Novel fluorescent techniques for investigating calcium handling properties of the cardiac sarcoplasmic reticulum (SR) and calsequestrin in the presence and absence of dantrolene

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Dantrolene sodium has long been recognised as a powerful inhibitor of SR calcium release in skeletal muscle. More recently there has been renewed interest in its potential to modulate SR calcium release in cardiac muscle, with the potential of it being used as a treatment of heart failure. Here we present novel methods using a 96-well array plate system (Flexstation 3, Molecular Devices) for making quantitative measurements of calcium fluxes associated with sarcoplasmic reticulum (SR) loading and release in isolated skinned cardiac myocytes and for monitoring the polymerisation of calsequestrin and its calcium binding properties using changes in its native fluorescence. The Flexstation 3 allows for the micro-sizing of assays and has the potential for taking 96 measurements under differing conditions on the same batch of cells. It can also measure multiple fluorescence wavelengths on a single assay allowing multiple parameters of an experiment to be monitored simultaneously.

Fresh sheep hearts were obtained from Hardwick's abattoirs Kyneton, Victoria, transported back to the laboratory on ice and the right ventricle was enzymatically digested to produce single cardiac cells. The cells were then allowed to adhere to a 96-well plate (black, clear bottom), by incubating overnight (5%CO_2, 37ºC). The cells were subsequently skinned (sarcolemma made leaky) for 10 to 45 minutes in a sodium-based solution containing 0.1% w/v saponin, 8mM ATP and 1mM $EGTA_{Total}$. After skinning the cells were washed in a solution containing 49.9mM $HDTA_{Total}$ + 0.1mM $EGTA_{Total}$ and resuspended in the same solution containing the Ca^{2+}-sensitive fluorescent dye Fluo4 (5μM). To investigate calcium dependence on SR loading and release a selection of calcium loading solutions was used (pCa = $-\log_{10}[Ca^{2+}]$ of 6.54 and 6.26, 0.1mM $EGTA_{Total}$, 8mM ATP, 1mM Mg^{2+} for 3.0 minutes). SR calcium release was induced by the addition of caffeine (24 mM final concentration). The fluorescent signal from Fluo4 (Ex 494, Em 516 nm) was continually monitored using the Flexstation 3 (read rate 1.15 s for up to 5 minutes). This was compared to the maximal calcium signal recorded after destroying all of the membranous compartments by exposure to 1% v/v Triton X100 for up to 5 minutes. Using these experimental conditions, the effect of dantrolene sodium (1μM) on SR calcium loading and release was investigated. The results show that dantrolene increased SR calcium loading at both pCa 6.54 and 6.26, but did not affect caffeine-induced SR calcium release.

The lipophilic nature of dantrolene makes it possible for it enter the SR and alter calcium binding to calsequestrin which is the major intra SR calcium binding protein. The effects of dantrolene on calcium binding by cardiac calsequestrin were investigated. A plasmid containing the human calsequestrin 2 gene (GeneCopoeia) was inserted into single step KRX competent cells (Promega) and expressed as per the protocol provided (Promega). Calsequestrin was purified using a GST affinity column (Sigma) and GST tag cleaved using TEV protease (Sigma). The native fluorescence properties of calsequestrin (Ex 280, Em 340, Absorbance 350 nm) were used to monitor calcium binding and polymerization in the absence and presence of 10μM dantrolene. Over the calcium range (10^{-7} - 10^{-2}M), dantrolene induced polymerization of calsequestrin at lower free $[Ca^{2+}]$ than in control conditions. Further it reduced free calcium in the solutions which suggests that dantrolene increases calcium binding by calsequestrin.

In conclusion, using these novel fluorescent assays, our data show that dantrolene alters the calsequestrin properties, which affects how calcium is stored and therefore how much is available for release in cardiac muscle.