Complex regulation of ryanodine receptors by calsequestrin in cardiac muscle: the effect of doxorubicin

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Ca^{2+} release from the internal sarcoplasmic reticulum (SR) Ca^{2+} store triggers muscle contraction and is an integral part of the excitation-contraction coupling process. Ca^{2+} flows through the SR Ca^{2+} release channel - the ryanodine receptor (RyR) - which forms a complex with three proteins within the SR lumen; calsequestrin (CSQ; the main SR Ca^{2+} binding protein) and triadin and junctin (which anchor CSQ to the RyR). In addition to providing a pool of Ca^{2+} for release through the RyR, CSQ acts as a luminal Ca^{2+} sensor for the channel, a role that is integral for stable RyR function. CSQ regulates the channel to ensure both robust Ca^{2+} release during systole, and minimal diastolic RyR2 leak (Dulhunty *et al.*, 2012). Several therapeutic agents bind to CSQ and modify its structure in cardiac and skeletal muscle (Subra *et al.*, 2012), namely the anti-psychotic agent trifluoperazine, the cholesterol lowering drugs statins and importantly doxorubicin, a chemotherapy drug used to treat breast cancer. Our aim was to determine how the binding of potent doxorubicin metabolite doxorubicinol (doxOL) to cardiac CSQ (CSQ2) impacts cardiac RyR (RyR2) function.

Sheep hearts were excised from anaesthetized ewes (5% pentobartitone (IV) then oxygen/hatothane), with SR prepared on a discontinuous sucrose gradient and CSQ2 purified by native preparative gel electrophoresis (Hanna *et al.*, 2011). SR vesicles (containing RyR2 channels) were reconstituted into artificial planar lipid bilayers that separate two chambers that are equivalent to the cytoplasmic and SR luminal compartments of the myocytes. The impact of doxOL on CSQ2's ability to bind Ca^{2+} was determined using a $^{45}Ca^{2+}$ binding assay (Wium *et al.*, 2012) and on formation of Ca^{2+}-induced polymers (required for protein function) was tracked using a CSQ2 turbidity assay (Wium *et al.*, 2012).

CSQ's role as a luminal Ca^{2+} sensor for RyR2 is due to its ability to bind Ca^{2+}, and to its Ca^{2+} dependent polymer structure, which promotes association with the channel. DoxOL (2.5μM) significantly reduced CSQ2's ability to bind Ca^{2+}, leading to a near 50% loss in the number of Ca^{2+} ions bound to the protein. In addition, the ability of CSQ2 to assemble into aggregated polymers was significantly impeded by 2.5μM doxOL in a time-dependent manner, which was reflected by a significant loss of CSQ2 association with the RyR2 complex over time. These data illustrate that doxOL alters both the Ca^{2+} binding capacity of CSQ2, and that long term doxOL exposure would depolymerize CSQ2, resulting in only monomers and dimers of CSQ2 attached to the RyR complex.

In lipid bilayers, native RyR2 activity increased as luminal Ca^{2+} was raised from 0.1mM to 1.5mM, which would allow robust release of Ca^{2+} during systole. Pre-treatment with 2.5μM doxOL completely abolished RyR2 sensitivity to changes in luminal [Ca^{2+}]. To determine whether doxOL binding to CSQ2 was responsible for the loss of Ca^{2+} sensitivity, CSQ2 was selectively dissociated from the native RyR2 by high Cs^{+} prior to incubation with or without doxOL. The luminal Ca^{2+} response curve from CSQ2-dissociated RyR2 was identical with and without doxOL, and was in stark contrast to the lack of Ca^{2+} sensitivity in doxOL treated native RyR2. Together, these data provide compelling evidence that doxOL binding to CSQ2 mediates the loss of RyR2 luminal Ca^{2+} sensitivity, and that long term, cellular doxOL accumulation would disrupt CSQ2 polymer structure, undoubtedly leading to an additional dysfunction in RyR2 regulation.

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