Platform: Excitation-Contraction Coupling

2073-Plat

3D Imaging of Nanoscopic Membrane Systems Regulating Cardiac Excitation-Contraction Coupling: Multi-Color Optical Super-Resolution and Serial Block-Face Scanning Electron Microscopy

Joseph Isaac S. Wong[1,2], David Baddeley[3], Eric A. Bushong[2,4], Mark Ellisman[2,4], Masahiko Hoshijima[1,2], Christian Soeller[3].
[1]Department of Medicine, University of California San Diego, La Jolla, CA, USA, [2]The Center for Research in Biological Systems, University of California San Diego, La Jolla, CA, USA, [3]Department of Physiology, School of Medical Sciences, University of Auckland, Auckland, New Zealand, [4]The National Center for Microscopy and Imaging Research, University of California San Diego, La Jolla, CA, USA.

Cardiac excitation contraction (E-C) coupling is controlled by ionic fluxes in couplons, nano-scale junctional structures between sarcolemma and sarcoplasmic reticulum (SR) membranes. The 3-dimensional (3D) geometry of cardiac couplons and their relationship to the transverse-tubule system (t-system) is complex with little 3D data available. Using super-resolution microscopy, we successfully imaged couplons in normal adult mouse ventricular myocytes with a resolution of about 30nm. A fluorescence microscope equipped with an insertable cylindrical lens and an EMCCD camera enabled multi-color 3-dimensional dSTORM imaging and recorded fluorophore ''blinking'': rapid transitions between a photon-emitting ''on''-state and laser-induced ''dark''-state. This data was analyzed with custom-written software to localize individual fluorophores and render super-resolution images. We used ryanodine receptor (RyR) to identify couplons and the sarcolemmal protein Caveolin-3 (CAV3) to visualize the t-system. Separately, we applied an advanced 3D electron microscopic technology, Serial Block-face Scanning Electron microscopy (SBFSEM), to cardiac muscle for the first time to image, segment, and annotate couplons and the t-system in mouse cardiac myocytes. Expectedly, the majority of RyR clusters aggregated near the t-system. The individual morphology and size of couplons visualized with dSTORM and SBFSEM were both highly variable, in agreement with our recent publications. Furthermore, both microscopies strongly suggested a significant enlargement in diameter of the t-system at loci where couplons accumulated. This enlargement may contribute to minimizing ion accumulation or depletion. Alternatively, it could reflect a geometrical requirement for forming larger junctional complexes. Collectively, the study revealed the strength of integrating new advanced 3D light and electron microscopies. Knowledge of nanoscale anatomy may be critical both to mechanistic understanding of E-C coupling and as a resource to generate realistic computational models that can simulate E-C coupling and other muscle biology.

2074-Plat

Junctional Cleft $[Ca]_i$ Measurements using Novel Cleft-Targeted Ca Sensors

Sanda Despa[1], Julie Bossuyt[1], Bo Shui[2], Michael I. Kotlikoff[2], Donald M. Bers[1].
[1]University of California Davis, Davis, CA, USA, [2]Cornell University, Ithaca, NY, USA.

Cardiac SR Ca release is controlled locally by $[Ca]_i$ in the junctional cleft. Despite its importance for excitation-contraction coupling (ECC), there is no method for direct cleft $[Ca]_i$ measurements. We constructed novel cleft-targeted $[Ca]_i$ sensors by attaching FKBP12.6 to the genetically-encoded Ca sensor GCaMP2. FKBPs12.6 binds with high affinity and specificity to ryanodine receptors (RyRs) and targets the sensor to the junctions. We also constructed a FKBP-GCaMP2 variant with low Ca affinity ($K_d \approx 10$ μM; FKBP-GCaMP-Low). Targeted or untargeted GCaMP2 sensors were expressed in rat cardiac myocytes by adenoviral infection. Both FKBP-GCaMP2s express in a striated pattern, spaced ~2 μm apart (at T-tubules), while the un-tagged GCaMP2s are more uniformly distributed. Ca transients recorded with FKBP-GCaMP-Low are ~2-fold larger and have a much faster upstroke (time-to-peak=46 ± 5 *vs.* 90 ± 7 ms) and decay (decay time=254 ± 19 *vs.* 421 ± 42 ms at 0.5 Hz) compared to those reported by un-targeted GCaMP-Low. Thus, we can detect a $[Ca]_{Cleft}$ transient with much larger amplitude and faster kinetics than global $[Ca]_{Bulk}$ transient during ECC. During diastole SR Ca leak (or influx) may raise local $[Ca]_{Cleft}$ above $[Ca]_{Bulk}$ (distant from Ca sources). We used high-affinity FKBP-GCaMP and GCaMP to compare diastolic $[Ca]_{Cleft}$ to $[Ca]_{Bulk}$. Blockade of SR Ca leak and sarcolemmal Ca influx (with 1 mM tetracaine & 0Na/0Ca solution) should equalize $[Ca]_{Cleft}$ to $[Ca]_{Bulk}$. Thus, a larger fluorescence decline ($\Delta F/F_0$) in $[Ca]_{Cleft}$ vs. $[Ca]_{Bulk}$ upon block provides a measure of this diastolic $[Ca]_i$ gradient. Indeed, $\Delta F/F_0$ was significantly larger for FKBP-GCaMP2 *vs.* GCaMP2 (0.55 ± 0.10 *vs.* 0.13 ± 0.02), indicating that $[Ca]_{Cleft} > [Ca]_{Bulk}$ during diastole. In conclusion, we developed sensors for measuring $[Ca]_{Cleft}$ vs. $[Ca]_{Bulk}$ and provided measures of a standing $[Ca]_i$ gradient during diastole and dynamic differences during ECC.

2075-Plat

Activation of Calcium Release from RyR Clusters Depends on their Distance from the Sarcolemma

Natalia S. Torres[1], Frank B. Sachse[1,2], Kenneth W. Spitzer[1,3], John H.B. Bridge[1,4].
[1]Nora Eccles Harrison Cardiovascular Research and Training Institute, University of Utah, Salt Lake City, UT, USA, [2]Department of Bioengineering, University of Utah, Salt Lake City, UT, USA, [3]Department of Physiology, University of Utah, Salt Lake City, UT, USA, [4]School of Medicine, University of Utah, Salt Lake City, UT, USA.

Summation of microscopic release events from RyRs underlies the upstroke of the calcium transient in mammalian ventricular cells. These release events are initiated when RyRs are gated by transmembrane calcium influx. In rabbit cardiac myocytes the majority of RyR clusters are closely associated with the sarcolemma where they form couplons. However, significant numbers of RyR clusters are non-junctional and do not form couplons. It is unclear if or by what mechanism non-junctional RyRs are activated. We analyzed the activation of both types of RyRs. We studied calcium transients in rabbit isolated ventricular cells that exhibit a sparse t-system with Fluo-4 and high-speed two-dimensional confocal microscopy. We constructed distance maps indicating that some intracellular regions are up to 3.5 μm away from the sarcolemma. Image sequences showed that transients were spatially and temporally inhomogeneous. Transients originated in regions where we detected sarcolemma and spread with a significant delay to areas lacking t-system where non-junctional RyRs are present. We found an increasing relationship between sarcolemmal distances and local activation times with activation times of 13.0 ± 6.6 ms at sarcolemmal sites, and 20.5 ± 5.1 ms at distances 3-3.5 μm. The relationship between sarcolemmal distances and maximal upstroke of the transient was more complex. Maximal upstroke at sarcolemmal sites was 13.2 ± 4.1 %/ms versus 8.8 ± 1.6 ms at 3-3.5 μm. To interpret these results we used a one-dimensional model of calcium diffusion, which explains the spread of upstroke that we see experimentally. We conclude that non-junctional RyRs within 1.5 μm of the sarcolemma are activated. Beyond 1.5 μm the rise in calcium could result from either diffusion or slow activation. In both cases, activation of non-junctional RyRs would occur by a mechanism different than local control, i.e. a modified common pool model.

2076-Plat

Pixel-Wise Fitting: Noise-Free Analytical 3D Visualisation of Calcium Handling in Cardiac Myocytes

Qinghai Tian, Lars Kaestner, **Peter Lipp**.
Saarland University, Homburg/Saar, Germany.

Realtime imaging of fast signaling events has yielded important insight and has greatly fostered our understanding of fundamental processes such as subcellular calcium signalling in cardiac and neuronal cells. Scanning technology has pushed the edge of acquisition speeds into dimensions in which the level of single pixel noise has become limiting for the interpretation of the data. Here, we demonstrate that for excitable cells, such as cardiac myocytes, for which signaling (i.e. excitation-contraction coupling, ECC) occurs on the millisecond timescale, such technical limitations can be overcome by a pixel-wise fitting approach using high-speed 2-dimensional confocal calcium data (280 nm lateral resolution and 146 frames/s) and a mathematical approach designed to approximate local calcium transients. Such an approach not only produces virtually noise-free single pixel fluorescence data originating from subcellular volumes as small as 0.08 fl. It also allows extraction of 2D information on signalling events such as calcium induced calcium release in a very robust manner. Using such an analytical approach enabled us to tackle important questions of cardiac ECC under physiological conditions but also helped us to reveal novel information about the pro-arrhythmogenic calcium alternans by demonstrating that microscopic alternans preceeds macroscopic alternans. The applicability of such an analytical, pixel-wise fitting approach can be transferred to other biological systems and may thus help to overcome present technical limitations such as diminished signal to noise levels in high-speed live cell imaging.
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2077-Plat

Identification of the First Mutations in the Human Triadin Gene, Associated to Catecholaminergic Tachycardia, a Pathology of the Cardiac Calcium Release Complex

Nathalie Roux-Buisson[1], Marine Cacheux[1], Anne Fourest-Lieuvin[1], Jérémy Fauconnier[2], Julie Brocard[1], Alain Lacampagne[2], Julien Fauré[1], Joël Lunardi[1], **Isabelle Marty**[1].
[1]Inserm U836, Grenoble, France, [2]Inserm U1046, Montpellier, France.