Phosphorylation of CSQ affects Ca^{2+} binding and interactions with anchoring protein junctin

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Depolarisation of the sarcolemma triggers Ca^{2+} release through ryanodine receptor (RyR) calcium release channels in the sarcoplasmic reticulum (SR) of skeletal muscle. Calsequestrin (CSQ) is the major Ca^{2+} binding protein found within the SR, and binds Ca^{2+} with a high capacity and moderate affinity. Recent studies have shown that CSQ also regulates RyRs. The best studied mechanism of CSQ-RyR interaction is indirect, thought to be mediated by anchoring proteins triadin and junctin and results in RyR inhibition (Szegedi *et al.*, 1999; Beard *et al.*, 2002). The relative importance of triadin and junctin in facilitating the interaction with the RyR is not clear, nor is the role *in vivo* of CSQ phosphorylation on the interaction between triadin, junctin and the RyR. Given that CSQ is isolated in both a phosphorylated and dephosphorylated form, it is conceivable that a CSQ phosphorylation/dephosphorylation cycle is important in regulating the RyR. Our hypothesis is that as the site of CSQ phosphorylation is believed to be close to the putative Ca^{2+} binding site, and triadin and junctin binding sites, that changes in phosphorylation may modify Ca^{2+} binding capacity and the ability of CSQ to interact with triadin and/or junctin.

We have investigated the effects of CSQ phosphorylation on CSQs role as a Ca^{2+} binding protein and regulator of the native RyR. Rabbit skeletal CSQ cDNA was subcloned into a pGex 5X-1 vector (containing a glutathione-*S*-transferase tag), transformed and expressed in *Escherichia coli* BL21(DE3) cells. CSQ was phosphorylated and dephosphorylated according to established methods (Cala & Jones, 1991).

We found that the Ca^{2+} binding capacity was significantly reduced in dephosphorylated CSQ (deP-CSQ), compared with phosphorylated CSQ (P-CSQ) over a range of [Ca^{2+}] from 100 nm – 5 mM. This was most evident at the physiological free [Ca^{2+}] (1 mM) and at 5 mM Ca^{2+} (a concentration shown to dissociate CSQ from the native RyR). Despite these changes in binding capacity, both P-CSQ and deP-CSQ caused similar significant inhibition of the native RyR (at 1 mM luminal Ca^{2+}; Beard *et al.*, 2005). As the putative major Ca^{2+} binding region and site of CSQs interaction with triadin and junctin are presumed identical (residues 354-367), we investigated what effects P-CSQ/deP-CSQ had on its interactions with triadin and junctin. Using CSQ-GST fusion protein affinity chromatography, we found that under close to physiological conditions (150 mM NaCl, 1 mM Ca^{2+}), both P-CSQ and deP-CSQ bound triadin and junctin. Not surprisingly, under conditions known to completely dissociate CSQ from the native RyR (5 mM Ca^{2+}), neither P-CSQ nor deP-CSQ interacted with a significant amount of triadin or junctin. Curiously, at low luminal Ca^{2+} (100 nM), CSQ binding to these two anchoring proteins was phosphorylation-dependent. P-CSQ bound significant amounts of both triadin and junctin, whilst deP-CSQ was found only to interact with triadin, but not with junctin. In experiments where 100 nM luminal Ca^{2+} was used to depolymerise P-CSQ and deP-CSQ from a solubilized SR sample, a significant proportion of both forms of CSQ remained tethered close to the RyR/triadin/junctin complex. The combination of these results suggests that an interaction with junctin is not required to tether CSQ close to the native RyR.

These results show firstly, that CSQ dephosphorylation reduces the ability of CSQ to bind Ca^{2+}. Although this does not affect overall CSQ regulation of the RyR, subtle effects of altering CSQs phosphorylation status on channel gating, or CSQs ability to act as a luminal Ca^{2+} sensor remain to be investigated. Secondly, these results illustrate that triadin, but not junctin, is essential for association of CSQ with the native RyR. Further investigation on whether the alteration in junctin binding results in altered regulatory effects on the native RyR may elucidate a specific role of junctin and the potential P-CSQ/deP-CSQ cycle on SR Ca^{2+} release. Thirdly, the results show that conformational changes that alter Ca^{2+} binding capacity do not necessarily alter CSQ binding to triadin and junctin and therefore the specific residues which comprise both the major Ca^{2+} binding site and triadin/junctin binding sites are not identical.

Beard, N.A., Casarotto, M.G., Wei, L., Varsányi, M., Laver, D.R. & Dulhunty, A.F. (2005) *Biophysical Journal* **88**, 3444-3454.

Beard, N.A., Sakowska, M.M., Dulhunty, A.F. & Laver, D.R. (2002) *Biophysical Journal* **82**, 310-320.

Cala, S.E. & Jones, L.R. (1991) *Journal of Biological Chemistry* **266**, 391-398.

Szegedi, C., Sarkozi, S., Herzog, A., Jona, I. & Varsanyi, M. (1999) *Biochemical Journal* **337**, 19-22.