A calsequestrin polymer is necessary for the Ca^{2+} binding protein to regulate RyR channels

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Calsequestrin (CSQ) forms a complex with the Ca^{2+} release channel ryanodine receptor (RyR) and anchoring proteins, triadin (Tri), and junctin (Jun) in the lumen of the sarcoplasmic reticulum (SR) of skeletal and cardiac muscles. CSQ acts to both regulate the RyR, and buffer the $[Ca^{2+}]_{free}$ inside the SR at 1 mM (Beard *et al.*, 2004). CSQ requires a compact structural conformation to achieve high capacity Ca^{2+} binding (He *et al.*, 1993). CSQ is thought to undergo a self-polymerisation as local $[Ca^{2+}]$ increases, and is believed to form a polymer at the physiological $[Ca^{2+}]$ (1 mM) in the SR. Our aim was to determine whether the CSQ polymer and its regulatory interaction within the complex would be disrupted and whether CSQ would be dissociated from the quaternary complex, when the intraluminal $[Ca^{2+}]$ falls to a low level at which CSQ is thought to be depolymerised. To achieve this, we examined the effects of low luminal Ca^{2+} on CSQs ability to regulate the RyR and correlated these effects with known Ca^{2+}-dependent changes in CSQ structure.

New Zealand male white rabbit were euthanized by a captive bolt and back and leg muscle used to prepare heavy SR vesicles. Heavy SR vesicles were reconstituted into artificial planar lipid bilayers, which separate two chambers, *cis* (cytoplasmic) and *trans* (luminal). *Trans* $[Ca^{2+}]$ was kept at 1 mM during incorporation and then lowered to 100 nM by the addition of BAPTA or EGTA. Channel activity was recorded at positive and negative potentials. To determine the effect of $[Ca^{2+}]$ on CSQ association with the RyR/Tri/Jun complex, SR vesicles were solubilised in 0.5% triton X-100, followed by an ultracentrifugation and resuspended in a solution containing 1 mM Ca^{2+}. The suspension was divided to three fractions and incubated at 4°C for 1 h in 1 mM, 1 μM and 100 nM Ca^{2+} after adjustment of $[Ca^{2+}]$ by BAPTA, prior to a second centrifugation. Resultant pellets and supernatants were subjected to SDS-PAGE and immunoblot analysis.

In single channel studies, a sudden delayed increase in activity was observed after the native RyR was exposed to low luminal Ca^{2+} (100 nM) for ~3 min. Returning *trans* $[Ca^{2+}]$ to 1 mM did not fully reverse the increase in activity to control levels, but addition of 16 μg/ml of exogenous CSQ to the *trans* chamber completely restored control channel activity. These changes were similar to those seen with dissociation of CSQ from the RyR/Tri/Jun, with high luminal ionic strength or Ca^{2+} (Beard *et al.*, 2004), but a longer exposure time was required before the sudden increase in activity was observed. The levels of CSQ in the membrane pellet of solubilised junctional face membrane (containing the RyR/Tri/Jun/CSQ complex) exposed to different $[Ca^{2+}]$, were compared with levels found in the original membrane fractions, to determine if CSQ was dissociated from the membrane. Increasing amounts of CSQ were dissociated from the membrane pellet as $[Ca^{2+}]$ fell, with 8.6%, 35.8% and 63% of the total CSQ dissociated by 1 mM, 1μM and 100 nM Ca^{2+}, respectively. This is in contrast to ~100% dissociation with high ionic strength or high Ca^{2+}. Additional single channel experiments showed that the response of RyRs associated with depolymerised CSQ to changes in luminal Ca^{2+} between 100 nM to 1 mM was typical of that seen in channels in which CSQ was fully dissociated.

Since CSQ is depolymerised with low $[Ca^{2+}]$ but retains its ability to bind to triadin and junctin (Shin *et al.*, 2000), we suggest that the CSQ remaining associated with the junctional face membrane was bound to triadin and junctin and that the dissociated CSQ was depolymerised and dissociated from the residual bound CSQ. Channel data suggests that the delayed RyR activation reflects depolymerisation and dissociation of CSQ not bound to triadin and junctin. The longer delay before the sudden increase in channel activity after exposure to low Ca^{2+} suggest a different physical process to the dissociation from triadin and junctin seen with high ionic strength and high Ca^{2+}. The results further suggest that the residual CSQ remaining associated with the RyR/Tri/Jun complex after depolymerisation was unable to regulate RyR activity in the normal manner until it formed a polymer with the excess exogenous CSQ. Therefore we concluded that a CSQ polymer is required for the calcium binding protein to regulate the RyR via triadin and junctin.

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