BTP2 negatively regulates Orai1 and ryanodine receptor function in skeletal muscle

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Research

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Abstract

Background. BTP2 is known to block Orai1, the Ca^{2+} channel of store-operated Ca^{2+} entry (SOCE) but no detailed analysis has been undertaken in skeletal muscle, where the drug has been used extensively to study SOCE.

Methodology. We trapped a Ca^{2+} sensitive dye in the tubular (t-) system of mechanically skinned fibres from rat to define the effect of BTP2 on SOCE in skeletal muscle fibres and used a cytoplasmic rhod-2 to track Ca^{2+} release in the presence of BTP2.

Results. In addition to blocking Orai1-dependent SOCE, we found a BTP2-dependent inhibition of a resting Ca^{2+} conductance, likely to be through the Orai1 channel. Intriguingly, increasing concentrations of BTP2 displayed a hormetic effect on resting [Ca^{2+}] in the t-system ($[Ca^{2+}]_{t-sys}$), shifting from inducing an accumulation of Ca^{2+} in the t-system presumably due to Orai1 channels blocking, to reducing the resting $[Ca^{2+}]_{t-sys}$. In absence of functional ryanodine receptors (RyRs), this biphasic effect was not observed, suggesting that above the hormetic zone, BTP2 impairs RyR function. Additionally, we found that BTP2 impairs the cytoplasmic Ca^{2+} transients during repetitive excitation-contraction coupling (EC coupling) cycles, independently of extracellular Ca^{2+} entry impairment. We determined that the release of Ca^{2+} through the RyR was inhibited by BTP2, strongly suggesting that the RyR was the point of inhibition during the cycles of EC coupling. Finally, we found that BTP2 inhibition of RyR-mediated Ca^{2+} release was independent of extracellular or intracellular application of the agent, indicating that BTP2 can impair RyR function in intact muscle.

Conclusion. Our results show that both Ca^{2+} channels, the Orai1 and RyR, are negatively regulated by BTP2, shedding new light on previous work that applied BTP2 to block SOCE in muscle.

Introduction

Store-operated Ca^{2+} entry (SOCE) is a retrograde Ca^{2+} regulatory mechanism, activated by a local depletion of calcium in the endo/sarcoplasmic reticulum that causes the influx of Ca^{2+} to the cell. Two components, an SR Ca^{2+} sensor (STIM1), and a Ca^{2+} channel in the plasma membrane (Orai1), mediate this process (Soboloff et al. 2006; Feske et al. 2006).

SOCE is well-described in non-excitable cells, where the process can take seconds following store-depletion to commence (Roos et al. 2005; Zhang et al. 2005; Feske et al. 2006; Soboloff et al. 2006). In contrast, SOCE in skeletal muscle can be activated in less than a millisecond following local Ca^{2+} release through the ryanodine receptor (RyR) on the sarcoplasmic reticulum (SR) (Launikonis and Ríos 2007; Duke et al. 2010; Cully et al. 2018; Koenig, et al. 2018; Koenig et al. 2019). For such rapid activation of SOCE, a sub-population of the STIM1 and the Orai1 molecules must be pre-positioned to respond immediately to a near-membrane depletion of Ca^{2+} inside the SR (Koenig et al. 2019). The intra-SR Ca^{2+}-

gradient generated by an increase in RyR permeability to Ca^{2+} causes Ca^{2+} dissociation from STIM1, inducing STIM1 interaction with the Orai1 Ca^{2+} channel on the immediately adjacent tubular (t-) system membrane (Launikonis et al 2003; Stiber et al 2008; Wei-Lapierre et al 2013), an invagination of the plasma membrane in muscle (Peachy 1965), resulting in SOCE activation.

The store-dependent Ca^{2+} influx in skeletal muscle is tiny in comparison to the Ca^{2+} released during a twitch, with 3–4 orders of magnitude difference between the fluxes (Launikonis et al 2010). This tiny SOCE flux has not been imaged during the release of Ca^{2+} from the SR in intact muscle fibre preparation because of problems separating it from the cytoplasmic Ca^{2+} released from the SR. SOCE in intact muscle fibre preparations has been studied through the use of pharmacological agents such as 2-APB, SKF-26365 and BTP2 that block SOCE. Since its discovery and characterization as an Orai1 inhibitor (Trevillyan et al. 2001; Zitt et al. 2003; Ishikawa et al. 2003), BTP2 ((3,5-bis(trifluoromethyl) pyrazole derivative) has been broadly used in the muscle and non-muscle fields to study cellular and physiological aspects of SOCE, representing an agent we can use to better understand how SOCE works in skeletal muscle. Defining the effects of BTP2 on SOCE would significantly aid our understanding of the physiological role of SOCE in skeletal muscle.

We characterized the effect of BTP2 on Ca^{2+} movements in skeletal muscle using mechanically skinned fibres, which provide a means of assessing Orai1 Ca^{2+} conductance in the presence of a fully functional SR (Launikonis et al 2003), where Ca^{2+} release through the RyR can be imaged simultaneously with SOCE-dependent depletion of t-system [Ca^{2+}] (Launikonis & Ríos, 2007). We observed that BTP2 blocked a t-system Ca^{2+} leak and, unexpectedly, the RyR Ca^{2+} leak in the resting muscle. Additionally, we found that BTP2 impaired RyR function in response to repetitive electrical stimulation and Mg^{2+} removal-induced Ca^{2+} release, regardless of whether BTP2 was applied intra- or extracellularly to the muscle fibre.

Methods

Muscle preparation for single fibre imaging

All experimental methods using rodents were approved by the Animal Ethics Committees at The University of Queensland. Male Wistar rats were killed by asphyxiation via CO_2 exposure and the extensor digitorum longus (EDL) muscles were rapidly excised from the animals. Muscles were then placed in a Petri dish under paraffin oil above a layer of Sylgard. In experiments where Ca^{2+} was released from the SR by stimulation with low $[Mg^{2+}]_{cyto}$ or activated by field stimulation, fibres were mechanically skinned and transferred to the experimental chamber and bathed in a cytoplasmic solution containing (mM): Mg^{2+}, 1; CaEGTA/EGTA, 1; Hepes, 90; K^+, 126; Na^+, 36; ATP, 8; creatine phosphate, 10; rhod-2, 0.01; and N-Benzyl-p-toluenesulfonamide (*BTS*), 0.05 with pH adjusted (with KOH) to 7.1. [Ca^{2+}] in solution was set to 100 nM or to 200 nM to load the SR with Ca^{2+}. In some experiments, [Mg^{2+}] was lowered to 0.01 mM to stimulate the thorough release of Ca^{2+} from the SR.

In other experiments, rhod-5N salt was trapped in the sealed t-system as originally described by Lamb et al (1995). Briefly, small bundles of fibres from EDL muscles were isolated using fine forceps and exposed to a Na^+-based physiological solution (external solution) containing (mM): rhod-5N, 2.5; $CaCl_2$, 2.5; NaCl, 132; $MgCl_2$, 1; KCl, 3.3; Hepes, 20 and the pH was adjusted to 7.4 with NaOH. The dye was allowed 10 min to diffuse into the t-system from the surrounding bubble of solution containing fluorescent dye. After this equilibration period, individual fibres that had been exposed to the dye solution were isolated from the bundle and mechanically skinned. In experiments where Ca^{2+} release was measured, the intact fibres were not exposed to a dye containing solution but immediately mechanically skinned. In all cases, after skinning, fibres were transferred to an experimental chamber containing a K^+-based internal solution which allowed the sealed t-system to generate a normal resting membrane potential (Lamb & Stephenson, 1990; 1994). The solution for “resting Ca^{2+} conductance experiments” contained (mM): Mg^{2+}, 1; CaEGTA/EGTA, 50; Hepes, 90; K^+, 126; Na^+, 36; ATP, 8; creatine phosphate, 10; and *BTS*, 0.05 with pH adjusted (with KOH) to 7.1. Free [Ca^{2+}] was set to 200 nM in this solution to promote a loaded SR. To release SR Ca^{2+}, a similar solution with 30 mM caffeine, [Mg^{2+}] lowered to 0.01 mM and no added Ca^{2+} was applied to skinned fibres (Cully et al 2016). All chemicals were obtained from Sigma (Australia). BTP-2 and BTS were prepared in stocks dissolved in DMSO. Equivalent levels of DMSO used in solutions containing BTP-2 were added as vehicle to solutions used in control experiments.

Extracellular administration of Fluo-5N and BTP2

Segments of single fibres of EDL immersed on paraffin oil were exposed to a Na^+-based physiological solution containing (mM): Fluo-5N, 2.5; $CaCl_2$, 2.5; NaCl, 132; $MgCl_2$, 1; KCl, 3.3; Hepes, 20 with or without BTP2, 10 mM (pH was adjusted to 7.4 with NaOH) using a 1 µL microcap pipette. The dye was allowed 2 min to diffuse into the t-tubules in contact with the bubble of solution containing Fluo-5N or Fluo-5N + BTP2. The section of fibre that was exposed to the extracellular solution was about 50 µm, leaving the rest of the fiber unexposed to fluo-5N or BTP2. The localised application of BTP2, so that the same fibre had a BTP2-exposed section and a non-exposed section, was possible through leveraging: (i) the restriction on diffusion set by the paraffin oil surrounding the intact fibre and physiological solution applied to it (Lamb et al 1995); and (ii) the restriction on longitudinal diffusion of small molecules within the t-system (Edwards & Launikonis, 2008). After the 2 min equilibration period, the fiber was mechanically skinned along its length, encompassing the extracellular solution exposed region and unexposed region. The preparation was transferred to a custom-built chamber and fixed under a bubble of K^+-based internal solution (described on *Muscle preparation for single fibre imaging* section) for imaging on the confocal microscope.

Confocal imaging

Mounted skinned fibres were imaged using an Olympus FV1000 confocal microscope equipped with an Olympus 0.9NA 40x Plan-Apochromat objective. Rhod-5N trapped in the sealed t-system or cytoplasmic rhod-2 was excited with 543 nm HeNe laser and the emission was filtered using the Olympus spectra detector. For tracking Ca^{2+} transients in the t-system or during direct activation of Ca^{2+} release with low Mg^{2+}, images were continuous recorded in xyt mode with an aspect ratio of 256 x 512, with the long aspect of the image parallel with that of the preparation. Temporal resolution of imaging in this mode where the fluorescence signal from within the borders of the fibre was 0.8 s. For imaging action potential-induced Ca^{2+} release, xt scanning was performed at 2 ms $line^{-1}$ with the line parallel to the long axis of the fibre. Scanning was always initiated prior to the field pulses. Field pulses were delivered at a rate of 0.5 Hz and strength of 30-50 V cm^{-2} (Posterino et al 2000) using a Grass stimulator box.

Image analysis for Ca^{2+} measurements

t-system rhod-5N fluorescence (t) ($F(t)$) was collected during continuous xyt imaging during multiple internal solution changes. At the end of the experiment each fibre was exposed to ionophore and 5 mM Ca^{2+}, followed by 0 Ca^{2+} to obtain the fluorescence maximum (Fmax) and minimum (Fmin), respectively. These values were used in conjunction with the previously determined K_D of rhod-5N in the t-system of 0.8 mM (Cully et al 2016) to determine $[Ca^{2+}]_{t\text{-}sys}$, with the relationship: $[Ca^{2+}]_{t\text{-}sys}(t) = k_{D,Ca} * (F(t) - F\text{min})/(F\text{max} - F(t))$.

Results are expressed as mean ± SD.

Results

Effect of BTP2 on Orai1

In mechanically skinned fibres, SOCE induces t-system Ca^{2+} ($[Ca^{2+}]_{t-sys}$) depletion during Ca^{2+} release from the SR. In order to determine the dose-dependence of BTP2 on the blockade of Orai1 in the t-system, we exposed mechanically skinned fibres with Rhod5N trapped inside the T-system to a release solution containing 50 mM EGTA, 30 mM caffeine, 0.01 mM Mg^{2+} and no added Ca^{2+} to induce SR Ca^{2+} release and a thorough depletion of the SR. As observed previously, the exposure of the preparation in the absence of BTP2 caused a rapid decline of $[Ca^{2+}]_{t-sys}$ with the activation of SOCE (Fig. 1). To test the effectiveness of BTP2, the skinned fibre was pre-equilibrated with 1, 5, 10 and 20 μM BTP2. (Control condition was pre-equilibration with DMSO vehicle).

A dose-dependent effect of BTP2 on the $[Ca^{2+}]_{t-sys}$ transient ($[Ca^{2+}]_{t-sys}(t)$) was observed, where $[Ca^{2+}]_{t-sys}(t)$ showed progressively slowed responses in the presence of release solution (Fig. 1A). Figure 1B shows the summary of the experiments from 8–12 fibres under each experimental condition. At rest, $[Ca^{2+}]_{t-sys}$ was 1.0945 mM; SOCE activation in absence of BTP2 induced a $[Ca^{2+}]_{t-sys}$ decrease to

0.0291 mM. In contrast, 5, 10 and 20 μM BTP2 partially inhibited the depletion of $[Ca^{2+}]_{t-sys}$ to 0.3544, 0.4223 and 0.6359 mM, respectively (SD = 0.05959, 0.1144 and 0.09096, respectively). Our results confirm that BTP2 blocks SOCE in a dose-dependent fashion.

The results described above show that BTP2 inhibits the activation of Orai1 channels in response to a physiological activation of SOCE. Others have shown that 5 μM BTP2 reduces a Ca^{2+} influx in normal, resting myotubes in the presence of the endogenous SR Ca^{2+} load (Eltit et al 2013). We tested if BTP2 was able to affect the resting Ca^{2+} conductance from the t-system in the presence of SR Ca^{2+} in fully differentiated skeletal muscle. To do this, we exposed fibres to increasing concentrations of BTP2 expecting that any blockade of a resting Orai1 channel Ca^{2+} conductance would result in the net increase of $[Ca^{2+}]_{t-sys}$. The high sensitivity of the $[Ca^{2+}]_{t-sys}$ to changes in the fluxes of Ca^{2+} across the t-system membrane provided the opportunity to assess whether BTP2 blocked a basal Orai1 Ca^{2+} flux (Cully et al 2018) (the pump-leak balance that sets the steady state $[Ca^{2+}]_{t-sys}$ is potentially changed by BTP2).

Figure 2Ai shows a representative trace of t-system rhod-5N signal from a fibre exposed to increasing concentrations of BTP2. We have also left the signal in arbitrary fluorescence units to maintain a higher signal-to-noise than would be possible if the signal was converted to $[Ca^{2+}]_{t-sys}$, to allow detection of small changes in t-system Ca^{2+} levels. 1 and 5 μM BTP2 induced an increase in t-system rhod-5N signal, suggesting an inhibition of resting Ca^{2+} efflux through Orai1. However, the administration of 10 and 20 μM induced the opposite effect, lowering the signal. This result suggests that increasing concentrations of BTP2 have a secondary effect that causes the $[Ca^{2+}]_{t-sys}$ to drop.

Previously, we have shown the dependence of $[Ca^{2+}]_{t-sys}$ on the resting SR Ca^{2+} leak through the RyR, which sets the $[Ca^{2+}]$ in the junctional space between the SR and t-system and determines t-system Ca^{2+} pump activity (Cully et al. 2018). Thus in the situation shown in Fig. 2Ai, the $[Ca^{2+}]_{JS}$ that is activating the t-system Ca^{2+} pumps is much greater than the bulk $[Ca^{2+}]_{cyto}$ of 200 nM that is set by the cytoplasmic bathing solution. If BTP2 affects the conductance of Ca^{2+} through the RyR, then the $[Ca^{2+}]_{JS}$ would drop and so would the $[Ca^{2+}]_{t-sys}$ (Cully et al 2018), as seen in Fig. 2A from 5 μM BTP2. To test whether the RyR Ca^{2+} leak of the resting fibre was affected by higher concentrations of BTP2, the same experiment as in Fig. 2Ai was performed in the constant presence of 1 mM tetracaine, which blocks the RyR resting leak (Fig. 2Aii). The blockade of the RyR Ca^{2+} leak with tetracaine allows the $[Ca^{2+}]_{JS}$ to equilibrate with the bulk $[Ca^{2+}]_{cyto}$ and remain constant.

Figure 2Aii shows that 1 and 5 μM BTP2 exerted a comparable rhod-5N signal increase as in the absence of tetracaine (Fig. 2Ai). In contrast, in the presence of tetracaine, at 10 and 20 μM of BTP2, the t-system rhod-5N signal remained steady, suggesting that under these conditions only a single effect of BTP2 on $[Ca^{2+}]_{t-sys}$ occurred that reached saturation at 5 μM BTP2; and that in the absence of tetracaine the rhod-

5N signal decline observed (Fig. 2Ai) was due to a negative modulation of RyR Ca^{2+} leak by BTP2 at > 5 μM.

To confirm that 10 μM BTP2 inhibits the resting RyR Ca^{2+} leak, t-system Ca^{2+} was continuously tracked while fibers were exposed to tetracaine, BTP2 or a combination of both drugs. Figure 3A shows a representative trace of $[Ca^{2+}]_{t-sys}$ on a fibre with Rhod-5N trapped within its t-system. Initially, the sealed t-system presented a $[Ca^{2+}]$ of 1.1305 mM when the fibre was bathed on 200 nM Ca^{2+} solution (described on the trace as “C” (control)). As described above, t-system Ca^{2+} depletion occurs upon activation of SOCE with 30 mM caffeine in a solution with 0.01 mM Mg^{2+}. The t-system is able to refill with Ca^{2+} following the exchange of the release solution for a solution containing 1 mM Mg^{2+} and 200 nM Ca^{2+} (control solution). As explained above, the $[Ca^{2+}]_{t-sys}$ depends on resting leak through RyR. A blockage of the RyR with 1 mM tetracaine reduced the resting $[Ca^{2+}]_{t-sys}$ from 1.1305 to 0.9674 mM. Following another cycle of release and “control” solutions, 10 μM BTP2 was added to control (“C”) solution, where a decrease in $[Ca^{2+}]_{t-sys}$ was observed. The reduction $[Ca^{2+}]_{t-sys}$ upon exposure of BTP2 was comparable to the one observed with tetracaine. Furthermore, addition of 1 mM tetracaine to the 10 μM BTP2 bathing solution did not produce any synergistic effect, strongly suggesting that both drugs share the same target. This observation supports the conclusions from Fig. 2, showing that 10 μM BTP2 blocks resting RyR Ca^{2+} leak.

Effect of BTP2 on RyRs and SR Ca^{2+} release

The unexpected finding that BTP2 could be affecting RyRs obliged us to explore this further. It was possible to examine the effect of BTP2 on RyR function in absence of any contribution of SOCE to the calcium content of the SR in the skinned fibre because the open cytoplasm provides an infinite pool for the SR to sequester Ca^{2+}. In other words, the $[Ca^{2+}]_{SR}$ of the fibre is buffered by the $[Ca^{2+}]_{cyto}$ (in the presence of 1 mM EGTA) in the bathing solution, making any role of Orai1 in providing Ca^{2+} to the cytoplasm during repetitive cycles of EC coupling negligible. Therefore, the effect of BTP2 on repetitive EC coupling without any influence of Ca^{2+} entry from the t-system on $[Ca^{2+}]_{SR}$ could be examined.

Figure 4 shows the cytoplasmic Ca^{2+} transients elicited at 0.5 Hz in mechanically skinned fibres imaged by confocal microscopy, as described previously (Posterino, et al. 2000; Choi, et al. 2017). The cytoplasmic Ca^{2+} transients were imaged with ms resolution by exciting rhod-2 present in the bathing cytoplasmic solution of skinned fibres along a single scanning line positioned parallel to the long axis of the fibre. After 20 s of field stimulation of the fibre in a control solution, BTP2 (0, 1, 5, 10 and 20 μM) was directly added to the bathing solution of the fibre (Fig. 4A). The amplitude of the action potential-elicited Ca^{2+} transients appear to decline as a function of [BTP2]. Additionally, the onset of effect of BTP2 on the Ca^{2+} transients occurred within a second or so at 10 and 20 μM BTP2.

Figure 4B shows the summary of the experiments under each experimental condition. The Ca^{2+} transients were insensitive to 1 μM BTP2. In contrast, the presence of 5 μM BTP2 caused a reduction of Ca^{2+} transient amplitude of 47.5% after 100 seconds of electrical stimulation (SD = 4.041). Both 10 and 20 μM BTP2 reduced the amplitude of the electrically evoked Ca^{2+} transients 86% after 100 seconds of stimulation (SD = 4.056 and 4.113, respectively). Ca^{2+} transient amplitude decline rate increased with increasing [BTP2] (Fig. 4B).

The inhibition of electrically evoked Ca^{2+} transients by BTP2 at 5 μM or greater was possibly due to a block of the RyR or another component involved in EC coupling. To test whether BTP2 was affecting RyR Ca^{2+} release, we examined the effect of BTP2 on direct stimulation of RyR, induced by lowering $[Mg^{2+}]_{cyto}$ (Lamb & Stephenson, 1991). To do this, skinned fibres were bathed in cytoplasmic “resting” solution containing 1 mM Mg^{2+} and 100 nM Ca^{2+}; then the fibres were exposed to a 0.01 mM Mg^{2+} solution (the rest of the components of this release solution remained unaltered compared to the resting solution). After this, we returned the fibre to the 1 mM Mg^{2+} solution to reload the SR and repeat the experiment to confirm the consistency of the amplitude of the Ca^{2+} transient induced by Mg^{2+} removal. This experiment was performed adding the different BTP2 concentrations after the first Mg^{2+} removal-induced Ca^{2+} transient and the amplitude of the second transient was compared to the first one (Fig. 5Ai-v). From this set of experiments, we observed a decrease in Ca^{2+} transient amplitude in presence of 10 (27% decrease; SD = 6.481) and 20 (60% decrease; SD = 11.11) μM BTP2.

Finally, we wished to determine whether the effect of BTP2 on the RyR in Fig. 5 was due to the intracellular application of BTP2. Typically, BTP2 is applied extracellularly in intact fibre preparations to inhibit Orai1. To determine whether extracellular application of BTP2 affected the RyR, we applied BTP2 to the intact fibre under paraffin oil, where it would be expected to diffuse throughout the t-system (Edwards & Launikonis, 2008). To trace the movement of the physiological solution containing BTP2 into the t-system, fluo-5N was added to the solution as the indicator of t-system exposure that could be later imaged on the confocal microscope. The physiological solution containing fluo-5N or fluo-5N + 10 μM BTP2 was applied to intact fibres with a microcap pipette so that the region of fibre exposed to the solution could be controlled. Care was taken to leave a segment of the fibre unexposed to the physiological external solution. The t-system of skeletal muscle is diffusionally restricted in the longitudinal direction, preventing the exposed transverse tubules of the t-system exchanging or equilibrating their luminal content with the adjacent regions of transverse tubules not directly exposed to physiological external solution at the intact sarcolemma (Edwards & Launikonis, 2008) (Fig. 6A). The fibre was mechanically skinned after at least a 2 min equilibration period with the physiological solution. The skinned fibres prepared in this manner were initially bathed in a K^+-based cytoplasmic resting solution containing 1 mM Mg^{2+}, 100 nM Ca^{2+} and rhod-2. An approximate 100 μm length of fibre was positioned in the microscope field of view with the border between the sections of the fibre with t-system with and without fluo-5N in approximately the centre of the field of view. The positioning of the fibre in

this way allowed the cytoplasmic rhod-2 fluorescence in both sections of the preparation to be imaged simultaneously.

To induce the release of SR Ca^{2+}, the resting solution was exchanged for a solution with 0.01 mM Mg^{2+}. The cytoplasmic rhod-2 fluorescence was imaged during the course of the exchange of solutions and during the ensuing Ca^{2+} transients. Ca^{2+} transients were observed in both fibre sections without or with fluo-5N + BTP2. The peak amplitude of the Ca^{2+} transients elicited by lowering Mg^{2+} in the presence or absence of t-system fluo-5N was the same (Fig. 6Bi). In contrast, the pre-exposure of 10 µM BTP2 to the intact section of the skinned fibre showed a significant reduction in the peak amplitude of the rhod-2 fluorescence transient (Fig. 6Bii) (F/F_0 = 2.77 and 1.92 in absence and presence of BTP2, respectively; SD = 0.5318 and 0.6297, respectively), suggesting that BTP2 applied extracellularly slowed flux of Ca^{2+} through the activated RyR.

Discussion

The experiments presented in this work provide novel evidence regarding the effect of BTP2 on skeletal muscle fibres. We show that very low µM concentrations of BTP2 block a t-system Ca^{2+} leak channel, likely to be Orai1. However, at concentrations above 5 µM, RyR Ca^{2+} leak and release was impaired by BTP2. The inhibitory effect of BTP2 on the RyR was observed following either extracellular or intracellular application of the agent. The sensitivity of low [BTP2] to a t-system Ca^{2+} leak channel has implications for understanding sarcolemmal Ca^{2+} leak, which is important in conditions such as muscular dystrophy (Turner et al 1991) and malignant hyperthermia (Eltit et al 2013; Cully et al 2018). Additionally, the promiscuity of BTP2 at commonly used doses highlight a need for re-evaluation of some conclusions regarding SOCE function in skeletal muscle that have been based under the premise of a specific action of BTP2 on Orai1.

BTP2 mechanism of action

The lack of effect of 1 mM BTP2 perfused into Jurkat cells cytoplasm in contrast to the inhibitory effect observed on CRAC channel when applied extracellularly suggests that, regardless of being a membrane permeant molecule, 1 mM BTP2 exerts its action through extracellular interaction with this channel (Zitt et al. 2003). In accordance with Zitt et al, the administration of cytosolic 1 mM BTP2 in this work did not exert a significant effect on Ca^{2+} movements, whereas higher concentrations such as 10 mM (where the RyR becomes affected) were needed to see an effect, coinciding with what is typically used in the skeletal muscle field (Zhao et al. 2005; Thornton et al. 2011; Wei-Lapierre et al. 2013). Experiments shown on figure 6 where 10 mM BTP2 was applied extracellularly strongly suggest that regardless of the nature of exposure, either cytosolic or extracellular administration of 10 mM BTP2 impairs the release of Ca^{2+} through the RyR.

Being a cell permeant molecule, it would not be surprising that 10 mM BTP2 could interact with both extracellular or cytosolic targets; importantly, the two possibilities are not mutually exclusive. In regards of putative BTP2 intracellular effectors, a chemico-genetic analysis revealed the F-actin binding cytosolic protein Drebrin as a direct ligand of BTP2 (Mercer et al. 2010). Intriguingly, Drebrin *knock down* leads to inhibition of SOCE at comparable levels to BTP2 treatment and the authors did not observe synergic effect when Drebrin *knock down* cells were treated with BTP2, suggesting that the SOCE inhibitory effect of BTP2 occurs through Drebrin inhibition. Anecdotally, when Zitt et al. perfused 1 mM BTP2 into Jurkat cells, they reported a drastic change in cell morphology that was not explored in detail. This observation goes on the same line of BTP2 as a modulator of the actin cytoskeleton, a well-known determinant of cell morphology.

The role of cytoskeleton on SOCE remains controversial. While some reports suggest that actin filaments participate in STIM1 rearrangement and association with SOC channels (Galán et al. 2011), previous ones did not observe an effect of actin cytoskeleton modulating SOCE (Ribeiro, et al. 1997). Interestingly, Rahman et al. observed no effect of BTP2 in STIM1 aggregation after SR Ca^{2+} depletion (Rahman and Rahman 2017), suggesting that BTP2 could be impairing Orai1 function independently of STIM1 modulation. In line with this, evidence of the modulation of actin cytoskeleton to TRP channels has been described for TRPC1, TRPC4 and TRPC5 (Rosado, et al. 2000; Tang et al. 2000). Therefore, one possibility is that BTP2 mechanism of action on Orai1 involves, as has been previously suggested, Drebrin inhibition, leading to the impairment of actin filaments interacting with SOC channels and subsequently the dysregulation of them. Structural studies of the interplay between actin filaments and SOC channels would improve our knowledge about how these components cooperate.

A question that is raised from our work is how BTP2 impairs RyR function. Following the evidence of actin cytoskeleton as a target of BTP2, it would not be surprising that the cytoskeletal negative modulation could lead to the impairment of the RyR. In a study performed on the neuroblastoma cell line NG115-401L, Bose et al found that actin disruption using cytochalasin D impairs the RyR-mediated ER Ca^{2+} release (Bose and Thomas, 2009). In the case of skeletal muscle, γ-actin has been found attached to the SR and its disruption leads to impairment of SR Ca^{2+} release, suggesting that actin cytoskeleton plays a modulatory role on RyR function in skeletal muscle (Gokhin and Fowler 2011). However, we cannot rule out a direct action of BTP2 on RyR function.

BTP2, Ca^{2+} leak and SOCE across the t-system membrane

In resting myotubes, Eltit et al (2013) found a similar inhibition of the influx of Ca^{2+} in the presence of 5 μM BTP2 or with the overexpression of dominant negative $Orai1^{E190Q}$. This similarity and the well-established effect of BTP2 on Orai1 suggests the BTP2-sensitive channel in the resting cell is likely to be at least partially through Orai1. Additionally, BTP2 may inhibit a resting Ca^{2+} conductance through TRP channels (Eltit et al 2013). Our results and those of Eltit et al (2013) indicate a basal conductance of Ca^{2+}

through Orai1/TRP channels in the absence of STIM1 activation. The level of Orai1 Ca^{2+} flux in muscle is amplified from its basal level by the dissociation of Ca^{2+} from STIM1 as the RyR Ca^{2+} conductance increases during action potential-induced Ca^{2+} release, probably to remain proportional the activation of Ca^{2+} efflux pathways across the t-system with increasing $[Ca^{2+}]_{JS}$ (Cully et al 2018; Koenig et al 2018; Azimi et al 2020).

The higher [BTP2] required to inhibit SOCE in skinned fibres (Fig. 1) than the t-system Ca^{2+} leak (Fig. 2) likely reflects the increased activation of the STIM1/Orai1 pathway as caffeine thoroughly depletes the SR of Ca^{2+}. Additionally, we note that the selective Orai1 enhancer, IA65, increased the t-system Ca^{2+} flux in the presence of low Mg^{2+} and caffeine in skinned fibres, confirming the molecular identity of the t-system SOCE channel as Orai1 (Azimi et al 2020). However, it remains possible that other channels also conduct SOCE in the muscle.

Pathophysiological Ca^{2+} entry in muscular dystrophy and malignant hyperthermia are both likely to have components that are dependent and independent of RyR Ca^{2+} leak (Turner et al 1991; Eltit et al 2013; Cully et al 2018). BTP2 and, for example, tetracaine, provide complimentary tools for deciphering the modes of excessive Ca^{2+} entry in muscle. Pathways for Ca^{2+} leak into the muscle that are blockable by GsMTx-4 and heavy metals also exist (Eltit et al 2013), indicating that the t-system is a compartment very leaky to Ca^{2+}. The t-system is dependent on the function of the SR in sequestering and concentrating cytoplasmic Ca^{2+} into the junctional space via RyR Ca^{2+} leak, where the local increase $[Ca^{2+}]$ at the t-system increases plasma membrane Ca^{2+} ATPase activity to maintain the steep t-system Ca^{2+} gradient (Cully et al 2018). Thus, the pathophysiological excessive Ca^{2+} influx under muscular dystrophies and RyR-related myopathies should be considered conditions of altered basal Ca^{2+} handling at the SR and t-system.

It is possible BTP2 also effects other RyR isoforms. In the sinoatrial node, Liu et al. showed a negative effect of BTP2 on spontaneous and caffeine-induced SR Ca^{2+} release (Liu et al. 2015). This observation made on intact cells may be attributed to a direct effect of BTP2 on RyR2 Ca^{2+} efflux or an effect on Orai1 Ca^{2+} current. However, the our demonstration of an effect of BTP2 on RyR1 it would seem that the results of Liu et al are at least in part due to a slowing of Ca^{2+} efflux through RyR2 after exposure to the drug. An evaluation of the effect of BTP2 on RyR2 is warranted.

Physiological significance of SOCE in skeletal muscle

A major hypothesis regarding the physiological role of SOCE in skeletal muscle is that SOCE provides resistance to fatigue, where it is proposed that a loss of Ca^{2+} from the stimulated muscle needs to be replenished by Ca^{2+} from outside the fibre via SOCE (eg. Zhao et al 2005; Thornton et al 2011; Wei-Lapierre et al 2013). To support this theory, researchers have followed two different strategies. The first one is related to the use of genetically modified animals where SOCE is impaired in constitutively Orai1 or

STIM1 *knock-out* mice. However, this approach presents developmental defects in the adult mutant muscle (Stiber et al. 2008) (Wei-Lapierre et al 2013) and thus complicates the comparison between wild-type and STIM1/Orai1 knock-out mice as to the action of SOCE during repetitive cycles of EC coupling. In contrast to this result, the only study of an inducible Orai1 knock-out mouse concluded that there is no significant role for acute SOCE in resisting fatigue in repetitive EC coupling cycles (Carrell et al. 2016).

The second strategy is based on the use of multiple pharmacological agents that impair SOCE. Some of the most popular ones have been BTP-2, 2-ABP and SKF-96365. 2-ABP and SKF-96365 have been shown to be unselective for the SOC channels (Launikonis and Ríos 2007; Olivera and Pizarro 2010). In respect of BTP2, the data presented here suggest that [BTP2] should not exceed 5 μM intracellularly to avoid RyR impairment. The BTP2 concentration chosen to block Orai1 in muscle experiments has typically been 10 μM, which in mechanically skinned fibres curtails the release of Ca^{2+} from the SR and the activation of SOCE. In these experiments the decline of the action potential-induced Ca^{2+} transients in the presence of BTP2 in intact and skinned fibres show decline (Fig 3; Wei-Lapierre et al 2013). While the patterns of decline where not identical (eg. Small shift in concentration dependence), this may be due to factors such as diffusion rates across the plasma membrane, potency of different batches of BTP2 or other minor differences in handling the agent across different labs. Importantly, the decline of the Ca^{2+} transient was not dependent on which side of the plasma membrane it was applied, nor was it dependent on number of action potentials stimulating the fibre, as a single direct stimulation of RyR opening was affected by exposure to BTP2 (Figs 5 & 6). It follows that an action of BTP2 on the RyR on intact fibre preparation is likely to be the cause of the Ca^{2+} transient decline in those experiments as well.

Additionally, the biophysical properties of Ca^{2+} release and SOCE flux in skeletal muscle do not provide a framework within which it is possible to model SOCE as a “store-refiller” during repetitive cycles of EC coupling. During EC coupling, SOCE is activated very rapidly and briefly following the release of Ca^{2+} from the SR, which causes the near-membrane depletion of Ca^{2+} in the SR terminal cisternae to activate the local STIM1 (Koenig et al 2018, 2019). The proportional contribution of the SR and t-system to the Ca^{2+} entering the cytoplasm during EC coupling is 99:1, making it difficult to argue that SOCE contributes significantly to the Ca^{2+} in the muscle cytoplasm during repetitive cycles of EC coupling (Koenig et al 2018). The development of fatigue is more likely due to the inhibition of the Ca^{2+} release mechanism of the muscle by the build-up of metabolites than an inherent loss of fibre calcium (Allen et al. 2008) (Olsson et al 2020). We also point out that the SOCE mechanism and kinetics in muscle is not different between slow- and fast-twitch fibres (Cully et al. 2016), thus making it unlikely that a role of SOCE is based around fatigue-resistance.

Ivarsson et al (2019) and Nelson et al (2019) have recently reported enlightening results around the role of SOCE signalling in muscle. Nelson et al. described a set of conserved phosphorylation events in mouse, rat and human in response to exercise. One protein whose phosphorylation was conserved in response to exercise was STIM1. Moreover, the authors demonstrated that STIM1 phosphorylation in response to exercise negatively regulates SOCE (Nelson et al. 2019). This is contradictory to the idea of

SOCE as a SR refiller to prevent fatigue during exercise. Interestingly, SOCE probably acts as a signal for muscle adaptation following exercise. Ivarsson et al (2019) showed increases in RyR Ca^{2+} leak following endurance exercise in mice to induce store-dependent influx during periods of rest and decreases in muscle STIM1 content as mice became fitter (Ivarsson et al. 2019). Near-membrane depletion of Ca^{2+} inside the SR due to the leaky RyR will cause Ca^{2+} dissociation from STIM1, providing physiological activation of SOCE while the SR Ca^{2+} content remains relatively high in the presence of a fully functional SR Ca^{2+} pump (Cully et al 2016, 2018).

Declarations

Ethical Approval

Work with rats was approved by The Animal Ethics Committee of The University of Queensland.

Availability of data and materials

Contact the corresponding author

Authors' contributions

AMH designed and performed experiments, analysed and interpreted data and wrote the paper. BSL designed experiments, interpreted data and wrote the paper.

Competing interests

There are no competing interests

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Figures

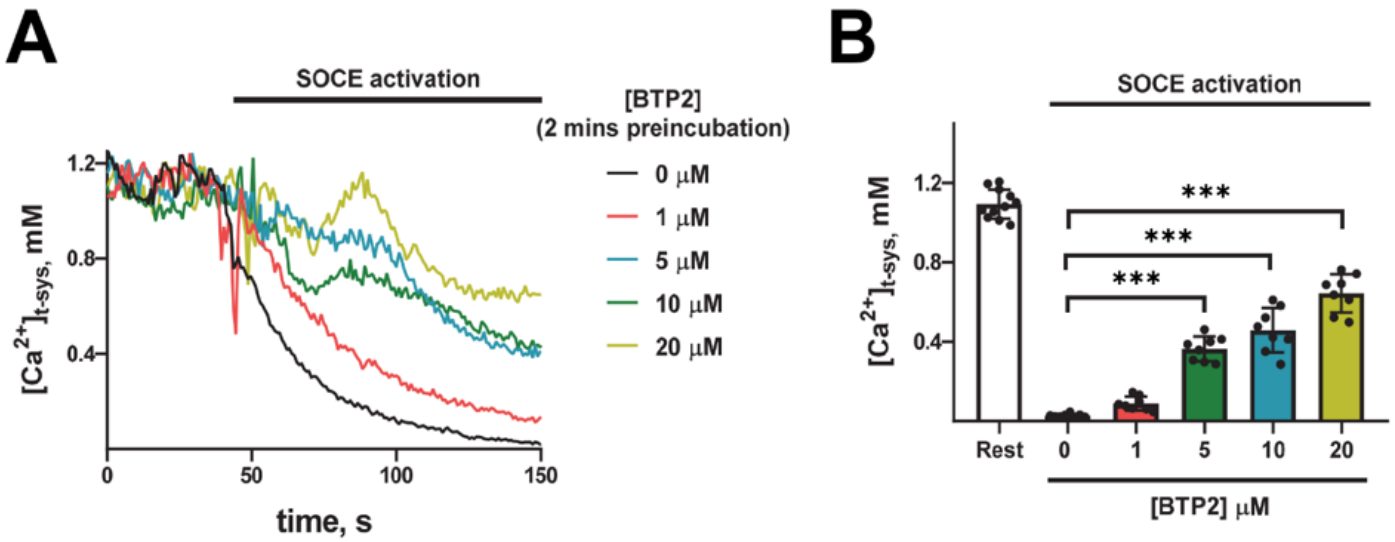

Figure 1

Effect of BTP2 in SOCE-induced [Ca2+]t-sys depletion. A. Representative SOCE-induced [Ca2+]t-sys depletion transients from fibers preincubated for 2 minutes with increasing concentrations of BTP2. SOCE was induced by exposing the fibres to 30 mM caffeine, causing a thorough depletion of SR Ca2+ and consequently SOCE activation. B. Mean of [Ca2+]t-sys at rest and after SOCE activation in presence of the different BTP2 concentrations. (n= 8-12 fibres). One way anova revealed significant difference between 0 vs 5 (p□0.001), 0 vs 10 (p□0.001) and 0 vs 20 (p□0.001) mM BTP2.

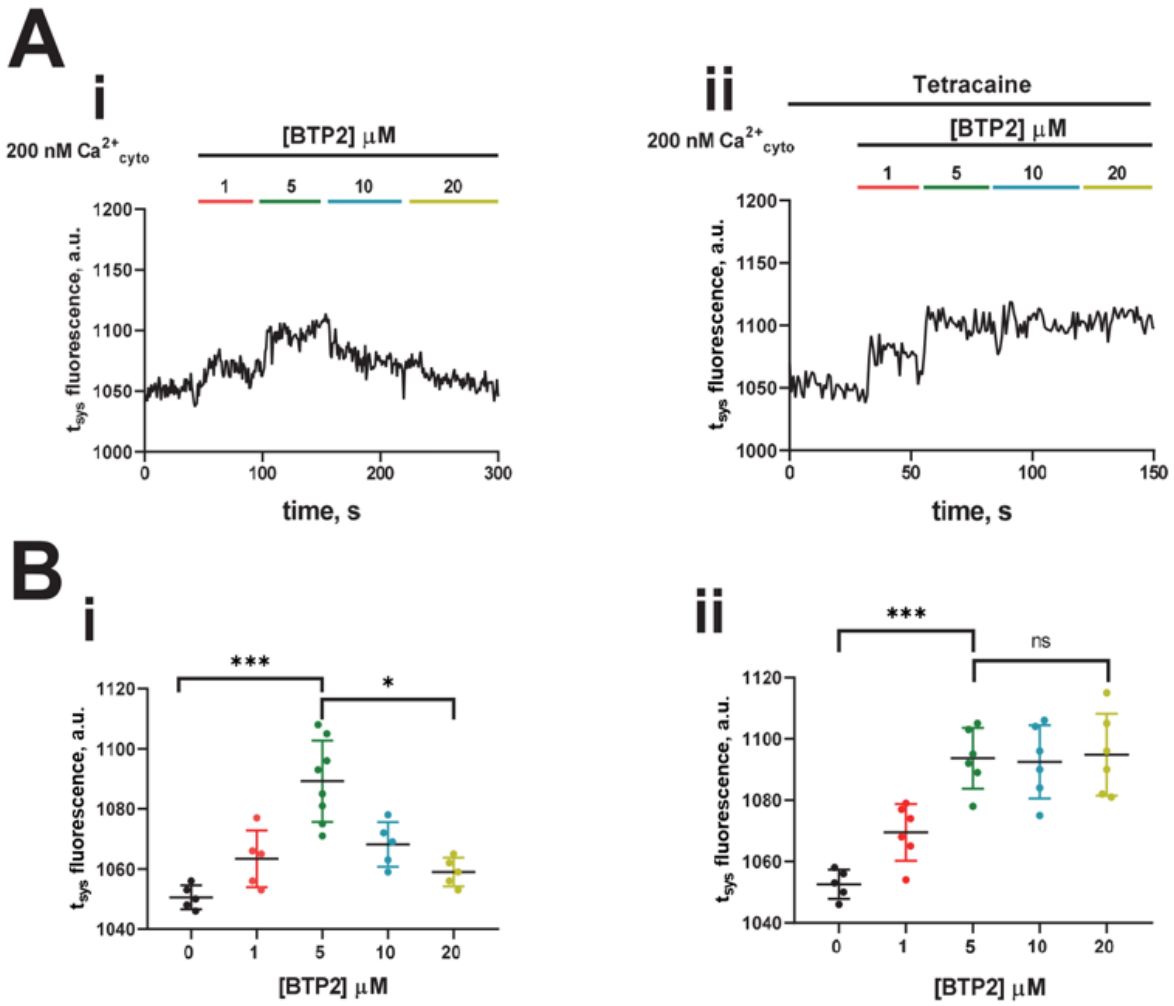

Figure 2

Effect of BTP2 in resting t-system trapped rhod5N signal. A.T-system trapped Rhod 5N signal from fibres exposed to increasing concentrations of BTP2 in absence (left) and presence (right) of tetracaine. B. Mean of T-system trapped Rhod 5N signal from fibers exposed to different concentrations of BTP2. (n = 5 – 8). One way anova revealed significant differences between 0 vs 5 µM BTP2 ($p = 0.0008$) and 5 vs 20 µM BTP2 ($p = 0.0201$) in absence of Tetracaine. In presence of tetracaine, one way anova revealed significant differences between 0 vs 5 µM BTP2 ($p = 0.0008$). No differences between 5 vs 20 µM BTP2 were found (p□0.999).

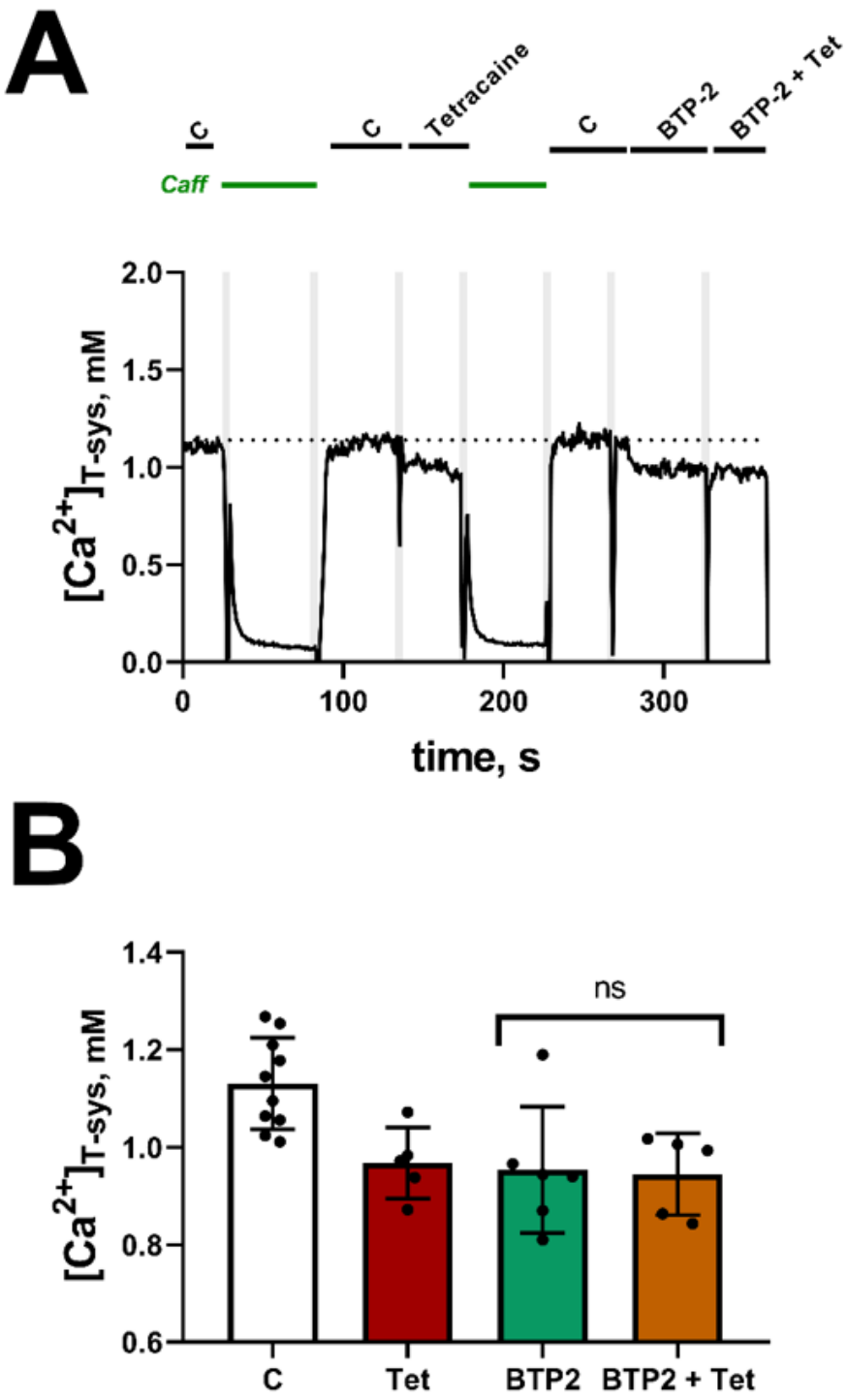

Figure 3

Effect of BTP2 on resting RyR Ca2+ leak. A. [Ca2+]t-sys transient during exposure to 200 nM [Ca2+]cyto, caffeine, 1 mM tetracaine and BTP2. The solution containing 200 nM Ca2+ is considered the control solution (indicated as “C”). Tetracaine and BTP-2 are added to “C”, as indicated. Note that the caffeine causes a chronic activation of SOCE following thorough depletion of the SR Ca2+. BTP-2 causes a block of the RyR Ca2+ leak, as indicated by the decline in the [Ca2+]t-sys transient to the same level as the

known RyR inhibitor, tetracaine. B. [Ca2+]t-sys mean of the different conditions (n = 5-10). No statistical significance was found comparing BTP2 treatment to BTP2 + Tetracaine treatment.

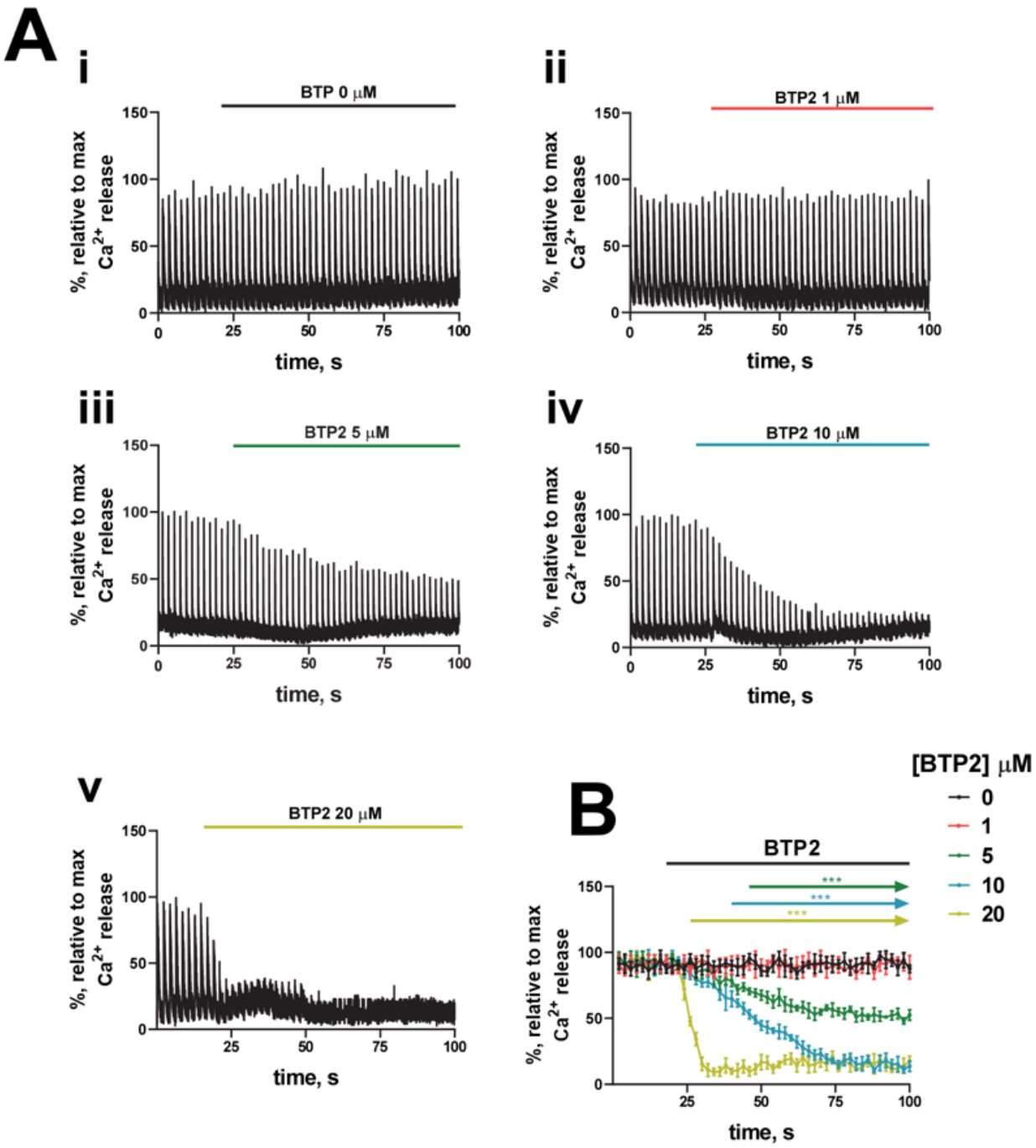

Figure 4

Effect of BTP2 on electrically evoked SR Ca2+ release. A. Original recordings of mechanically skinned fibres exposed to increasing concentrations of BTP2 (i-v) obtained by confocal line scans parallel to the fiber long axis. Normalization was done using the maximum value as 100% and the minimum as 0%. B.

Mean of transients amplitude over time (n= 4 - 7). Arrows indicate the starting point where amplitude starts to be significantly decreased (p□0.005) compared to 0 µM BTP2 treatment.

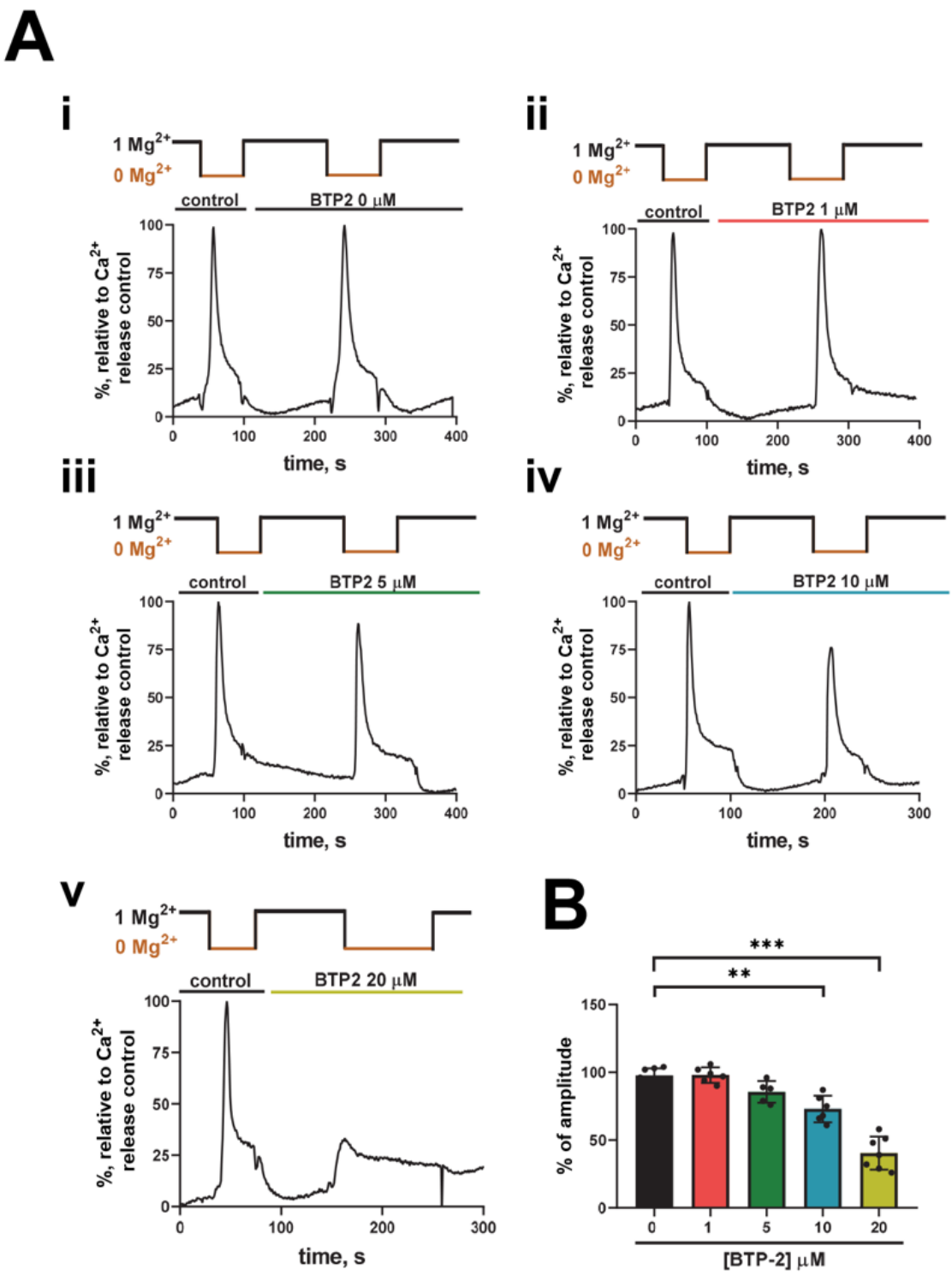

Figure 5

ffect of BTP2 on Mg2+ removal SR calcium release. A. Spatially averaged Rhod2 cytoplasmic fluorescence in mechanically skinned fibres exposed to increasing concentrations of BTP2 (i-v). Normalization was done using the maximum value as 100% and the minimum as 0%. B. Means of

transients amplitude after exposure to different BTP2 concentrations (n= 4 - 7). One way anova revealed a significant amplitude decrease in fibres exposed to 10 (p = 0.0048) and 20 (p□0.0001) μM BTP2.

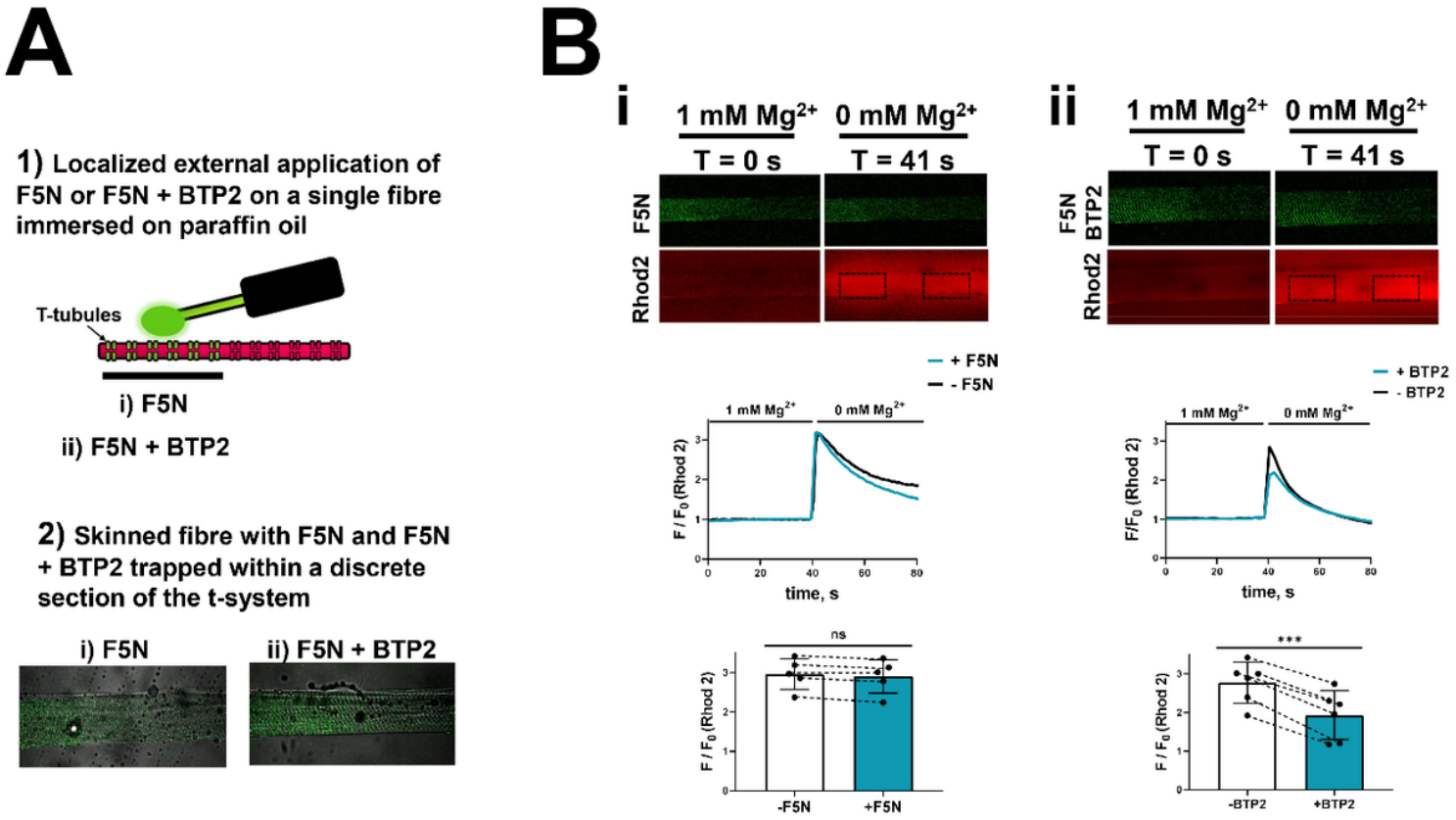

Figure 6

Effect of extracellular administration of BTP2 on Mg2+ removal-induced SR calcium release. A. 1) Diagram describing the protocol of extracellular administration of BTP2 (10 μM) on a single fibre isolated from EDL set on paraffin oil (fibre is partially exposed to F5N or F5N +10 μM BTP2). 2) Fibres exposed to these solutions were skinned and transferred to the chamber custom-built for confocal microscopy. B. (Top) Representative images of F5N and Rhod-2 channels in two different time points (0 and 41 s) corresponding to cytosolic 1 mM Mg2+ and 0 mM Mg2+, respectively. (Middle) Spatially averaged profile of xyt recordings of Ca2+ transients in the skinned fibres evoked by removal of Mg2+ in absence (black) and presence (blue) of F5N (i) or F5N + BTP2 (ii). (Bottom) Mean ± SD of normalized peak amplitude values (F/F0) in absence and presence of 10 □M BTP2. Data from each half of the same fibre are indicated by the joining dotted lines (n = 5 and 6, for i and ii, respectively). Statistical significance was assessed by a paired t-test (***p < 0.001).