The effect of novel ryanodine receptor modulators on Ca^{2+} leak in skeletal muscle fibres

D.P. Singh,[1] R.T. Rebbeck,[2] R.L. Cornea[2] and B.S. Launikonis,[1] [1]School of Biomedical Sciences, The University of Queensland, Brisbane, QLD 4072, Australia and [2]Department of Biochemistry, Molecular Biology and Biophysics, University of Minnesota, MN 55455, USA.

The ryanodine receptor (RyR) is the Ca^{2+} release channel of skeletal muscle. It is located at the terminal cisternae of the sarcoplasmic reticulum (SR) and activated directly by the conformational change of the voltage sensor on the tubular (t-) system membrane following depolarization. In the resting fibre, the RyR leaks Ca^{2+} to the cytoplasm and this is constantly resequestered by the SR Ca^{2+} pump. Under pathological conditions, including inherited RyR or other mutations, through ageing, or muscle disuse, the RyR can become excessively leaky. The excess RyR Ca^{2+} leak can lead to elevated resting Ca^{2+}, which is cytotoxic. This makes the RyR a potential therapeutic target to alleviate leak-related muscle weakness. Therefore, we aimed to test novel RyR modulators – recently identified using a high throughput screening (HTS) platform (Rebbeck *et al*., 2016) – in a skeletal muscle fibre preparation, where the RyR leak activity can be detected by tracking movements of Ca^{2+} into and out of the closely apposed t-system membrane (Cully *et al*., 2016).

All experiments performed were approved by The University of Queensland Animal Ethics Committee. Male Wistar and Sprague-Dawley rats (3-12 months old) were euthanized by overdose of CO_2. The extensor digitorm longus (EDL) muscles were then removed and pinned to Sylgard set in a Petri dish containing paraffin oil. Bundles of muscle fibres were isolated and exposed to a Na^+-based Ringer solution containing 2 mM rhod-5N salt. Individual fibres were then isolated and mechanically skinned to seal the t-system and trap the Ca^{2+}-sensitive dye within. Skinned fibres were transferred to a custom built chamber with a coverslip base and bathed in an internal solution containing (in mM): K^+ (136); Na^+ (36); Mg^{2+}(1); Ca^{2+} (0.00005); ATP (8); creatine phosphate (10); and EGTA (50); with pH adjusted to 7.1 ± 0.1 with KOH. SR Ca^{2+} was released with 30 mM caffeine in an internal solution with no Mg^{2+}, and Ca^{2+} was loaded into the t-system and SR in a solution with 200 nM Ca^{2+} while being continuously imaged on the confocal microscope in xyt mode (see Cully et al 2016). T-system rhod-5N fluorescence was converted to t-system $[Ca^{2+}]$ ($[Ca^{2+}]_{t\text{-}sys}$) as described (Cully et al 2016). RyR modulators identified by HTS (myricetin and chloroxine) where dissolved as stock solutions in DMSO and added to internal solution containing 200 nM Ca^{2+}.

The steady-state $[Ca^{2+}]_{t\text{-}sys}$ was expected to be raised by the accumulation of Ca^{2+} in front of the t-system membrane directly due to RyR leak. Therefore, a change in RyR leak rate was expected to be reflected as a change in steady-state $[Ca^{2+}]_{t\text{-}sys}$. The presence of 100 μM chloroxine in internal solutions depressed the steady-state $[Ca^{2+}]_{t\text{-}sys}$ compared to control (1.48 mM ± 0.07 *vs* 1.22 mM ± 0.06, control and chloroxine, respectively. *T*-test, $P < 0.05$). The reduction in $[Ca^{2+}]_{t\text{-}sys}$ in the presence of chloroxine was similar to that caused by a known RyR inhibitor, tetracaine (1.18 mM ± 0.05). In other experiments, $[Ca^{2+}]_{t\text{-}sys}$ fluctuated in the presence of 200 nM Ca^{2+} in 5 out of 14 fibres isolated from rats aged 6-12 months. A depression of $[Ca^{2+}]_{t\text{-}sys}$ moved as an apparent wave through the t-system at a rate of 11 – 110 μm/s. This rate of Ca^{2+} wave propagation cannot be supported by the t-system due to the much slower longitudinal diffusional limit of Ca^{2+} movement through the t-system (Edwards & Launikonis, 2008). The introduction of either tetracaine or chloroxine interrupted the waves and significantly increased the level of $[Ca^{2+}]_{t\text{-}sys}$. This suggests that excessively leaky RyRs were present in the fibres displaying spontaneous wave behaviour. These Ca^{2+} waves were likely being driven through the SR lumen with subsequent Ca^{2+} release activating store-operated Ca^{2+} entry, which resulted in transiently depressed $[Ca^{2+}]_{t\text{-}sys}$. Taken together, these results show that tracking $[Ca^{2+}]_{t\text{-}sys}$ allows the detection of RyR activity and its modulation by exogenous compounds.

Cully TR, Edwards JN, Murphy RM & Launikonis BS (2016). A quantitative description of tubular system Ca^{2+}-handling properties in fast- and slow-twitch fibres. *J Physiol* **594**, 2795-2810.

Edwards JN & Launikonis BS (2008). The accessibility and interconnectivity of the tubular system in toad skeletal muscle. *J Physiol.* **586**, 5077-5089.

Rebbeck RT, Essawy MM, Nitu FR, Grant BD, Gillispie GD, Thomas DD, Bers DM, Cornea RL (2017). High-throughput screens to discover small-molecule modulators of ryanodine receptor calcium release channels. *SLAS Discov*. **22**, 176-186.