Cross-bridge blocker BTS permits direct measurement of SR Ca\(^{2+}\) pump ATP utilization in toadfish swimbladder muscle fibers

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**Young, Iain S., Claire L. Harwood, and Lawrence C. Rome.** Cross-bridge blocker BTS permits direct measurement of SR Ca\(^{2+}\) pump ATP utilization in toadfish swimbladder muscle fibers. *Am J Physiol Cell Physiol* 285: C781–C787, 2003.—Because the major processes involved in muscle contraction require rapid utilization of ATP, measurement of ATP utilization can provide important insights into the mechanisms of contraction. It is necessary, however, to differentiate between the contribution made by cross-bridges and that of the sarcoplasmic reticulum (SR) Ca\(^{2+}\) pumps. Specific and potent SR Ca\(^{2+}\) pump blockers have been used in skinned fibers to permit direct measurement of cross-bridge ATP utilization. Up to now, there was no analogous cross-bridge blocker. Recently, N-benzyl-p-toluene sulfonamide (BTS) was found to suppress force generation at micromolar concentrations. We tested whether BTS could be used to block cross-bridge ATP utilization, thereby permitting direct measurement of SR Ca\(^{2+}\) pump ATP utilization in saponin-skinned fibers. At 25 μM, BTS virtually eliminates force and cross-bridge ATP utilization (both <4% of control value). By taking advantage of the toadfish swimbladder muscle’s unique right shift in its force-Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]) relationship, we measured SR Ca\(^{2+}\) pump ATP utilization in the presence and absence of BTS. At 25 μM, BTS had no effect on SR pump ATP utilization. Hence, we used BTS to make some of the first direct measurements of ATP utilization of intact SR over a physiological range of [Ca\(^{2+}\)] at 15°C. Curve fits to SR Ca\(^{2+}\) pump ATP utilization vs. pCa indicate that they have much lower Hill coefficients (1.49) than that describing cross-bridge force generation vs. pCa (≈5). Furthermore, we found that BTS also effectively eliminates force generation in bundles of intact swimbladder muscle, suggesting that it will be an important tool for studying integrated SR function during normal motor behavior.

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tions, BTS markedly reduced force generation in fast-twitch fibers while having little effect on the Ca$^{2+}$ transient (3). It seemed likely that BTS could be used as a specific cross-bridge blocker that would permit the direct measurement of SR Ca$^{2+}$ pumps in muscle fibers over a broad range of [Ca$^{2+}$].

Here we show that BTS can knock out cross-bridge ATP utilization as well as force at low concentrations (~25 μM). Furthermore, by utilizing the special properties of the swimbladder muscle and working below the threshold [Ca$^{2+}$] for cross-bridge activation, we show that 25 μM BTS has no effect on SR Ca$^{2+}$ pump ATP utilization rate. After establishing the efficacy of BTS as a specific cross-bridge blocker, we used it to make some of the first direct measurements of SR Ca$^{2+}$ pump ATP utilization in skinned fibers over a full physiological range of [Ca$^{2+}$] (9). Our data show that SR Ca$^{2+}$ pumping has a low apparent level of cooperativity [i.e., the Hill coefficient ($n_H$) is 1.49] compared with that of cross-bridge force generation ($n_H = ~5$ in swimbladder). Finally, preliminary results show that BTS not only allows one to measure the rate of ATP utilization of intact SR in saponin-skinned fibers but can block cross-bridge function in intact fibers as well. Hence, we surmise that BTS will be a valuable tool for studying integrated SR function and Ca$^{2+}$ cycling in muscle during normal motor behavior.

METHODS

Animals and Preparation

Toadfish were kept at 15°C in flow-through seawater tanks at the Marine Biological Labs (MBL) or in filtered seawater at the University of Pennsylvania and fed ad libitum during their captivity. They were sedated in ice-cooled water until unresponsive and then killed by cervical sectioning and double-pithing according to guidelines set out by the Institutional Animal Use and Care Committees of the University of Pennsylvania and the MBL. Swimbladder muscle was isolated from the toadfish and dissected as described previously (19). The swimbladder was quickly removed and placed in chilled Ringer solution (composition in mM: 132 NaCl, 2.6 KCl, 1 MgCl$_2$, 2.7 CaCl$_2$, 10 imidazole, 10 sodium pyruvate, pH 7.7 at 15°C), and bundles of this pure fiber type were dissected out and checked for strong twitches by electrical stimulation. Responsive bundles of ~100 fibers were then depolarized in a high-potassium solution (composition in mM: 7.8 MgCl$_2$·6H$_2$O, 50 K$_2$EGTA, 1 KH$_2$PO$_4$, 6.2 sodium ATP, 58.2 TES, pH 7.1 at 15°C). Fibers were dissected down to small bundles of two to four fibers (fiber diameter ~40–50 μm) and “skinned” with 50 μg/ml saponin (20 min at 4°C), which permeabilizes the cell membrane (10, 17, 20) but does not affect the Ca$^{2+}$ pumps or SR membrane (10). The fiber bundle was secured with foil clips between a force transducer (400 series, Aurora Scientific) and a fixed hook. Sarcomere length, determined by microscopy (18), was set at 2.2–2.3 μm.

ATP Utilization Measurements

ATP utilization was measured with a fluorescent coupled assay (20) in a temperature-controlled (15 ± 0.1°C), vigorously stirred 5.5-μl chamber (16, 21). The Ca$^{2+}$-EGTA solutions were the same as those used previously (16). To block SR Ca$^{2+}$ pumping a cocktail of 20 μM TBQ and 20 μM CPA was used (as in Ref. 16). Stock solutions of TBQ (7.5 mM), CPA (7.5 mM), and BTS (20 mM) were made in DMSO. We carried out preliminary experiments to confirm that DMSO alone, in the final concentrations used in this study (0.13% for BTS alone and 0.4% with BTS, TBQ, and CPA) had no effect on the rate of either cross-bridge or SR Ca$^{2+}$ pump ATP utilization. No effect was observed even up to DMSO concentrations of 1%.

All solutions contained 5 mM caffeine to prevent buildup of Ca$^{2+}$ in the SR and consequent back-inhibition of SR Ca$^{2+}$ pumping. Previous direct tests of the effect of caffeine concentration on fiber ATP utilization showed that it was constant over caffeine concentrations ranging from 2 to 20 mM (20).

We performed three sets of experiments. The first two sets of experiments were designed to test the effect of BTS on cross-bridge and SR Ca$^{2+}$ pump ATP utilization, respectively. Having shown that BTS blocks cross-bridge ATP utilization without affecting SR Ca$^{2+}$ pump ATP utilization, in the third set of experiments we measured the effect of [Ca$^{2+}$] on SR Ca$^{2+}$ pumping. These different protocols are described in RESULTS. At the end of each experiment, the fiber bundle was dried, the clips were removed, and the weight of the bundle was determined with a Cahn microbalance (model C-35). Its wet weight was calculated with a conversion factor of 8 (16), and intact fiber volume was calculated by assuming a fiber density of 1.05 kg/l. All data are reported as means ± SE. Statistical significance was set at the $P = 0.05$ level.

RESULTS

BTS Effectively Blocks Cross-Bridge ATP Utilization and Force Generation

In this set of experiments a dose-response relationship was determined for ATP utilization and force generation of the cross bridges. To accomplish this the SR Ca$^{2+}$ pump ATP utilization was first completely blocked by a combination of two Ca$^{2+}$ pump inhibitors (20 μM TBQ and 20 μM CPA) (16). Figure 1 shows that ~85% of the control cross-bridge ATP utilization and force are lost with only 5 μM BTS (note that even at 40 mM, BDM does not produce as large a decline; Ref. 22). The decrease in cross-bridge ATP utilization and force appeared similar up to 10 μM. As BTS concentration was increased above 20 μM, however, the force became indistinguishable from zero while the ATP utilization rate fell to a constant low level (~4%).

It is unclear whether this small difference between cross-bridge force generation and cross-bridge ATP utilization represents a differential response to high BTS concentrations. The swimbladder muscle generates not only about one-tenth the stress of other skeletal muscles (15). Because our technique requires vigorous mixing and small bundle sizes for accurate measurement of the swimbladder’s rapid ATP utilization, it is difficult to resolve small forces representing only a small percentage of a bundle’s low maximum force. Several additional experiments were performed with larger preparations to improve force resolution. Although these preparations were too large for accurate measurements of ATP utilization, we found that a force of ~2% of control remained at 25 μM BTS. This is close to

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CROSS-BRIDGE BLOCKER BTS REVEALS Ca\textsuperscript{2+} PUMP ATP\textsubscript{ase}

Having demonstrated that BTS does not affect SR Ca\textsuperscript{2+} pumping at concentrations that effectively block cross-bridge ATP utilization, we were able to measure SR Ca\textsuperscript{2+} pump ATP utilization over a wide range of [Ca\textsuperscript{2+}] values. In what we believe are among the first direct measurements of their kind in a skinned fiber (9), we found that the rate of SR Ca\textsuperscript{2+} pump ATP utilization increased with increasing [Ca\textsuperscript{2+}] up to \(p\text{Ca} 5.2\) (Fig. 3). Furthermore, this relationship could be described by the Hill equation with a relatively low \(n_H (1.49 \pm 0.04; n = 9)\), and the 50\% maximum pumping rate occurs at pCa 6.08 \(\pm 0.015 (n = 9)\) or at a [Ca\textsuperscript{2+}] of \(\sim 0.83\ \mu\text{M}\). To prove that this ATP utilization

the remaining percentage of ATP utilization, so it appears that BTS has a similar effect on both cross-bridge ATP utilization and force generation. These results are consistent with, and thus do not differentiate between, two possible mechanisms of action: BTS reduces the number of attached cross bridges without affecting the kinetics and/or BTS slows the attachment rate constant \(f(3)\).

BTS Does Not Affect SR Ca\textsuperscript{2+} Pump ATP Utilization

Previous studies have shown that in swimbladder muscle the ATP utilization of the Ca\textsuperscript{2+} pumps can be obtained without interference of the cross bridges up to a [Ca\textsuperscript{2+}] of \(p\text{Ca} 5.8\) (16). Hence, to determine the effect of BTS on SR Ca\textsuperscript{2+} pumping, we measured ATP utilization at pCa 6.2, pCa 6, and pCa 5.8 without BTS (Fig. 2) and then remeasured the values after addition of 25 \(\mu\text{M}\) BTS. Each run was performed in duplicate, and the values were averaged. Figure 2 shows that although SR Ca\textsuperscript{2+} pump ATP utilization increased with increasing [Ca\textsuperscript{2+}], there was no difference in the average values between control and 25 \(\mu\text{M}\) BTS. The average ratios of the control values to the BTS values from individual muscle bundles (\(n = 9\)) were 0.98 \(\pm 0.05\), 0.98 \(\pm 0.04\), and 0.99 \(\pm 0.08\) for pCa 6.2, pCa 6, and pCa 5.8, respectively. These values were found to be not significantly different from 1.0.

SR Ca\textsuperscript{2+} Pumping as a Function of [Ca\textsuperscript{2+}]

Having demonstrated that BTS does not affect SR Ca\textsuperscript{2+} pumping at concentrations that effectively block cross-bridge ATP utilization, we were able to measure SR Ca\textsuperscript{2+} pump ATP utilization over a wide range of [Ca\textsuperscript{2+}] values. In what we believe are among the first direct measurements of their kind in a skinned fiber (9), we found that the rate of SR Ca\textsuperscript{2+} pump ATP utilization increased with increasing [Ca\textsuperscript{2+}] up to \(p\text{Ca} 5.2\) (Fig. 3). Furthermore, this relationship could be described by the Hill equation with a relatively low \(n_H (1.49 \pm 0.04; n = 9)\), and the 50\% maximum pumping rate occurs at pCa 6.08 \(\pm 0.015 (n = 9)\) or at a [Ca\textsuperscript{2+}] of \(\sim 0.83\ \mu\text{M}\). To prove that this ATP utilization

Fig. 1. Twenty-five micromolar \(N\)-benzyl-p-toluene sulfonamide (BTS) eliminates almost all force and cross-bridge ATP utilization. Control force and cross-bridge ATP utilization were measured in small saponin-skinned swimbladder bundles without BTS. The mean maximal cross-bridge ATP utilization was 0.79 \(\pm 0.07\) mmol \(\cdot\) s\(^{-1}\) \(\cdot\) kg\(^{-1}\) (\(n = 9\)). Subsequent measurements for a BTS dose-response curve were made in the presence of increasing BTS and normalized to the control values. We found that cross-bridge force generation and cross-bridge ATP utilization declined by a similar proportion as BTS concentration was increased. Note that the sarcoplasmic reticulum (SR) Ca\textsuperscript{2+} pump ATP utilization was completely blocked with a combination of 20 \(\mu\text{M}\) cyclopiazonic acid (CPA) and 20 \(\mu\text{M} 2,5\text{-di-(tert-butyl)-1,4-benzohydroquinone (TBQ) (see METHODS/RESULTS); hence, the remaining ATP utilization was from the cross bridges. Values are means \(\pm\) SE (\(n = 9\)), and in most cases the SE is smaller than the symbols. Note that the fibers were preincubated with each BTS concentration for a minimum of 5 min before measurements were made.

Fig. 2. Twenty-five micromolar BTS has no effect on ATP utilization by the SR Ca\textsuperscript{2+} pumps. SR Ca\textsuperscript{2+} pump ATP utilization was measured in small saponin-skinned swimbladder bundles at pCa of 6.2, 6.0, and 5.8 in the absence (control) and presence of 25 \(\mu\text{M}\) BTS. A: original fluorescence records of ATP utilization in 1 bundle (exposure to resting and preactivating solutions is denoted by R and P, respectively; these slopes are subtracted from slopes at various Ca\textsuperscript{2+} concentrations ([Ca\textsuperscript{2+}]) values to provide the rate of ATP utilization). B: mean \(\pm\) SE values (\(n = 8\) muscle bundles). At these [Ca\textsuperscript{2+}] values, the cross bridges do not generate force or utilize ATP, hence the ATP utilization measured is that of the SR Ca\textsuperscript{2+} pumps. As [Ca\textsuperscript{2+}] increased, there was an increase in the absolute rate of ATP utilization; however, there was no difference between the control value and the value in the presence of 25 \(\mu\text{M}\) BTS. Note that for each condition ATP utilization was determined on 2 runs and the values were averaged.
were excluded from the Hill equation curve fit (shown in gray). The values shown are from 1 typical muscle bundle (pCa50 = 6.09, nH = 1.47) compared with mean values of pCa50 = 6.08, nH = 1.49 for 9 bundles. Finally, an averaged force-pCa curve from Ref. 19 is shown (dashed line, B) for comparison. Interestingly, the maximum activity of both the SR and cross bridges appeared at a pCa of ~5; the fact that a much higher [Ca^{2+}] was required for initial cross-bridge force generation results in a much steeper force-pCa curve. Note that in the present ATP utilization experiments the force-pCa curve is likely shifted somewhat to the left of that shown because of the presence of caffeine to prevent back-inhibition of the SR. Caffeine results in leftward shifting of the force-pCa curve (23). Caffeine was not used in Ref. 19.

![Graph A](https://example.com/graph.png)

**Fig. 3.** SR Ca^{2+} pump ATP utilization of intact SR in small saponin-skinned swimbladder muscle bundles as a function of [Ca^{2+}]. A: original fluorescence records (note that the slope is equivalent to the rate of ATP utilization). B: mean ATP utilization rates from 2 runs (increasing and decreasing [Ca^{2+}] values) on the same muscle bundle. All experiments were performed in the presence of 25 μM BTS to remove >95% of the cross-bridge ATP utilization. The fiber was exposed to increasing [Ca^{2+}] starting with pCa 7.2 and increasing with 0.2-pCa increments to pCa 5.0. The fiber was then exposed to pCa 4.8, 4.6, and 4.2. As [Ca^{2+}] increased, so did the ATP utilization rate (increasing slope in A), reaching a maximum at ~pCa 5.2. Note that, as in Fig 2, the averaged slope obtained in resting and preactivating solutions (not shown, approximately equal to ½ that at pCa 7.2) was subtracted from each measurement. All values were normalized to that at pCa 5.2 (B) and fit by the following Hill equation written in SigmaPlot software: %Force = 100(10^{10^{-pC50-pH}}) where nH is the Hill coefficient. At [Ca^{2+}] values >pCa 5.0, there appeared to be a small but consistent decline in ATP utilization; thus these points were excluded from the Hill equation curve fit (shown in gray). The values shown are from 1 typical muscle bundle (pCa50 = 6.09, nH = 1.47) compared with mean values of pCa50 = 6.08, nH = 1.49 for 9 bundles. Finally, an averaged force-pCa curve from Ref. 19 is shown (dashed line, B) for comparison. Interestingly, the maximum activity of both the SR and cross bridges appeared at a pCa of ~5; the fact that a much higher [Ca^{2+}] was required for initial cross-bridge force generation results in a much steeper force-pCa curve. Note that in the present ATP utilization experiments the force-pCa curve is likely shifted somewhat to the left of that shown because of the presence of caffeine to prevent back-inhibition of the SR. Caffeine results in leftward shifting of the force-pCa curve (23). Caffeine was not used in Ref. 19.

DISCUSSION

**SR Ca^{2+} ATP Utilization as a Function of [Ca^{2+}]**

For approximately a decade, several potent SR pump inhibitors (e.g., TBQ, CPA) have been used to permit direct measurements of the rate of cross-bridge ATP utilization. Here we demonstrate that the potent and specific cross-bridge inhibitor BTS can be used to knock out cross-bridge ATP utilization in saponin-skinned fibers (Fig. 1) without affecting SR Ca^{2+} pump ATP utilization (Fig. 2). This permits the direct measurement of SR Ca^{2+} pump ATP utilization as a function of [Ca^{2+}] (Fig. 3). The impact and future uses of BTS are discussed below.

We found that the Ca^{2+} pumping rate vs. [Ca^{2+}] relationship was quite shallow (i.e., nH was small: 1.49) compared with the value for cross bridges (nH = ~5; Ref. 19). Values of nH ranging from 1 to 2 have been found previously for SR Ca^{2+} pumping with different techniques, muscle preparations, and temperatures. For instance, using skinned Xenopus fibers at 4.3°C, Stienen and colleagues (20) found an nH of 2. Without the benefit of a specific cross-bridge blocker, rather than making direct measurements, Stienen and colleagues (20) had to determine the rate of SR Ca^{2+} pump ATP utilization by calculating the difference between the total ATP utilization and the ATP utilization in fibers treated with Triton (which removes the SR Ca^{2+} pump ATP utilization). In addition, they did not measure the rate of ATP utilization between pCa 5.8 and pCa 4.4 and hence may have missed the peak rate for SR Ca^{2+} pump ATP utilization (see Fig. 3), which in turn may have caused an overestimation of nH.

Using an entirely different technique, Kurebayashi and Ogawa (9) extracted troponin C (TnC) from skinned guinea pig fast-twitch muscle fibers, thereby preventing the activation of cross bridges and hence cross-bridge ATP utilization. They found that, although ATP utilization due to SR Ca^{2+} pumping had a pCa50 (6.15 at 20°C) similar to what we observed, there was a very low level of cooperativity (nH = ~1). A difference between this study and our study is that Kurebayashi and Ogawa used an unstirred ATP utilization assay with relatively low enzyme activities, both of which may have reduced the value obtained for maximum flux and thereby affected the estimation of nH.

Because the swimbladder is one of only a few muscles in which the number of SR Ca^{2+} pumps has been determined by ultrastructural morphometrics (1), an
accurate determination of intrinsic Ca\(^{2+}\) pump function is possible. The maximum value we obtained was 1 mmol ATP\(\cdot s^{-1}\)\cdot kg muscle\(^{-1}\). From this value and the density of pumps (1), the pump turnover rate for the sarco(endo)plasmic reticulum Ca\(^{2+}\)-ATPase (SERCA)1 pumps in swim bladder is \(-2.5 \text{ s}^{-1}\), very similar to values found in biochemical vesicle studies for SERCA1 pumps in mammals (Ref. 8; adjusted to 15°C according to Ref. 7). This similarity between the maximum pumping rates found for SERCA1 pumps in these divergent animals reinforces the notion that SERCA1 is highly conserved and thus the maximum Ca\(^{2+}\) pumping rate of fast muscles is increased predominantly by adding more pumps, not by changing the kinetics of the pump (16). This stands in contrast to cross-bridge function, which is altered almost exclusively by changing kinetics because the number of myosin heads in a sarcomere varies little.

One further interesting feature of the SR Ca\(^{2+}\) pump ATP utilization is that at \([\text{Ca}^{2+}]_o > \text{pCa } 5\), we found that there was a small but consistent reduction in the rate of ATP utilization. Slower SR Ca\(^{2+}\) pump ATP utilization (and consequently Ca\(^{2+}\) uptake by the SR) at high \([\text{Ca}^{2+}]_o\) values has been observed previously (9), and this reduction generally has been considerably larger than that observed here. It has been proposed that the mechanism for the decline at very high \([\text{Ca}^{2+}]_o\) may be the increase in intra-SR \([\text{Ca}^{2+}]\) (6), which has been shown to decrease pump rate due to back-inhibition. Although this was not tested explicitly (as we did not have the means of determining the free \([\text{Ca}^{2+}]\) within the lumen of the SR of our saponin-skinned fibers), it appears unlikely under our experimental conditions. The 5 mM caffeine that we used was shown previously to keep the intra-SR \([\text{Ca}^{2+}]_o\) sufficiently low to prevent slowing of SR pumping (20). Furthermore, we did not observe any time-dependent slowing of SR ATP utilization, which would be expected if back-inhibition was occurring.

**BTS as a Tool and Swimbladder as a Model for Studying Integrated SR Function and Ca\(^{2+}\) Handling During Normal Motor Behavior**

BTS permits study of intact SR in skinned fibers. What advantages are offered by measurements on skinned fibers over measurements on more reduced SR preparations? Saponin skinning permeabilizes the sarcolemma but is not thought to affect SR Ca\(^{2+}\) pumping or other SR function except for increasing the open time of the Ca\(^{2+}\)-release channels (10). Thus these experiments, along with Ca\(^{2+}\) accumulation studies on mechanically skinned fibers (2, 11), provide some of the first functional measurements of an intact SR (9, 20). There are several important differences between a structurally intact SR and reduced SR preparations (i.e., vesicles). Hence, to obtain quantitative understanding of Ca\(^{2+}\) cycling in normally functioning muscles, it is necessary to integrate results from reduced preparations with those from intact SR preparations. One important advantage in studying intact SR preparations (vs. vesicles) is that the measurement of SR Ca\(^{2+}\) pump function will better approximate the value in intact muscle. There are several reasons for this. First, during homogenization and purification to produce vesicles, some SR Ca\(^{2+}\) pumps may be damaged and hence the measured rate of SR Ca\(^{2+}\) pumping (or ATP utilization) may be reduced compared with intact SR. This could explain the lower values previously obtained in toadfish swimbladder vesicle studies \([-0.5 \text{ mmol Ca}^{2+}\cdot s^{-1}\cdot kg \text{ muscle}^{-1}\] (4); original measurements at 23°C corrected for 15°C by using a Q10 of 3\) than we observed here \((-2 \text{ mmol Ca}^{2+}\cdot s^{-1}\cdot kg \text{ muscle}^{-1}\); a stoichiometry of 2 Ca\(^{2+}\) per ATP is assumed).

In addition, most vesicles are made from either “heavy” SR (taken from the terminal cisterna) or “light” SR (taken from the longitudinal segment sitting between the terminal cisternae at either end of the sarcomere) (12–14). The distributions of Ca\(^{2+}\)-handling proteins differ in the two types of vesicles. In the heavy SR, the Ca\(^{2+}\)-release channels (ryanodine receptors) and the intra-SR Ca\(^{2+}\) binding protein calsequestrin are in high concentration. In contrast, light SR is devoid of Ca\(^{2+}\)-release channels and has little calsequestrin but contains a higher concentration of SR Ca\(^{2+}\) pumps (6). The differing ratios of SR Ca\(^{2+}\) pumps (by which Ca\(^{2+}\) enters SR lumen) to Ca\(^{2+}\)-release channels and calsequestrin (by which Ca\(^{2+}\) either leaves the lumen or is taken out of solution by binding) confer differing physiological properties. Vesicles of light SR have only a slow leakage of Ca\(^{2+}\) that is inhibited by high external \([\text{Ca}^{2+}]_o\), whereas vesicles of heavy SR have an order of magnitude higher leakage rate that is stimulated by external \([\text{Ca}^{2+}]_o\) (12). Hence, one would anticipate finding differing function in vesicles made from different types of SR (e.g., more back-inhibition in the light SR). Thus, by using an intact SR (as in our saponin-skinned fibers), we study both types of SR at once and hence we are observing their combined function in a quantitatively (and spatially) appropriate manner.

It is interesting to note that in swimbladder, force generation does not start until much higher \([\text{Ca}^{2+}]_o\) values than SR Ca\(^{2+}\) pumping (Fig. 3). However, because the force-pCa relationship is much steeper than the pumping-pCa relationship, both cross bridge and SR Ca\(^{2+}\) pump ATP utilization reach their maximal value at about the same \([\text{Ca}^{2+}]_o\) (pCa ~5; Fig. 3). This lack of overlap is unusual. In normal fast-twitch muscles, force is generated at lower \([\text{Ca}^{2+}]_o\) values (i.e., the force-[Ca\(^{2+}\)] relationship is shifted leftward to higher affinity) and hence overlaps with SR Ca\(^{2+}\) pumping more fully. However, it is necessary to emphasize that it is not the unusual properties of the swimbladder muscle that make BTS work; on the contrary, we have simply taken advantage of these properties to demonstrate the efficacy of BTS. This evidence that BTS can be used to block cross-bridge ATP utilization without affecting SR Ca\(^{2+}\) pump ATP utilization (Figs. 1 and 2) also suggests that BTS will be highly effective in permitting measurements of SR Ca\(^{2+}\) pumping in more typical fast-twitch fibers in which the SR Ca\(^{2+}\) pump-
ing overlaps with cross-bridge force generation. Indeed, this is confirmed by our own preliminary experiments (unpublished data) in fast-twitch mammalian fibers.

Comparing Ca\(^{2+}\) pumping in skinned fibers with in vivo Ca\(^{2+}\) cycling in swimbladder muscle. Although swimbladder muscle was chosen because its unique properties permitted us to test the effect of BTS on SR Ca\(^{2+}\) pump ATP utilization without interference from the cross bridges, the toadfish swimbladder muscle also represents a near-ideal system to explore the principles of Ca\(^{2+}\) handling during normal motor behavior (16). The data obtained in this study partially explain an interesting discrepancy between our previous measurement of the rate of SR Ca\(^{2+}\) pump ATP utilization and that which might be expected given the tremendous speed at which swimbladder muscle functions. If one assumes that during each twitch sufficient Ca\(^{2+}\) is released (and taken back up) to saturate and desaturate TnC (~35 M; following Refs. 24 and 25 we assume that [TnC] is equal to one-half the myosin heavy chain concentration, which is 67 M (Ref. 15)), then at 100 Hz, the Ca\(^{2+}\) pumping rate would need to be ~7 mmol·s\(^{-1}\)·kg muscle\(^{-1}\) (i.e., 2 Ca\(^{2+}\) per TnC × 100 Hz) to keep up (16). We previously reported (16) that SR Ca\(^{2+}\) pumps use ATP at a rate of 0.45 mmol·s\(^{-1}\)·kg muscle\(^{-1}\) at pCa 5.8, which is equivalent to a Ca\(^{2+}\) pumping rate of 0.9 mmol·s\(^{-1}\)·kg muscle\(^{-1}\) (or nearly 8-fold less than that required). It was assumed that the rate of pumping had reached a maximum level at pCa 5.8 because a similar value was obtained at pCa 4.4, where the difference of total fiber ATP utilization (cross bridge + SR Ca\(^{2+}\) pumps) and cross-bridge ATP utilization was determined. Although this was a reasonable conclusion, we now demonstrate that the SR Ca\(^{2+}\) pump ATP utilization continues to increase up to pCa 5.2 and then declines again at pCa 4.4 (Fig. 3).

This behavior may have contributed to an underestimate of the maximum rate of SR Ca\(^{2+}\) pump ATP utilization of swimbladder muscle (16).

With BTS we were able to study a full range of [Ca\(^{2+}\)] values and thus determine the maximum SR Ca\(^{2+}\) ATP utilization rate. The maximum value was obtained at pCa 5.2 and was about twofold higher (~1 mmol·s\(^{-1}\)·kg muscle\(^{-1}\)) than reported previously. Assuming a stoichiometry of 2 Ca\(^{2+}\) pumped per ATP utilized, this is equivalent to a Ca\(^{2+}\) pumping rate of ~2 mmol·s\(^{-1}\)·kg muscle\(^{-1}\). This faster Ca\(^{2+}\) pumping rate helps to explain part of the discrepancy between the hypothesized Ca\(^{2+}\) cycling rate at 100 Hz and the relatively low SR Ca\(^{2+}\) pumping rate. However, even after accounting for this approximately twofold increase, there remains a three- to fourfold difference. This is likely explained by either (or a combination) of two additional mechanisms (16): 1) some of the Ca\(^{2+}\) binds to parvalbumin during the 300-ms call and then is pumped back at a slow rate during the long 5- to 10-s intercall interval; and/or 2) the force oscillations of the swimbladder muscle during calling involve only small changes in troponin occupancy and thus only a small amount of Ca\(^{2+}\) (i.e., ~5/70 μmol/kg muscle) is released and taken back up during each stimulus. The reduced Ca\(^{2+}\) release per twitch is regulated by SR Ca\(^{2+}\) channel inactivation (5); a result of the high frequency of muscle stimulation.

Future use of BTS in intact fibers will permit measurement of in vivo Ca\(^{2+}\) cycling. Although fundamental to normal Ca\(^{2+}\) handling, these proposed mechanisms cannot be tested without having the ability to measure SR Ca\(^{2+}\) pump function during contraction in intact fibers. Up to now, partitioning the ATP utilization rate has been very difficult in intact muscle. Preliminary experiments, however, show that BTS can knock out cross-bridge force generation (and thus cross-bridge ATP utilization) nearly as effectively (reduces to ~5%) in large (~150 mg) swimbladder bundles suitable for intact energetics experiments as in skinned toadfish fibers. Hence, measuring the SR Ca\(^{2+}\) pump ATP utilization during contraction in intact fibers, and combining this with a stoichiometry of 2 Ca\(^{2+}\) pumped per ATP, would enable one to determine the rate, time course, and overall magnitude of Ca\(^{2+}\) release and reuptake during normal contractions. This may prove to be the most important use of BTS, and as such, BTS will have a large impact on the field of muscle energetics and on helping us to understand Ca\(^{2+}\) cycling and the role of parvalbumin in living skeletal muscle.

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