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Sarcoplasmic Reticulum Phospholipid Fatty Acid Composition and Sarcolipin Content in Rat Skeletal Muscle

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Abstract In a previous study, we reported lower sarcoplasmic reticulum (SR) Ca^{2+} pump ionophore ratios in rat soleus compared to red and white gastrocnemius (RG, WG) muscles which may be indicative of greater SR Ca^{2+} permeability in soleus. Here we assessed the lipid composition of the SR membranes obtained from these muscles to determine if SR docosahexaenoic acid (DHA) content and fatty acid unsaturation could help to explain the previously observed differences in SR Ca^{2+} permeability. Since we have shown previously that sarcolipin may also influence SR Ca^{2+} permeability, we also examined the levels of sarcolipin in rat muscle. We found that SR membrane DHA content was significantly higher in soleus (5.3 ± 0.2 %) compared to RG (4.2 ± 0.2 %) and WG (3.3 ± 0.2 %). Likewise, total SR membrane unsaturation and unsaturation index (UI) were significantly higher in soleus (% unsaturation: 59.1 ± 2.4; UI: 362.9 ± 0.8) compared to RG (% unsaturation: 55.3 ± 1.0; UI: 320.9 ± 2.5) and WG (% unsaturation: 52.6 ± 1.1; UI: 310. ± 2.2). Sarcolipin protein was 17-fold more abundant in rat soleus compared to RG and was not detected in WG; however, comparisons between soleus, RG, and WG in sarcolipin-null mice revealed that, in the absence of sarcolipin, ionophore ratios are still lowest in soleus and highest in WG. Overall, our results suggest that SR membrane DHA content and unsaturation, and, in part, sarcolipin expression may contribute to SR Ca^{2+} permeability and, in turn, may have implications in muscle-based metabolism and diet-induced obesity.

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Introduction

Biological membranes are essential cellular constituents that act as semi-permeable barriers housing vital proteins required for many physiological processes. Membrane lipids and properties may influence membrane permeability thereby altering the function of the integral proteins. Increased percentage of docosahexaenoic acid (DHA) in membrane phospholipids and membrane unsaturation, in particular, can increase Na^+ and H^+ permeability thereby promoting futile Na^+/K^+ pump activity and mitochondrial proton leak (Hulbert and Else 1999; Hulbert et al. 2005). However, whether DHA and membrane unsaturation could have similar effects and induce futile cycling of the Ca^{2+} pump via enhancement of the sarcoplasmic reticulum (SR) membrane Ca^{2+} permeability remains unknown.

SR Ca^{2+} permeability can be estimated by the ratio of Ca^{2+} pump activity measured in the presence of the Ca^{2+} ionophore A23187, to Ca^{2+} pump activity measured in the absence of ionophore. This ratio is termed the ionophore ratio. The ionophore increases membrane Ca^{2+} permeability and prevents back-inhibition of the Ca^{2+} pump, thereby increasing maximal Ca^{2+} pump activity (Bombardier et al. 2013b). An ionophore ratio of 1 is the minimum value of the ratio and is suggestive of a fully permeable membrane; conversely, a high ionophore ratio (>1) equates to lower Ca^{2+} permeability. In a previous study from our laboratory (Holloway et al. 2006), it was shown that SR Ca^{2+} permeability was higher in rat soleus than either the red or white portions of the gastrocnemius muscles (RG and WG) based on differences in the ionophore ratio (soleus, 2.0; RG, 3.8; WG, 4.1). Interestingly, lipid

analysis from whole muscle homogenate preparations has shown that DHA content and total unsaturation was lowest in the soleus compared to RG and WG muscles (Stark et al. 2007). However, differences in membrane lipid composition of the various subcellular organelles may not be reflected at the whole muscle level (Stefanyk et al. 2010). It is possible that the SR membranes from rat soleus could have greater DHA content and membrane unsaturation compared to rat RG and WG, especially given the muscle differences in SR Ca^{2+} permeability. However, the lipid composition of the SR membranes obtained from rat soleus, RG, and WG muscles has never been assessed.

Sarcolipin is a small (31 amino acid) integral SR protein that uncouples Ca^{2+} pump-mediated Ca^{2+} transport from ATP hydrolysis (Bombardier et al. 2013b) and plays a primary role in mediating muscle-based adaptive thermogenesis (for review see Gamu et al. 2014). We have previously shown that ionophore-supported Ca^{2+}-ATPase activity was not different between the soleus muscles of sarcolipin-null ($Sln^{-/-}$) mice and their wild-type littermates (Tupling et al. 2011). In contrast, maximal Ca^{2+}-ATPase activity without ionophore was significantly lower in the soleus muscles from $Sln^{-/-}$ mice which we attributed to sarcolipin causing slippage of the Ca^{2+} pump and preventing back-inhibition (Bombardier et al. 2013b). Indeed, increasing the sarcolipin:Ca^{2+} pump molar ratio in lipid vesicles significantly augmented the rate of Ca^{2+} slippage from the pump (Smith et al. 2002). Therefore, it is possible that differences in sarcolipin content between soleus, RG, and WG, which have yet to be assessed, may also relate to the previously observed differences in Ca^{2+} pump ionophore ratio between these muscles (Holloway et al. 2006).

In this study, we analyzed the phospholipid and fatty acid composition of SR membranes obtained from rat skeletal muscles to investigate the hypothesis that DHA content and membrane unsaturation would be highest in muscles with the highest SR Ca^{2+} permeability (i.e., soleus) as determined previously (Holloway et al. 2006). Given the putative effect of sarcolipin on ionophore ratio, we also assessed sarcolipin protein expression across rat soleus, RG, and WG homogenates to determine whether sarcolipin protein could contribute to the differences in ionophore ratio previously observed (Holloway et al. 2006). Similar to DHA content and membrane unsaturation, we hypothesized that sarcolipin protein expression would be greatest in soleus muscles.

Methods

Animals

Four male adult Sprague–Dawley rats (416 ± 3 g) were used to characterize membrane phospholipid composition in the different muscles and four female adult Sprague–Dawley rats (294 ± 12 g) were used to assess sarcolipin protein expression. Four male adult $Sln^{-/-}$ mice (35.9 ± 1.5 g) were used to determine ionophore ratios in soleus, RG, and WG muscles in the absence of sarcolipin protein. Animals were fed water and laboratory chow ad libitum and were housed in an environmentally controlled room (temperature 22–24 °C, 40–60 % relative humidity) on a reverse 12:12-h light–dark cycle until the time of the study. All animal procedures were reviewed and approved by the Animal Care Committee of the University of Waterloo and are consistent with the guidelines established by the Canadian Council on Animal Care.

Collection of Tissues and Sarcoplasmic Reticulum Vesicle Isolation

All rats were anesthetized using intraperitoneal injections of pentobarbital sodium (6 mg/100 g body wt) and select hind limb skeletal muscles (soleus, RG, WG) were extracted and homogenized. SR vesicles were isolated as previously performed in our laboratory (Tupling et al. 2001a). Enrichment of SR vesicles results in a 19–22 fold increase in Ca^{2+}-ATPase activity normalized to protein weight (data not shown). Contamination from mitochondrial membranes, assessed by determining the percentage of citrate synthase activity measured in the SR fractions versus activity measured in the crude homogenates (data not shown), is 5.8 ± 0.8 %. Homogenates and SR vesicles were stored at −80 °C until Western blotting, lipid extraction and analysis were performed.

Lipid Extraction and Analysis

Isolated SR vesicles (25 µl, 2.5–6.0 mg protein/ml) were homogenized in 3.0 ml of ultra-pure grade chloroform–methanol (2:1 vol/vol, Folch and Lees 1957) containing 50 µg/ml butylated hydroxytoluene (Sigma-Aldrich, St. Louis, MO, USA) and 1 µl of a 100 µM internal standard (equimolar mix of 14:1/14:1 phosphatidylcholine, PC; 14:0/14:0 phosphatidylethanolamine, PE; 14:0/14:0 phosphatidylglycerol, PG). This was followed by the addition of 0.5 ml of aqueous buffer (0.2 M sodium phosphate, pH 7.3) and brief centrifugation to separate the aqueous and organic phases. The organic phase containing the lipids was collected, dried, and reconstituted in chloroform for storage (−80 °C) until analysis. The fatty acid composition of total lipids and the acyl species of phospholipids were determined using a non-targeted semi-quantitative approach to allow for an initial characterization of glycerophospholipids of the SR. Through this approach, absolute concentrations of individual phospholipids and fatty acids are not possible, but differences across muscle types can be examined. Lipid samples in chloroform were directly infused using a Harvard syringe pump into the electrospray

source (10 µl/min) of a Micromass ZQ Spectrometer with MassLynx 4.0 software (Waters Corporation, Milford, MA) for electrospray ionization mass spectrometry. The capillary charge was set at 2.8 kV and the source temperature and desolvation temperature were kept at 100 and 150 °C, respectively. The radio frequency lens and Extractor voltage are set at 0.1 and 4 V, respectively. The fatty acid composition of the lipid extracts was determined using in-source, collision-induced dissociation to isolate the acyl fragments. The cone voltage was set at 120 V and negative ion masses in the 100–400 *m/z* range were measured (Sforza et al. 2004). The acyl species of intact phospholipids were detected using a cone voltage of 60 V for both negative and positive ions and mass spectra were measured in the 600–1000 *m/z* range. Neutral (PC and PE) and anionic (phosphatidyl inositol, PI; phosphatidylserine, PS; phosphatidic acid, PA) phospholipids in the chloroform extracts of SR membrane fractions were analyzed in the positive and negative ion modes, respectively. Phospholipids and fatty acyls were semi-quantified by comparisons of the individual ion peak intensity with internal standard (PE, PG, and PC) after correction for ^{13}C isotope effect (Han and Gross 2003) with the different cone voltage settings. Total amounts of phospholipid classes were determined by summing the individual phospholipid acyl species. The unsaturation index (UI) was calculated from SR membrane phospholipid composition as $\Sigma m_i \times n_i$, where m_i is the weight percentage and n_i is the number of carbon–carbon double bonds of the fatty acid (Stefanyk et al. 2010).

Western Blot Analysis

Western blot analysis was performed to detect relative differences in sarcolipin protein expression in soleus, RG, and WG as previously described (Bombardier et al. 2013b). Whole muscle homogenate was used to detect differences in sarcolipin content since sarcolipin is localized only in the SR membrane (Gramolini et al. 2004). Muscle homogenates from Sprague–Dawley rats were solubilized into 1× solubilizing buffer (0.1 % 2-mercaptoethanol, 0.0005 % bromophenol blue, 10 % glycerol, 2 % SDS, 63 mM Tris-HCl pH 6.8), and 40 µg of total protein was loaded onto a 13 % polyacrylamide Tris-Tricine gel, and separated by SDS-PAGE at constant voltage (120 V) for 70 min. Proteins were subsequently transferred onto 0.20 µm nitrocellulose membranes and later incubated in blocking solution (Tris base, 137 mM NaCl, and 0.1 % (v/v) Tween 20, pH 7.5, with 5 % (w/v) non-fat dry milk) for 1 h to block all non-specific binding sites. The membranes were then incubated for 1 h in 5 % milk-TBST containing sarcolipin antibody (1:100, Lampire Biological Laboratories, PA, USA). The membranes were then washed and incubated for 1 h in 5 % milk-TBST containing goat anti-rabbit IgG (peroxidase conjugated) with a 1:2000 dilution. Membranes were washed again and antibody-antigen complexes were visualized with a Chemi Genius2 Bio Imaging system (Syngene, MD, USA) after addition of Supersignal West Femto™ HRP substrate (Thermo Scientific, IL, USA). Membranes were also probed for α-actin (1:10 000, A2172, Sigma Aldrich, MO, USA) to control for variances in protein loading. Antibody-antigen complexes for α-actin were visualized with a Chemi Genius2 Bio Imaging system (Syngene, MD, USA) after addition of ECL Western Blot Substrate (BioVision, CA, USA).

Ca^{2+}-ATPase Activity and Ionophore Ratios

Ca^{2+}-ATPase activity was assessed via an enzyme-coupled assay in crude homogenates prepared from soleus, RG, and WG muscles from $Sln^{-/-}$ mice in the presence (without ionophore A23187) or absence (with 4 µM ionophore A23187) of a Ca^{2+} gradient using a spectrophotometric plate reader assay which has been described previously (Duhamel et al. 1985). The values for maximal Ca^{2+}-ATPase activity in the presence and absence of ionophore were obtained over a *p*Ca range of 5.2–4.5 and used to calculate the ionophore ratio (maximal Ca^{2+}-ATPase activity with ionophore A23187/maximal Ca^{2+}-ATPase activity without ionophore A23187) (Tupling et al. 2001b).

Statistical Analysis

All values are expressed as mean ± standard error. Differences in the phospholipid class content, fatty acid composition, and phospholipid acyl species of SR from soleus, RG, and WG muscles were analyzed using a one-way ANOVA. When necessary a Tukey post hoc analysis was performed to localize specific differences. Similarly, calculated ionophore ratios from $Sln^{-/-}$ mouse soleus, RG, and WG muscle homogenates were analyzed using a one-way ANOVA followed by a Tukey post hoc test. Assumptions for normality were verified using the Shapiro–Wilk test, and when data did not meet this assumption, Kruskal–Wallis ANOVA by ranks was performed. When necessary a Dunn's post hoc multicomparison analysis was performed to localize specific differences. Differences in sarcolipin protein expression between RG and soleus muscles were analyzed using a Student's *t* test. The level of statistical significance was set at $p \leq 0.05$ for all analyses and GraphPad Prism software was used.

Results

SR Membrane Total DHA Content

By utilizing a high cone voltage during ESI to induce acyl dissociation, we were able to use the MS to examine the distribution of the predominant fatty acids from SR

Table 1 Percent composition of the predominant fatty acids from total lipids of SR membranes across rat skeletal muscle types

Fatty acid	Soleus	RG	WG
16:0	13.1 ± 0.6	14.3 ± 0.8	15.9 ± 0.6*
18:0	27.8 ± 0.2	30.4 ± 1.0*	31.5 ± 0.7*
18:1	12.2 ± 0.9†	9.2 ± 0.2*	8.2 ± 0.4*
18:2n-6	26.1 ± 0.7†	24.5 ± 0.1*	18.6 ± 0.2*,†
20:4n-6	12.4 ± 0.4	13.9 ± 0.2	18.4 ± 0.2*
22:4n-6	0.5 ± 0.1†	0.8 ± 0.0*	0.9 ± 0.0*
22:5n-3, 22:5n-6	2.7 ± 0.1	2.7 ± 0.2	3.3 ± 0.2*
22:6n-3	5.3 ± 0.2†	4.2 ± 0.2*	3.3 ± 0.1*,†

Values are percent weight and are expressed as mean ± standard error. *RG* red gastrocnemius, *WG* white gastrocnemius, *SOL* soleus

* Significantly different from soleus, $p \leq 0.05$

† Significantly different from red gastrocnemius, $p \leq 0.05$

membranes across rat skeletal muscle types irrespective of the individual phospholipid classes (Table 1). Focusing on percent total DHA (22:6n-3) content, it was found to be highest in soleus SR membranes, followed by RG, and lowest in WG SR membranes.

SR Membrane Phospholipid DHA Content and Distribution

The phospholipid profile of the SR-enriched membranes from rat skeletal muscle in this study was composed mainly of PC (67–77 %), PE (8–12 %), and to a lesser extent, PI (6–9 %), PA (2–6 %), and PS (2–3 %). Consistent with total DHA content, four of the five predominant phospholipid species (PC, PE, PS, and PA) in SR membranes had greater relative DHA content in soleus compared with RG and WG (Table 2). In addition, comparisons among the

Table 2 Percent composition of the phospholipid acyl species from SR membranes across rat skeletal muscle types

Fatty acid	Soleus	RG	WG
PC			
16:0/18:1	11.1 ± 0.0†	19.0 ± 0.3*	19.0 ± 0.2*
16:0/18:2	7.4 ± 0.2	8.6 ± 0.8	8.2 ± 1.1
16:0/20:4	11.5 ± 0.2†	19.9 ± 0.3*	25.9 ± 0.5*,†
18:0/18:2	8.7 ± 0.0†	7.8 ± 0.2*	5.9 ± 0.1*,†
16:0/22:6n-3	9.0 ± 0.2	9.1 ± 0.2	7.2 ± 0.1*,†
18:1/20:4	5.5 ± 0.1†	4.8 ± 0.0*	5.4 ± 0.1†
18:0/20:4	9.2 ± 0.1†	5.7 ± 0.0*	4.8 ± 0.1*,†
18:0/22:4	4.3 ± 0.1†	2.2 ± 0.1*	1.0 ± 0.3*,†
PE			
18:0/18:2, 18:1/18:1	2.5 ± 0.2	2.6 ± 0.3	2.1 ± 0.2
16:0/22:6n-3	1.7 ± 0.1†	1.1 ± 0.1*	0.9 ± 0.0*
18:0/20:4	5.3 ± 0.5	3.7 ± 0.4	3.3 ± 0.3*
18:0/22:6n-3	2.7 ± 0.1†	1.4 ± 0.1*	1.5 ± 0.1*
PS			
16:0/18:0	1.7 ± 0.1†	1.1 ± 0.1*	0.9 ± 0.0*
18:0/22:6n-3	1.7 ± 0.1†	1.1 ± 0.1*	0.9 ± 0.0*
PA			
18:0/20:4	2.8 ± 0.1†	0.8 ± 0.1*	0.6 ± 0.0*
18:0/22:6n-3, 18:1/22:5n-3	3.4 ± 0.2†	1.8 ± 0.4*	2.3 ± 0.1*
PI			
18:0/20:4	5.0 ± 0.5	5.4 ± 0.2	7.6 ± 0.5*
18:0/22:6n-3	0.6 ± 0.0	0.6 ± 0.0	0.5 ± 0.0
18:0/22:5n-3	0.7 ± 0.0	0.7 ± 0.0	0.8 ± 0.0

The sum of individual percentages in each muscle do not equate to 100 % because of the presence of unidentified peaks

Values are percent weight and are expressed as mean ± standard error. *RG* red gastrocnemius, *WG* white gastrocnemius, *SOL* soleus, *PC* phosphatidylcholine, *PE* phosphatidylethanolamine, *PS* phosphatidylserine, *PA* phosphatidic acid, *PI* phosphatidylinositol

* Significantly different from soleus, $p \leq 0.05$

† Significantly different from red gastrocnemius, $p \leq 0.05$

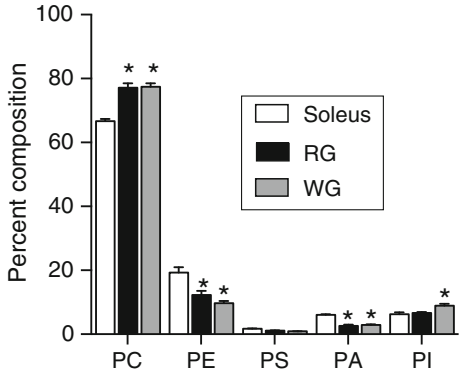

Fig. 1 Percent composition of phospholipid classes in sarcoplasmic reticulum-enriched membranes across skeletal muscle types (soleus, $n = 4$; *RG* red gastrocnemius, $n = 3$; *WG* white gastrocnemius), *PC* phosphatidylcholine, *PE* phosphatidylethanolamine, *PS* phosphatidylserine; *PA* phosphatidic acid, *PI* phosphatidylinositol). *Significantly different from soleus, $p \leq 0.05$ assessed by one-way ANOVA and Tukey post hoc test

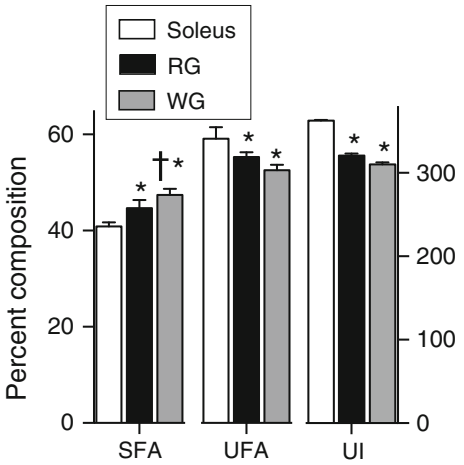

Fig. 2 Fatty acid indices of saturation and unsaturation in sarcoplasmic reticulum-enriched membranes across skeletal muscle types (soleus, $n = 4$; *RG* red gastrocnemius, $n = 3$, *WG* white gastrocnemius, $n = 4$) independent of phospholipid species. *SFA* saturated fatty acids (% of total fatty acids), and *UFA* unsaturated fatty acids (% of total fatty acids), calculated from the total SR fatty acid composition. *UI* unsaturation index, calculated from SR membrane phospholipid composition by multiplying the fraction of each PL species by its number of double bonds and summating these values within each muscle type and is plotted on the right *y*-axis. *Significantly different from soleus, $p \leq 0.05$; †significantly different from red gastrocnemius, $p \leq 0.05$

skeletal muscle types revealed that soleus had significantly higher percent composition of PE and PA, but lower PC compared to RG and WG (Fig. 1).

SR Membrane Saturation Profile

From our acyl chain determinations, we assessed the saturation profile which included percent of total fatty acid saturation (SFA), unsaturation (UFA), and unsaturation index (UI) to give a sense of the fluid nature of the SR-enriched membranes. Comparisons among the skeletal muscle types showed that soleus SR membranes had the highest percent unsaturation and UI and the lowest percent saturation (% unsaturation: 59.1 ± 2.4; UI: 362.9 ± 0.8) compared to both RG (% unsaturation: 55.3 ± 1.0; UI: 320.9 ± 2.5) and WG (% unsaturation: 52.6 ± 1.1; UI: 310. ± 2.2) (Fig. 2).

Sarcolipin Protein Expression

Western blot analyses revealed that sarcolipin protein expression was highest in the soleus muscles followed by RG and was not detected in WG muscles (Fig. 3a). A significant ~17-fold difference between soleus and RG sarcolipin expression was detected (Fig. 3b).

Ionophore Ratios in *Sln*$^{-/-}$ Mice

By determining maximal Ca^{2+}-ATPase activity in the presence and absence of the Ca^{2+} ionophore A23187, we found that, in the absence of sarcolipin, the soleus ionophore ratios were the lowest, being significantly different from WG ($p = 0.004$) and approaching significant difference from RG ($p = 0.08$) (Fig. 4). Ionophore ratios tended to be lower in *Sln*$^{-/-}$ RG muscle homogenates compared to WG (Fig. 4, $p = 0.06$). Thus, in the absence of sarcolipin, the ionophore ratio hierarchy among rodent skeletal muscles appears to be maintained (soleus < RG < WG).

Discussion

In the present study, we examined SR membrane composition semi-quantitatively and sarcolipin protein expression of rat soleus, RG, and WG muscles to test the hypotheses that DHA content, membrane unsaturation, and sarcolipin would be different between muscles and would correspond with the previously determined differences in SR Ca^{2+} permeability (Holloway et al. 2006). As hypothesized, we found the highest levels of total DHA content in the SR membranes from soleus muscles compared to those from both RG and WG muscles. In addition, of the five predominant phospholipid classes found in the SR membranes, four of them (PC, PE, PS, and PA) had greater DHA content in soleus muscles. The remaining phospholipid, PI, also had greater DHA content in soleus muscles compared to WG; however, this only approached statistical significance ($p = 0.051$). With respect to membrane unsaturation, soleus muscles had the highest levels of unsaturation as indicated by percent total fatty acid unsaturation and UI, and the lowest levels of total fatty acid

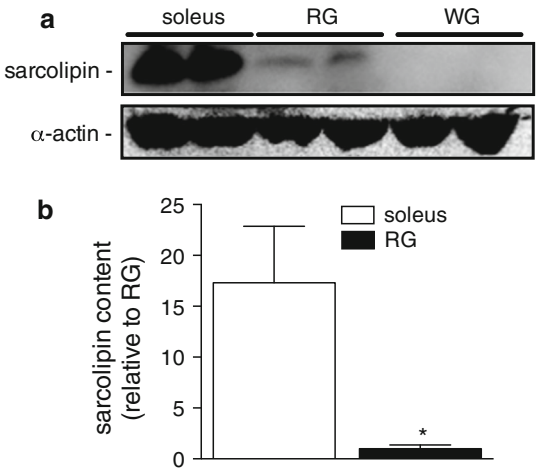

Fig. 3 Sarcolipin protein expression in rat soleus, red gastrocnemius (*RG*), and white gastrocnemius (*WG*) muscles. **a** Representative Western blot illustrating sarcolipin protein expression in rat soleus, RG, and WG muscle. For soleus, RG, and WG, 40ug of total protein was loaded. **b** Densitometric analysis of sarcolipin content normalized to α-actin and expressed relative to RG. *Significantly different from soleus, $p \leq 0.05$ using Student's t test

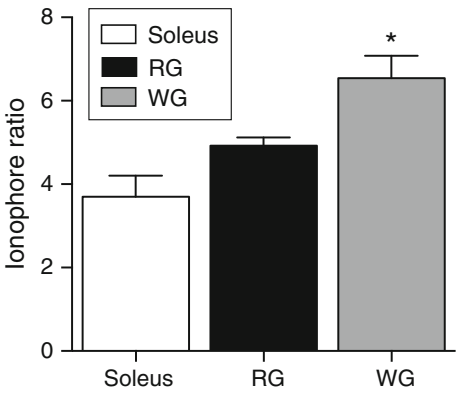

Fig. 4 Ionophore ratios calculated from $Sln^{-/-}$ mouse soleus, red gastrocnemius (RG), and white gastrocnemius (WG) muscle homogenates. *Significantly different from soleus, $p \leq 0.05$ using a one-way ANOVA and Tukey post hoc test

percent saturation. Thus, from our cross-sectional comparisons of SR membrane properties of different rat skeletal muscles, it appears that membrane DHA content and percent unsaturation may influence SR Ca^{2+} permeability. It is important to recognize that the phospholipid and fatty acid composition data presented herein were semi-quantitative and the ability to compare individual lipid levels within a sample type is limited; however, the present composition results are comparable with quantitative results published previously (Fiehn et al. 1971).

Apart from the phospholipid fatty acyl composition, the phospholipid head group may also influence membrane fluidity and permeability. Specifically, unlike PC, PE and PA are non-lamellar phospholipids that have the tendency to induce tighter packing of membrane lipids as well as reduce membrane fluidity (Cullis and de Kruijff 1979; Dalton et al. 1998; Fajardo et al. 2011; Starling et al. 1996), which would be expected to reduce membrane permeability (Lande et al. 1995). Furthermore, PE may actually prevent passive leak of Ca^{2+} through the SR Ca^{2+} pumps (Gould et al. 1987a, b). Therefore, our observation of higher PE and PA content and lower PC content in the most permeable SR membrane (i.e., soleus) suggests that relative to other membrane properties, the distribution of phospholipid classes and their effect on membrane packing is unlikely to have a major influence on the Ca^{2+} permeability of SR membranes in vivo. Alternatively, the differences in membrane phospholipid composition could aid in SR Ca^{2+} permeability via differences in the propensity to retain DHA in the lipid bilayer. For example, PE has been shown to have higher DHA content relative to other phospholipids (Metherel et al. 2009; Seigneuret and Devaux 1984; Stefanyk et al. 2010; Tsalouhidou et al. 2006). The observed differences in membrane phospholipid distribution reported in the present study could also be associated with other SR properties apart from SR Ca^{2+} permeability. Phospholipid distribution differences may be reflective of differences in SR membrane morphology with fiber types as reported previously (Schiaffino et al. 1970).

In the present study, we assessed whether differences in sarcolipin protein expression may also contribute to the previously observed differences in SR Ca^{2+} permeability across the soleus, RG, and WG muscles (Holloway et al. 2006) as previous data from our laboratory showed that sarcolipin can influence the ionophore ratio (Bombardier et al. 2013b; Tupling et al. 2011). Here, our Western blot data shows that rat soleus muscles have the highest protein expression of sarcolipin when compared to both RG and WG muscles which supports the view that sarcolipin may contribute to the lower ionophore ratios previously seen across these muscles (Holloway et al. 2006). However, when we calculate the ionophore ratios in soleus, RG, and WG muscle homogenates from $Sln^{-/-}$ mice we find the differences between muscles are similar to what was observed previously for rat isolated SR vesicles (Holloway et al. 2006). Therefore, it appears that sarcolipin may only account for part of the differences in ionophore ratios previously observed in rat (Holloway et al. 2006).

Collectively, our findings are suggestive of a potential influence of membrane DHA content, unsaturation, and sarcolipin levels on ionophore ratios and SR membrane Ca^{2+} permeability, and as such, could have implications in muscle-based thermogenesis and diet-induced obesity. In this context, both sarcolipin (Bal et al. 2012; Bombardier et al. 2013a; Maurya et al. 2015) and DHA (Delarue et al. 2004; Li et al. 2008; Lorente-Cebrian et al. 2013; Rossmeisl et al. 2009; Ruzickova et al. 2004; Vasickova et al. 2011) have been shown to stimulate energy expenditure and reduce/

prevent diet-induced obesity. Mechanistically, with respect to sarcolipin, our lab has found that its interaction with the Ca^{2+} pump reduces Ca^{2+} transport efficiency (Ca^{2+} uptake/Ca^{2+}-ATPase activity) likely by inducing Ca^{2+} slippage (Bombardier et al. 2013b). Conversely, DHA has been shown to enhance Na^+ and H^+ membrane permeability thereby leading to greater energy expenditure by the Na^+/K^+ pump and mitochondrial uncoupling (Hendriks et al. 1976; Hulbert and Else 1999; Hulbert et al. 2005; Stillwell et al. 1997; Stillwell and Wassall 2003). Thus, our data here suggest that another mechanism by which DHA can increase energy expenditure may be by enhancing SR Ca^{2+} permeability and inducing a futile cycle of the SR Ca^{2+} pump. This effect has the potential to dramatically alter muscle metabolism, since the SR Ca^{2+} pump accounts for 40–50 % of resting muscle energy expenditure (Smith et al. 2013). In support of this view, we have shown recently that after 8 weeks of dietary DHA supplementation, SR Ca^{2+} transport efficiency in rat skeletal muscle was significantly decreased, presumably due to a significant increase in SR Ca^{2+} permeability as indicated by a lower ionophore ratio (Fajardo et al. 2015). Since dietary DHA supplementation did not lead to a significant increase in % membrane unsaturation or UI, the increase in SR Ca^{2+} permeability was mostly attributed to SR DHA incorporation. Thus, future studies are required to fully elucidate the role of membrane unsaturation on SR Ca^{2+} permeability.

In summary, we found greater SR DHA content and membrane unsaturation, along with higher whole muscle sarcolipin in soleus compared to RG and WG muscles. As the soleus muscle exhibits greater SR Ca^{2+} permeability based on previous results using ionophore ratio, it seems as though DHA content, membrane unsaturation, and sarcolipin may have a role in influencing SR Ca^{2+} permeability and futile Ca^{2+} pumping. However, sarcolipin may only account for part of the differences in ionophore ratio as comparisons in soleus, RG, and WG muscles from mice lacking sarcolipin maintained the expected hierarchy. Combined with our recently published work, the results from the present study provide evidence that DHA can influence SR Ca^{2+} permeability and may induce futile Ca^{2+} cycling which has implications for both muscle energetics and diet-induced obesity. Future studies should aim to examine the potential role of membrane unsaturation on SR Ca^{2+} permeability and Ca^{2+} transport efficiency.

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