ATP consumption by sarcoplasmic reticulum Ca\(^{2+}\) pumps accounts for 50% of resting metabolic rate in mouse fast and slow twitch skeletal muscle

Sarah Michelle Norris,* Eric Bombardier,* Ian Curtis Smith, Chris Vigna, and Allan Russell Tupling

Department of Kinesiology, University of Waterloo, Waterloo, Ontario, Canada

Submitted 27 October 2009; accepted in final form 11 December 2009

Norris SM, Bombardier E, Smith IC, Vigna C, Tupling AR. ATP consumption by sarcoplasmic reticulum Ca\(^{2+}\) pumps accounts for 50% of resting metabolic rate in mouse fast and slow twitch skeletal muscle. Am J Physiol Cell Physiol 298: C521–C529, 2010. First published December 16, 2009; doi:10.1152/ajpcell.00479.2009.—In this study, we aimed to directly quantify the relative contribution of Ca\(^{2+}\) cycling to resting metabolic rate in mouse fast-twitch (extensor digitorum longus, EDL) and slow-twitch (soleus) skeletal muscle. Resting oxygen consumption of isolated muscles (\(\dot{V}_{\text{O}}\), \(\mu\text{g} \cdot \text{g wet wt}^{-1} \cdot \text{s}^{-1}\)) measured polarographically at 30°C was ~25% higher in soleus (0.61 ± 0.03) than in EDL (0.46 ± 0.03). To quantify the specific contribution of Ca\(^{2+}\) cycling to resting metabolic rate, cyclopiazonic acid (CPA), a highly specific inhibitor of sarco(endo)plasmic reticulum Ca\(^{2+}\) ATPases (SERCAs), was added to the bath at different concentrations (1, 5, 10, and 15 \(\mu\text{M}\)). There was a concentration-dependent effect of CPA on \(\dot{V}_{\text{O}}\), with increasing CPA concentrations up to 10 \(\mu\text{M}\) resulting in progressively greater reductions in muscle \(\dot{V}_{\text{O}}\). There were no differences between 10 and 15 \(\mu\text{M}\) CPA, indicating that 10 \(\mu\text{M}\) CPA induces maximal inhibition of SERCAs in isolated muscle preparations. Relative reduction in muscle \(\dot{V}_{\text{O}}\) in response to CPA was nearly identical in EDL (1 \(\mu\text{M}\), 10.6–3.0%; 5 \(\mu\text{M}\), 33.2 ± 3.4%; 10 \(\mu\text{M}\), 49.2 ± 2.9%; 15 \(\mu\text{M}\), 50.9 ± 3.8%) and soleus (1 \(\mu\text{M}\), 11.2 ± 1.5%; 5 \(\mu\text{M}\), 37.7 ± 2.4%; 10 \(\mu\text{M}\), 50.0 ± 1.3%; 15 \(\mu\text{M}\), 49.9 ± 1.6%). The results indicate that ATP consumption by SERCAs is responsible for ~50% of resting metabolic rate in both mouse fast- and slow-twitch muscles at 30°C. Thus SERCA pumps in skeletal muscle could represent an important control point for energy balance regulation and a potential target for metabolic alterations to oppose obesity.

oxygen consumption; in vitro isolated skeletal muscle; energy expenditure; calcium pumps

Skeletal muscle represents ~40% of body weight and accounts for ~20–30% of whole body basal metabolic rate (45, 60). Like other cell types, a portion of basal metabolic rate in skeletal muscle can be attributed to the cycling of Ca\(^{2+}\) across cell membranes, but very few studies have attempted to quantify the relative contribution of Ca\(^{2+}\) cycling to resting muscle metabolism. Under basal conditions in skeletal muscle, sarco(endo)plasmic reticulum Ca\(^{2+}\) ATPases (SERCAs) are responsible for maintaining a >100-fold Ca\(^{2+}\) concentration gradient across the sarcoplasmic reticulum (SR) membrane and for keeping the cytosolic free Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]) at or below 100 nM (53). Under optimized states, SERCAs transport 2 mol of Ca\(^{2+}\) across the SR membrane upon the hydrolysis of 1 mol of ATP (15, 31, 51). Early estimates of the energy consumed by SERCAs in muscle under basal conditions were based on measurements of the rate of Ca\(^{2+}\) influx from isolated SR vesicles and then the amount of ATP that would be required to reuptake that Ca\(^{2+}\) was calculated assuming an optimal Ca\(^{2+}\)-to-ATP coupling ratio. This approach yielded values for the energetic cost of SR Ca\(^{2+}\) pumping of ~3.4–7% of resting muscle metabolism (13, 27).

Although direct measurements are limited, there is evidence to suggest that these values largely underestimate the energetic cost of SR Ca\(^{2+}\) pumping in resting skeletal muscle. For example, another approach that has been employed to determine the relative contribution of SR Ca\(^{2+}\) cycling to resting energy expenditure in skeletal muscle is to measure the decrease in energy expenditure following exposure of the muscle to chemicals that indirectly inhibit SERCAs by inhibiting Ca\(^{2+}\) leakage from SR Ca\(^{2+}\) release channels (CRCs). Using this approach with direct calorimetry, Chinet et al. (12) found that ~12–24% of resting energy expenditure in mouse soleus is related to Ca\(^{2+}\) cycling across the SR membrane. Similar experiments on mouse soleus and extensor digitorum longus (EDL) muscles showed that 18–22% of resting energy expenditure in both muscles is related to SR Ca\(^{2+}\) uptake (20). However, for several reasons, these studies also likely underestimate the actual contribution of SERCA activity to resting metabolic rate in skeletal muscle. First, there is no certainty that Ca\(^{2+}\) leakage through CRCs was completely blocked by the CRC inhibitors employed in those studies (2,3-butanedione monoxime, dantrolene sodium); second, SERCA pumps themselves are a very significant pathway for leakage of Ca\(^{2+}\) out of the SR (42); and third, SERCAs will be active in resting skeletal muscle as long as some Ca\(^{2+}\) is present in the cytoplasm, unless SERCA activity is directly inhibited.

Surprisingly, no study has used specific inhibitors of SERCAs to directly quantify the relative contribution of SR Ca\(^{2+}\) pumping to resting metabolic rate in intact skeletal muscle. Chinet et al. (12) attempted this approach but found it problematic to separate the effects of blocking SR Ca\(^{2+}\) uptake from the resulting rise in [Ca\(^{2+}\)]) and development of contracture on muscle energy expenditure. To accurately quantify the contribution of Ca\(^{2+}\) cycling to resting metabolic rate in skeletal muscle, SERCA activity must be inhibited directly in conjunction with myosin ATPase activity. This can be accomplished pharmacologically using the myosin II inhibitor N-benzyl-p-toluene sulfonamide (BTS), which appears to selectively inhibit cross-bridge cycling but only in fast-twitch muscles (11, 49, 58). An alternative method that should be equally effective in fast- and slow-twitch muscles is to simply prestretch the muscle to eliminate filament overlap prior to inhibiting SERCA activity (6, 43, 56). Here, we used cyclopiazonic acid (CPA), a highly specific inhibitor of SERCA activity (23, 48), in conjunction with the prestretch technique, to more accurately quantify the contribution of SR Ca\(^{2+}\) pumping to resting metabolic rate in mouse fast- and slow-twitch skeletal muscle.
For comparison, some experiments with fast-twitch muscles were also performed with BTS.

**MATERIALS AND METHODS**

This study was approved by the Animal Care Committee at the University of Waterloo, and all procedures were performed in accordance with the Canadian Council on Animal Care.

**Animal description and experimental conditions.** A total of 93 sexually mature (8–12 wk) male C57BL/6 mice weighing an average of 23.8 ± 0.4 g were used in this study. Animals were housed two to four per cage in an environmentally controlled room with a standard 12:12-h light-dark cycle and allowed access to food (Tekland 22/5 rodent diet, Harlan-Teklad, Madison, WI) and water ad libitum. Animals were euthanized, and the EDL or soleus muscles were carefully removed from both hindlimbs with tendons intact. Because of the time required for each experiment, only one muscle (EDL or soleus) could be assessed from each mouse. In total, 40 EDL muscles and 40 soleus muscles were randomly assigned to one of five experimental conditions consisting of control (i.e., no CPA) and four CPA conditions (i.e., 1, 5, 10, and 15 μM). Control trials were conducted with the CPA solvent dimethyl sulfoxide (DMSO). An additional five mice were used for EDL experiments with BTS, and an additional eight mice were used for experiments to assess the effects of time on muscle VO\textsubscript{2} in prestretched resting soleus and EDL.

**Oxygen consumption in isolated intact mouse skeletal muscles.** The isolated EDL and soleus muscles were mounted in the TIOX tissue bath system (Hugo Sachs Electronik-Harvard Apparatus, March-Hugstetten, Germany) for the measurement of resting muscle oxygen consumption (VO\textsubscript{2}). The TIOX tissue bath system consists of a moveable platform that supports the muscle and a force transducer (F30 type 372) for measuring contractile force. A moveable jacketed chamber that is fitted with a temperature probe and a Clarke-type PO\textsubscript{2} electrode (model 1302) are also mounted onto the platform. The jacketed reservoir is connected to a thermocirculator to maintain constant chamber temperatures. Two parallel platinum plate electrodes are located on either side of the muscle for stimulation, enabling simultaneous measurement of force production and VO\textsubscript{2}. All components of the TIOX system are connected and controlled by individual PLUGSYS modules, and the data acquired from the individual modules are compiled, filtered, displayed, and stored by HSE-HA ACAD data-acquisition software (Hugo Sachs Electronik-Harvard Apparatus).

Using 4/0 surgical silk, we mounted the isolated muscles onto a fixed lower hook attached to the platform and a top hook that passes through a hole in the lid of the platform and connects to the external force transducer. After mounting the muscle, we closed the tissue chamber to the atmosphere by gently raising it to the platform and tightly sealing it with screws and wing nuts to fasten the chamber in place. The system was made air tight by closing off the hole in the lid of the platform using high-vacuum grease (Dow Corning, Midland, MI). The chamber was then filled with Ringer solution (in mM: 121 NaCl, 5 KCl, 1.8 Ca\textsubscript{Cl}\textsubscript{2}, 0.5 Mg\textsubscript{Cl}\textsubscript{2}, 0.4 NaH\textsubscript{2}PO\textsubscript{4}, 24 NaHCO\textsubscript{3}, 5.5 glucose, and 0.1 EDTA, pH 7.3), which was preheated to 30°C and aerated with 95% oxygen and 5% carbon dioxide, through a port on the bottom of the bath until it overflowed out of the ventilation cap in the lid of the platform, which was then sealed. The contents of the chamber were constantly stirred with a magnetic bar and stirrer located directly under the oxygen electrode to ensure consistent oxygen concentration throughout the solution. The muscle length was adjusted to achieve optimal length (\textit{L}_\text{o}) for twitch force production, and then the muscle was given 10 min to equilibrate inside the chamber before we initiated data collection.

PO\textsubscript{2} measurements were recorded every 4 s during each of three separate experimental trials at 30°C designed to quantify resting muscle VO\textsubscript{2} and SERCA pump energetics. The PO\textsubscript{2} in the bath (~620 Torr) should enable adequate diffusive oxygen supply to support resting muscle metabolism of mouse soleus and EDL (8); however, to prevent the formation of hypoxia in the core of the muscle, all experiments were terminated before the PO\textsubscript{2} of the Ringer solution fell below 580 Torr. First, the decrease in PO\textsubscript{2} of the Ringer solution was recorded in the presence of a resting muscle at \textit{L}_\text{o} for 30 min. Second, the muscle was lengthened to ~1.42 \textit{L}_\text{o} to eliminate sarcomere overlap and prevent activation of myosin ATPase activity during CPA trials. The bath was then emptied and refilled with fresh Ringer solution, and the PO\textsubscript{2} was recorded for 30 min. Finally, with the muscle still prestretched, the bath was emptied and refilled with fresh Ringer solution and either CPA (1, 5, 10, or 15 μM) or vehicle (DMSO) was added with a Hamilton syringe through the vent in the lid of the platform and PO\textsubscript{2} was recorded for 30 min. After this final 30-min period, the muscle was returned to its \textit{L}_\text{o}, and a single twitch was applied to ensure continued viability following CPA exposure. To further demonstrate the viability of each muscle preparation and the adequacy of O\textsubscript{2} diffusion, after the final twitch, muscles were removed and frozen immediately in liquid nitrogen for later analysis of high-energy phosphates [ATP and phosphocreatine (PCr)] and muscle metabolites [IMP and creatine (Cr)]. Because the CPA trial was always performed last, it was also important to demonstrate that muscle VO\textsubscript{2} in resting soleus and EDL is constant over the full duration of the experiment (i.e., 90 min). Therefore, in a subset of experiments (\textit{n} = 8), muscle VO\textsubscript{2} in prestretched resting soleus and EDL was recorded for 90 min in the absence of CPA (5 × 30-min trials). Finally, in a subset of experiments with EDL (\textit{n} = 5), BTS was used to inhibit myoinositol ATPase activity as opposed to prestretch. For these experiments, after the measurement of resting VO\textsubscript{2} for 30 min, EDL muscles were incubated with 25 μM BTS in (DMSO) for 45 min to inhibit myoinositol ATPase activity, followed by incubation with both 25 μM BTS and 10 μM CPA for an additional 30 min. The muscles were maintained at \textit{L}_\text{o} for all of these incubations.

**Calculation of muscle VO\textsubscript{2}**. The muscle VO\textsubscript{2} was calculated by multiplying the measured drop in PO\textsubscript{2} with time by the solubility of oxygen in Ringer solution at 30°C and the chamber volume (12.7 ml). The solubility of oxygen at 30°C was calculated to be 0.001203 M BTS and 10 μM CPA for an additional 30 min. The muscles were maintained at \textit{L}_\text{o} for all of these incubations.

The solubility of oxygen at 30°C was calculated using the following equation:

\[
kH = kH_o + e^{-\frac{Ah}{RT}}
\]

where \(kH\) is solubility constant of O\textsubscript{2} at experimental temperature, \(kH_o\) is the solubility constant of O\textsubscript{2} in pure water at standard temperature of 25°C (0.00113 M/atm), \(\Delta H\) is the enthalpy to dissolve O\textsubscript{2} (gas) in water (11.7 kJ/mol), \(R\) is the gas constant (0.008312 kJ·mol\(^{-1}\)·K\(^{-1}\)), \(T\) is the experimental temperature (30°C or 303.15 K), and \(T_o\) is standard temperature (25°C or 298.15 K).

In reality, the TIOX system is not completely closed to the atmosphere, resulting in leak of oxygen out of the solution even with no muscle mounted inside the chamber. Therefore, to account for the oxygen leak, blank trials were done at the beginning and at the end of daily data collection. A blank trial measures the rate of oxygen loss in an empty chamber (i.e., no muscle). The average daily rate of oxygen leak is then subtracted from the oxygen loss in the presence of the muscle to give the muscle VO\textsubscript{2}. A sample PO\textsubscript{2} tracing of a leak, rest, and PO\textsubscript{2} leak is then subtracted from the oxygen loss in the presence of the muscle to give the muscle VO\textsubscript{2}. A sample PO\textsubscript{2} tracing of a leak, rest, and PO\textsubscript{2} leak is then subtracted from the oxygen loss in the presence of the muscle to give the muscle VO\textsubscript{2}.

**High-energy phosphates and muscle metabolites.** Muscle metabolites were extracted from freeze-dried tissue (25, 26), and both adenosine triphosphate (ATP) and inosine monophosphate (IMP) were measured by using ion-pair reversed-phase high-performance liquid chromatography procedures originally developed by Ingebretson et al. (32) and subsequently modified by Green et al. (25).
The twitch and passive force responses recorded for an EDL muscle through-out a single experiment are shown in Fig. 2. The force responses for soleus were nearly identical to the response shown in Fig. 2 (data not shown). Twitch force was measured at the start of each experiment at $L_o$ (Fig. 2A) and then at progressively longer muscle lengths up to ~1.42 $L_o$ (Fig. 2B) where minimum twitch force was achieved (~7% of twitch force measured at $L_o$). There was a slow but steady decline in passive tension during prestretch trials (without CPA) probably due to relaxation of series and parallel elastic elements within the muscle. Although active twitch force could not be completely eliminated by prestretch, importantly passive muscle tension remained fairly constant during CPA trials (Fig. 2D), indicating minimal activation of myosin ATPase activity. Twitch force measured at $L_o$ at the end of each experiment following the CPA trial (Fig. 2E) was ~76% of initial twitch force measured at $L_o$. These force responses are similar to what has been reported previously for mouse EDL under similar conditions (8).

Resting muscle $\dot{V}O_2$. The rates of $\dot{V}O_2$ of the soleus and EDL muscles were measured at $L_o$ for 30 min at 30°C ($n$ = 40 each). The average resting $\dot{V}O_2$ (μg·g wet wt $^{-1}$·s$^{-1}$) at 30°C was 25% higher ($P < 0.05$) in soleus (0.61 ± 0.03) than in EDL (0.46 ± 0.03) (Fig. 3, open bars). Prestretching the muscles had no effect ($P > 0.1$) on muscle $\dot{V}O_2$ for either soleus or EDL muscles (Fig. 3). Resting $\dot{V}O_2$ of both soleus and EDL was stable with time, as the rates measured from 0 to 30 min of incubation were not different ($P > 0.05$) from measurements taken from 60 to 90 min of incubation (Fig. 4).
Control trials were conducted with the CPA solvent DMSO, high-energy phosphates (ATP, PCr) and metabolites (IMP, Cr). Drial function, which was assessed indirectly by measuring muscle inhibition of SERCA activity without compromising mitochondria, determine the optimal concentration that would elicit maximal 30 min. \( V_O2 \) (at 23°C and 1 atm) is expressed relative to muscle wet weight (\( \mu g^{-1}s^{-1} \)). Values are means ± SE (n = 40). *Significantly different from EDL (\( P < 0.05 \)).

Oxygen cost related to SR \( Ca^{2+} \) pumping in EDL and soleus muscles. The percent contribution of \( Ca^{2+} \) pumping by SERCAs to resting \( V_O2 \) was calculated by dividing the difference between the prestretch trial and the CPA trial by prestretch \( V_O2 \) (at \( L_o \)). A range of CPA concentrations (1, 5, 10, and 15 \( \mu M \)) was used to determine the optimal concentration that would elicit maximal inhibition of SERCA activity without compromising mitochondrial function, which was assessed indirectly by measuring muscle high-energy phosphates (ATP, PCr) and metabolites (IMP, Cr). Control trials were conducted with the CPA solvent DMSO, which had no effect on muscle \( V_O2 \) (\( P > 0.1 \)) in either EDL or soleus muscles (Fig. 5). In both EDL and soleus, there was a concentration-dependent effect of CPA on \( V_O2 \) with increasing CPA concentrations up to 10 \( \mu M \), which resulted in progressively greater reductions in muscle \( V_O2 \) (Fig. 5). There were no differences (\( P > 0.1 \)) between 10 and 15 \( \mu M \) CPA, indicating that 10 \( \mu M \) CPA induces maximal inhibition of SERCAs in isolated muscle preparations. The relative reduction in muscle \( V_O2 \) in response to CPA was nearly identical in EDL (1 \( \mu M \), 10.6 ± 3.0%; 5 \( \mu M \), 33.2 ± 3.4%; 10 \( \mu M \), 49.2 ± 2.9%; 15 \( \mu M \), 50.9 ± 2.1%) and soleus (1 \( \mu M \), 11.2 ± 1.5%; 5 \( \mu M \), 37.7 ± 2.4%; 10 \( \mu M \), 50.0 ± 1.3%; 15 \( \mu M \), 49.9 ± 1.6%). Importantly, when BTS was used to inhibit myosin ATPase activity in EDL muscles instead of prestretch, similar results were obtained where 10 \( \mu M \) CPA caused a 50.9 ± 7.4% reduction in muscle \( V_O2 \) (Fig. 6).

Muscle metabolites. High-energy phosphates (ATP, PCr) and metabolites (IMP, Cr) were measured in both EDL and soleus muscles, which were freeze clamped after the CPA trial in the TIOX. Metabolite measurements were made on the freeze-dried tissue. Figure 7 shows the high-energy phosphate and metabolic levels for the DMSO and 10 \( \mu M \) CPA conditions. There were no differences (\( P > 0.1 \)) between the DMSO and 10 \( \mu M \) CPA conditions for any of the metabolites measured in either EDL or soleus muscle. SERCA density and isoform distribution in soleus and EDL muscles. The content and relative distribution of SERCA pump isoforms (SERCA1a + SERCA2a) were compared in EDL and soleus by quantitative Western blotting as described in MATERIALS AND METHODS (e.g., Fig. 8). The amount of SERCA1a (mg/g total muscle protein) was 254 ± 23 and 2.8 ± 0.4, respectively, in EDL and 49 ± 3 and 3.6 ± 1, respectively, in soleus. Therefore, the ratio of SERCA1a to SERCA2a was 6:1 in EDL compared with ~14:1 in soleus; in total, there were ~4.9-fold more SERCA pumps (SERCA1a + SERCA2a) in EDL than in soleus.

Fig. 3. Resting oxygen consumption (\( V_O2 \)) of EDL and soleus (SOL) muscles at \( L_o \) compared with \( V_O2 \) during stretch trial (no sarcomere overlap) at 30°C for 30 min. \( V_O2 \) (at 23°C and 1 atm) is expressed relative to muscle wet weight (\( \mu g^{-1}s^{-1} \)). Values are means ± SE (n = 40). *Significantly different from EDL (\( P < 0.05 \)).

Fig. 4. Resting \( V_O2 \) of EDL and soleus muscles measured at 30°C during 0–30 and 60–90 min of incubation. \( V_O2 \) (at 23°C and 1 atm) is expressed relative to muscle wet weight (\( \mu g^{-1}s^{-1} \)). Values are means ± SE (n = 8).

Fig. 5. Relative reduction (%) of muscle \( V_O2 \) compared with rest after inhibition of SERCA activity by 1, 5, 10, and 15 \( \mu M \) CPA and in the control (DMSO) trial in both EDL and soleus muscles. Values are means ± SE (n = 8 each group). *Significantly different from DMSO (\( P < 0.05 \)). #Significantly different from 1 \( \mu M \) CPA (\( P < 0.05 \)). ΔSignificantly different from 5 \( \mu M \) CPA (\( P < 0.05 \)).
DISCUSSION

Few studies have attempted to quantify the specific contribution of SR Ca\(^{2+}\) pumping to resting metabolic rate in muscle. This is the first study to use a direct inhibitor of SERCA (i.e., CPA) to quantify the contribution of SERCA activity to resting energy expenditure in mouse skeletal muscle. The approach used in previous studies involved the use of CRC blockers (i.e., BDM and dantrolene) to inhibit SR Ca\(^{2+}\) release, thus lowering [Ca\(^{2+}\)]\(_{f}\) and indirectly inhibiting SERCA activity (12, 20). We hypothesized that this approach likely underestimates the ATP turnover by SERCAs in resting muscle because, even if BDM and dantrolene completely inhibit Ca\(^{2+}\) release through CRCs, SERCAs would remain active and contribute to energy consumption at rest (4, 42). Indeed, our results indicate that ATP consumption by SERCAs is responsible for \(~50\%\) of resting metabolic rate in both EDL and soleus, a quantity that is at least 2-fold higher than previously reported (12, 20). However, consistent with our results, Dulloo et al. (20) found that the relative (%) contribution of Ca\(^{2+}\)-dependent ATP turnover to resting metabolic rate was equal between EDL and soleus.

In this study, we measured rates of \(\dot{V}_{O_2}\) at 30°C in resting mouse EDL and soleus muscle using the TIOX tissue bath system. With the exception of at least one other study (14), our finding that resting \(\dot{V}_{O_2}\) was \(~25\%\) higher in soleus than in EDL is in agreement with most previous studies (18, 20, 56). The differences in muscle \(\dot{V}_{O_2}\) that we observed between EDL and soleus correspond to an absolute ATP turnover rate by SERCAs at the whole muscle level that is \(~24\%\) lower in EDL (159 \pm 8.8 mmol·g\(^{-1}\)·s\(^{-1}\)) than in soleus muscle (209 \pm 11.5 mmol·g\(^{-1}\)·s\(^{-1}\)). With the assumption that [Ca\(^{2+}\)]\(_{f}\) is constant in resting skeletal muscle, basically two main factors determine the ATP turnover rate by SERCAs in resting muscle: 1) the rate of Ca\(^{2+}\) leakage out of the SR and 2) the stoichiometry of Ca\(^{2+}\) uptake by SERCAs (i.e., Ca\(^{2+}\)-to-ATP ratio). Measurements in mechanically skinned fibers from rat EDL and soleus indicate that, under normal resting conditions, Ca\(^{2+}\) leaks out of the SR at approximately the same absolute rate in both muscles (38, 39). CRCs and SERCAs represent the major pathways for Ca\(^{2+}\) leakage from the SR, although SERCAs appear to be of primary importance in this regard (38, 39, 42). It is well known that the density of CRCs is about two- to threefold higher in EDL than in soleus (3, 19) and that the SERCA density is approximately three- to eightfold higher in soleus than in EDL (4, 42).

![Graph showing \(\dot{V}_{O_2}\) of EDL muscles measured at 30°C at \(L_o\) in the absence of inhibitors (rest), in the presence of 25 \(\mu\)M N-benzyl-p-toluene sulfonamide (BTS), and in the presence of 25 \(\mu\)M BTS plus 10 \(\mu\)M CPA (BTS + CPA). \(\dot{V}_{O_2}\) (at 23°C and 1 atm) is expressed relative to muscle wet weight (\(\mu\)g\(^{-1}\)·s\(^{-1}\)). Values are means \pm SE (\(n = 5\)). *Significantly different from both rest and BTS (\(P < 0.05\)).](image1)

![Graph showing high-energy phosphate and metabolite levels in soleus and EDL muscles after exposure to 10 \(\mu\)M CPA or DMSO (control) in the TIOX.](image2)
in EDL than in soleus (42, 55, 57). In this study, we found that mouse EDL contained 4.9-fold more SERCA pumps than shown in soleus. Therefore, it might seem surprising that the rate of Ca\(^{2+}\) leakage from the SR is the same in EDL and soleus; however, Murphy et al. (42) recently showed that the high content of calsequestrin in EDL muscle fibers helps to keep the [Ca\(^{2+}\)]\(_{\text{SR}}\) at sufficiently low levels to prevent high rates of Ca\(^{2+}\) leakage through the high density of SERCA pumps. Assuming that rat and mouse fibers are comparable and that the SR Ca\(^{2+}\) leak rates are the same in mouse EDL and soleus, the differences that we observed between EDL and soleus in ATP turnover by SERCAs would therefore suggest that Ca\(^{2+}\) uptake by SERCAs is less efficient in soleus than in EDL, at least under resting conditions.

A 2:1 ratio of Ca\(^{2+}\) transport to ATP hydrolysis by SERCAs is considered to be optimal as it corresponds to the stoichiometry of two Ca\(^{2+}\) binding sites and one ATP binding site on each SERCA pump (40, 52). However, our values for ATP turnover by SERCAs strongly suggest that the Ca\(^{2+}\)-to-ATP coupling ratio is actually much lower than 2:1 under physiological conditions. If the coupling ratio was 2 Ca\(^{2+}\) to 1 ATP, the rate of Ca\(^{2+}\) uptake (which is equal to the rate of Ca\(^{2+}\) leakage in resting muscle) would be ~318 nmol·g\(^{-1}\)·s\(^{-1}\) in EDL and ~418 nmol·g\(^{-1}\)·s\(^{-1}\) in soleus, which is ~22- to 26-fold higher than what has been measured in mechanically skinned EDL and soleus fibers from the rat (35, 38, 39). Taking into account the ATP turnover rates that we measured in this study and the SR Ca\(^{2+}\) leak rate reported by Lamb and Cellini (35), for rat EDL (which would be equal in soleus), we can calculate coupling ratios of ~0.09 Ca\(^{2+}\)/1 ATP in EDL and ~0.07 Ca\(^{2+}\)/1 ATP in soleus. Interestingly, the calculated coupling ratios for mouse EDL and soleus are similar to measurements made in SR preparations isolated from canine cardiac muscle (10, 21), human skeletal muscle (28), and various rat skeletal muscles (29). However, we are likely underestimating the true SR Ca\(^{2+}\) leak rates in EDL and soleus under the experimental conditions employed in this study; therefore, these calculations also likely underestimate the coupling ratios in EDL and soleus. The amount of slippage/leakage of SERCA pumps increases with increasing temperature (16) and ADP concentration (38). Compared with the skinned fiber study by Lamb and Cellini (35), both the experimental temperature (23 vs. 30°C) and the ADP concentration in the fibers (~0.1 vs. ~10 \(\mu\)M) were higher in the present study. Higher coupling ratios have been reported for rabbit skeletal muscle preparations, and, in contrast to our calculations, the Ca\(^{2+}\)-to-ATP ratio was higher in slow-twitch muscle SR vesicles (i.e., 0.98 Ca\(^{2+}\)/1 ATP) than in fast-twitch skeletal muscle SR vesicles (i.e., 0.37 Ca\(^{2+}\)/1 ATP) (44). The coupling ratio differences found between slow- and fast-twitch muscle were attributed to differences in SERCA isoform expression since SR vesicles from rabbit fast-twitch skeletal muscle only expressed SERCA1a, whereas vesicles from rabbit slow-twitch skeletal muscle expressed preferentially SERCA2a (44). In contrast, we found that over 90% of the SERCA molecules found in both mouse EDL and soleus were SERCA1a, which could explain why the calculated coupling ratios for each muscle are closer to the ratios that were...
reported for rabbit fast-twitch skeletal muscle vesicles in the study by Reis et al. (44).

One possible explanation for reduced Ca$^{2+}$ transport efficiency of SERCAs in mouse soleus compared with EDL is related to differences in the expression of both phospholamban (PLN) and sarcoclinin (SLN), two homologous SERCA binding proteins that regulate SERCA activity by lowering the apparent Ca$^{2+}$ affinity of the pump. Comparisons between wild-type and PLN-null mice have shown that PLN also decreases the Ca$^{2+}$-to-ATP coupling ratio in cardiac SR preparations, specifically at low [Ca$^{2+}$]$_i$ (21). Likewise, reconstitution experiments have shown that SLN uncouples ATP hydrolysis from Ca$^{2+}$ transport by SERCAs (51) and increases the amount of heat released per mole of ATP hydrolyzed (41). These results could be explained by SLN causing an increased rate of slippage on SERCAs (41, 51), which would decrease the fraction of energy released during ATP hydrolysis that is converted into work and increase the amount of heat released (16, 17). Both PLN and SLN are expressed abundantly in mouse soleus (5, 50) but not in EDL. This could explain why the absolute ATP turnover by SERCAs is higher in mouse soleus than in EDL under resting conditions; however, because resting VO$_2$ is higher in soleus, the relative contribution of SERCA activity to muscle VO$_2$ is the same in EDL and soleus.

We are confident in the validity of our results and our conclusion that SERCA pumps account for ~50% of resting muscle metabolic rate for several reasons. First, we used a range of CPA concentrations to ensure that maximal inhibition of SERCA activity was achieved in the isolated muscle preparations. The reduction in muscle VO$_2$ due to CPA exposure reached a plateau at 10 µM CPA in both EDL and soleus, suggesting that SERCAs were completely inhibited at this concentration. If SERCA inhibition was incomplete, even at 15 µM CPA, this would mean that, if anything, we could be underestimating the contribution of SERCA activity to resting muscle VO$_2$. Second, to prevent the expected contracture of the muscle following the addition of CPA, the prestretch method was used to inhibit cross-bridge formation. Importantly, prestretch had no effect on muscle VO$_2$ in either EDL or soleus, suggesting that SERCA activity was not altered by prestretch. As further support for this view, we have found that 10 µM CPA inhibits resting VO$_2$ in mouse EDL by ~50%, regardless of whether prestretch or BTS was used to inhibit myosin ATPase activity. It is also important to point out that, even if prestretch and/or BTS did not completely prevent activation of myosin ATPase activity as a result of elevated [Ca$^{2+}$]$_i$ during CPA trials, this would mean that we are in fact underestimating the contribution of SERCAs to resting muscle metabolic rate. Similarly, we could be underestimating the contribution of SERCAs to resting muscle metabolism if [Ca$^{2+}$]$_i$ was increased during CPA exposure and if Ca$^{2+}$ directly increased mitochondrial respiration (8). Third, 10 µM CPA should have highly specific effects on SERCAs in skeletal muscle preparations (30, 48). Nevertheless, we assessed muscle metabolic levels in EDL and soleus as a marker of mitochondrial function to rule out the possibility that the effects of CPA on muscle VO$_2$ were due to impaired oxidative phosphorylation. CPA had no effects on muscle high-energy phosphates (ATP, PCr) or related by-products (IMP, Cr), and the metabolite concentrations found in both EDL and soleus are within the normal range for resting mouse muscle (33). These results indicate that diffusive oxygen supply in these experiments was adequate to support resting metabolism, and importantly we can rule out the possibility that the effects of CPA on muscle VO$_2$ involved impairment of mitochondrial VO$_2$. Finally, since resting VO$_2$ in both EDL and soleus was stable for at least 90 min, we can conclude that the effects of CPA on muscle VO$_2$ were specifically due to inhibition of SERCAs and not a result of a coinciding progressive decrement in muscle metabolic rate with time.

In adult C57BL/6 mice, the EDL contains >90% type II fibers (mostly IIB) and <10% type I fibers, whereas the soleus contains ~50% type I and type II fibers (mostly IIA) (4, 2). Our results do not identify the contribution of the different fiber types to muscle VO$_2$ or the relative contribution of SERCA activity to the metabolic rate of the different fiber types. It is worth noting that Murphy et al. (42) reported that virtually all of the SERCAs found in rat soleus muscles were present in the fast-twitch fibers. Interestingly, the relative abundance of SERCA1a and SERCA2a mRNA levels in mouse (strain 129Sv/Swiss 50/50) soleus appears to match closely the fiber-type distribution (i.e., 65% SERCA2 and 42% SERCA1) (85). Importantly, the protein contents of the different SERCA isoforms in mouse soleus have not been quantified previously. In this study, SERCA1a and SERCA2a were quantified by comparing the Western blot band intensities of mouse muscle samples relative to purified proteins obtained from rat muscles. Although it is quite possible that the SERCA antibodies were differentially sensitive to the mouse and rat SERCA proteins, which would have caused an unknown level of error in the quantification, based on our results showing that over 90% of the SERCA molecules found in mouse soleus were SERCA1a, it is likely that SERCA1a is present in at least some type I mouse soleus fibers. Future studies should examine SERCA isoform protein expression in individual fiber types from mouse muscles to test this postulate.

It is well known that SERCAs play an important role in thermogenesis (9, 16), but SERCAs may also play an important role in the regulation of whole body energy balance. With the assumption that skeletal muscle accounts for 30% of basal metabolic rate in mice (45) and that the results of our present study can be extrapolated to all mouse muscles at physiological temperature, SERCA activity can explain ~15% of whole animal resting VO$_2$. It is also well known that SERCAs contribute ~30–40% to the energy cost associated with muscle contraction (for review, see Ref. 7). Therefore, given that basal metabolic rate and physical activity account for ~60% and 30% of total daily energy expenditure, respectively (36, 45), and that skeletal muscle accounts for ~90% of the increased energy requirements associated with physical activity (45), SERCAs in skeletal muscle may explain ~17–20% of whole body total daily energy expenditure.

In summary, the results from this study support the conclusion that ATP consumption by SERCAs is responsible for ~50% of resting metabolic rate in both mouse EDL and soleus muscles at 30°C. These results have important implications for the basic understanding of muscle energetics and suggest that the SERCA pump in skeletal muscle represents an important control point for energy balance regulation and a potential target for metabolic alterations to oppose obesity and other metabolic disorders.
ACKNOWLEDGMENTS

We thank Dr. Richard Hughson and Dr. James Rush for helpful comments on the manuscript.

GRANTS

This work was supported by Grant MOP-86618 from the Canadian Institutes of Health Research (to A. R. Tulip). S. M. Norris was supported by a Natural Sciences and Engineering Research Council of Canada Postgraduate Scholarship M award. E. Bombardier, I. C. Smith, and C. Vigna are all supported by a Natural Sciences and Engineering Research Council of Canada Postgraduate Scholarship D award.

DISCLOSURES

No conflicts of interest are declared by the author.

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