

# BTP2 negatively regulates Orai1 and ryanodine receptor function in skeletal muscle

Aldo Meizoso-Huesca

University of Queensland

Bradley Launikonis (✉ [b.launikonis@uq.edu.au](mailto:b.launikonis@uq.edu.au))

University of Queensland <https://orcid.org/0000-0003-0700-4867>

---

## Research

**Keywords:** BTP-2, Orai1, ryanodine receptor, store-operated Ca<sup>2+</sup> entry, Ca<sup>2+</sup> release, fatigue, skeletal muscle, excitation-contraction coupling

**Posted Date:** January 24th, 2020

**DOI:** <https://doi.org/10.21203/rs.2.21863/v1>

**License:**   This work is licensed under a Creative Commons Attribution 4.0 International License.

[Read Full License](#)

---

# Abstract

**Background.** BTP2 is known to block Orai1, the Ca<sup>2+</sup> channel of store-operated Ca<sup>2+</sup> 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<sup>2+</sup> 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<sup>2+</sup> release in the presence of BTP2. **Results.** In addition to blocking Orai1-dependent SOCE, we found a BTP2-dependent inhibition of Orai1 channel resting Ca<sup>2+</sup> conductance. Intriguingly, increasing concentrations of BTP2 displayed a hormetic effect on resting [Ca<sup>2+</sup>] in the t-system ([Ca<sup>2+</sup>] t-sys), shifting from inducing an accumulation of Ca<sup>2+</sup> in the t-system presumably due to Orai1 channels blocking, to reducing the resting [Ca<sup>2+</sup>] t-sys. This biphasic effect is not observed in presence of a ryanodine receptor (RyR) inhibitor, suggesting that above the hormetic zone, BTP2 impairs RyR function. Additionally, we found that BTP2 impairs the cytoplasmic Ca<sup>2+</sup> transients during repetitive excitation-contraction coupling (EC coupling) cycles independent of extracellular Ca<sup>2+</sup> entry. We determined that the release of Ca<sup>2+</sup> through the RyR was inhibited by BTP2, strongly suggesting that the RyR was the point of inhibition during the cycles of EC coupling. **Conclusion.** Our results show that both Ca<sup>2+</sup> 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<sup>2+</sup> entry (SOCE) is a retrograde Ca<sup>2+</sup> regulatory mechanism, activated by the depletion of calcium in the endo/sarcoplasmic reticulum that causes the influx of Ca<sup>2+</sup> to the cell. Two components, an SR Ca<sup>2+</sup> sensor (STIM1), and a Ca<sup>2+</sup> channel in the plasma membrane (Orai1), mediate this process (Soboloff et al. 2006; Feske et al. 2006).

SOCE is well-described in non-excitabile 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 during local Ca<sup>2+</sup> 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 channels must be pre-positioned to respond immediately to a near-membrane depletion of Ca<sup>2+</sup> inside the SR (Koenig et al. 2019). The intra-SR Ca<sup>2+</sup>-gradient generated by an increase in RyR permeability to Ca<sup>2+</sup> causes Ca<sup>2+</sup> dissociation from STIM1, inducing STIM1 interaction with the Orai1 Ca<sup>2+</sup> 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<sup>2+</sup> influx in skeletal muscle is tiny in comparison to the Ca<sup>2+</sup> 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  $\text{Ca}^{2+}$  from the SR in intact muscle fibre preparation because of problems separating it from the release of  $\text{Ca}^{2+}$  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 field to study cellular and physiological aspects of SOCE, representing an agent we can use to better understand how SOCE works in skeletal muscle. However, the absence of an available direct measurement of SOCE during  $\text{Ca}^{2+}$  release in intact fibre preparations, coupled with assumptions of specificity of action of pharmacological blocking agents at Orai1 generate a level of uncertainty in many of the conclusions drawn in regards to the roles of SOCE in skeletal muscle that have been proposed following such experimental approaches (Cully & Launikonis, 2013). Defining the effects of BTP2 on SOCE would significantly aid our understanding of the physiological role of SOCE in skeletal muscle. Of course, in skeletal muscle, SOCE requires the simultaneous action of STIM1, Orai1 and RyRs in muscle for physiological relevant activation (Cully et al 2018; Koenig et al 2018). Importantly, the flux of  $\text{Ca}^{2+}$  through the RyR grades the flux of  $\text{Ca}^{2+}$  through Orai1 (Koenig et al 2018) and thus any effect of BTP2 on RyR would directly affect Orai1 activity.

We characterized the effect of BTP2 on  $\text{Ca}^{2+}$  movements in skeletal muscle using mechanically skinned fibres, which provide a means of assessing Orai1  $\text{Ca}^{2+}$  conductance in the presence of a fully functional SR (Launikonis et al 2003), where  $\text{Ca}^{2+}$  release through the RyR can be imaged simultaneously with SOCE-dependent depletion of t-system  $[\text{Ca}^{2+}]$  (Launikonis & Ríos, 2007). We observed a dose-dependent inhibition of SOCE by BTP2 and show a t-system  $\text{Ca}^{2+}$  accumulation at rest in response to BTP2, strongly suggesting the presence of a resting  $\text{Ca}^{2+}$  efflux through Orai1 channels on the t-system. Unexpectedly, we found that increasing concentrations of BTP2 exert the opposite effect in  $[\text{Ca}^{2+}]_{\text{t-sys}}$ , lowering t-system calcium levels. Interestingly, this biphasic effect is not observed in the presence of the RyR antagonist tetracaine. We additionally provide evidence from experiments with mechanically skinned fibres where the cytosolic  $\text{Ca}^{2+}$  is provided by the bathing solution, and therefore SOCE becomes physiologically redundant, to show that 10 and 20  $\mu\text{M}$  BTP2 impair RyR function in response to repetitive electrical stimulation and  $\text{Mg}^{2+}$  removal.

## 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  $\text{CO}_2$  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  $\text{Ca}^{2+}$  was released from the SR by stimulation with low  $[\text{Mg}^{2+}]_{\text{cyto}}$  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.

## 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 continuously recorded in xyt mode with an aspect ratio of  $256 \times 512$ , 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  $2 \text{ ms line}^{-1}$  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  $30\text{--}50 \text{ V cm}^{-2}$  (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

(F<sub>max</sub>) and minimum (F<sub>min</sub>), respectively. These values were used in conjunction with the previously determined K<sub>D</sub> of rhod-5N in the t-system of 0.8 mM (Cully et al 2016) to determine [Ca<sup>2+</sup>]<sub>t-sys</sub>, with the relationship: [Ca<sup>2+</sup>]<sub>t-sys</sub> (t) = k<sub>D,Ca</sub> \* (F (t) - F<sub>min</sub>)/(F<sub>max</sub> - F (t)).

Results are expressed as mean ± SD.

## Results

### Effect of BTP2 on Orai1

In mechanically skinned fibres, SOCE induces t-system Ca<sup>2+</sup> ([Ca<sup>2+</sup>]<sub>t-sys</sub>) depletion during Ca<sup>2+</sup> 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<sup>2+</sup> and no added Ca<sup>2+</sup> to induce SR Ca<sup>2+</sup> 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<sup>2+</sup>]<sub>t-sys</sub> 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<sup>2+</sup>]<sub>t-sys</sub> transient ([Ca<sup>2+</sup>]<sub>t-sys</sub> (t)) was observed, where [Ca<sup>2+</sup>]<sub>t-sys</sub> (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<sup>2+</sup>]<sub>t-sys</sub> was 1.0945 mM; SOCE activation in absence of BTP2 induced a [Ca<sup>2+</sup>]<sub>t-sys</sub> decrease to 0.0291 mM. In contrast, 5, 10 and 20 μM BTP2 partially inhibited the depletion of [Ca<sup>2+</sup>]<sub>t-sys</sub> 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. We next tested if BTP2 was able to affect the resting Ca<sup>2+</sup> conductance of these channels in the absence of SR Ca<sup>2+</sup> depletion. To do this, we exposed fibres to increasing concentrations of BTP2 expecting that any blockade of a resting Orai1 channel Ca<sup>2+</sup> conductance would result in the net increase of [Ca<sup>2+</sup>]<sub>t-sys</sub>. The high sensitivity of the [Ca<sup>2+</sup>]<sub>t-sys</sub> to changes in the fluxes of Ca<sup>2+</sup> across the t-system membrane provided the opportunity to assess whether BTP2 blocked a basal Orai1 Ca<sup>2+</sup> flux (Cully et al 2018) (the pump-leak balance that sets the steady state [Ca<sup>2+</sup>]<sub>t-sys</sub> is potentially changed by BTP2).

Previously we have shown the dependence of [Ca<sup>2+</sup>]<sub>t-sys</sub> on the resting SR Ca<sup>2+</sup> leak through the RyR, which sets the [Ca<sup>2+</sup>] in the junctional space between the SR and t-system and determines t-system Ca<sup>2+</sup> pump activity (Cully et al. 2018). To test whether the RyR Ca<sup>2+</sup> leak of the resting fibre could be affected

by the treatment of increasing 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). When applied, Tetracaine induces a decrease in t-system Rhod 5N signal, however, we normalized the starting signal to 1050 arbitrary units for comparative purposes as in the start of the recording in Fig. 2 Ai. In this experiment, 1 and 5  $\mu$ M BTP2 exerted a comparable rhod-5N signal increase as before (Fig. 2Ai). In contrast, when the fibres were exposed to 10 and 20  $\mu$ M of BTP2, the signal remained steady, suggesting that the rhod-5N signal decline observed in Fig. 2Ai when the RyRs were functional was due to a negative modulation of RyR by BTP2. It is interesting to point that the fact that the rhod-5N signal in presence of tetracaine did not increase with 10 and 20  $\mu$ M BTP2 as observed with 1 and 5  $\mu$ M, suggesting that in our preparation, 5  $\mu$ M BTP2 is sufficient to reach a saturation of Orai1 channels resting conductance inhibition.

To confirm that 10  $\mu$ M BTP2 (a commonly used concentration in the field) inhibits the resting RyR  $\text{Ca}^{2+}$  leak, t-system  $\text{Ca}^{2+}$  was continuously tracked while fibers were exposed to tetracaine, BTP2 or a combination of both drugs. Figure 3A shows a representative trace of  $[\text{Ca}^{2+}]_{\text{t-sys}}$  on a fibre with Rhod-5N trapped within its t-system. Initially, the sealed t-system presented a  $[\text{Ca}^{2+}]$  of 1.1305 mM when the fibre was bathed on 200 nM  $\text{Ca}^{2+}$  solution (described on the trace as “C” (control)). As described above, t-system  $\text{Ca}^{2+}$  depletion occurs upon activation of SOCE with 30 mM caffeine 0.01 mM  $\text{Mg}^{2+}$  solution. The t-system is able to refill by removing the release solution and exposing the fibre to 200 nM  $\text{Ca}^{2+}$  control solution. As explained above, resting  $[\text{Ca}^{2+}]_{\text{t-sys}}$  depends on resting leak through RyR. A blockage of this channel with 1 mM tetracaine reduced the resting  $[\text{Ca}^{2+}]_{\text{t-sys}}$  from 1.1305 to 0.09674 mM. Following another cycle of Caffeine and “control” solution, 10  $\mu$ M BTP2 was added to C solution, where a decrease in  $[\text{Ca}^{2+}]_{\text{t-sys}}$  was observed. The reduction  $[\text{Ca}^{2+}]_{\text{t-sys}}$  upon exposure of BTP2 was comparable to the one observed with tetracaine. Furthermore, addition of 1 mM tetracaine to the 10  $\mu$ M 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, suggesting that 10  $\mu$ M BTP2 blocks resting RyR  $\text{Ca}^{2+}$  leak.

## Effect of BTP2 on RyRs and SR $\text{Ca}^{2+}$ release

The unexpected finding that BTP2 could be affecting RyRs obliged us to explore this further. It is possible to examine the effect of BTP2 on RyR function in absence of a physiological contribution of SOCE during excitation-contraction coupling because the open cytoplasm of the skinned fibre provides an infinite pool for the SR to sequester  $\text{Ca}^{2+}$ , making any role of Orai1 providing  $\text{Ca}^{2+}$  during repetitive cycles of EC coupling redundant.

Therefore, we performed a set of experiments where skinned fibres were stimulated at 0.5 Hz to elicit repetitive cycles of EC coupling in increasing concentrations of BTP2. To do this, mechanically skinned EDL fibres were bathed in a  $\text{K}^+$ -based cytoplasmic solution and excited by field stimulation, as described previously (Posterino, et al. 2000; Choi, et al. 2017). The cytoplasmic  $\text{Ca}^{2+}$  transients were imaged 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  $\mu$ M) was directly added to the bath solution. Examples of this experiment are shown in Fig. 3A. A dose-dependent effect of BTP2 on the amplitude of the action potential-elicited  $\text{Ca}^{2+}$  transients was observed.

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

The results provided here suggest that BTP2 at high doses impairs the function of the RyR in mechanically skinned fibres.

## Discussion

The experiments presented in this work provide novel evidence regarding the effect of BTP2 on skeletal muscle fibres. We show that very low  $\mu$ M concentrations of BTP2 block the t-system Orai1 channel in isolation but at concentrations above 5  $\mu$ M, RyR function is impaired in parallel to Orai1. Overall, the results and techniques employed here offer an original and highly sensitive tool that can be used to study Orai1 resting  $\text{Ca}^{2+}$  conductance in muscle fibres. Importantly, given the promiscuity of BTP2 at commonly used doses, our results highlight a need for re-evaluation of some conclusions regarding SOCE function in skeletal muscle that have been based on the premise of a specific action of BTP2 on Orai1.

## BTP2 mechanism of action

Initial studies perfusing 1  $\mu$ M BTP2 into Jurkat cells proposed that the Orai1 inhibition site was located at the extracellular region of the channel (Zitt et al. 2003). In contrast to this, the general body of skeletal muscle researchers use BTP2 at 10  $\mu$ M concentrations (Zhao et al. 2005; Thornton et al. 2011; Wei-Lapierre et al. 2013). In addition, we must keep in mind we do not have a direct comparison of protein expression levels between different cell systems such as Jurkat cells and skeletal muscle fibres, an issue that can complicate the extrapolation of conclusions from one system to another.

The use of BTP2 on mechanically skinned fibres implies that the effects observed come from two possible scenarios: 1. BTP2 binding to intracellular ligands or 2. BTP2 crossing the t-tubule membrane and interacting with the extracellular domain of Orai1 (as proposed by Zitt et al), which in our preparation resides within the sealed t-tubules. BTP2 is a cell permeant molecule; therefore, we can assume with confidence that the two possibilities can occur in any cell-based biological model (cell culture, intact fibres or skinned fibres) and importantly, they 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 is mediated by Drebrin inhibition. Anecdotally, when Zitt et al. perfused  $1 \mu\text{M}$  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  $\text{Ca}^{2+}$  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; this could lead to the impairment of actin filaments interacting with Store Operated  $\text{Ca}^{2+}$  (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 to achieve their functions.

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. Working with neurons, Bose et al found that actin disruption using cytochalasin D impairs the RyR-mediated ER  $\text{Ca}^{2+}$  release (Bose and Thomas, 2009). In the case of skeletal muscle,  $\gamma$ -actin has been found attached to the SR and the disruption of it leads to impairment of SR  $\text{Ca}^{2+}$  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.

## 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  $\text{Ca}^{2+}$  from the stimulated muscle needs to be replenished by  $\text{Ca}^{2+}$  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 are 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 1  $\mu\text{M}$  to avoid RyR impairment. The BTP2 concentration chosen to block Orai1 in muscle experiments has typically been 10  $\mu\text{M}$ , which in mechanically skinned fibres curtails the release of  $\text{Ca}^{2+}$  from the SR and the activation of SOCE. In these experiments the decline of the action potential-induced  $\text{Ca}^{2+}$  transients in the presence of BTP2 in intact and skinned fibres is very similar (Fig. 3; Wei-Lapierre et al 2013). It follows that an action of BTP2 on the RyR on intact fibre preparation is likely to be the cause of the  $\text{Ca}^{2+}$  transient decline in those experiments as well. In this matter, Liu et al. showed a negative effect of BTP2 on spontaneous and caffeine-induced SR  $\text{Ca}^{2+}$  on intact fibres isolated from the sinoatrial node (Liu et al. 2015). This observation made on intact cells supports the findings presented here and suggests that BTP2 could also impair not only the RyR1 isoform but also RyR2. However, we acknowledge that the dose-dependent effects of BTP2 on the RyRs of intact and skinned fibre preparations may have some differences.

Additionally, the biophysical properties of  $\text{Ca}^{2+}$  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. The proportional contribution of the SR and t-system to the  $\text{Ca}^{2+}$  entering the cytoplasm during EC coupling is 99:1, making it difficult to argue that SOCE contributes significantly to the  $\text{Ca}^{2+}$  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  $\text{Ca}^{2+}$  release mechanism of the muscle by the build-up of metabolites than an inherent loss of fibre calcium (Allen et al. 2008). 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<sup>2+</sup> 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<sup>2+</sup> inside the SR due to the leaky RyR will cause Ca<sup>2+</sup> dissociation from STIM1, providing physiological activation of SOCE while the SR Ca<sup>2+</sup> content remains relatively high in the presence of a fully functional SR Ca<sup>2+</sup> 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

### **Funding**

This work was supported by an Australian Research Council Discovery Project to BSL (DP180100937).

### **Acknowledgments.**

We thank Cedric Lamboley, Luke Pearce, Daniel Singh and Crystal Seng for helpful comments on the manuscript.

## **References**

A.M., Thornton, Zhao X., Weisleder N., Brotto L.S., Bougoin S., Nosek T.M., Reid M., et al. 2011. "Store-Operated Ca(2+) Entry (SOCE) Contributes to Normal Skeletal Muscle Contractility in Young but Not in

Aged Skeletal Muscle." *Aging* 3 (6): 621–34. <http://ovidsp.ovid.com/ovidweb.cgi?T=JS&PAGE=reference&D=emed13&NEWS=N&AN=362914291>.

Allen, D. G., G. D. Lamb, and H. Westerblad. 2008. "Skeletal Muscle Fatigue: Cellular Mechanisms." *Physiological Reviews* 88 (1): 287–332. <https://doi.org/10.1152/physrev.00015.2007>.

Carrell, Ellie M., Aundrea R. Coppola, Helen J. McBride, and Robert T. Dirksen. 2016. "Orai1 Enhances Muscle Endurance by Promoting Fatigue-Resistant Type I Fiber Content but Not through Acute Store-Operated Ca<sup>2+</sup> Entry." *FASEB Journal* 30 (12): 4109–19. <https://doi.org/10.1096/fj.201600621R>.

Choi, Rocky H., Xaver Koenig, and Bradley S. Launikonis. 2017. "Dantrolene Requires Mg<sup>2+</sup> to Arrest Malignant Hyperthermia ." *Proceedings of the National Academy of Sciences* 114 (18): 4811–15. <https://doi.org/10.1073/pnas.1619835114>.

Cully, Tanya R., Rocky H. Choi, Andrew R. Bjorksten, D. George Stephenson, Robyn M. Murphy, and Bradley S. Launikonis. 2018. " Junctional Membrane Ca<sup>2+</sup> Dynamics in Human Muscle Fibers Are Altered by Malignant Hyperthermia Causative RyR Mutation ." *Proceedings of the National Academy of Sciences* 115 (32): 8215–20. <https://doi.org/10.1073/pnas.1800490115>.

Cully, Tanya R, Joshua N Edwards, Robyn M Murphy, and Bradley S Launikonis. 2016. "TECHNIQUES FOR PHYSIOLOGY A Quantitative Description of Tubular System Ca<sup>2+</sup> Handling in Fast- and Slow-Twitch Muscle Fibres" 11: 2795–2810. <https://doi.org/10.1113/JP271658>.

Duke, Adrian M., Philip M. Hopkins, Sarah C. Calaghan, Jane P. Halsall, and Derek S. Steele. 2010. "Store-Operated Ca<sup>2+</sup> Entry in Malignant Hyperthermia-Susceptible Human Skeletal Muscle." *Journal of Biological Chemistry* 285 (33): 25645–53. <https://doi.org/10.1074/jbc.M110.104976>.

Feske, Stefan, Yousang Gwack, Murali Prakriya, Sonal Srikanth, Sven Holger Puppel, Bogdan Tanasa, Patrick G. Hogan, Richard S. Lewis, Mark Daly, and Anjana Rao. 2006. "A Mutation in Orai1 Causes Immune Deficiency by Abrogating CRAC Channel Function." *Nature* 441 (7090): 179–85. <https://doi.org/10.1038/nature04702>.

Galán, Carmen, Natalia Dionisio, Tarik Smani, Ginés M. Salido, and Juan A. Rosado. 2011. "The Cytoskeleton Plays a Modulatory Role in the Association between STIM1 and the Ca<sup>2+</sup> Channel Subunits Orai1 and TRPC1." *Biochemical Pharmacology* 82 (4): 400–410. <https://doi.org/10.1016/j.bcp.2011.05.017>.

Gokhin, David S., and Velia M. Fowler. 2011. "Cytoplasmic  $\gamma$ -Actin and Tropomodulin Isoforms Link to the Sarcoplasmic Reticulum in Skeletal Muscle Fibers." *Journal of Cell Biology* 194 (1): 105–20. <https://doi.org/10.1083/jcb.201011128>.

Ishikawa, Jun, Keiko Ohga, Taiji Yoshino, Atsushi Ichikawa, Hirokazu Kubota, Toshimitsu Yamada, and Email Alerts. 2003. "A Pyrazole Derivative, YM-58483, Potently Inhibits Store-Operated Sustained Ca<sup>2+</sup>

Influx and IL-2 Production in T Lymphocytes." *Journal of Immunology*.

<https://doi.org/10.4049/jimmunol.170.9.4441>.

Ivarsson, Niklas, C. Mikael Mattsson, Arthur J. Cheng, Joseph D. Bruton, Björn Ekblom, Johanna T. Lanner, and Håkan Westerblad. 2019. "SR Ca<sup>2+</sup> Leak in Skeletal Muscle Fibers Acts as an Intracellular Signal to Increase Fatigue Resistance." *Journal of General Physiology* 151 (4): 567–77.

<https://doi.org/10.1085/jgp.201812152>.

Koenig, Xaver, Rocky H. Choi, Klaus Schicker, Daniel P. Singh, Karlheinz Hilber, and Bradley S. Launikonis. 2019. "Mechanistic Insights into Store-Operated Ca<sup>2+</sup> Entry during Excitation-Contraction Coupling in Skeletal Muscle." *Biochimica et Biophysica Acta - Molecular Cell Research* 1866 (7): 1239–48.

<https://doi.org/10.1016/j.bbamcr.2019.02.014>.

Koenig, Xaver, Rocky H. Choi, and Bradley S. Launikonis. 2018. "Store-Operated Ca<sup>2+</sup> Entry Is Activated by Every Action Potential in Skeletal Muscle." *Communications Biology* 1 (1).

<https://doi.org/10.1038/s42003-018-0033-7>.

Launikonis, Bradley S., and Eduardo Ríos. 2007. "Store-Operated Ca<sup>2+</sup> entry during Intracellular Ca<sup>2+</sup> release in Mammalian Skeletal Muscle." *Journal of Physiology* 583 (1): 81–97.

<https://doi.org/10.1113/jphysiol.2007.135046>.

Liu, Jie, Li Xin, Victoria L. Benson, David G. Allen, and Yue Kun Ju. 2015. "Store-Operated Calcium Entry and the Localization of STIM1 and Orai1 Proteins in Isolated Mouse Sinoatrial Node Cells." *Frontiers in Physiology* 6 (MAR): 1–12. <https://doi.org/10.3389/fphys.2015.00069>.

Mercer, Jason C, Qian Qi, Laurie F Mottram, Mankit Law, Danny Bruce, Archana Iyer, J Luis Morales, et al. 2010. "Chemico-Genetic Identification of Drebrin as a Regulator of Calcium Responses." *International Journal of Biochemistry and Cell Biology* 42 (2): 337–45. <https://doi.org/10.1016/j.biocel.2009.11.019>.

Nelson, Marin E, Benjamin L Parker, James G Burchfield, Nolan J Hoffman, Elise J Needham, Kristen C Cooke, Timur Naim, et al. 2019. "Phosphoproteomics Reveals Conserved Exercise-stimulated Signaling and AMPK Regulation of Store-operated Calcium Entry." *The EMBO Journal*, 1–20.

<https://doi.org/10.15252/emj.2019102578>.

Olivera, J. Fernando, and Gonzalo Pizarro. 2010. "Two Inhibitors of Store Operated Ca<sup>2+</sup> Entry Suppress Excitation Contraction Coupling in Frog Skeletal Muscle." *Journal of Muscle Research and Cell Motility* 31 (2): 127–39. <https://doi.org/10.1007/s10974-010-9216-7>.

Peachy, L. D. 1965. "The Sarcoplasmic Reticulum and Transverse Tubules of the Frog's Sartorius." *The Journal of Cell Biology* 25: 209–31.

Posterino, G. S.; Lamb, G. D.; Stephenson D. G. 2000. "Twitch and Tetanic Force Responses and Longitudinal Propagation of Action Potentials in Skinned Skeletal Muscle Fibres of the Rat." *Journal of*

*Physiology* 527 (1): 131–37. <https://doi.org/10.1111/j.1469-7793.2000.t01-2-00131.x>.

Rahman, Saifur, and Taufiq Rahman. 2017. “Unveiling Some FDA-Approved Drugs as Inhibitors of the Store-Operated Ca<sup>2+</sup> Entry Pathway.” *Scientific Reports* 7 (1): 1–13. <https://doi.org/10.1038/s41598-017-13343-x>.

Ribeiro, Carla M. Pedrosa, Jeffrey Reece, and James W. Putney. 1997. “Role of the Cytoskeleton in Calcium Signaling in NIH 3T3 Cells. An Intact Cytoskeleton Is Required for Agonist-Induced [Ca<sup>2+</sup>]<sub>i</sub> Signaling, but Not for Capacitative Calcium Entry.” *Journal of Biological Chemistry* 272 (42): 26555–61. <https://doi.org/10.1074/jbc.272.42.26555>.

Roos, Jack, Paul J. DiGregorio, Andriy V. Yeromin, Kari Ohlsen, Maria Lioudyno, Shenyuan Zhang, Olga Safrina, et al. 2005. “STIM1, an Essential and Conserved Component of Store-Operated Ca<sup>2+</sup> Channel Function.” *Journal of Cell Biology* 169 (3): 435–45. <https://doi.org/10.1083/jcb.200502019>.

Rosado, Juan A., Susanne Jenner, and Stewart O. Sage. 2000. “A Role for the Actin Cytoskeleton in the Initiation and Maintenance of Store-Mediated Calcium Entry in Human Platelets. Evidence for Conformational Coupling.” *Journal of Biological Chemistry* 275 (11): 7527–33. <https://doi.org/10.1074/jbc.275.11.7527>.

Soboloff, Jonathan, Maria A Spassova, Xiang D Tang, Thamara Hewavitharana, Wen Xu, and Donald L Gill. 2006. “Orai1 and STIM Reconstitute Store-Operated Calcium Channel Function \*.” *Journal of Biological Chemistry* 281 (30): 20661–65. <https://doi.org/10.1074/jbc.C600126200>.

Stiber, Jonathan, April Hawkins, Zhu Shan Zhang, Sunny Wang, Jarrett Burch, Victoria Graham, Cary C. Ward, et al. 2008. “STIM1 Signalling Controls Store-Operated Calcium Entry Required for Development and Contractile Function in Skeletal Muscle.” *Nature Cell Biology* 10 (6): 688–97. <https://doi.org/10.1038/ncb1731>.

Tang, Yufang, Jisen Tang, Zhangguo Chen, Claudia Trost, Veit Flockerzi, Min Li, Vijaya Ramesh, and Michael X. Zhu. 2000. “Association of Mammalian Trp4 and Phospholipase C Isozymes with a PDZ Domain-Containing Protein, NHERF.” *Journal of Biological Chemistry* 275 (48): 37559–64. <https://doi.org/10.1074/jbc.M006635200>.

Thomas, Diptiman D. Bose and David W. 2009. “THE ACTIN CYTOSKELETON DIFFERENTIALLY REGULATES NG115-401L CELL RYANODINE RECEPTOR AND INOSITOL 1,4,5- TRISPHOSPHATE RECEPTOR INDUCED CALCIUM SIGNALING PATHWAYS.” *Biochemical and Biophysical Research Communications* 379 (2): 594–99. <https://doi.org/10.1371/journal.pone.0178059>.

Trevillyan, James M, X Grace Chiou, Yung-wu Chen, Stephen J Ballaron, Michael P Sheets, Morey L Smith, Paul E Wiedeman, et al. 2001. “Potent Inhibition of NFAT Activation and T Cell Cytokine Production by Novel Low Molecular Weight Pyrazole Compounds \*.” *The Journal of Biological Chemistry* 276 (51): 48118–26. <https://doi.org/10.1074/jbc.M107919200>.

Wei-Lapierre, Lan, Ellie M. Carrell, Simona Boncompagni, Feliciano Protasi, and Robert T. Dirksen. 2013. "Orai1-Dependent Calcium Entry Promotes Skeletal Muscle Growth and Limits Fatigue." *Nature Communications* 4: 1–12. <https://doi.org/10.1038/ncomms3805>.

Zhang, Shenyuan L., Ying Yu, Jack Roos, J. Ashot Kozak, Thomas J. Deerinck, Mark H. Ellisman, Kenneth A. Stauderman, and Michael D. Cahalan. 2005. "STIM1 Is a Ca<sup>2+</sup>-sensor That Activates CRAC Channels and Migrates from the Ca<sup>2+</sup>-store to the Plasma Membrane." *Nature* 437 (7060): 902–5. <https://doi.org/10.1038/nature04147>.

Zhao, Xiaoli, Morikatsu Yoshida, Leticia Brotto, Hiroshi Takeshima, Noah Weisleder, Yutaka Hirata, Thomas M. Nosek, Jianjie Ma, and Marco Brotto. 2005. "Enhanced Resistance to Fatigue and Altered Calcium Handling Properties of Sarcalumenin Knockout Mice." *Physiological Genomics* 23 (1): 72–78. <https://doi.org/10.1152/physiolgenomics.00020.2005>.

Zitt, Christof, Bettina Strauss, Eva C Schwarz, Nicola Spaeth, Georg Rast, Armin Hatzelmann, Markus Hoth, S W J Biol Chem, and S W Cell Immunol. 2003. "Potent Inhibition of Ca<sup>2+</sup> Release-Activated Ca<sup>2+</sup> Channels and T-Lymphocyte Activation by the Pyrazole Derivative BTP2\*." *The Journal of Biological Chemistry* 279 (13): 12427–37. <https://doi.org/10.1074/jbc.M309297200>.

## Figures

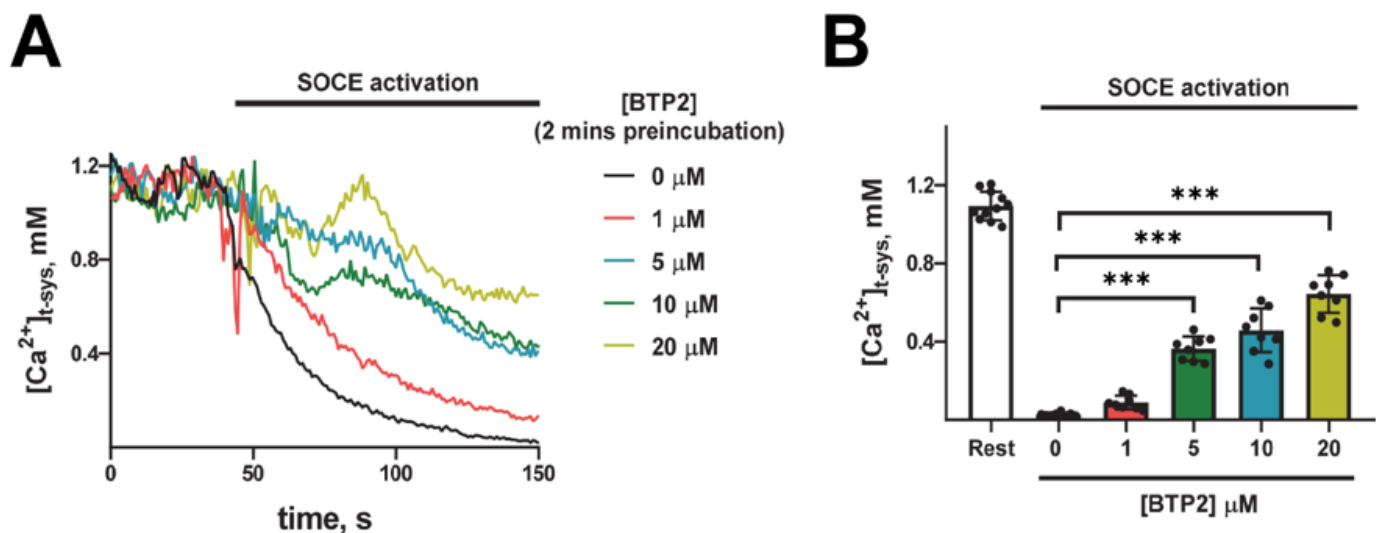
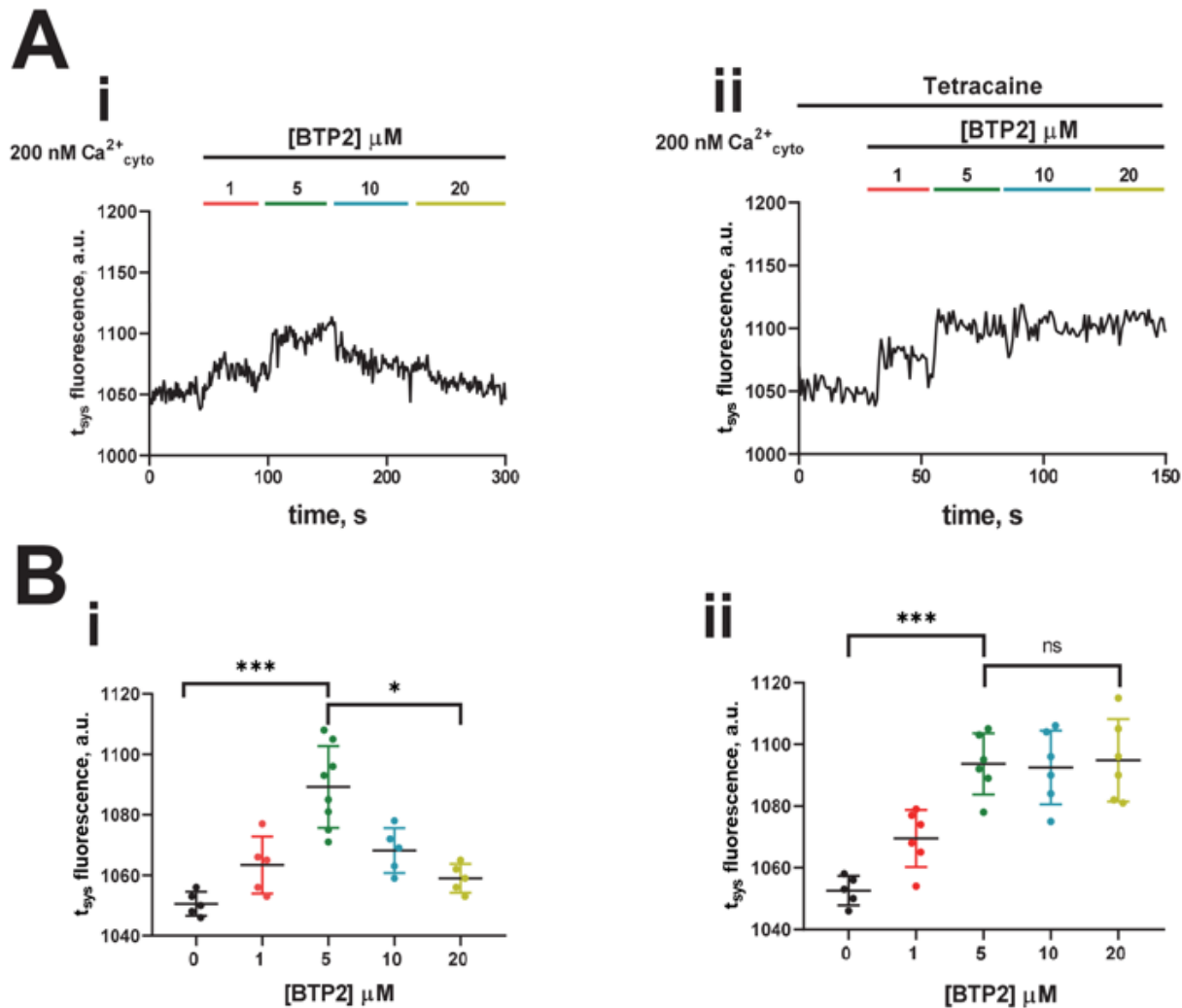


Figure 1

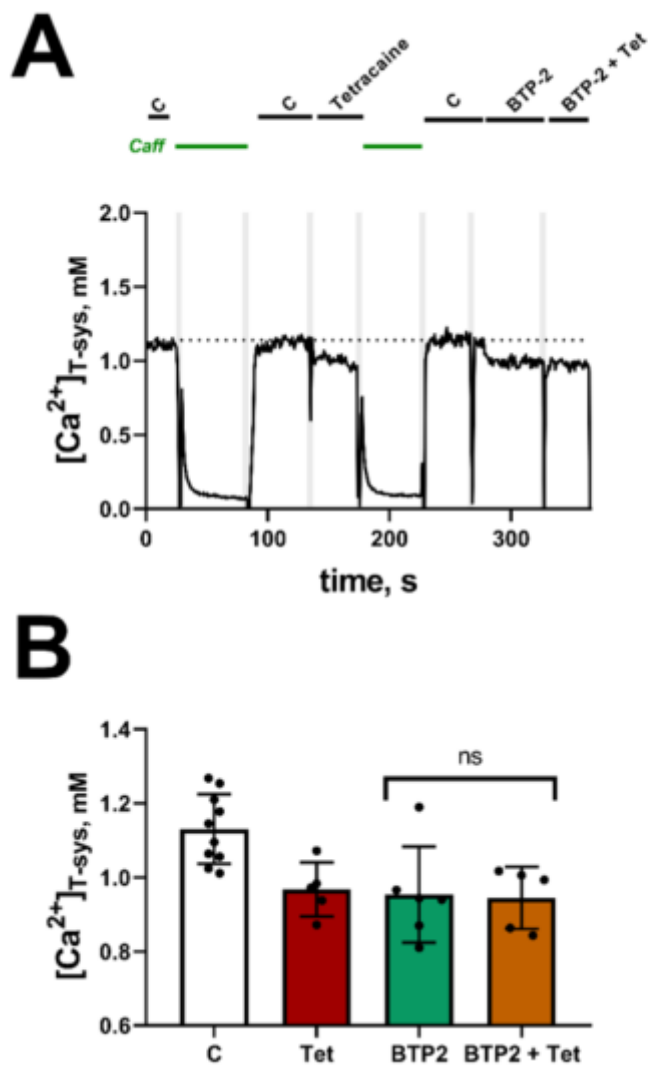
Effect of BTP2 in SOCE-induced [Ca<sup>2+</sup>]<sub>t-sys</sub> depletion. A. Representative SOCE-induced [Ca<sup>2+</sup>]<sub>t-sys</sub> depletion transients from fibers preincubated for 2 minutes with increasing concentrations of BTP2.

SOCE was induced by exposing the fibres to 30 mM caffeine, causing a thorough depletion of SR Ca<sup>2+</sup> and consequently SOCE activation. B. Mean of [Ca<sup>2+</sup>]<sub>t-sys</sub> 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**

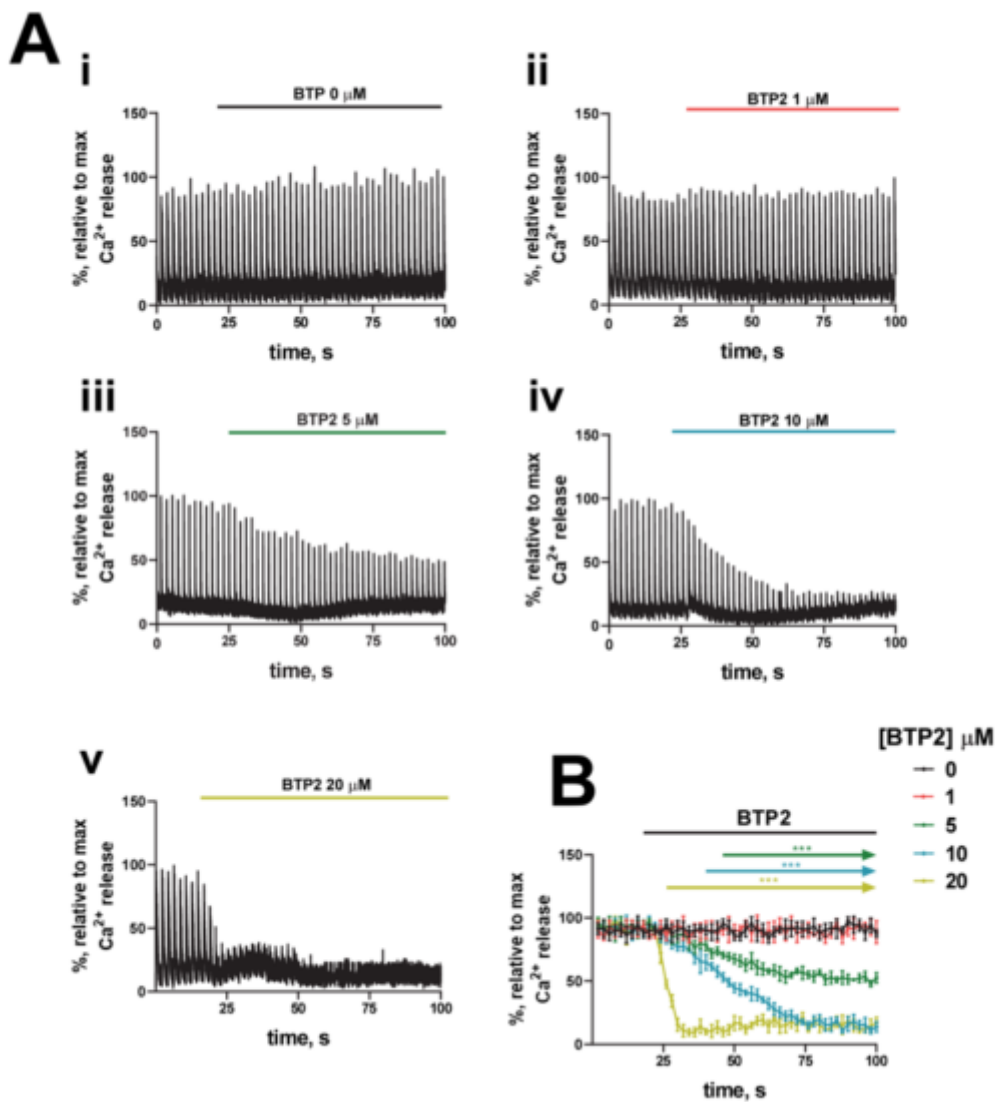
Effect of BTP2 in resting t-system trapped rhod5N signal. A. T-system trapped Rhod 5N signal from fibres exposed to increasing concentrations of BTP2 in absence (left) and presence (right) of tetracaine. B. Mean of T-system trapped Rhod 5N signal from fibers exposed to different concentrations of BTP2. (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**

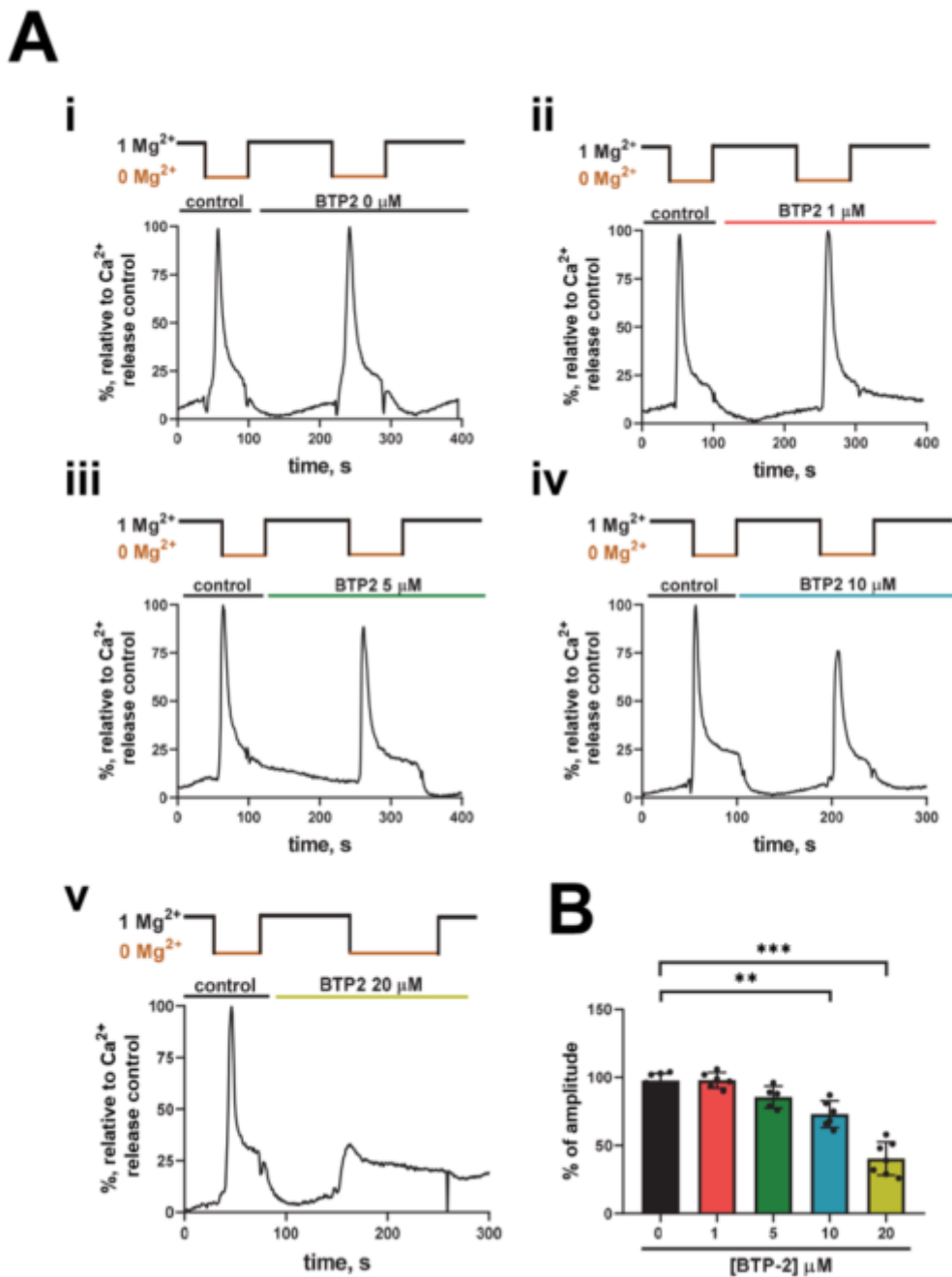
Effect of BTP2 on resting RyR  $\text{Ca}^{2+}$  leak. A.  $[\text{Ca}^{2+}]_{\text{T-sys}}$  transient during exposure to 200 nM  $[\text{Ca}^{2+}]_{\text{cyto}}$ , caffeine, 1 mM tetracaine and BTP2. The solution containing 200 nM  $\text{Ca}^{2+}$  is considered the control solution (indicated as "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  $\text{Ca}^{2+}$ . BTP-2 causes a block of the RyR  $\text{Ca}^{2+}$  leak, as indicated by the decline in the  $[\text{Ca}^{2+}]_{\text{T-sys}}$  transient to the same level as the known RyR inhibitor, tetracaine. B.  $[\text{Ca}^{2+}]_{\text{T-sys}}$  mean of the different conditions (n = 5-10). Non statistical significance was found comparing BTP2 treatment to BTP2 + Tetracaine treatment.





**Figure 4**

Effect of BTP2 on electrically evoked SR Ca<sup>2+</sup> 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**

Effect of BTP2 on  $\text{Mg}^{2+}$  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 ( $p \leq 0.0001$ )  $\mu\text{M}$  BTP2.