SI Appendix

Detailed Materials and Methods

tsA201 cell culture and plasmid constructs

tsA201 cells were kept in Dulbecco's Modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin in a cell culture incubator (37°C, 95:5 % O_2:CO_2). Cells were passaged every 4-5 days and transfected at ~70% confluence using JetPRIME DNA transfection reagent (Polyplus Transfection, New York, NY, USA). After 6-8 h of transfection, cells were plated at low density onto glass coverslips and used for experiments the following day ~16 h later.

For expression of $Ca_V1.2$ in tsA201 cells, 1000 ng each of the following plasmid constructs were transfected: rabbit cardiac isoform of $Ca_V1.2$ α1c (GenBank accession number: NP_001129994.1; a gift from William Catterall, University of Washington, Seattle, WA, USA), rat $Ca_V\alpha_2\delta$ (AF286488), rat $Ca_V\beta_3$ (M88751; both auxiliary subunits were gifts from Dr. Diane Lipscombe; Brown University, Providence, RI, USA). The pore-forming α_{1c} construct was tagged at its C-terminus with a monomeric GFP(A206K), a tagRFP or CFP to permit confirmation of transfection and to monitor channel localization. 1000 ng of either type 1 muscarinic receptor (M1R) or the angiotensin receptor type 1 (AT1R) were also transfected for some experiments. The cDNA clone for AT1R was obtained from the cDNA Resource Center (www.cdna.org) and was subsequently subcloned into an mCherry vector by GenScript. $PH_{PLC\delta1}$ plasmids were gifts from Dr. Tamas Balla (National Institutes of Health). For expression of the rapamycin dimerization system, 200 ng each of pseudojanin-FKBP-RFP (GenBank accession number: NM_054014, a gift from Robin Irvine) and a PM targeted Lyn11-FRB (Addgene plasmid # 20147; provided by Tobias Meyer)) were transfected. Some cells were transfected with 200 ng of 5-phosphatase-dead and Lyn11-FRB-CFP (GenBank accession number NM_002350.3, provided by Robin Irvine; Addgene plasmid # 38003).

TIRF imaging of tsA201 cells

In tsA201 cell TIRF experiments, appropriately transfected cells were perfused with Ringer's solution (160 mM NaCl, 2.5 mM KCl, 2 mM $CaCl_2$, 1mM $MgCl_2$, 10 mM HEPES and 8 mM glucose; pH adjusted to 7.4 with NaOH) and imaged in TIRF mode at 0.2 Hz using an Olympus IX83 inverted microscope equipped with a Cell-TIRF MITICO and a 60x or 100x/1.49 NA TIRF objective

lens. Ringer's supplemented with the appropriate drug was then perfused. Image stacks were subjected to a 50-pixel rolling ball background subtraction, bleach correction, and a 1-pixel median filter using ImageJ/Fiji. The TIRF footprint of the cell was outlined, and the z-axis profile was measured over time and then normalized to the control period.

In experiments designed to assess the Ca^{2+}-dependence of the AngII-triggered channel endocytosis, a modified Ringer's solution in which 2 mM $CaCl_2$ was replaced with 2 mM $BaCl_2$ was used. Transfected cells were perfused with the Ba^{2+}-containing external for at least 15 min before the experiment proceeded as above, stimulating with 100 nM AngII, and then washing out. Cells were then perfused with 2 mM Ca^{2+} Ringers and the experiment was repeated in the presence of Ca^{2+}.

Drugs/reagents used for tsA201 cell TIRF experiments were as follows: 10 μM Oxotremorine-m (MilliporeSigma, Rockville, MD, USA; 63939-65-1), 100 nM Angiotensin II (MilliporeSigma; A9525), 1 μM Rapamycin (MilliporeSigma; SIAL-553210), 10 μM SII AngII (GenScript, Piscataway, NJ, USA), and 80 μM Dynasore (MilliporeSigma; D7693).

Isolation of mouse ventricular myocytes

Adult mouse ventricular myocytes were isolated from male and female C57BL/6J mice obtained from The Jackson Laboratory (Sacramento, CA, USA). Mice were euthanized at 12-24 weeks old by intraperitoneal injection of pentobarbital solution (> 100 mg/kg; Beuthanasia-D Special; Merck Animal Health, Madison, NJ, USA). The heart was removed by sharp dissection and then placed in ice-cold digestion buffer (130 mM NaCl, 5 mM KCl, 3 mM Na-pyruvate, 25 mM HEPES, 0.5 mM $MgCl_2$, 0.33 mM NaH_2PO_4, and 22 mM glucose) supplemented with 150 μM EGTA. Myocytes were enzymatically digested and isolated using the Langendorff technique as previously described (1, 2). In brief, the aorta was cannulated, and the heart was perfused with 37°C digestion buffer until clear of blood, at which point the perfusion was switched to a digestion buffer containing 50 μM $CaCl_2$ (Thermo Fisher Scientific, Rockford, IL, USA), 0.04 mg/ml protease (MilliporeSigma), and 1.4 mg/ml type 2 collagenase (Worthington Biochemical, Lakewood, NJ, USA). Complete digestion was assessed by appearance and firmness of the heart. The atria and ventricles were separated, and the ventricles were cut into small pieces and placed in warm digestion buffer supplemented with 0.96 mg/mL collagenase, 0.04 mg/mL protease, 100 μM $CaCl_2$, and 10 mg/ml BSA. Individual cardiomyocytes were gently separated from the tissue by a transfer pipette. Cells were pelleted by centrifugation for 3 min at 300 rpm. The digestion buffer

was then removed and replaced with wash buffer consisting of digestion buffer supplemented with 10 mg/ml BSA and 250 μM $CaCl_2$. The cells were centrifuged once more, the supernatant removed, and the cells resuspended in appropriate solution for experimentation.

Single Molecule Localization Microscopy (SMLM)

No. 1.5 coverslips were sonicated for 20 mins in 2 N NaOH to remove contaminants and rinsed several times with de-ionized water to thoroughly removed any base. Coverslips were then coated with poly-L-lysine (0.01%; MilliporeSigma) and laminin (20 μg mL^{-1}; Life Technologies, Carlsbad, CA, USA) and isolated myocytes were plated and placed into a 37°C incubator to adhere for 45 min. Adherent myocytes were treated for 5 min with either PBS (control) or 100 nM AngII at 37°C. Cells were then fixed in ice-cold 100% methanol (Fisher Scientific) for 5 min at -20°C, before thorough washing in PBS, and blocking for 1 h at room temperature in 20% SEA Block (Thermo Fisher Scientific) and 0.5% v/v Triton X-100 (MilliporeSigma) in PBS. Primary antibody incubation was performed overnight at 4°C in rabbit polyclonal anti-$Ca_V1.2$ FP1 antibody (provided by Johannes Hell; (3, 4)) diluted 1:100, or rabbit polyclonal anti-$Ca_V1.2$ antibody (CACNA1C, ACC-003, Alomone Labs, Jerusalem, Israel) diluted 1:300 in blocking buffer. The next day, the primary antibody was removed and rinsed thoroughly with PBS and incubated in secondary antibody Alexa Fluor 647-conjugated goat anti- rabbit (Life Technologies) diluted 1:1000 in PBS for 1 h at room temperature in the dark. After rinsing with PBS coverslips were mounted onto glass depression slides (neoLab, Heidelberg, Germany) with a cysteamine (MEA)-catalase/glucose/glucose oxidase (GLOX) imaging buffer containing TN buffer (50 mM Tris pH 8.0, 10 mM NaCl), a GLOX oxygen scavenging system (0.56 mg/mL glucose oxidase, 34 μg/mL catalase, 10% w/v glucose) and 100 mM MEA. Twinsil dental glue (Picodent, Wipperfürth, Germany) and aluminum tape (T205-1.0 - AT205; Thorlabs Inc., Newton, NJ, USA) were used to secure the coverslip to the slide and to keep out oxygen. Cells were imaged on a Leica 3D-GSD-SR microscope (Leica Microsystems, Wetzlar, Germany) in TIRF mode with a penetration depth of 150 nm, using an oil-immersion HC PL APO 160 ×/1.43 NA TIRF objective (Leica). Ground State Depletion (GSD) was performed, and dye-blinking was provoked using a 642 nm/500 mW laser. Photon emission was detected with an iXon3 EM-CCD camera (Andor). Raw blinking images were collected at 100 fps for 38,000 frames using Leica Application Suite software and visualized in a live localization map. 10 nm pixel localization maps were used to determine $Ca_V1.2$ cluster areas and the number of events per pixel using ImageJ/Fiji (NIH).

Surface Biotinylation

Isolated male ventricular myocytes from at least two animals were pooled together in PBS, evenly distributed into tubes of two groups, and then treated with 100 nM AngII (MilliporeSigma) or PBS (vehicle control) for 5 min at 37°C. Immediately following the incubation, the tubes were plunged into an ice-bath and ice-cold PBS was added on top of the cells. The tubes were then centrifuged 300 x g for 10 min at 4°C, the resulting supernatant removed, and the cell pellet was resuspended in 1ml of ice-cold PBS and washed followed by a repeat centrifugation.

The cell pellets were treated by resuspension in 500 µL of biotinylation buffer (1 mg/mL of Sulfo-NHS-LC-LC-biotin and 1 mg/mL of Maleimide-PEG2-biotin solution in PBS) on ice for 1 hr during which cells were mixed by resuspension after 30 min and at 1 hr. After the final resuspension, the cells were immediately centrifuged for 10 min. The biotinylation of each pelleted sample was then quenched by 5 cycles of resuspension of cell pellets in 500 µL of ice-cold 100 mM Glycine in PBS buffer followed by a 3 min incubation on ice and centrifugation essentially as described before (5). Each cell pellet was then washed with 1x PBS and then resuspended in RIPA cell lysis buffer (50 mM Tris-Cl pH 7.4, 150 mM NaCl, 5 mM EGTA pH 7.4, 10 mM EDTA pH 8.0, 1 % NP-40, 10 % Glycerol, 0.05 % SDS, 0.4 % deoxycholate, 34 µg/mL PMSF, 1 µg/mL Pepstatin A, 0.1 µg/mL Leupeptin/Aprotinin) for 10 min, which was then transferred to an ultracentrifuge tube and ultracentrifuged for 30 min at 40,000 g, 4° C. The resulting lysate supernatant was removed, and the protein concentration of each was quantified using a BCA assay.

All biotinylated samples were treated with the same method from this step forward essentially as previously described (4). Supernatant sample containing matched amounts of protein was loaded onto 40 µL of a 50:50 slurry of NeutrAvidin-conjugated Sepharose beads (Thermo Fisher Scientific) and rotated head-over-head at 4°C for 4 hrs. The biotinylated proteins bound to the beads were washed 3 times with ice-cold RIPA buffer containing a cocktail of protease inhibitors. For the fourth and final wash, 10 mM Tris buffer (10 mM Tris, pH 7.4; 10 mM EDTA, pH 8.0; 10 mM EGTA, pH 7.4) was used. All protein samples were eluted and denatured in equal amounts of Laemmli SDS sample buffer by incubation at 65° C for 20 min. Proteins were fractionated by 10% acrylamide SDS-PAGE, then transferred onto polyvinylidene difluoride membranes (PVDF; Bio-Rad, Hercules, California, USA) via electrophoresis in transfer buffer (95.9 mM glycine, 12.5 mM Tris, 150 mL MeOH, up to 1 L H_2O) at 50 V for 10 hrs.

PVDF membranes were stained Ponceau S and imaged, then cut into separate sections spanning the molecular masses of the proteins of interest. The membrane sections were incubated in blocking buffer (BB) consisting of 150 mM NaCl, 10 mM Tris–HCl, pH 7.4 (TBS) with 0.10% Tween (TBST), and either 2% bovine serum albumin (BSA; RPI Corp., Mount Prospect, IL, USA), or 10% milk for 1 hr at RT before incubation with primary antibodies in BB overnight at 4° C. $Ca_V1.2$ was detected using either FP1 or CNC1 antibodies ((3)). Probing with antibodies against the cytosolic proteins GAPDH (MilliporeSigma), vinculin (Cell Signaling Technology, Inc., Danvers, MA), and α-tubulin (Santa Cruz Biotechnology Inc., Dallas, TX, USA), was used as a negative control for surface biotinylation and to control for variations in lysate protein concentration. Membranes were washed for at least 30 min with at least five exchanges of TBST. Membranes were then incubated with horseradish peroxidase-conjugated secondary antibodies diluted in TBS-T, rocking for 1 hr at RT. Secondary antibodies were then washed for 90 min with at least 6 exchanges of solution. The membranes were developed using ECL reagents (Immobilon Classico Western HRP substrate, Millipore Sigma; SuperSignal™ West Femto Maximum Sensitivity Substrate, Thermo Fisher) and a film developer (Medical Film Processor, SRX-101A, Konica Minolta, Tokyo, Japan) followed by quantification of unsaturated protein band signals in the linear range as described (5, 6).

Lipid Mass Spectrometry

Adult mouse hearts were dissected and cannulated on a Langendorff apparatus. Either control PBS or 100 nM AngII in PBS was perfused through the heart for 5 min. Each heart was then snap-frozen in liquid nitrogen before preparing lysates. Hearts were homogenized in RIPA buffer (R26200, Research Products International, Mount Prospect, IL USA) containing protease inhibitor, 4 µM microcystin (Insolution microcystin-LR, Thermo Fisher Scientific) and 1 mM sodium fluoride using tubes pre-filled with beads and centrifugation in a “Bullet Blender Storm 24” centrifuge (Next Advance; Troy, NY, USA) in a cold room for 20 min. The supernatant was extracted, and protein concentration was determined by BCA assay. 20 % of the volume of this lysate was designated for mass spec.

Phosphoinositide levels were then quantified by a lipid mass spectrometry procedure as previously described (7, 8). Endogenous lipids from whole heart samples and internal standards were extracted with N-butanol and chloroform. Samples were derivatized via methylation and separated on a C4 column in an acetonitrile formic acid gradient. Column eluate was infused with NaFormate, then monitored by a Waters XEVO TQ-S MS/MS in multiple reaction monitoring

mode (MRM) using electrospray and positive ion mode. Quantification of total integrated areas under peaks from sample and standard eluate profiles was performed using MassLynx software. Peak area of phosphoinositide species from biological samples were normalized to synthetic standards. Total protein was used to further correct for tissue amount.

PIP_2 Shuttle

For the PIP_2 supplementation experiments, a $PI(4,5)P_2$ shuttle kit (P-9045; Echelon Biosciences, Inc., Salt Lake City, UT, USA) was used. Equimolar quantities of unlabeled carrier 2 (Histone H1 carrier) and $PI(4,5)P_2$ diC16 were incubated at RT for 10 min (100 µM concentration in PBS each). The complex was diluted in PBS and added to isolated myocytes to a final concentration of 10 µM on the cells. Cells were plated in the same manner as described in preparation for super-resolution microscopy and incubated at 37°C for 25 min. Then, cells were treated, fixed, immunolabeled, and imaged using SMLM as described previously.

TIRF imaging of transduced ventricular myocytes

Three-week-old mice, under isoflurane anesthesia, were retro-orbitally injected with AAV9-$Ca_V\beta_{2a}$-paGFP. Mice were euthanized 7-12 weeks later and myocytes were isolated using the Langendorff method described above. Individual myocytes were allowed to attach to poly-L-lysine coated glass coverslips and were imaged on an Olympus IX83 inverted microscope equipped with a Cell-TIRF MITICO and a 60×/1.49 NA or a 100x/1.49 NA TIRF objective lens. Cells were perfused with Tyrode's solution (140 mM NaCl, 5 mM KCl, 10 mM HEPES, 10 mM glucose, 2 mM $CaCl_2$, and 1 mM $MgCl_2$; pH adjusted to 7.4 with NaOH) at 10.34 fps. A 300-frame control period was followed by 2 min of 100 nM AngII. The paGFP was photoactivated by 405 nm epifluorescent light, and a 488 nm laser light was then used to excite activated GFP. TIRF footprints were imaged at a 152 nm penetration depth.

ImageJ/FIJI was used for image analysis. Image stacks were subjected to a bleach correction followed by a 20-pixel rolling ball background subtraction, and a 10-frame moving average. To observe and quantify the internalized, recycled, and static population of AAV9-$Ca_V\beta_{2a}$-paGFP, maximum intensity z-projections of the 300-frame control period and the final 300 frames of the AngII period were generated, thresholded and made binary. Next, the 'image calculator' function in FIJI was used to generate a map of channels that were: i) inserted/recycled into the membrane during AngII, the binary control image was subtracted from the binary AngII image, ii) internalized during AngII, the binary AngII image was subtracted from the binary control image, and iii) static

population, the binary control and AngII images were multiplied. The integrated density of each population was then calculated and taken as a percentage of the total of all populations.

Immunostaining and Airyscan microscopy

Freshly isolated ventricular myocytes were plated onto poly-L-lysine and laminin-coated glass coverslips and allowed to adhere for 45 mins at room temperature. Myocytes were washed with PBS before a 5 min treatment with either PBS (control), 100 nM AngII, or 10 μM SII AngII. Myocytes were then fixed in a 4% paraformaldehyde solution with a cytoskeletal preserving buffer (PEM) containing 80 mM PIPES, 5 mM EGTA, and 2 mM $MgCl_2$ at 37°C for 10 min. Cells were rinsed with PBS and incubated for 10 mins in 0.5% Triton-X 100 (MilliporeSigma) prior to blocking for 1 hr at room temperature in blocking solution containing 50% SEA Block (Thermo Fisher Scientific) and 0.5% v/v Triton-X100 in PBS. Primary antibodies were diluted in blocking solution and applied directly to the coverslip overnight at 4 °C. Two primary antibodies were used simultaneously for double labelling. The following primary antibodies were used: rabbit polyclonal IgG anti-$Ca_V1.2$ (1:300; Alomone labs ACC-003), mouse monoclonal anti-EEA1 IgG1 (1:250; BD Bioscience 610456), mouse monoclonal IgG1 anti-Rab11a (1:250; Santa Cruz Biotechnology Inc., Dallas, TX, USA; sc-166523), mouse monoclonal IgG1 anti-Rab7 (1:250; Santa Cruz sc-376362). After thorough washing with PBS, cells were incubated in Alexa Fluor-conjugated secondary antibodies (1:1000; Life Technologies) for 1 hr at room temperature. The following secondary antibodies were used: Alexa Fluor 488 goat anti-rabbit and Alexa Fluor 647 goat anti-mouse IgG1. After thorough washing in PBS, coverslips were mounted onto glass slides and examined on a Zeiss LSM 880 super-resolution microscope equipped with an Airyscan detector and a Plan-Apochromat 63×/1.40 oil DIC M27 objective (Carl Zeiss Microscopy, LLC., White Plains, NY, USA). Images were acquired using Zen software (Carl Zeiss Microscopy, LLC).

Perforated patch clamp electrophysiology

$Ca_V1.2$ channel currents were recorded from freshly isolated myocytes using borosilicate glass pipettes fire-polished to a resistance of 1-3 MΩ and filled with internal solution (87 mM Cs-aspartate, 20 mM CsCl, 1 mM $MgCl_2$, 10 mM HEPES, 10 mM EGTA, 5 mM MgATP and pH adjusted to 7.2 with CsOH). For perforated patch clamp experiments, 50 μM escin was added to the patch pipette. Initial perfusion with external Tyrodes solution (140 mM NaCl, 5 mM KCl, 10 mM HEPES, 10 mM glucose, 2 mM $CaCl_2$, 1 mM $MgCl_2$ and pH adjusted to 7.4 with NaOH) was performed. This solution was switched to one containing 5 mM CsCl, 10 mM HEPES, 10 mM Glucose, 140 mM NMDG, 1 mM $MgCl_2$ and 2 mM $CaCl_2$ (pH adjusted to 7.3 with HCl) upon

successful perforation. Series resistance was monitored throughout, and a successful perforated configuration was assumed when the series resistance fell below 15 MΩ. Currents were elicited using a single-step protocol in which myocytes were held at -80 mV, stepped to -40 mV for 100 ms, then to the test potential of 0 mV for 300 ms. After seven stable control sweeps, external solution supplemented with 100 nM AngII was perfused for three minutes. Currents were sampled at a frequency of 10 kHz, and low-pass filtered at 2 kHz using an Axopatch 200B amplifier (Molecular Devices, Sunnyvale, CA, USA), digitized using a Digidata 1550B plus Humsilencer (Molecular Devices) and acquired using pClamp, version 10.7 (Molecular Devices). Clampfit software (Molecular Devices) was used for analysis and the current-voltage relationship was plotted using Prism (GraphPad Software Inc., La Jolla, CA, USA). Membrane potentials were corrected for liquid junction potential.

Adult cardiomyocyte culture and adenoviral transduction

Freshly isolated myocytes were suspended in plating media consisting of MEM (11090-081, Gibco - Thermo Fisher Scientific) supplemented with 0.1 mL/L ITS (Insulin transferrin selenium, Gibco - Thermo Fisher Scientific), 1 % penicillin/streptomycin, 2 mM Glutamax (Gibco, Thermo Fisher Scientific), 4 mM $NaHCO_3$ (Thermo Fisher Scientific), 10 mM HEPES (Thermo Fisher Scientific), 0.2 % BSA (Sigma-Aldrich), 25 µM Blebbistatin (MilliporeSigma), and 5 % FBS (Gibco - Thermo Fisher Scientific). Cells were subsequently plated (in plating media) onto cleaned, sterile, poly-L-lysine and laminin coated coverslips at a concentration of 5 x 10^4 cells/mL and placed in a cell culture incubator (37°C, 95:5 % O_2:CO_2). Two hours later, the media was exchanged with FBS-free plating media and Ad-RFP-$PH_{PLC\delta1}$ (5 x 10^7 viral particles/million cells) and incubated overnight. The following day, the media was exchanged again with fresh, FBS-free media. Myocytes were used for experiments ~24 hrs later (i.e. ~48 hrs after isolation).

Cultured myocytes were imaged on an Olympus IX83 inverted microscope with Andor W1-spinning disk confocal equipped with a 60X/1.49 objective lens and 561 nm laser light to excite the transduced RFP-$PH_{PLC\delta1}$. In a time-lapse protocol, cells were continuously perfused with Tyrode's solution supplemented with 2 mM ATP and 15 mM BDM or 25 µM Blebbistatin and z-stacks through the cell depth were captured before, during, and after addition of 100 nM AngII. ImageJ/FIJI was used for image analysis. In brief, maximum intensity z-projections underwent bleach correction and background subtraction (150-pixel rolling ball), and the time course of fluorescence intensity emission was measured from membrane ROIs.

Calcium Transients

Myocytes were loaded with 10 μM Fluo4-AM (Thermo Fisher Scientific Scientific) for 20 min. Cells were then centrifuged for 2 min at 300 rpm, resuspended in Tyrode's solution (140 mM NaCl, 5 mM KCl, 10 mM HEPES, 10 mM glucose, 2 mM $CaCl_2$ and 1 mM $MgCl_2$; pH adjusted to 7.4 with NaOH) and incubated for 20 min to allow complete de-esterification of intracellular AM esters. A Myopacer field stimulator (IonOptix LLC., Westwood, MA, USA) was used to pace cells at 1 Hz and the resultant Ca^{2+} transients were recorded using a line-scanning Zeiss LSM 880 and a Plan-Apochromat 63×/1.40 oil DIC M27 objective. 488-nm laser light was used to excite the Fluo-4 and the brightness of the fluorescent signals represents the relative level of intracellular [Ca^{2+}]. Cells were continuously perfused with Tyrode's solution and paced to steady state before imaging commenced. Immediately following the control recording, 100 nM AngII was perfused for 1 min before recording the second set of transients. Analysis was performed in ImageJ/FIJI and Clampfit.

Chemicals and statistical analysis

Chemical reagents were obtained from MilliporeSigma unless noted otherwise. Data are reported as the mean ± SEM. *N* indicates the number of animals used in a dataset, while *n* reflects the number of cells. Student's t-test was used for paired, or unpaired dataset comparison if the data passed a normality test. If not, a non-parametric Wilcoxon test was performed. One-way/two-way analysis of variance (ANOVA) was performed for multiple comparisons with appropriate post hoc tests. $P < 0.05$ was considered statistically significant.

References

1. D. W. Ito *et al.*, β-adrenergic-mediated dynamic augmentation of sarcolemmal $Ca_V1.2$ clustering and co-operativity in ventricular myocytes. *J Physiol* **597**, 2139-2162 (2019).
2. S. G. Del Villar *et al.*, beta-Adrenergic control of sarcolemmal $Ca_V1.2$ abundance by small GTPase Rab proteins. *Proc Natl Acad Sci U S A* **118** (2021).
3. O. R. Buonarati, P. B. Henderson, G. G. Murphy, M. C. Horne, J. W. Hell, Proteolytic processing of the L-type Ca^{2+} channel $\alpha_1$1.2 subunit in neurons. *F1000Research* **6**, 1166-1166 (2017).
4. M. A. Davare, M. C. Horne, J. W. Hell, Protein phosphatase 2A is associated with class C L-type calcium channels ($Ca_V1.2$) and antagonizes channel phosphorylation by cAMP-dependent protein kinase. *J Biol Chem* **275**, 39710-39717 (2000).
5. M. Turner *et al.*, alpha-Actinin-1 promotes activity of the L-type Ca^{2+} channel $Ca_V1.2$. *EMBO J* **39**, e102622 (2020).
6. P. Bartels *et al.*, Half-calcified calmodulin promotes basal activity and inactivation of the L-type calcium channel $Ca_V1.2$. *J Biol Chem* 10.1016/j.jbc.2022.102701, 102701 (2022).
7. A. Traynor-Kaplan *et al.*, Fatty-acyl chain profiles of cellular phosphoinositides. *Biochim Biophys Acta Mol Cell Biol Lipids* **1862**, 513-522 (2017).
8. O. Vivas, S. A. Tiscione, R. E. Dixon, D. S. Ory, E. J. Dickson, Niemann-Pick Type C Disease Reveals a Link between Lysosomal Cholesterol and PtdIns(4,5)P_2 That Regulates Neuronal Excitability. *Cell Rep* **27**, 2636-2648 e2634 (2019).

Supplemental Figures and Legends

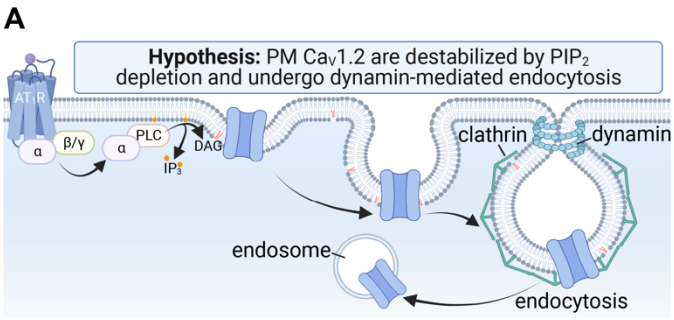

Figure S1. Model of PIP_2 depletion-triggered destabilization and dynamin-dependent endocytosis of $Ca_V1.2$. A, Illustration of the hypothesis where AngII-mediated PIP_2 depletion destabilizes $Ca_V1.2$ channels, triggering their removal from the PM via dynamin-dependent endocytosis. In this process, PM cargo is sequestered into membrane invaginations called clathrin-coated vesicles, which are ultimately pinched off by dynamin to become endosomes.

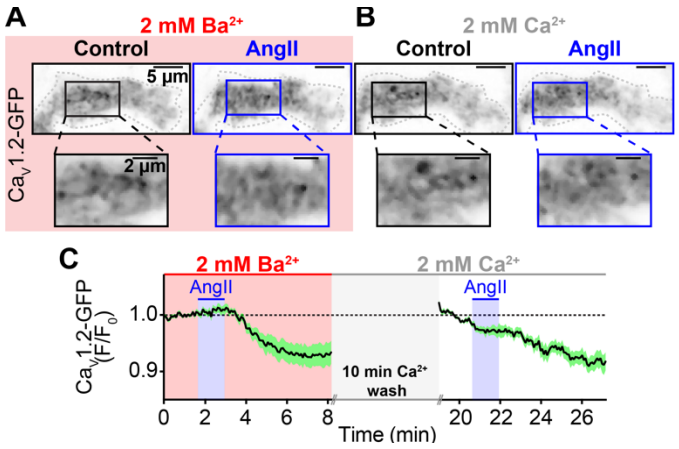

Figure S2. AngII-stimulated $Ca_V1.2$ channel internalization is Ca^{2+} independent. A, Representative TIRF images showing $Ca_V1.2$-GFP localization in transfected tsA201 cells before and after 100 nM AngII treatment in the presence of a (nominally Ca^{2+}-free) 2 mM Ba^{2+} containing bath solution and **B**, after 10 min wash with 2 mM Ca^{2+} Ringer's solution. **C**, Time-course (mean ± S.E.M) of the AngII stimulated changes in normalized $Ca_V1.2$-GFP fluorescence emission (F/F_0) in the TIRF footprint during these perfusing-ion substitution experiments (n = 15).

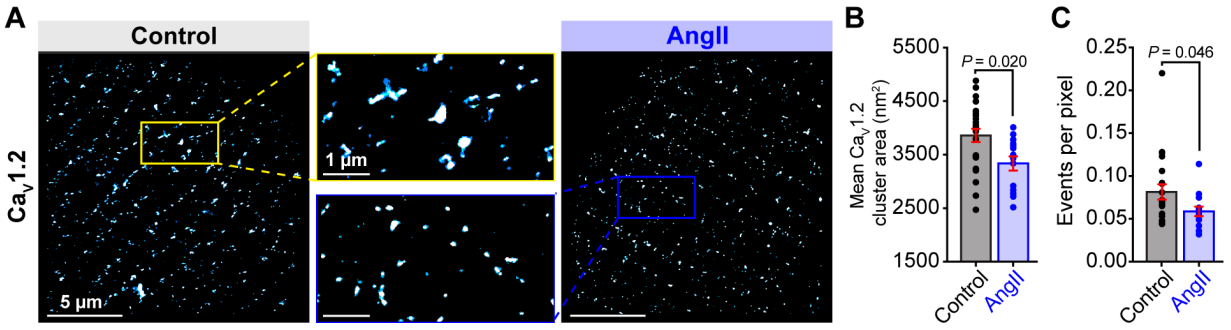

Figure S3. AngII also stimulates a decrease in t-tubular $Ca_V1.2$ cluster area and overall PM expression in females A, SMLM localization maps of fixed female ventricular myocytes immunostained to examine $Ca_V1.2$ channel distribution under untreated control (*left*) or 100 nM AngII-stimulated (*right*) conditions (control: N = 5, n = 21; AngII: N = 3, n = 14). **B**, Aligned dot plots showing mean $Ca_V1.2$ channel cluster area, and **C**, the events per pixel in each condition. Data in B and C were analyzed with unpaired, two-tailed Student's t-tests. Error bars indicate SEM.

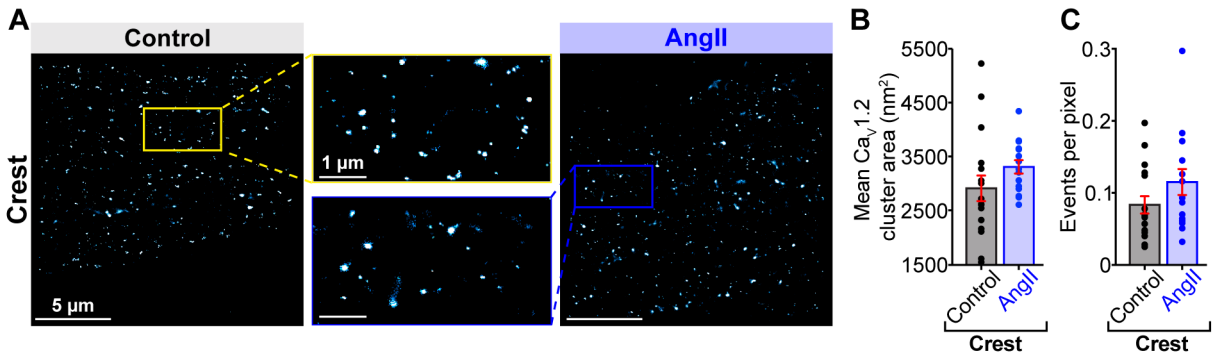

Figure S4. AngII does not affect $Ca_V1.2$ cluster area nor overall PM expression in the sarcolemma crest. A, SMLM localization maps of fixed ventricular myocytes immunostained to examine $Ca_V1.2$ channel distribution in the sarcolemmal crest under untreated control (*left*) or 100 nM AngII-stimulated (*right*) conditions (control: $N = 3$, $n = 17$; AngII: $N = 3$, $n = 15$). **B**, Aligned dot plots showing mean $Ca_V1.2$ channel cluster area, and **C**, the events per pixel in each condition. Data in B and C were analyzed with unpaired, two-tailed Student's t-tests. Error bars indicate SEM.

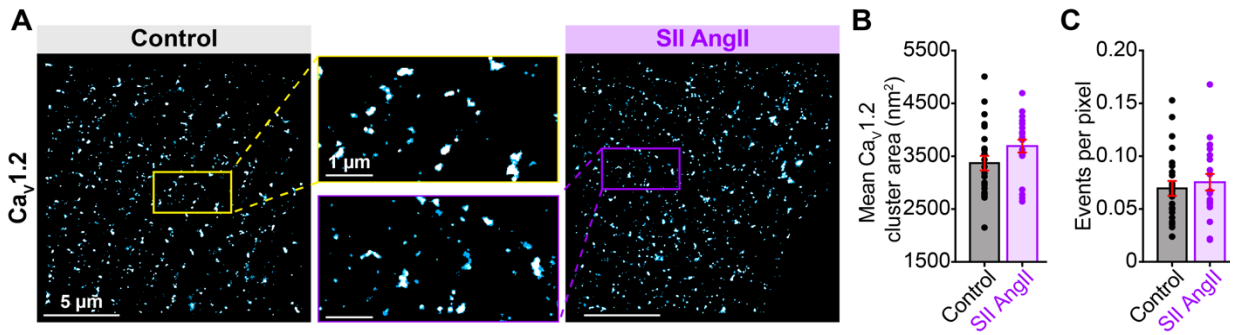

Figure S5. SII AngII treatment does not alter Ca$_V$1.2 channel cluster area or PM expression. **A**, SMLM localization maps of fixed ventricular myocytes immunostained to examine Ca$_V$1.2 channel distribution in the sarcolemmal crest under untreated control (*left*) or *β*-arrestin biased AT1R agonist SII AngII-stimulated (10 µM; *right*) conditions (control: *N* = 3, *n* = 24; SII AngII: *N* = 3, *n* = 21). **B**, Aligned dot plots showing mean Ca$_V$1.2 channel cluster area, and **C**, the events per pixel in each condition. Data in B and C were analyzed with unpaired, two-tailed Student's t-tests. Error bars indicate SEM

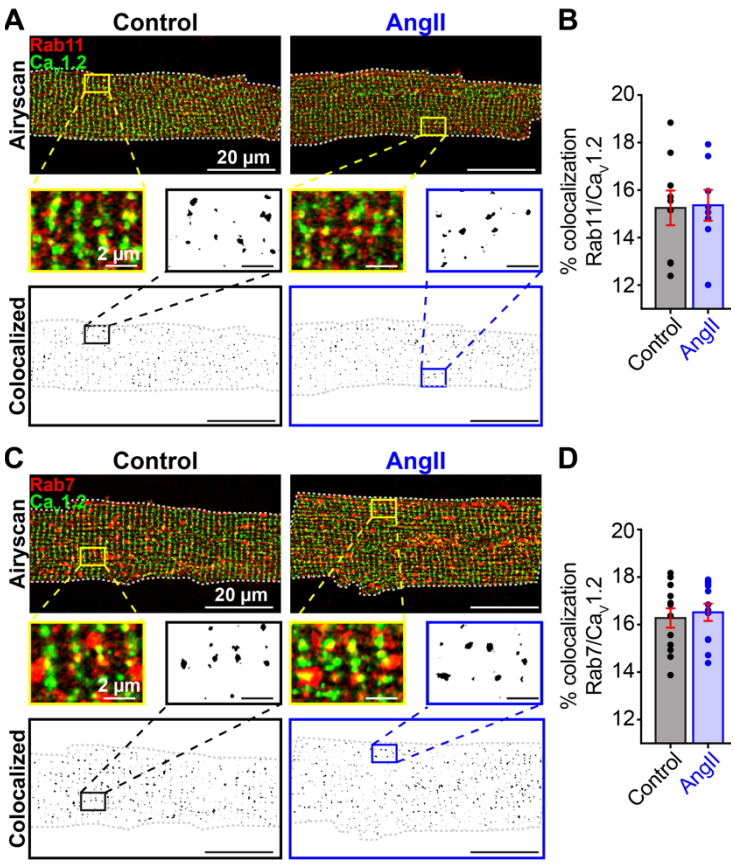

Figure S6. AngII-triggered endocytosis of $Ca_V1.2$ channels does not affect recycling or late endosome channel pools. A, Immunostaining of $Ca_V1.2$ and $Rab11^+$ recycling endosomes, and **B**, accompanying histogram summarizing results from control (*N* = 3, *n* = 9) and AngII conditions (*N* = 3, *n* = 8). **C**, Immunostaining of $Ca_V1.2$ and $Rab7^+$ late endosomes. **D**, Histogram summarizing results from control (*N* = 3, *n* = 12) and AngII-stimulated (*N* = 3, *n* = 12) myocytes. Data in B and D were analyzed with unpaired Students t-tests. Error bars indicate SEM