Supporting Information

Perni et al. 10.1073/pnas.1716461115

SI Materials and Methods

The expression plasmids for ECFP– and EYFP–$Ca_V1.1$ (1), EYFP–$Ca_V1.2$, Stac3–YFP, and unlabeled β1a (2), and ECFP– and EYFP–RyR1 (3) were described earlier. EYFP–$Ca_V1.1$–N617D was created from EYFP–$Ca_V1.1$ by using quick-change mutagenesis with forward primer ACGGGTGAGGACTGGGACTCCGTGATGTACAAC and reverse primer GTTGTACATCACGGAGTCCCAGTCCTCACCCGT. To produce a hygromycin-selectable RyR1 construct ("RyR1–pCEP4"), the RyR1 coding sequence was excised with HindIII and MfeI from ECFP–RyR1 and inserted into the multiple cloning sites of the pCEP4 plasmid (Invitrogen). mCherry-$Ca_V1.2$ was created by replacing the EYFP sequence in YFP–$Ca_V1.2$ with mCherry from pmCherry-C1 (Clontech) using NheI and HindIII. Stac3–tagRFP was obtained by using BamHI and NotI to replace EYPF in the Stac3–YFP plasmid with tagRFP from pTagRFP-N (Evrogen). Human JP2 inserted into the pcDNA3.1+/C-(K)DYK expression vector was obtained from Genscript. The JP2 sequence from this plasmid was excised with HindIII and XbaI and substituted for the RyR1 sequence, removed with the same enzymes from EYFP–RyR1 or ECFP–RyR1, to produce EYFP–JP2 and ECFP–JP2, respectively. Unlabeled α2δ-subunit was provided by William A. Sather, University of Colorado, Anschutz Medical Campus, Aurora, CO. $Ca_V1.2$-N739D was provided by Symeon Papadopoulos, University of Cologne, Cologne, Germany. The Stac3 cDNA used as the basis for our tagged Stac3 constructs was provided by Eric N. Olson, University of Texas Southwestern Medical Center, Dallas.

1. Papadopoulos S, Leuranguer V, Bannister RA, Beam KG (2004) Mapping sites of potential proximity between the dihydropyridine receptor and RyR1 in muscle using a cyan fluorescent protein-yellow fluorescent protein tandem as a fluorescence resonance energy transfer probe. *J Biol Chem* 279:44046–44056.
2. Polster A, Perni S, Bichraoui H, Beam KG (2015) Stac adaptor proteins regulate trafficking and function of muscle and neuronal L-type Ca^{2+} channels. *Proc Natl Acad Sci USA* 112:602–606.
3. Hanaichi T, et al. (1986) A stable lead by modification of Sato's method. *J Electron Microsc (Tokyo)* 35:304–306.

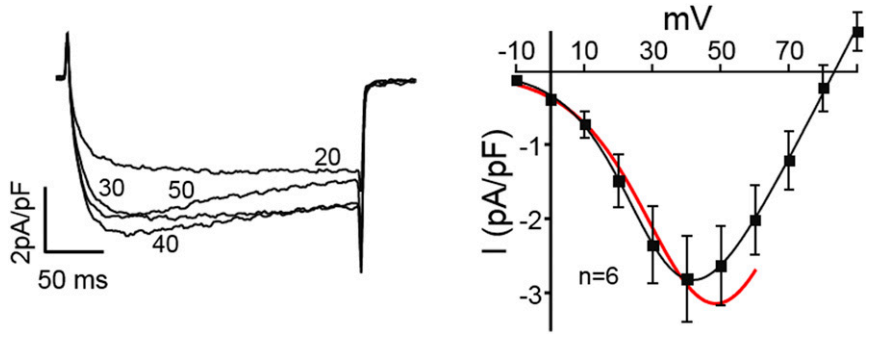

Fig. S1. Representative currents for the indicated test potentials (*Left*) and average peak current vs. voltage relationship (*Right*) measured with the perforated patch technique in tsA201 cells transiently transfected with YFP–$Ca_V1.1$, β1a, Stac3–RFP, and JP2 without α2–δ1. The red curve is replotted from Fig. 2*H* and corresponds to the peak *I–V* relationship for cells transfected with YFP–$Ca_V1.1$, β1a, Stac3–RFP, and JP2 plus α2–δ1, measured under slightly different conditions (whole-cell technique, external Ca^{2+} = 10 mM vs. 2.6 mM for the perforated patch technique).

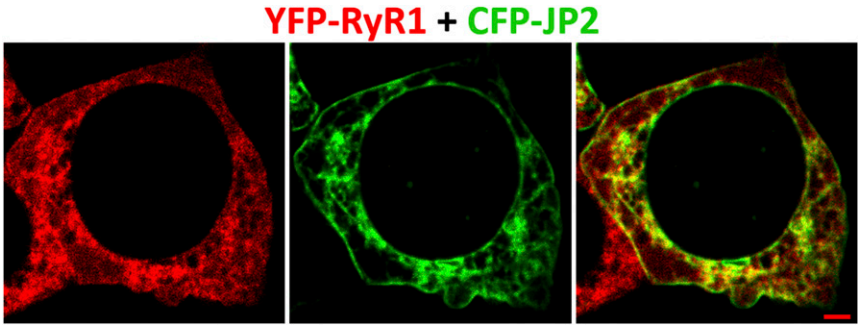

Fig. S2. JP2 alone is sufficient to cause some RyR1 to become associated with the cell surface (tsA201 cell transfected with YFP–RyR1 and CFP–JP2). (Scale bar, 2 μm.)

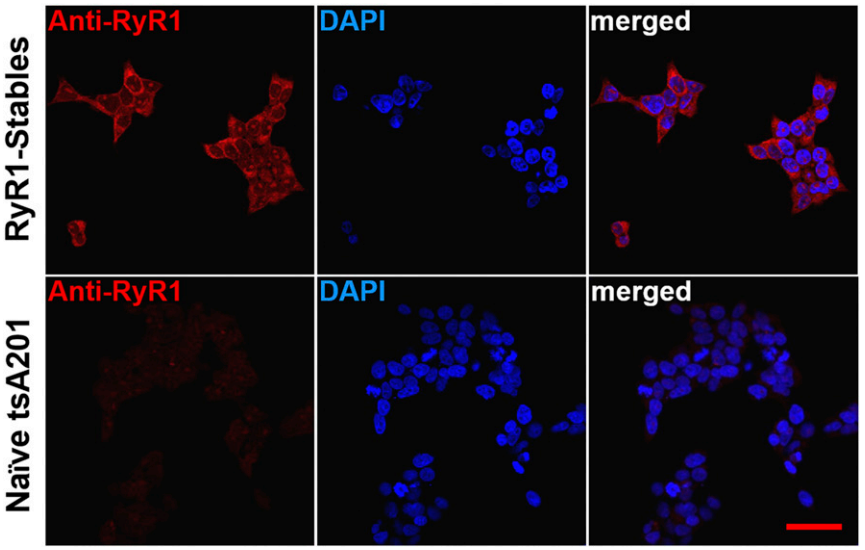

Fig. S3. Immunostaining indicates that RyR1 is expressed in close to 100% of tsA201 cells stably transfected with RyR1 (RyR1-stable cells). With identical immunostaining and image acquisition parameters, little signal was detected in naïve tsA201 cells (*Materials and Methods*). (Scale bar, 50 μm.) Cells were fixed for ≥20 min with 4% paraformaldehyde in PBS, washed 3× with PBS containing 1% (wt/vol) BSA, and permeabilized/blocked for 1.5 h at room temperature with PBS containing 1% BSA, 10% (vol/vol) goat serum, and 0.5% (wt/vol) Triton X-100. The cells were incubated overnight at 4 °C with monoclonal 34C (Developmental Hybridoma Studies Bank, University of Iowa) diluted 1:50 in PBS/BSA 1%/Triton X-100 0.5%, washed 3×, exposed for 1.5 h at room temperature to Alexa 568-conjugated goat anti-mouse (Molecular Probes) 1:1,000 in PBS/BSA 1%/Triton X-100 0.5%, washed 3× with PBS, and mounted with DAPI supplemented mounting medium (Vectashield; Vector Laboratories Inc.).

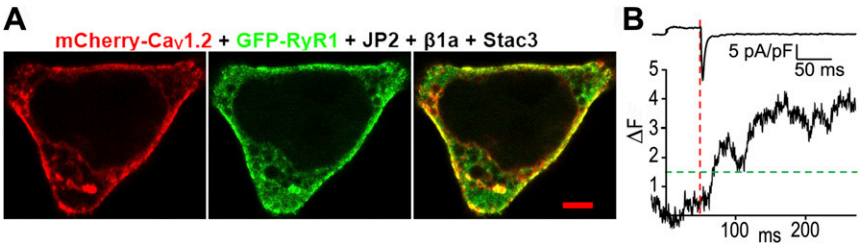

Fig. S4. $Ca_V1.2$ colocalizes at the surface with RyR1 and supports Ca^{2+}-entry dependent Ca^{2+} transients, in tsA201 cells transfected with constructs for $Ca_V1.2$, β1a, Stac3, JP2, and RyR1. (*A*) Midlevel confocal section of a tsA201 cell transiently transfected with the indicated constructs. (Scale bar, 5 μm.) (*B*) Superimposed current (*Upper*) and Fluo-3 fluorescence change (*Lower*) in response to a 50-ms step to +90 applied via a perforated patch to an RyR1-stable cell transfected with YFP–$Ca_V1.2$, β1a, Stac3–RFP, and JP2. The red dashed line indicates the time of repolarization to the holding potential (−60 mV). The resulting, large inward tail current triggered a rapid increase in cytoplasmic Ca^{2+}. The green dashed line indicates the threshold ($\Delta F \geq 1.5$ during the 200-ms interval after the onset of depolarization) which was used to determine whether to include fluorescence data for calculation of average responses.

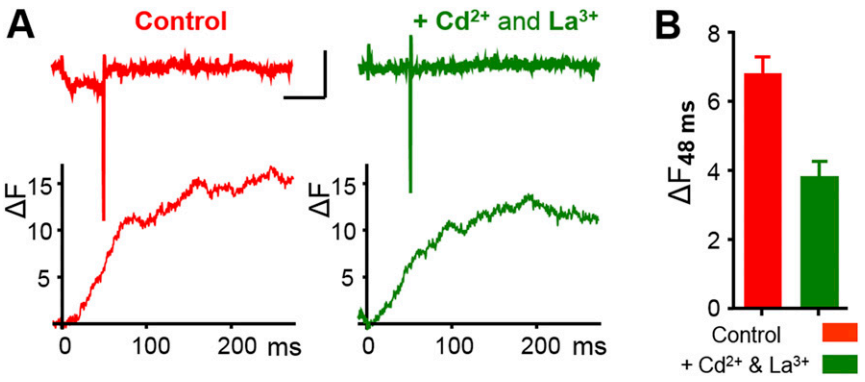

Fig. S5. Depolarization elicits intracellular Ca^{2+} transients that do not require Ca^{2+} entry in RyR1-stable cells transfected with $Ca_V1.1$, β1a, Stac3, and JP2. (*A*) Ca^{2+} currents (upper traces) and Ca^{2+} transients (lower traces) acquired before and after addition of 0.5 mM Cd^{2+} and 0.1 mM La^{3+} to the solution bathing an RyR1-stable cell transfected with $Ca_V1.1$, β1a, Stac3, and JP2. The cell had been loaded with Fluo-3 AM and was depolarized for 50 ms to +30 mV via a perforated patch. Calibration for the current traces: 2 pA/pF (vertical), 50 ms (horizontal). (*B*) Average fluorescence change (48 ms after onset of depolarization to +30 mV) measured in five cells (including the one illustrated in *A*) before (control) and after addition of Cd^{2+} and La^{3+} to the bath. As noted in *Results*, cells not exposed to Cd^{+2} and La^{3+} also produced smaller transients in response to the second of two identical stimuli.

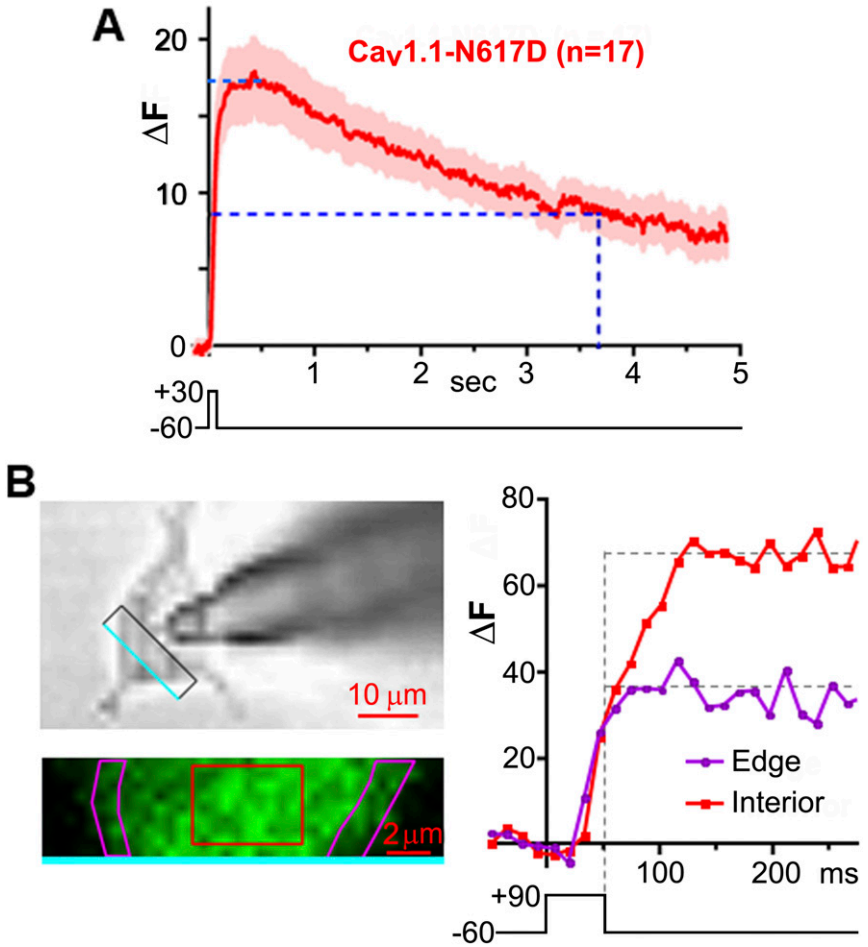

Fig. S6. Calcium removal and release kinetics in tsA201 cells transfected with five triadic proteins. (*A*) Average change in Fluo-3 fluorescence (±SEM) obtained in long-duration recordings of the response to a 50-ms step to +30 applied via a perforated patch to RyR1-stable cells transfected with YFP–$Ca_V1.1$–N617D, β1a, Stac3–RFP, and JP2. The fluorescence decayed with a half-time of ~3 s, indicating that Ca^{2+} removal processes are slow in these cells. (*B*) Comparison of fluorescence signals at the edge and interior of an RyR1-stable cell transfected with YFP–$Ca_V1.1$–N617D, β1a, Stac3–RFP, and JP2, and depolarized for 50 ms to +90 mV via a perforated patch. Superimposed on the transmitted light image (*Upper Left*) is a rectangle indicating the region subsequently subjected to repetitive confocal scanning (72.7 Hz with each scan lasting 6.87 ms): A single scan obtained ~50 ms after repolarization is shown in *Lower Left*. In *Right*, the superimposed dotted lines were drawn by eye to facilitate comparison of the average fluorescence change within the two regions of interest indicated in *Lower Left* (baselines adjusted to be zero before depolarization). The rising phase of the fluorescence increase was more prolonged in the interior than at the edge. Similar results were obtained in a total of five cells, three loaded with Fluo-8 (as for the cell illustrated here) and two with Fluo-3 (which produced smaller and noisier signals).

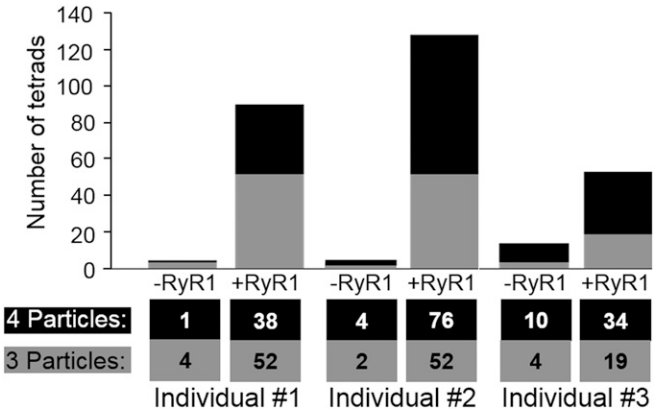

Fig. S7. Numbers of three- or four-particle tetrads as determined by three individuals from freeze-fracture images of naïve tsA201 cells ("-RyR1") or RyR1-stable cells ("+RyR1") which had been transfected with YFP–$Ca_V1.1$, β1a, Stac3, and JP2. The three individuals were provided 20 unidentified micrographs, 10 each from transfected naïve and RyR1-stable cells, which had similar densities of large particles (1,055 and 1,153 particles per μm^2, respectively). The individuals were not informed about how many different conditions were represented.

Table S1. Morphometric analysis of naïve and YFP–JP2-transfected tsA201 cells

Cell type	Cells with ≥2 junctions, %	Junctions per cell, mean ± SEM	Maximum junctions per cell	Junction length, μm, mean ± SEM	Maximum junction length, μm	$\frac{\Sigma \text{junction length}}{\text{Cell perimeter length}}$, %, mean ± SEM
Naïve tsA201	8 (n = 88)	2.14 ± 0.14 (n = 7)	3	0.148 ± 0.016 (n = 15)	0.28	0.9 ± 0.1 (n = 7)
YFP-JP2 transfected	19 (n = 95)	16.78 ± 2.67** (n = 18)	37	0.303 ± 0.014 (n = 302)	2.4	14.4 ± 2.1** (n = 18)

**P < 0.0001 compared with naïve cells.

Table S2. Fit parameters

Data	Fitted equation	Reference	$V_{1/2}$, mV	k, mV	Max
I–V	$I = \frac{G_{max}(V - V_{rev})}{\{1 + exp[(V_{1/2} - V)/k]\}}$	Fig. 2*D*	29.75	9.71	3.15 (pS/pF)
		Fig. 2*H*	30.21	9.97	87.3 (pS/pF)
		Fig. 4*C*: $Ca_V1.2$-N739D	35	14.59	23.7 (pS/pF)
		Fig. S1	30.36	9.97	89.2 (pS/pF)
Q_V	$Q_{on} = \frac{Q_{max}}{\{1 + exp[(V_{1/2} - V)/k]\}}$	Fig. 2*B*	3.25	14.62	3.75 (nC/μF)
		Fig. 2*F*	3.06	13.89	5.57 (nC/μF)
ΔF–V	$\Delta F = \frac{\Delta F_{max}}{\{1 + exp[(V_{1/2} - V)/k]\}}$	Fig. 5*B*	1.94	7.87	6.01

Where I, Q_{on}, and ΔF are peak current, on charge movement, and change in fluorescence, respectively; G_{max}, Q_{max}, and ΔF_{max} are the maximum values of conductance, Q_{on}, and ΔF, respectively; V is the test potential; V_{rev} is the reversal potential for current; $V_{1/2}$ is the midpoint potential; and k is the slope factor.