanized by cervical dislocation, and the TA muscles were rapidly excised. Cane toads were stunned with a blow to the head and double J mice were euthanized by cervical dislocation, and the TA muscles were rapidly excised. The preparation was transferred to a custom-built chamber and placed under oil above a layer of Sylgard.

All chemicals were obtained from Sigma-Aldrich. Ryanodine, Tetracaine, CPA, and N-benzyl-p-toluene sulfonamide (BTS) were prepared in stocks dissolved in dimethyl sulfoxide (DMSO).

Localized Inhibition of RyR1s within a Single Muscle Fiber. Single fibers from TA muscles were isolated by using fine forceps. To expose discrete segments of single fibers immersed on paraffin oil to Ryanodine, the fibers were mechanically skinned, leaving an approximate 500-μm subsection of the fiber with the sarcolemma intact. The intact section of the fiber was exposed to a solution containing 50 μM Ryanodine, (the following in mM) Fluo-5N, 1; Fluo-5N acetoxymethyl (AM) was used to monitor SR Ca\(^{2+}\) concentration. Single fibers were bathed in cytoplasmic solution (same formulation as above) followed by a solution with 0 Ca\(^{2+}\) to obtain the fluorescence maximum (Fmax) and minimum (Fmin), respectively. The previously determined Kp of Fluo-5N in the SR [0.4 mM (42)] was used to determine [Ca\(^{2+}\)]\(_{ISR}\), with the relationship:

\[
[Ca^{2+}]_{ISR} = \frac{F_{t} - F_{min}}{F_{max} - F_{min}} \times K_{p,Fluo-5N} 
\]

with F and [Ca\(^{2+}\)], the respective free Ca\(^{2+}\) concentration in the cytoplasm (100 mM) and the SR (calibrated in an independent set of experiments), respectively.

ERTY vs. [Ca\(^{2+}\)]\(_{Cyto}\) Calibration Curves. Fibers were initially bathed in 500 mM Ca\(^{2+}\) solution and progressively exposed to decreasing [Ca\(^{2+}\)]\(_{cyto}\) solutions (500—400—300—250—200—150—100—50 mM Ca\(^{2+}\)). Each Ca\(^{2+}\) bathing solution was maintained for either a minute or for the necessary time for the ERTY fluorescence intensity to reach a plateau. These experiments were performed in constant presence of 1 mM Tetracaine to discard any contribution of RyR1 channels.

SR Ca\(^{2+}\) Pump ATP Consumption Modeling. The SR Ca\(^{2+}\) pump ATP consumption rates were modeled establishing a sigmoidal relationship between [Ca\(^{2+}\)]\(_{ISR}\), and the rate of the SR Ca\(^{2+}\) pumping (46). For this model, we estimated the maximum rate of SR Ca\(^{2+}\) pumping from the rate of skeletal muscle heat production during an isometric contraction (47).

Statistical Analysis. Statistical analysis was performed with GraphPad Prism 8. Paired t test was used to compare the CPA-induced ERTY transients in the presence and absence of Ryanodine treatment within the same preparation. A one-way ANOVA with Tukey’s multiple comparisons was used to compare the RyR1-dependent and RyR1-independent ΔERTY upon SR Ca\(^{2+}\) pump inhibition across the different genotypes, as well as ERTY fluorescence intensity and [Ca\(^{2+}\)]\(_{ISR}\) across mild increases in [Ca\(^{2+}\)]\(_{cyto}\) and local [Ca\(^{2+}\)]\(_{isotonic}\) at the SR...
Ca\(^{2+}\) pump, the SR Ca\(^{2+}\) pump ATP consumption rate, and the SR Ca\(^{2+}\) pump-dependent heat production across the three genotypes. For all cases, differences were considered statistically significant at \(P < 0.05\).

Data Availability. All study data are included in the article and/or supporting information.

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