1557-Pos

Association of Triadin to the Junctional Sarcoplasmic Reticulum of Skeletal Muscle Cells

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The junctional sarcoplasmic reticulum (jSR) of skeletal muscle cells contains several proteins that participate to the mechanisms of Ca2+ release in the process of excitation-contraction coupling. Among these proteins, ryanodine receptor, triadin, junctin and calsequestrin have been found to associate into a stable complex. We recently reported that assembly of jSR domains is accompanied by a strong decrease in the mobility fraction of jSR proteins (Cusimano et al., PNAS 2009). In particular, we found that the mobility of triadin appeared to be mediated by its intraluminal region (aa 232-729). In order to identify the minimal regions required for association of triadin to the jSR, deletion mutants of the luminal domain (triadin Δ232-440 and triadin Δ441-729) were generated and expressed in primary muscle myotubes. Analysis of the mobility fraction of these mutants showed that they do not differ from wild type triadin, indicating that either one of the two regions is sufficient to provide a strong association of the protein to the jSR. Interestingly, we found that the luminal region of triadin contains several defined domains, including a coiled coil region and short amino acids repeats, that are present in the region between aa 232-440 and aa 441-729, where they may mediate protein-protein interactions. Results on the role of these amino acid repeats in mediating triadin association with the jSR will be reported.

1558-Pos

Time-Resolved FRET Detection of Structural Distributions Involving FKBP12.6 and Calmodulin Bound Within Macromolecular RyR Channels

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We used multi-exponential analysis of time-resolved (TR) fluorescence decays to investigate the array of distances underlying fluorescence resonance energy transfer (FRET) between donors and acceptors bound within functional RyR channels in sarcoplasmic reticulum membranes. Previously, we have used steady-state (SS) FRET to demonstrate that CaM is oriented with the N-lobe proximal to the FKBP12.6 subunit on each lateral face of the RyR tetrameric complex (Cornea et al. 2009). Here, we used cysteine mutagenesis and sulfhydryl-specific fluorescent labeling to attach Alexa Fluor (AF) donor probes to single-cysteines at FKBP12.6 sites 49 or 85 (denoted D49 and D85), and acceptor probes to calmodulin (CaM) N-lobe sites 26 or 34 (denoted A26 and A34). The fluorescence of RyR-bound donor-labeled FKBP12.6 was decreased in the presence of saturating acceptor-labeled CaM, indicating FRET. Förster analysis of TR- and SS-FRET data of donor/acceptor pairs AF350/AF488 and AF488/AF568 (with different ranges of sensitivity) yielded similarly-ranked energy transfer efficiencies: D85/A26 $\geq$ D49/A26 $>$ D49/D34 $>$ D85/34. Distances calculated from these FRET efficiencies are remarkably similar for the two different donor/acceptor probe pairs. However, this kind of analysis can extract only averaged distances from an RyR sample in which distinct biophysical states are known to co-exist. Further multi-exponential analysis of the donor-only (AF350-FKBP12.6) and donor+acceptor (AF350-FKBP12.6/AF488-CaM) TR-FRET data sets resolved at least two simultaneous distance populations for each pair of labeled sites. These distances and their distribution responded to changes in [Ca^{2+}]. We conclude that TR-FRET provides a powerful approach for resolving dynamic transitions between structural states within functional, multimeric RyR channels. Further studies will aim to determine whether these findings reflect transitions between different conformations of CaM itself, or of the underlying RyR channel.

1559-Pos

FKBP12.6 Inhibits Resting Ryanodine Receptor Activity but PKA-Dependent Phosphorylation Does Not Alter FKBP12.6-RyR2 Binding in Rat Permeabilized Myocytes

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FK506-binding proteins FKBP12.6 and FKBP12 are associated with the cardiac ryanodine receptor (RyR2), and PKA dependent hyperphosphorylation of RyR2 has been proposed to interrupt the FKBP12.6-RyR2 interaction and activate RyR2 opening. However, the physiological function of FKBP12.6/12.0 in cardiac myocytes and the role of PKA-dependent RyR2 phosphorylation are controversial. We used permeabilized rat ventricular myocytes, and fluorescently-labeled FKBP12.6/12.0 to directly measure in situ binding of FKBP12.6/12.0 to RyR2, with simultaneous Ca sparks measurements as an RyR2 functional index. We found that both FKBP12.6 and FKBP12 concentrate at the Z-line, consistent with RyR2 binding. However, only FKBP12.6 inhibits resting RyR2 activity. Assessment of fluorescent FKBP binding at the Z-line of permeabilized myocytes revealed a high affinity of FKBP12.6-RyR2 (Kd = 0.97 ± 0.1 nM) and much lower affinity of FKBP12-RyR2 (Kd = 206 ± 70 nM). Fluorescence recovery after photobleach of FKBP in myocytes confirmed these different affinities and showed that the main difference was in koff. Activation of RyR2 phosphorylation by PKA had no significant affect on either binding kinetics or affinity of FKBP12.6/12-RyR2. Using quantitative immunoblots, we determined the concentration of endogenous FKBP12 in intact myocytes was ~1 μM, while FKBP12.6 is at most ~150 nM. Taken together, our data suggest that FKBP12.6 binds to and stabilizes the resting RyR2 but cAMP-dependent RyR2 phosphorylation does not dissociate FKBP12.6 (or FKBP12) from RyR2 in the myocyte environment. More important, this study highlights the importance of in situ binding properties measurement and clarifies some aspects of controversy.

1560-Pos

FKBP12 and FKBP12.6 Exert Opposing Actions on the Single-Channel Behaviour of Both RyR1 and RyR2

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It is widely believed, that in striated muscle, FKBP12.6 selectively binds to RyR2 but that FKBP12 is the binding partner for RyR1. Few studies have addressed whether FKBP12 can modulate RyR2 function or whether FKBP12.6 can affect RyR1. Our recent single-channel studies show that FKBP12 activates RyR2 with nanomolar affinity but because binding is rapidly reversible, this interaction is unlikely to be detected by Western blot analysis. We have therefore compared how FKBP12 and FKBP12.6 affect the single-channel properties of RyR1 and RyR2 reconstituted into bilayers. Using Ca^{2+} as the permeant ion, cytosolic addition of 500 nM FKBP12 significantly decreased the open probability (Po) of RyR1 from 0.021 ± 0.005 (SEM; n = 4) in controls to 0.001 ± 0.001 (SEM; n = 4; $P<0.05$). This effect was irreversible after perfusing away the FKBP12. In contrast, 200 nM and 1 μM FKBP12.6 increased RyR1 Po. This effect was also irreversible after washout of FKBP12.6 but could be antagonised by addition of FKBP12. In comparison, FKBP12 is a reversible activator of RyR2 whereas FKBP12.6 has little intrinsic action itself but can antagonise FKBP12-induced activation. The presence or absence of FKBP12/12.6 did not lead to the appearance of sub-conductance gating states in RyR2 but, after FK-506 treatment or addition of FKBP12.6 to RyR1, sub-conductance states were frequently observed. Since FKBP12 activates RyR2 but is antagonised by FKBP12.6, and FKBP12.6 activates RyR1 but is antagonised by FKBP12, the ratio of FKBP12/FKBP12.6 levels in the cytoplasm will be critical in determining RyR activity *in situ*. Changes in the cytoplasmic FKBP12/FKBP12.6 ratio may be important in heart failure, where it has been suggested that less FKBP12.6 binds to RyR2.

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1561-Pos

Use of Rapamycin Reveals Evidence of the Physiological Roles of FKBP12 and FKBP12.6 In Cardiac Excitation-Contraction Coupling

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A huge body of evidence suggests that FKBP12.6, which binds tightly to RyR2, is an important physiological regulator of cardiac excitation-contraction (EC) coupling yet its mechanism of action remains elusive. Our recent single-channel studies now reveal that RyR2 activity may be controlled by the opposing actions of FKBP12.6 and FKBP12. To investigate how these proteins regulate RyR2 function in cardiac cells we have used rapamycin to dissociate FKBP12 and FKBP12.6 from RyR2 in isolated rat permeabilised ventricular myocytes. In control myocytes perfused with Fluo-5F, spontaneous waves of Ca^{2+}-induced Ca^{2+}-release were induced by 234 nM Ca^{2+} in the mock cytoplasmic solution. Treatment for 4-6 min with 20 μM rapamycin, reduced the frequency of the Ca^{2+}-waves from 0.57 ± 0.07 Hz to 0.18 ± 0.05 Hz (SEM; n=5; $P<0.002$) and led to the appearance of 'mini-waves' that did not propagate throughout the cell. Rapamycin treatment also increased baseline Fluo-5F fluorescence intensities by 204 ± 47% (SEM; n=8; $P<0.05$), an effect that was completely reversed by perfusion with a physiological level of FKBP12 (3 μM; n=4; $P<0.001$) but not with 200 nM FKBP12.6 (n=4). In rapamycin-treated cells, FKBP12 (from 0.15 ± 0.06 Hz to 0.27 ± 0.07 Hz (SEM; n=6; $P<0.025$) was