In vitro characterization of interactions between junctin and ryanodine receptors

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Junctin is an integral sarcoplasmic reticulum (SR) membrane protein in skeletal and cardiac muscle, with a short cytoplasmic N-terminal and a bulky, highly charged C-terminal domain located in the SR lumen. For many years, junctin was considered to play a major role only in connecting calsequestrin (CSQ) to the ryanodine receptor (RyR). However, recent *in vitro* and *in vivo* studies (Altschafl *et al.*, 2011) show a strong correlation between junctin and SR Ca^{2+} content, indicating a more complex role for junctin in regulating RyR activity and SR Ca^{2+} handling. Notably, junctin protein expression is significantly reduced in failing human heart, suggesting it may be involved in the dysregulation of SR Ca^{2+}cycling in heart failure. Despite its importance, the specific physiological roles of junctin remain obscure. Full length junctin added to the luminal solution directly activates purified RyRs in lipid bilayers (Gyorke *et al.*, 2004; Wei *et al.*, 2009). It was assumed that this effect depended on luminal interactions between the proteins. Any contribution of cytoplasmic RyR-junctin interaction to RyR regulation was not considered.

To explore the specific regions of junctin that regulate RyR, we synthesized a peptide corresponding to junctin's cytoplasmic N-terminal domain (Njun), expressed the luminal C-terminal domain (Cjun), while full length junctin (FLjun) was either isolated from skeletal muscle or expressed in bacterial cells. The effect of adding these constructs to solubilised RyR channels in lipid bilayers was examined. As with previous findings (Gyorke *et al.*, 2004; Wei *et al.*, 2009), FLjun in luminal solutions increased RyR1 and RyR2 activity. We predicted that luminal addition of Cjun would similarly activate RyRs if interactions occur only between their luminal domains. Unexpectedly, luminal Cjun elicited strong inhibition of both RyR1 and RyR2. Also unexpectedly, cytoplasmic addition of Njun enhanced activity to significantly greater levels than luminal FLjun. Neither luminal Njun nor scrambled Njun in cytoplasmic solutions altered channel activity, suggesting a specific cytoplasmic effect. The specificity was further confirmed as cytoplasmic Njun did not significantly change the activity of native RyRs, which contain endogenous junctin. Significantly, excess activation by cytoplasmic Njun was attenuated to FLjun levels by the subsequent luminal addition of Cjun, suggesting the cytoplasmic interaction dominates the functional effect of junctin on RyRs. In contrast, when Cjun was first associated with the luminal domain of RyR, the ability of Njun to activate the channel was abolished. We further examined the interactions between RyRs and a KEKE motif in Cjun (residues 85-106). The KEKE motif peptide added to luminal solution inhibited the channels in the same way as Cjun. This suggested that the KEKE motif in Cjun bound to the RyRs to produce the functional effects of Cjun on RyR activity.

Further affinity chromatography studies indicated that (i) both Cjun and Njun bind to the full length RyRs, (ii) Cjun, but not Njun, bind to the luminal domains of the RyRs and specifically to the pore loop and to the M5-M6 luminal linker (iii) Njun, but not Cjun, bind to the cytoplasmic domain of the RyRs, specifically within the residues 183-2156.

Together, these results demonstrate that junctin regulates both RyR1 and RyR2 via two distinct interactions, involving both cytoplasmic and SR luminal domains. The combined effects of Njun and Cjun imply that the interaction between Njun and RyR dictates the functional consequences of the full length protein binding to RyR channels, and allows Cjun to modulate the channel *in vivo*.

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