SUPPLEMENTAL DATA

Conversion factors and concentrations for calculations with the Ca^{2+} model

Biochemical measurements of myoplasmic protein content are usually expressed per kg muscle wet weight. For the model calculations, these values must be converted to molar concentrations referred to the water volume of the relevant intracellular location. The scaling factors for the conversions derived here are for swimbladder fibres of male toadfish since male fish were used predominantly in this study. The approach is similar to that developed for frog fibres (Baylor *et al.*, 1983) but takes into account the more complex anatomy of swimbladder fibres, which have a central core that is devoid of myofilaments but contains mitochondria and SR (Appelt *et al.*, 1991). Based on the work of Desmedt (1953), Baylor et al. (1983) assumed that 1 g of muscle wet weight contains 0.125 g of extracellular water, 0.662 g of fibre water, and 0.213 g solid material, and that fibre water is divided between myoplasm, SR, and mitochondria in proportion to the volume fraction of each compartment. According to the ultra-structural measurements of Appelt et al. (1991), the volume fraction of the myofibrillar region (non-central-core region) of male swimbladder fibres is 0.69 and that of the mitochondria, which are found largely in the central core, is 0.04. The volume fraction of the non-myofibrillar, non-mitochondrial region (referred to as the cytoplasmic region by Appelt *et al.*, 1991) is, therefore, 0.27. About 0.3 of the myofibrillar volume is occupied by SR (Clara Franzini-Armstrong, personal communication) from which myoplasmic constituents such as parvalbumin will be excluded. The volume fraction of myoplasmic water in the myofibrillar region is therefore 0.483 (= 0.69x0.7). The fraction of the cytoplasmic region occupied by SR can be estimated from the measured areas of SR membrane per unit volume reported by (Appelt *et al.*, 1991): 10.45

m^2/m^3 in the myofibrillar region and 6.45 m^2/m^3 in the cytoplasmic region. If the volume fraction of the SR is proportional to area, the SR then occupies about 0.185 (= 0.3x6.45/10.45) of the cytoplasmic region; correspondingly, the volume fraction of myoplasmic water in the cytoplasmic region is 0.22 (= 0.27x(1-0.185)). Since parvalbumin (and the SR Ca^{2+} pumps) is found in both the myofibrillar and cytoplasmic regions, the parvalbumin concentration expressed relative to fibre wet weight should be multiplied by the factor 2.15 (= 1/(0.662*(0.483+0.22))). The equivalent factor for troponin concentration is 3.13 (= 1/(0.662*0.483)), since troponin is restricted to the myofibrillar region. An additional factor, to correct the amplitude of measured F/F_R signals, takes into account that resting F comes from indicator in both the myofibrillar and cytoplasmic regions whereas the brief F stimulated by an action potential will arise predominantly in the myofibrillar region. (For example, the large parvalbumin concentration in swimbladder fibres will minimize the change in free $[Ca^{2+}]$ in the core region where no Ca^{2+} release takes place). The measured F/F signals were thus scaled by the factor 1.46 (= (0.483+0.22)/0.483) to refer them to the change in the myofibrillar region.

Concentrations in the myofibrillar region, expressed in moles per liter of myoplasmic water (M), were calculated for the following model constituents:

• Troponin. The troponin content reported in this article, 33.8 moles per kg wet weight, corresponds to 106 M if scaled by the factor 3.13 derived above. This is close to the 120 M value estimated for frog fibres (Baylor *et al.*, 1983). Since the troponin concentration should largely be set by the geometrical properties of the myofilament lattice, the similarity of these two numbers suggests that the morphological correction factors are reasonably accurate.

• Parvalbumin. The parvalbumin content of swimbladder fibres was measured to be 13.6 g per kg

wet weight (Tikunov & Rome, 2009). This corresponds to 1.24 mmoles per kg wet weight if the molecular weight of parvalbumin is 11,000. The estimated concentration referred to the myofibrillar water volume is then 2.67 mM (= 1.24x2.15), the value used in the model. This is in reasonable agreement with two other estimates from the literature. Hamoir et al. (1980) reported 1.61 mmoles per kg fresh weight, which corresponds to 3.46 mM (= 1.61x2.15) referred to the myoplasmic water volume. Appelt at al. (1991) reported a range for parvalbumin content in male toadfish, 40 to 84 g mg^{-1} dry weight. With 11,000 as parvalbumin's molecular weight, the midpoint of this range corresponds to 1.2 mmoles per kg wet weight (= 62x0.213/11), or 2.58 mM (= 1.2x2.15) referred to the myoplasmic water volume.

• Calcium pump. An anatomical estimate of the concentration of SR Ca^{2+} pumps in the myofibrillar region can be made from the SR membrane area (10.45 m^2/ m^3) and the pump density (32,000 per m^2) (Appelt *et al.*, 1991). This gives a concentration of 555 moles per liter of myofibrillar volume. The fraction of this volume occupied by myoplasmic water is 0.57 (= 0.7x0.81) so that the appropriate concentration of SR pump sites is 979 M. The factor 0.81 (= 0.662/(0.662 + 0.213/1.38)) is the estimated volume fraction of fibre water derived under the assumptions that average protein density is 1.38 g/cm^3 and that the same volume fraction for fibre water applies to all fibre compartments (Baylor *et al.*, 1983). This gives the larger of the two values used for the pump concentration in the model, 980 M. This concentration of Ca^{2+} pumps, in combination with the model's reaction scheme for Ca^{2+} binding and for the maximal turnover rate by the pump (Hollingworth *et al.*, 2006), is consistent with the maximal rate of Ca^{2+} pumping reported for skinned swimbladder fibres (Young *et al.*, 2003). The other pump concentration, 190 M, is based on the biochemical measurements of Feher et al. (1998), who reported that the

pump concentration in toadfish swimbladder muscle is 1.6 times that in rodent extensor digitorum longus muscle, for which a modeling value of 120 M was used previously (Baylor & Hollingworth, 2007).

Measurement of lactate production

As oxygen consumption was not measured in these experiments, the chamber was open to the atmosphere and the Ringer's was gassed continuously with a calibrated mixture of 75% O_2 and 25% N_2. Aliquots of 250 µl were removed from the chamber at 2-5 minute intervals and replaced with the same volume of fresh Ringer's solution (Rome & Kushmerick, 1983). These samples were immediately frozen for later analysis by placing them in an aluminum block on dry ice. The lactate concentration in each sample was determined using a blood lactate diagnostics kit (Sigma catalogue no. 826-A). Lactate and NAD are converted to pyruvate and NADH respectively by the action of LDH (lactate dehydrogenase). To ensure that the reaction went to completion, the pyruvate in the usual Ringer's solution was replaced with glucose. Experiments showed that there was no difference in O_2 whether pyruvate or glucose was used as a substrate. Lactate was measured as the NADH absorbance at a wavelength of 340 nm. lactate was converted into units of high-energy phosphate (~P) using a conversion factor 1.5.

Analysis of assumptions underlying the Recovery Oxygen consumption in the presence of BTS

The recovery-oxygen approach involves six assumptions: (1) at the concentration used in this study (25 BTS blocks all of the crossbridge ATPase, (2) 25 M BTS does not affect Ca^{2+} release (Methods, Supplemental Data), (3) the Ca^{2+} that is released is eventually pumped back into the SR over the time course of the oxygen measurements, (4) the ~P:O_2 ratio is

approximately 6 (Methods), (5) two Ca^{2+} molecules are pumped into the SR for each molecule of ATP consumed (Methods), and (6) the non-crossbridge ATP utilization is exclusively due to Ca^{2+} pumping. To the extent that these assumptions are inaccurate, generally simple linear errors likely apply. Many of these assumptions were dealt with in the Methods. Relevant additional points about assumptions 1, 2, 3, and 6 are discussed here.

We have previously shown that assumption 1 is valid; in saponin-skinned swimbladder fibers, 25 M BTS knocks out both force and crossbridge ATPase by more than 96% (Young *et al.*, 2003). A greater than 96% reduction in force was observed in intact fibers bundles used in the current experiments, indicating that at least 96% of the crossbridge ATP utilization has been eliminated. Because in the absence of BTS, the ATP utilized by the crossbridges is ~25 moles per kg muscle per twitch (Fig. 5), the assumption of complete elimination of ATP consumption by the crossbridges may give rise to an error of not more than 1 mole ATP per kg muscle per twitch. This corresponds to only 2 moles Ca^{2+} released per kg muscle per twitch.

A fundamental assumption of our approach is that BTS does not affect Ca^{2+} release per twitch (assumption 2). Cheung et al. (2002) reported that, at a concentration of 5 M, BTS does not affect the Ca^{2+} transient of frog muscles. However, they also reported that, at a concentration of 100 M, the refractory period for repetitive stimulation was lengthened, likely indicating action potential (AP) prolongation. In theory, a prolongation of the AP could lead to longer open times per twitch for the SR Ca^{2+} release channels and thus a greater Ca^{2+} release than in normal muscle. However, Woods et al. (2004) reported that, at a [BTS] of 50 , the prolongation of the AP in mouse fast-twitch fibers was slight, with little or no change in the Ca^{2+} transient, and therefore little or no change in the SR Ca^{2+} release. This conclusion is also supported by the finding in

mouse fast-twitch muscle that activation heat is the same whether measured by inhibiting crossbridge activity with 25 M BTS or by a reduction in filament overlap (Barclay *et al.*, 2008).

Hence it is unlikely that the 25 M concentration of BTS used in this study resulted in a significant change in SR Ca^{2+} release. Also of note, we have previously shown in saponin-skinned fibres that BTS has no direct effect on the pumping rate of the SR Ca^{2+} pumps (Young *et al.*, 2003).

Regarding assumption 3, it is important to note that toadfish call all night long, producing up to thousands of boatwhistle calls in an evening. If there were a significant net loss or net gain of Ca^{2+} to the SR with each call, the sheer magnitude of the number of contractions would likely lead to either depletion of SR or overfilling of the SR, neither of which would permit the muscle to continue to function in the observed way. Hence, it is reasonable to assume that the amount of Ca^{2+} pumped by the SR equals the amount released .

Regarding assumption 6, we estimate that consumption of ATP by the Na/K pump is ~1.3 mole kg^{-1} per twitch. Our estimate is based on the following assumptions: (i) the area of the exterior membranes (surface and t-tubular membranes) of a typical swimbladder fibre is 5,300 cm^2 per cm^3 of fibre volume (see, e.g., Fig. 1 of Appelt *et al.*, 1991), (ii) the electrical capacitance of these membranes is 1 F per cm^2, (ii) the exterior membranes undergo a 100 mV action potential with each twitch, (iv) the net influx of sodium with each action potential is larger than the minimum net influx calculated from the membrane capacitance and the action potential amplitude by a factor of 2 (Hodgkin & Horowicz, 1959, 20°C), (v) the elevation in intracellular sodium due to a high-frequency train of action potentials is reversed with a time constant of about 1 hour (Hodgkin & Horowicz, 1959), and (vi) the density of the fibre is 1.07 kg per L (Baylor *et al.*,

1983).

If calculated as a % of the ATP consumption per twitch attributed to Ca^{2+} pumping, this corresponds for an 80 Hz stimulus (Fig. 5, red bars), to a 1% error for twitch #1, a 2% error for twitches 2-4, a 3% error for twitches 5-10, and a 5% error for twitches beyond the 10^{th}. Overall these errors are small.

Comparison of two methods summed over the first 10 stimuli

It is also useful to compare the total Ca^{2+} released over the first 10 twitches. One of the measurements described to generate the data shown in Fig 5 was O_2 for ten-twitch trains (see Fig 5 legend). We obtained a value of 1.07 ±0.07 S.E.M. (*N*=9 muscles) mmoles Ca^{2+} kg^{-1} using the recovery oxygen approach (80 Hz stimulus). The values from the release modeling (83.3 Hz stimulus, when expressed in comparable units, mmoles kg^{-1}) were 0.78 mmole kg^{-1} (low [pump], slow PARV kinetics; Fig 6) ; 0.84 mmole kg^{-1} (high [pump], slow PARV kinetics; Fig 7A) and 1.03 mmoles kg^{-1}, (high [pump], fast PARV kinetics; Fig 7B). If the recovery-oxygen consumption estimate is reduced for small contributions from the crossbridges (20 moles kg^{-1}) and the Na^+/K^+ pumps (26 moles kg^{-1}), the corrected mean value, 1.03 mmoles/kg, falls within the range of estimates obtained with the release modeling.

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