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β-adrenergic-mediated dynamic augmentation of sarcolemmal CaV1.2 clustering and co-operativity in ventricular myocytes

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Key points
• Prevailing dogma holds that activation of the β-adrenergic receptor/cAMP/protein kinase A signalling pathway leads to enhanced L-type CaV1.2 channel activity, resulting in increased Ca2+ influx into ventricular myocytes and a positive inotropic response. However, the full mechanistic and molecular details underlying this phenomenon are incompletely understood.
• CaV1.2 channel clusters decorate T-tubule sarcolemmas of ventricular myocytes. Within clusters, nanometer proximity between channels permits Ca2+-dependent co-operative gating behaviour mediated by physical interactions between adjacent channel C-terminal tails.
• We report that stimulation of cardiomyocytes with isoproterenol, evokes dynamic, protein kinase A-dependent augmentation of CaV1.2 channel abundance along cardiomyocyte T-tubules, resulting in the appearance of channel ‘super-clusters’, and enhanced channel co-operativity that amplifies Ca2+ influx.
• On the basis of these data, we suggest a new model in which a sub-sarcolemmal pool of pre-synthesized CaV1.2 channels resides in cardiomyocytes and can be mobilized to the membrane in times of high haemodynamic or metabolic demand, to tune excitation–contraction coupling.

Abstract Voltage-dependent L-type CaV1.2 channels play an indispensable role in cardiac excitation–contraction coupling. Activation of the β-adrenergic receptor (βAR)/cAMP/protein kinase A (PKA) signalling pathway leads to enhanced CaV1.2 activity, resulting in increased Ca2+ influx into ventricular myocytes and a positive inotropic response. CaV1.2 channels exhibit a clustered distribution along the T-tubule sarcolemma of ventricular myocytes where nanometer proximity between channels permits Ca2+-dependent co-operative gating behaviour mediated by dynamic, physical, allosteric interactions between adjacent channel C-terminal tails.

Danica Ito is a third year PhD student in the Molecular, Cellular and Integrative Physiology (MCIP) program at the University of California, Davis. She has a BS in Kinesiology and Biology (Westmont College, 2014) and a MS in Exercise Physiology (San Diego State University, 2016). Her ongoing graduate research focuses on the effect of β-adrenergic receptor stimulation on CaV1.2 channel clustering and co-operativity in ventricular myocytes.
This amplifies Ca\(^{2+}\) influx and augments myocyte Ca\(^{2+}\) transient and contraction amplitudes. We investigated whether βAR signalling could alter Ca\(_{V1.2}\) channel clustering to facilitate co-operative channel interactions and elevate Ca\(^{2+}\) influx in ventricular myocytes. Bimolecular fluorescence complementation experiments reveal that the βAR agonist, isoproterenol (ISO), promotes enhanced Ca\(_{V1.2}\)–Ca\(_{V1.2}\) physical interactions. Super-resolution nanoscopy and dynamic channel tracking indicate that these interactions are expedited by enhanced spatial proximity between channels, resulting in the appearance of Ca\(_{V1.2}\) ‘super-clusters’ along the z-lines of ISO-stimulated cardiomyocytes. The mechanism that leads to super-cluster formation involves rapid, dynamic augmentation of sarcolemmal Ca\(_{V1.2}\) channel abundance after ISO application. Optical and electrophysiological single channel recordings confirm that these newly inserted channels are functional and contribute to overt co-operative gating behaviour of Ca\(_{V1.2}\) channels in ISO stimulated myocytes. The results of the present study reveal a new facet of βAR-mediated regulation of Ca\(_{V1.2}\) channels in the heart and support the novel concept that a pre-synthesized pool of sub-sarcolemmal Ca\(_{V1.2}\) channel-containing vesicles/endosomes resides in cardiomyocytes and can be mobilized to the sarcolemma to tune excitation–contraction coupling to meet metabolic and/or haemodynamic demands.

**Introduction**

Ca\(_{V1.2}\) channels are the most abundant L-type calcium channel in the heart, where they represent the primary Ca\(^{2+}\) entry pathway across the plasma membrane and are thus indispensable for cardiac excitation–contraction (EC) coupling. Electron microscopy (Franzini-Armstrong et al., 1998), scanning ion-conductance microscopy (Bhargava et al., 2013) and, most recently, super-resolution nanoscopy (Dixon et al., 2015; Dixon et al., 2017) approaches have revealed an inherent tendency of these channels to form discrete clusters on the T-tubule sarcolemma of ventricular myocytes. We have previously reported that groups of channels within these clusters are capable of functionally interacting with their juxtaposed neighbors in a Ca\(^{2+}\)/calmodulin (Ca\(^{2+}\)/CaM)-dependent manner to open and close together (i.e. co-operative gating) (Dixon et al., 2015). In this scheme, Ca\(^{2+}\)/CaM formed when Ca\(^{2+}\) flows through the pore of one channel facilitates physical association between adjacent channels by bridging C-terminal pre-IQ motifs. This association allows channels to allosterically communicate with one another, such that the opening of one channel can influence the opening of those physically associated with it. This leads to enhanced open probability (\(P_o\)) of the adjoined channels and a resultant amplification of Ca\(^{2+}\) influx. An important question that has remained unaddressed is whether co-operative gating behaviour is subject to regulatory control by key signalling pathways.

In the heart, β-adrenergic receptor (βAR) activation initiates a key regulatory pathway, stimulated by the sympathetic nervous system during the fight-or-flight response. This triggers a signalling cascade that results in positive chronotropic, inotropic and lusitropic effects, equipping the heart to deal with the increased haemodynamic and metabolic requirements during physically demanding, stressful or emotional situations. The molecular details of how βAR activation leads to an inotropic response have been the subject of intense investigation for over 50 years after it was first appreciated that catecholamines can vastly enhance Ca\(^{2+}\) permeability in cardiomyocytes by increasing the magnitude of the Ca\(^{2+}\) current flowing through voltage-dependent L-type Ca\(^{2+}\) channels (Ca\(_{V1.2}\)) (Tsien et al., 1972; Sperelakis & Schneider, 1976; Reuter & Scholz, 1977). It is now well established that activation of cAMP-dependent protein kinase A (PKA), downstream of βAR stimulation, leads to phosphorylation of Ca\(_{V1.2}\) channels (Osterrieder et al., 1982; Hartzell et al., 1991). This results in increased channel \(P_o\) as a result of potentiation of longer-duration ‘mode 2’ openings (Yue et al., 1990) and an increased number of functional channels (Bean et al., 1984).

Within Ca\(_{V1.2}\) clusters, we have previously reported that channels with a higher \(P_o\) dictate the activity of adjoined channels. This was demonstrated in experiments where high \(P_o\) Timothy syndrome mutant Ca\(_{V1.2}\) channels (Ca\(_{V1.2}\)\(^{G436R}\)) were forced to physically interact with lower \(P_o\) wild-type (WT) channels using a light activated dimerization system (Dixon et al., 2012). The ensuing amplification of Ca\(^{2+}\) influx was attributed to an increase in the \(P_o\) of the lower activity WT channels, driven by the adjoined higher activity Timothy syndrome channels. An important implication of this work is that a small number of high \(P_o\) channels can have a disproportionately large effect on Ca\(^{2+}\) influx.
by communicating with other channels in the cluster and exerting a dominant gating influence over them. Applying this same logic to high $P_o$ PKA-phosphorylated channels, a small number of phosphorylated channels could have a big effect on $Ca^{2+}$ influx if they stably interact with lower $P_o$ unphosphorylated channels. However, it is unknown whether $\beta$AR signalling can affect CaV1.2 channel clustering and co-operativity.

Given the reliance of CaV1.2 co-operativity on $Ca^{2+}$, we hypothesized that enhanced $Ca^{2+}$ influx through a subset of high $P_o$ PKA-phosphorylated channels, subsequent to $\beta$AR stimulation, would promote augmented co-operativity of the channels and produce an amplification of $Ca^{2+}$ influx that could contribute to the inotropic response seen during fight-or-flight. We set out to rigorously test this hypothesis using a unique toolbox including electrophysiology, live-cell imaging, super-resolution nanoscopy, bimolecular fluorescence complementation (BiFC), stepwise photobleaching and $Ca^{2+}$ sparklet recordings. Unexpectedly, our data reveal a mechanistic model that extends beyond the initial assumption that $\beta$AR-mediated enhancement of $Ca^{2+}$ influx leads to more CaV1.2 channel co-operativity. We found that stimulation of cardiomyocytes with the non-selective $\beta$AR agonist isoproterenol (ISO), evokes dynamic augmentation of CaV1.2 channel clustering along the T-tubules of cardiomyocytes, resulting in the appearance of channel ‘super-clusters’. Within these super-clusters, enhanced levels of physical proximity promote functional interactions between channels. Dynamic live-cell imaging in freshly isolated adult mouse cardiomyocytes reveals that the ISO-stimulated super-clustering that enhances channel co-operativity occurs at least partly as a result of an increased abundance of the channels at the sarcolemma. On the basis of these data, we suggest a new model in which a readily insertable, sub-sarcolemmal pool of pre-synthesized CaV1.2 channels resides in cardiomyocytes and can be mobilized to the membrane in times of high haemodynamic or metabolic demand to tune EC-coupling.

**Isolation of ventricular myocytes**

Wild-type C57BL/6J mice were purchased from The Jackson Laboratory (Sacramento, CA, USA) and housed in approved vivarium facilities at UC Davis where they were given *ad libitum* access to food and water. Mice were killed with a single lethal dose of a phenytoin and pentobarbital solution (>100 mg kg$^{-1}$; Beuthanasia-D Special; Merck Animal Health, Madison, NJ, USA) delivered by intraperitoneal injection. Both male and female young (8–16 weeks old) adult mice were used in the study. Isolations were performed using a Langendorff apparatus as described previously (Dixon et al., 2012; Drum et al., 2013; Dixon et al., 2015). Briefly, hearts were excised and rinsed in a 150 μM EGTA-containing, chilled digestion buffer with the composition: 130 mM NaCl, 5 mM KCl, 3 mM Na-pyruvate, 25 mM Heps, 0.5 mM MgCl$_2$, 0.33 mM NaH$_2$PO$_4$ and 22 mM glucose. The aorta was cannulated and the heart was hung on a Langendorff perfusion apparatus, where it was perfused with warmed (37°C) 150 μM EGTA digestion buffer. When the perfusate was clear of blood, the perfusing solution was changed to a second digestion buffer-based solution (no EGTA) supplemented with 50 μM CaCl$_2$, 0.04 mg mL$^{-1}$ protease (XIV) and 1.4 mg mL$^{-1}$ type 2 collagenase (Worthington Biochemical, Lakewood, NJ, USA) until judged to be adequately digested (appraised by visual inspection of pallor and palpation of the heart to assess loss of rigidity). The atria were then removed via sharp dissection and the remaining ventricles were sliced and placed into 37°C digestion buffer supplemented with 0.96 mg mL$^{-1}$ collagenase, 0.04 mg mL$^{-1}$ protease, 100 μM CaCl$_2$ and 10 mg mL$^{-1}$ BSA. A transfer pipette was used to gently agitate the tissue and release the ventricular myocytes. Cells were left to gravity pellet for 15–20 min. The enzyme-containing supernatant was then removed with a transfer pipette and the pelleted cells were washed in digestion buffer supplemented with 10 mg mL$^{-1}$ BSA and 250 μM CaCl$_2$, and allowed to gravity pellet once more for 15–20 min. The washing supernatant was discarded, and cells were finally suspended in the appropriate solution for the experimental series. For example, for whole cell patch clamp experiments, cells were resuspended at room temperature in Tyrode’s solution containing 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).
Cell culture and transient transfection

TsA-201 cells originally obtained from Sigma-Aldrich (St Louis, MO, USA) were maintained in a cell culture incubator (37°C, 95:5% O2:CO2) in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin. Cells were passaged at confluence (every 3–4 days). For heterologous expression of plasmid DNA, cells were grown to 70% confluence and transiently transfected with the cationic transfection reagent, jetPEI (Polyplus Transfection, New York, NY, USA). Cells were incubated in the reagent/DNA mixture for 24 h and re-plated onto the appropriate coverglass the next day, ~16 h before experiments to allow appropriate recovery and adhesion of the cells.

Plasmid and viral constructs

In the present study, we used pcDNA clones of the pore-forming subunit of the rabbit cardiac isoform of CaV1.2 α1c (GenBank accession number: NP_001129994.1; provided by William Catterall, University of Washington, Seattle, WA, USA) and rat auxiliary subunits CaVα2δ (AF286488) and CaVβ3 (M88751; provided by Dr Diane Lipscombe; Brown University, Providence, RI, USA). Several CaV1.2 C-terminal fusion constructs are used in the study including CaV1.2-EGFP(A206K), CaV1.2-VC155, CaV1.2-VN155(I152L) [venus fragments were gifts from Chang-Deng Hu (Kodama & Hu, 2010); Addgene plasmid # 22011 and # 27097; Addgene, Cambridge, MA, USA]. These C-terminal fusion constructs were generated using standard PCR techniques as described previously (Dixon et al., 2015). For expression of CaV1.2 channels in tsA-201 cells, 800 ng of α1c, 400 ng of α2δ, 800 ng of β3 and 200 ng of protein kinase C (PKC)α was added to the jetPEI transfection reagent and the manufacturer’s protocol was followed. In BiFC experiments, 400 ng of CaV1.2-VC155 and of 400 ng CaV1.2-VN155(I152L) were used to make up the 800 ng of α1c.

In vivo viral transduction of cardiomyocytes

Because cardiomyocytes are impervious to chemical transfection, to visualize CaV1.2 channels in live cell dynamic imaging and stepwise photobleaching experiments, we used an in vivo viral transduction approach. The most cardiotropic adeno-associated virus serotype 9 (AAV9) (Fang et al., 2012) was used to deliver rat CaVβ2a (GenBank accession number: NM_053851.1), with a C-terminal photoactivatable GFP (paGFP) fusion tag via retro-orbital injections. This serotype has been used previously by our group (Dixon et al., 2015) and others (Fang et al., 2012; Drum et al., 2016) to successfully deliver cardiac genes in mice. AAV9-CaVβ2a-paGFP was engineered in an in-house core facility (Molecular Construct and Packaging Core) at UC Davis. WT mice were anaesthetized using an isoflurane vaporizer, and a small gauge insulin syringe was used to deliver 4 × 10^{-12} vg mL^{-1} AAV9-CaVβ2a-paGFP into the retro-orbital sinus. Petrolatum ophthalmic ointment was applied to the injected eye and the mice were returned to their cage once they regained consciousness. Injection volumes were restricted to 100–200 μL. Mice were killed 2–5 weeks post-injection and successful transduction was assessed by photo-activating the CaVβ2a-paGFP with 405 nm laser light. Prior to photo-activation, no GFP fluorescence emission was detected upon excitation with 488 nm laser light but, after photo-activation, robust GFP fluorescence emission was observed.

BiFC

Physical interactions between CaV1.2 channels were assayed using BiFC as described previously (Dixon et al., 2015; Moreno et al., 2016). Briefly, this protein–protein interaction assay was performed in tsA-201 cells transfected with ‘split venus’ tagged channels as described above [CaV1.2-VC155 and CaV1.2-VN155(I152L)]. The I152L mutation in the N-terminal portion of the split venus protein has been reported to reduce the level of spontaneous self-assembly between VN and VC, effectively reducing background fluorescence and increasing the signal-to-noise ratio of the assay (Kodama & Hu, 2010). In imaging experiments, tsA-201 cells were transiently transfected with CaV1.2-VN and CaV1.2-VC and the relevant auxiliary subunits described above. Total internal reflection fluorescence (TIRF) imaging was then performed on the coverslip plated cells, which were bathed in supplemented DMEM culture medium throughout and maintained at physiological temperature (37°C) for a period of hours. Overnight time series image acquisitions (20 frames min^{-1}) were performed on the cells mounted in a temperature-controlled stage-top CO2 incubator on a W1-spinning Disc confocal (Andor, Belfast, UK) with a Borealis modification and TIRF module, built around an IX83 inverted microscope (Olympus, Tokyo, Japan) equipped with a 60×1.49 NA TIRF objective lens. In experiments designed to evaluate the effect of βAR stimulation on physical interactions between CaV1.2 channels, 100 nM ISO was added at time zero at the onset of imaging. In controls for this experimental series, the overnight imaging proceeded in the absence of ISO. Analysis of the resultant image stacks was performed using ImageJ/Fiji open source image processing software (NIH, Bethesda, MD, USA).

Super-resolution nanoscopy

To remove fluorescent contaminants, #1.5 coverglass (VWR International, Radnor, PA, USA) was sonicated for
20 min in 2 N NaOH, thoroughly rinsed with de-ionized water, and subsequently sterilized and stored in 70% EtOH until use. Isolated myocytes were plated onto laminin (20 μg mL⁻¹; Life Technologies, Carlsbad, CA, USA) and poly-L-lysine (0.01%; Sigma-Aldrich) coated, cleaned coverglass and then placed in a 37°C incubator to adhere for 45 min. To stimulate endogenous βARs, adherent myocytes were treated with 100 nM ISO for 8 min, whereas control cells were left untreated for this time period. For labelling experiments on tsA-201 cells, transfected cells expressing Cav1.2 channels were plated onto cleaned, poly-L-lysine coverslips but otherwise treated in an identical manner to the myocytes.

In experiments designed to test the dependence of Cav1.2 clustering responses to ISO on PKA activity, from each isolation performed, the groups of coverslip mounted cells that were prepared comprised: (i) control, unstimulated myocytes; (ii) 100 nM ISO-stimulated myocytes (as described above); (iii) myocytes incubated for 10 min with 10 μM H-89 only; (iv) myocytes incubated for 10 min with 10 μM H-89, followed by 8 min with H-89 and 100 nM ISO; (v) myocytes incubated for 1 h with 5 μM protein kinase inhibitor peptide (PKI) only; and (vi) myocytes incubated for 1 h with 5 μM PKI, followed by 8 min with PKI and 100 nM ISO. Groups 1 and 2 served as positive controls to confirm that the super-clustering response was present in cells from each isolation.

For immunostaining, coverslip adherent cells were fixed with ice-cold 100% methanol (Fisher Scientific, Fair Lawn, NJ, USA) for 5 min at −20°C, then thoroughly washed, and blocked for 1 h at room temperature in 50% SEA Block (Thermo Fisher Scientific, Rockford, IL, USA) and 0.5% v/v Triton X-100 (Sigma-Aldrich) in PBS. Primary antibody incubation in rabbit polyclonal anti-Cav1.2 FP1 antibody (provided by Johannes Hell; Davare et al., 2000; Buonarati et al., 2017) and/or chicken anti-GFP (Life Technologies) was performed overnight at 4°C in antibody solutions diluted to 10 μg mL⁻¹ in blocking buffer (20% SEA BLOCK, 0.5% Triton X-100). After thorough washing in PBS, secondary antibody incubation was performed for 1 h at room temperature with Alexa Fluor 647-conjugated donkey anti-rabbit and/or Alexa Fluor 555-conjugated goat anti-chicken (2 μg mL⁻¹; Life Technologies) in PBS. Final thorough washing in PBS was performed to remove excess antibody. Coverglass was then 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⁻¹ glucose oxidase, 34 μg mL⁻¹ catalase, 10% w/v glucose) and 100 mM MEA. Twinsil dental glue (Picodont, Wipperfürth, Germany) and aluminum tape (T205-1.0 - AT205; Thorlabs Inc., Newton, NJ, USA) was used to seal the coverglass in place and to exclude oxygen.

Cells were imaged in TIRF mode with 150 nm penetration depth, on a super resolution-ground state depletion (GSD) microscope (Leica Microsystems, Wetzlar, Germany) equipped with an oil-immersion HC PL APO 160 ×/1.43 NA super-resolution objective (Leica), four laser lines (405 nm/30 mW, 488 nm/300 mW, 532 nm/500 mW and 642 nm/500 mW) and an iXon3 electron multiplying charge coupled device (EM-CCD) camera (Andor). Images were collected at a frame rate of 100 Hz for 60,000 frames using Leica Application Suite software. For two-colour images, Alexa-647 was imaged first, followed by Alexa-555. Post-acquisition analysis was performed on images with a 10 nm pixel size and cluster area sizes were determined using binary masks of the images in ImageJ.

**Dynamic imaging in live cardiomyocytes**

Myocytes were isolated from AAV9-Cavβ2a-paGFP transduced cardiomyocytes and plated onto poly-L-lysine and laminin-coated glass coverslips. After a 10–15 min settling period to permit cell attachment to the laminin on the coverglass, myocytes were imaged on an IX83 inverted microscope (Olympus) with Cell-TIRF MITICO and an 60×/1.49 NA TIRF objective lens with an additional 1.6× magnification. Light at 405 nm was used to photo-activate the GFP on the Cavβ2a and a 488 nm laser was then used to excite GFP and a 488/561 nm dual band filter cube optimized for TIRF applications (Chroma Technology Corp., Bellows Falls, VT, USA) was used to detect any GFP fluorescence. Cells were perfused with Tyrode’s solution (140 mM NaCl, 5 mM KCl, 10 mM Hepes, 10 mM glucose, 2 mM CaCl₂ and 1 mM MgCl₂; pH adjusted to 7.4 with NaOH) and TIRF time series images were acquired at a rate of 10 frames s⁻¹. The first 300 frames of each experiment were collected under control conditions, then, at frame 301, 100 nM ISO was applied, and the remaining 1200–1600 frames were collected to determine the effect of ISO of channel mobility and expression. ImageJ/Fiji was used for image analysis to quantify the GFP fluorescence in the background subtracted TIRF footprint over the course of the experiments. For display purposes, TIRF images shown in Fig. 3A (see also the Supporting information, Movie S1) were stabilized to compensate for cell movement using the ‘Image Stabilizer’ plugin for ImageJ. A 10-pixel rolling ball background subtraction was applied, followed by 10 frame moving average and, finally, a minimum intensity projection was subtracted from all frames in the stack. In addition, super-resolution reconstructions were generated from the raw, unprocessed TIRF time series images using the NanoJ-SRRF plugin freely available in ImageJ/Fiji (Gustafsson et al., 2016). The super-resolution radial fluctuations (SRRF) algorithm can be used to analyse conventional diffraction-limited images (such as our TIRF images) to generate super-resolution...
reconstructions by splitting the individual pixels of the image into smaller units and then examining each pixel for radiality. The idea is that fluorophores emit from a point source, convolved with a point spread function that has an intrinsic radiality. This distinguishes them from the background, which should not have radiality. The algorithm then highlights the pixels with high radiality to achieve a super-resolution reconstruction. Resolutions in the range of ~64 nm have been reported using this algorithm on conventional TIRF images (Gustafsson et al., 2016).

Stepwise photobleaching

Isolated, coverglass-plated AAV9-CaVβ2α1-paGFP transduced myocytes were fixed for 10 min in 4% paraformaldehyde, washed and subsequently imaged on a DMi6000 B TIRF microscope (Leica) using a 160×/1.43 NA objective. The paGFP was photoactivated with a 405 nm laser and then excited with 488 nm laser light illumination during the collection of 2000 frame image stacks acquired at a rate of 30 Hz. Data were analysed and bleaching steps were manually counted as described previously (Dixon et al., 2015).

Electrophysiology

Single CaV1.2 channel currents (iCa) were recorded from transiently transfected tsA-201 cells using the cell-attached configuration of the patch clamp technique. Borosilicate glass pipettes with 5–10 MΩ resistance were filled with a solution containing 20 mM CaCl2, 10 mM Hepes and 500 nM BayK-8644 (pH adjusted to 7.2 using TEA-Cl) and used to obtain a gigaseal. Cells were bathed in a high K+ solution (145 mM KCl, 2 mM MgCl2, 0.1 mM CaCl2, 10 mM Hepes and 10 mM glucose; pH adjusted to 7.4 with HCl). Currents were elicited with a series of 300 ms depolarizing pulses from a holding potential of −80 mV to test potentials ranging from −60 to +80 mV. The resultant current–voltage relationships were plotted using Prism (GraphPad Software Inc., La Jolla, CA, USA). The voltage-dependence of conductance was obtained by converting currents to conductances using the equation, G = ICa/[test pulse potential − reversal potential of ICA], normalizing (G/Gmax) and plotting conductance vs. the test potential. Gating currents were also measured in tsA-201 cells before and after application of 100 nM ISO to attempt to visualize a recapitulation of the βAR-mediated regulation of the channels seen robustly in ventricular myocytes. In these experiments, pipettes were filled with a Cs-based internal solution (7 mM Cs-aspartate, 20 mM CsCl, 1 mM MgCl2, 10 mM Hepes, 10 mM EGTA and 5 mM MgATP; pH adjusted to 7.2 with CsOH) and bathed in an external solution with the composition: 5 mM CsCl, 10 mM Hepes, 10 mM glucose, 113 mM NMDG, 1 mM MgCl2 and 20 mM CaCl2 (pH adjusted to 7.4 with HCl). Currents were elicited with a series of 300 ms depolarizing pulses from a holding potential of −80 mV to test potentials ranging from −60 to +80 mV. The resultant current–voltage relationships were plotted using Prism (GraphPad Software Inc., La Jolla, CA, USA). The voltage-dependence of conductance was obtained by converting currents to conductances using the equation, G = ICa/[test pulse potential − reversal potential of ICA], normalizing (G/Gmax) and plotting conductance vs. the test potential. Gating currents were also measured in tsA-201 cells before and after application of 100 nM ISO, by first running an I–V protocol, and calculating the reversal potential for Ca2+ from the I–V plot. Leak and capacitive currents were compensated for. The voltage protocol was then modified to step to ECa and record currents in the absence of ion flux, leaving only the gating current. We integrated the gating current to obtain the ON gating charge (QON).
long voltage step to –40 mV immediately preceded the depolarization pulses to inactivate voltage-gated Na\(^+\) channels.

Ca\(^{2+}\) sparklet recording and analysis

Optical recordings of Ca\(_V\)1.2 channel openings in transiently transfected tsA-201 cells or ventricular myocytes were performed by voltage clamping the cells at –80 mV using the whole cell configuration of the patch clamp technique, and dialysing in the tripotassium salt of the Ca\(^{2+}\) indicator Rhod-2 (200 \(\mu\)M) via the patch pipette. The membrane and near membrane area of these cells were imaged using an IX83 inverted microscope (Olympus) with Cell-TIRF MITICO and an 60\(\times\)/1.49 NA TIRF objective lens. This enabled capture of sub-sarcosomal Ca\(^{2+}\) signals (Ca\(^{2+}\) sparklets). Stacks of images were acquired at a rate of ~100 Hz using an iXon Ultra 888 back thinned EM-CCD camera (Andor). Cells were continuously perfused with the 20 \(\mu\)M Ca\(^{2+}\), 10 \(\mu\)M EGTA external solution described above. For experiments performed in ventricular myocytes, the sarco/endoplasmic reticulum Ca\(^{2+}\)-ATPase pump inhibitor thapsigargin (10 \(\mu\)M) was used to reduce SR Ca\(^{2+}\) load and prevent Ca\(^{2+}\) sparks to ensure specific recording of Ca\(^{2+}\) sparklets (Zima et al., 2008). TIRF imaging to create a shallow evanescent field, along with the Ca\(^{2+}\) indicator and EGTA buffer combination, permits the fluorescence labelling of Ca\(^{2+}\) as it enters the cell and binds to the fast Ca\(^{2+}\) indicator Rhod-2, before EGTA buffers Ca\(^{2+}\) to restrict the signal to the point of entry. Fluorescence signals were converted to intracellular Ca\(^{2+}\) concentrations using the \(F_{\text{max}}\) equation (Maravall et al., 2000). Sparklets were identified and their time courses analysed using a custom-written MATLAB script (MathWorks Inc., Natick, MA, USA). Sparklet activity was quantified as \(n/P\), where \(n\) is the number of quantal levels and \(P\) is the probability that a sparklet occurs. Another parameter, sparklet site density, is defined as the number of observable sparklet sites per unit of cell footprint area.

Western blots

Wild-type C57BL/6J mice were administered either an i.p. injection of saline (control) or 10 mg kg\(^{-1}\) ISO. After 20 min, mice were killed with an anaesthetic overdose of isoflurane (5%) and hearts were collected via sharp dissection. The hearts were subsequently homogenized in osmotic lysis buffer (25 mmol L\(^{-1}\) Tris-HCl, pH 7.4, 5 mmol L\(^{-1}\) EDTA, pH 8.0, 1 mmol L\(^{-1}\) phenylmethylsulphonyl fluoride (PMSF), 2 \(\mu\)g mL\(^{-1}\) aprotinin and 2 \(\mu\)g mL\(^{-1}\) leupeptin). Cell debris and nuclei were removed by centrifugation at 950 \(g\) for 5 min at 4\(^\circ\)C. The supernatant was then centrifuged at 37,500 \(g\) for 30 min at 4\(^\circ\)C using a Type 70.1 Ti Fixed-Angle Titanium Rotor (Beckman Coulter, Indianapolis, IN, USA). Pellets representing the sarclemma/plasma membrane (PM) fraction were resuspended in Triton lysis buffer (25 mmol L\(^{-1}\) Hepes, pH 7.4, 5 mmol L\(^{-1}\) EDTA, 150 mmol L\(^{-1}\) NaCl, 1% Triton X-100, and protease inhibitors containing 2 mmol L\(^{-1}\) Na\(_3\)VO\(_4\), 1 mmol L\(^{-1}\) PMSF, 10 mmol L\(^{-1}\) NaF, 10 \(\mu\)g mL\(^{-1}\) aprotinin, 5 mmol L\(^{-1}\) bestatin, 10 \(\mu\)g mL\(^{-1}\) leupeptin and 2 \(\mu\)g mL\(^{-1}\) pepstatin A) for western blotting. The supernatant was further centrifuged at 200,000 \(g\) for 1 h at 4\(^\circ\)C; the pellets obtained in this step contained internal membrane compartment fractions and were resuspended in Triton lysis buffer, as described above, for western blotting. Equal amounts of protein were resolved by SDS-PAGE and detected with anti-Ca\(_V\)1.2 FP1 antibody, anti-Rab4A antibody (D-20; rabbit polyclonal IgG; SC-312; Santa Cruz Biotechnology, Dallas, TX, USA) and anti-insulin receptor \(\beta\) antibody (L55B10; mouse monoclonal IgG1; 3020; Cell Signaling Technology, Danvers, MA, USA). All primary antibodies were revealed with IRDye 800 CW secondary antibodies using Odyssey detection system (Li-Cor Biosciences, Lincoln, NE, USA). The optical density of the bands was analysed with ImageJ (https://imagej.nih.gov/ij).

Chemicals and statistical analysis

Chemical reagents were obtained from Sigma-Aldrich unless otherwise stated. Data are reported as the mean ± SEM. \(N\) reflects the number of animals used in a dataset, whereas \(n\) reflects the number of cells. Actual \(P\) values are provided unless the \(P\) value is extremely low, when it is simply stated as \(P < 0.001\). Comparison of paired datasets was performed using Student’s \(t\) tests if the data passed a normality test. Otherwise, a non-parametric test was performed. \(P < 0.05\) was considered statistically significant (* \(P < 0.05\); ** \(P < 0.01\); *** \(P < 0.001\)).

Results

\(\beta\)AR stimulation promotes enhanced Ca\(_V\)1.2–Ca\(_V\)1.2 channel interactions

Ca\(_V\)1.2 channel co-operativity requires physical interactions between adjacent channels (Dixon et al., 2012; Dixon et al., 2015). Thus, our first experimental series investigated the effect of \(\beta\)AR stimulation on Ca\(_V\)1.2–Ca\(_V\)1.2 channel interactions using an optical assay of protein–protein interactions, namely BiFC (Fig. 1). We have previously used this approach to assay the Ca\(^{2+}\) and CaM-dependence of Ca\(_V\)1.2–Ca\(_V\)1.2 interactions in tsA201 cells (Dixon et al., 2015; Moreno et al., 2016). For this, we generated two fusion proteins, Ca\(_V\)1.2-VC155 and Ca\(_V\)1.2-VN155(I152L), by fusing the N- or C- terminal fragment of the venus fluorescent protein (FP) onto the
C-terminal of the Cav1.2 α1c subunit. In the present study, the channels were transiently transfected into tsA201 cells and the degree of venus reconstitution was assayed under control and ISO stimulated conditions. BiFC complex formation occurs when the two fragments of the FP interact and fold upon association. This leads to the irreversible reconstitution of the mature venus FP and a visible fluorescence read-out of the protein–protein interaction in the form of venus fluorescence as summarized by the cartoon in Fig. 1A. The time taken for protein folding varies but can be quite a protracted affair as demonstrated previously in a study examining the kinetics of the YFP BiFC complex formation, which reported that, although complexes could be appreciated in as little as 10 min, they continued to form over a period of at least 8 h (Robida & Kerppola, 2009). Because folding of venus fragments occurs at an accelerated rate compared to YFP (Nagai et al., 2002), we therefore monitored venus fluorescence from the TIRF footprint of tsA201 cells expressing Cav1.2-VN155(I152L) and Cav1.2-VC155 over a period of 2 h in cells maintained at 37°C with 95:5 O2:CO2 in a stage top incubator. Under control conditions, venus F/F0 increased with a τ of 18.57 min (n = 18) (Fig. 1B and D). Spontaneous interactions such as these in unstimulated cells are not unexpected, given that tsA-201 cells have quite a depolarized resting

Figure 1. β-AR stimulation promotes enhanced Cav1.2–Cav1.2 channel interactions

A, BiFC experimental strategy. Left: non-interacting channels with a C-terminal fusion of N- or C-terminal venus FP fragments. Right: application of 100 nM ISO stimulates endogenous β2-ARs, increasing cAMP and activating PKA that acts on the channels to promote physical interactions, resulting in reconstitution of the intact venus protein and fluorescence emission. B–C, TIRF image time series obtained from tsA-201 cells expressing Cav1.2-VN and Cav1.2-VC, incubated at 37°C without (control) (B) or with 100 nM ISO (C). Images received a one pixel median filter for display purposes. D, time course of the changes in normalized venus fluorescence emission (F/F0) over 2 h for control (black/grey) and ISO stimulated (blue) conditions. Circles indicate mean venus intensity at each time point, dashed lines and area fills indicate the SEM. Data were fit with mono-exponential functions to calculate the time constant (τ) of the interactions. E, time course of the ISO sensitive component of the interactions.
membrane potential of \( \sim -25 \) mV (Kirkton & Bursac, 2011), which would permit a small amount of \( \text{Ca}^{2+} \) influx through these voltage-gated channels and could facilitate \( \text{Ca}^{2+} \)-dependent physical interactions. Application of 100 nM ISO (\( n = 10 \)) at the onset of the experiment stimulated endogenous \( \beta_2 \)-ARs (Atwood et al., 2011) and resulted in a significantly larger increase in venus \( F/F_0 \) compared to the control (ISO = 2.84 ± 0.46 \( F/F_0 \); \( \text{control} = 1.71 \pm 0.21 \ F/F_0; \ P = 0.015 \)) (Fig. 1C and D). \( \text{CaV}_{1.2} \)-\( \text{CaV}_{1.2} \) interactions increased steadily over the time-course of the experiment with a \( \tau \) of 27.31 min, notably slower than that of the unstimulated control. To calculate the ISO-sensitive component of the interactions, we subtracted the control data from the ISO data to generate the data in Fig. 1E. The time constant of the ISO-sensitive interactions was 35.76 min. Collectively, these BiFC data suggest that \( \beta_2 \)-AR stimulation triggers enhanced levels of physical C-tail to C-tail association between \( \text{CaV}_{1.2} \) channels.

**ISO promotes super-clustering of \( \text{CaV}_{1.2} \) channels**

Enhanced spatial proximity between channels would be predicted to increase \( \text{CaV}_{1.2} \)-\( \text{CaV}_{1.2} \) interaction probability. Thus, to investigate the spatial distribution of \( \text{CaV}_{1.2} \) channels under control and ISO stimulated conditions, we examined fixed, anti-\( \text{CaV}_{1.2} \) immunostained cardiomyocytes and transfected tsA-201 cells using super-resolution nanoscopy. Under control conditions, the average \( \text{CaV}_{1.2} \) channel area in ventricular myocytes was 3698 ± 59 nm\(^2\) (\( N = 11, n = 54 \) cells) (Fig. 2A and D). In myocytes stimulated with 100 nM ISO for 8 min prior...
to fixation, the average CaV1.2 cluster area was ~17% larger than controls (4327 ± 92 nm², N = 11, n = 61) (Fig. 2A and D), with large ‘super-clusters’ evident in the GSD images. A significant, ISO-induced augmentation in CaV1.2 channel cluster area was also evident in CaV1.2 expressing tsA-201 cells (Fig. 3). These results suggest that βAR stimulation initiates a nanoscale redistribution of CaV1.2 channels within cardiomyocytes and tsA-201 cells that favours enhanced levels of physical interactions between the channels.

**Super-clustering in ventricular myocytes is PKA-dependent**

To determine whether the observed ISO-induced super-clustering of CaV1.2 channels depended on PKA activity, further super-resolution nanoscopy experiments were performed on isolated ventricular myocytes in the absence and presence of the PKA inhibitors H-89 (Hidaka & Kobayashi, 1992) or the more specific, membrane-permeable, myristoylated 14–22 amide (referred to in the present study as PKI) (Murray, 2008). Positive controls were performed on each batch of isolated cells to confirm that the super-clustering response to ISO was present in the absence of PKA inhibition and to give added strength to any observed effects. Average CaV1.2 channel cluster area was not significantly altered by ISO treatment when PKA was inhibited with either 10 μM H-89 (controlH-89 3879 ± 103 nm², n = 31; ISOH-89 3818 ± 172 nm², n = 31; P = 0.99, N = 4) (Fig. 2B and D) or 5 μM PKI (controlPKI 3657 ± 123 nm², n = 16; ISOPKI 3526 ± 165 nm², n = 16; P = 0.99, N = 3) (Fig. 2C and D). A one-way ANOVA with Tukey’s multiple comparison test indicated that all of the PKA-inhibited groups were not significantly different from the control (no inhibitor) group, although they were significantly different from the ISO-stimulated (no inhibitor) group (Fig. 2D). These results suggest that the super-clustering response to ISO is a PKA-dependent phenomenon.

**ISO-stimulated dynamic augmentation of sarcolemmal channel abundance**

There are at least two possible, not necessarily mutually exclusive mechanisms that could explain the enhanced CaV1.2 clustering response to ISO. First, smaller CaV1.2 channel clusters could fuse together to form super-clusters. Alternatively, enhanced trafficking and insertion of CaV1.2 channels into the sarcolemma could explain these results. To distinguish between these two scenarios, we aimed to examine the dynamics of CaV1.2 channels in freshly dissociated cardiomyocytes. These cells are impervious to chemical transfection techniques and rapidly lose their unique ion channel complement and specialized architecture as they dedifferentiate in culture (Louch et al., 2011). Thus, expression of FP-tagged channels in cardiomyocytes to monitor their trafficking and dynamics presents a technical challenge. We have previously demonstrated successful transduction of mouse cardiomyocytes in vivo with various FP-tagged constructs using retro-orbital injections of AAV9 (Dixon et al., 2015; Drum et al., 2016; Moreno et al., 2016). This approach allows us to circumvent the need to culture the cells and lets the transduction proceed in vivo. A limitation of AAV9s is their restrictive packaging capacity of ~5 kb (Vinge et al., 2008; Louch et al., 2011). This precludes expression of the ~6.6 kb α1c pore-forming subunit of the channel. We therefore chose to package one of the smaller (1.8 kb) auxiliary subunits of the CaV1.2 channel, CaVβ2a, which binds to the α1c with a 1:1 stoichiometry (Dalton et al., 2005). We fused CaVβ2a to a pEGFP to permit live-cell tracking of the subunit when expressed in cardiomyocytes. Note that the recent use of this approach by our group revealed CaVβ2a-associated fluorescence with similar cluster size and distributions to those observed in the present study (Dixon et al., 2015).

Transduced cardiomyocytes were imaged using TIRF microscopy to narrow the evanescent field and focus on channels at the plasma membrane. After photoactivation, a time series of TIRF images was collected at a rate of 10 frames s⁻¹ to monitor CaV1.2-paGFP movements during an initial 300 frame control period before wash-in of 100 nM ISO and a subsequent 1200–1600 frames to
capture the movements in the presence of this agonist. In the mean intensity analysis, presented in Fig. 4C, the sum of the intensity values of all the pixels in the TIRF footprint of the cell is divided by the number of pixels in the footprint. To obtain the \( F/F_0 \), the values are further normalized to the mean intensity measured in the first frame of the time series. If the super-clustering response was generated solely by individual or small aggregates of \( \text{CaV}_\beta_{2a} \)-paGFP fusing with one another to form larger, more readily detectable clusters then one would predict that the mean intensity would not change over the course of the experiment, rather the distribution of the existing FPs would be altered. We find instead that ISO stimulated a dynamic increase in \( \text{CaV}_\beta_{2a} \)-paGFP abundance in the TIRF footprint of cardiomyocytes (\( N = 2, n = 6 \)) (Fig. 4A–C; see also the Supporting information, Movie S1). Indeed, the average maximal response to ISO amounted to a 44.9 ± 6.2% increase \( (F/F_0) \) within the time-course of the experiment (Fig. 4C). This dynamic elevation in GFP intensity in the TIRF footprint can be visualized (see the Supporting information, Movie S1) as the appearance of discrete clusters of fluorescence presumably originating from a site deeper within the cell, outside the TIRF illumination field. This finding does not preclude the possibility that there is some aggregation of already present \( \text{CaV}_\beta_{2a} \)-paGFP, although our data suggest that this does not form the major basis of the super-clustering response.

To further investigate whether this dynamic channel insertion results in super-clustering, we generated super-resolution reconstructions from our TIRF image stacks using the NanoJ-SRRF plugin for ImageJ, allowing us to resolve discrete clusters of \( \text{CaV}_\beta_{2a} \)-paGFP (Fig. 4A–C) (Gustafsson et al., 2016). This open source plugin permits the post-acquisition extraction of live-cell super-resolution information from stacks of images acquired using diffraction-limited microscopy techniques such as TIRF or confocal microscopy. It is clear from these images that myocyte stimulation with ISO triggers dynamic augmentation of sarcolemmal \( \text{CaV}_\beta_{2a} \)-paGFP expression and super-clustering.

Overexpression of \( \text{CaV}_\beta_{2a} \) in cardiomyocytes does not affect basal \( \text{CaV}_1.2 \) clustering

Because we are using an overexpression paradigm to visualize \( \text{CaV}_\beta_{2a} \)-paGFP in these cells, on a background with native, non-fluorescent \( \text{CaV}_\beta_{2a} \), we are at best, observing only a small subset of the \( \text{CaV}_\beta_{2a} \) expression on the sarcolemma. Endogenously expressed \( \text{CaV}_\beta_{2a} \) probably constitutes the vast majority of the population of this subunit interacting with the \( \alpha_{1c} \). This probably explains why complete T-tubule localization pattern is not seen for \( \text{CaV}_\beta_{2a} \)-paGFP. However, upon stimulation with ISO, examination of the super-resolution reconstructions revealed discrete regions of the cells in which the pattern of \( \text{CaV}_\beta_{2a} \)-paGFP expression is consistent with the expected physiological range of sarcomere lengths and a separation of 1.6–2.3 \( \mu \)m between T-tubules (Fig. 4B).

To further examine the distribution of transduced \( \text{CaV}_\beta_{2a} \)-paGFP and its relative colocalization with endogenous \( \text{CaV}_1.2 \) channels, super-resolution GSD experiments were performed before and after ISO using an anti-\( \text{CaV}_1.2 \) and anti-GFP double labelling approach. In this dataset, the mean \( \text{CaV}_1.2 \) channel cluster area was 3253 ± 157 nm\(^2\) under control conditions (\( n = 5 \)), swelling to 4086 ± 151 nm\(^2\) in ISO treated myocytes (\( n = 6, P = 0.004 \)) (Fig. 4D–F). The magnitude of the response to ISO (≈26%), and the basal and ISO stimulated cluster areas were not significantly different from those observed in control untransduced cells (\( P = 0.058 \)), indicating that overexpression of the \( \text{CaV}_\beta_{2a} \) subunit did not itself artificially increase basal channel cluster sizes or the dynamic augmentation response to ISO. As in the dynamic imaging experiments, the distribution of \( \text{CaV}_\beta_{2a} \)-paGFP did not display a complete T-tubule localization pattern, although the added perspective given by the \( \text{CaV}_1.2 \) channel co-label confirms that the transduced subunits are indeed occupying positions along the T-tubes where they colocalize with \( \text{CaV}_1.2 \) clusters (Fig. 4D–F). Stimulation with 100 nM ISO prior to fixation also increased the mean \( \text{CaV}_\beta_{2a} \)-paGFP cluster area from 4141 ± 535 nm\(^2\) in control (\( n = 5 \)) to 5675 ± 453 nm\(^2\) (\( n = 6; P = 0.026 \)), reflecting the increased expression and larger post-ISO \( \text{CaV}_\beta_{2a} \)-paGFP cluster sizes observed in the dynamic experiments.

Further quantification of the \( \text{CaV}_\beta_{2a} \)-paGFP response to ISO was performed by examining single-particle photobleaching in transduced cardiomyocytes (Ulbrich & Isacoff, 2007). The number of \( \text{CaV}_\beta_{2a} \)-paGFP molecules within individual clusters was determined by counting the number of steps in the fluorescence as the fused paGFP tag bleached. Discrete bleaching steps become difficult to resolve in clusters containing more than five channels (Ulbrich & Isacoff, 2007). Therefore, instead, we measured the amplitude of a single paGFP bleaching-step by constructing an all-points histogram of all resolvable bleaching-steps (104 clusters, \( n = 11 \) cells). The cluster distribution was then fit with a two-component Gaussian function, and the centre of the peak corresponding to the smaller step size (23.61 ± 0.32 AU) was assumed to represent the quantal amplitude of a single paGFP photobleaching-step (Fig 5A). The number of \( \text{CaV}_\beta_{2a} \)-paGFP molecules per cluster was then calculated by subtracting the initial from the final fluorescence intensity of each cluster and dividing the result by the quantal value. An important caveat of this experiment is that we may be underestimating the number of channels per cluster. The first underestimation factor is a result.
**Figure 4. ISO stimulates dynamic augmentation of CaV1.2 channel sarcolemmal abundance**

A, top: diffraction-limited TIRF images of photoactivated GFP fluorescence emission from CaVβ2α-paGFP transduced cardiomyocytes before (left) and after application of 100 nM ISO (right). Middle row: NanoJ-SRRF generated super-resolution images of the same cell. Bottom row: composites of the TIRF and NanoJ-SRRF images. B, magnified NanoJ-SRRF images before and after ISO. Locations on the larger images are indicated by yellow boxes in (A). Intensity plot profiles across the dotted yellow lines labelled (i) and (ii) are displayed below where the black trace represents the control plot and the blue represents a plot from the same region after ISO. C, time course of the changes in normalized photoactivated CaVβ2α-paGFP emission (F/F0) during a 30 s control period and a subsequent 120 s exposure to ISO. D–E, super-resolution images of fixed control (D) or ISO-stimulated (E) ventricular myocytes double labelled to show CaV1.2 (left) and GFP (middle) distribution. Colocalized pixels appear yellow in the merged image (right) and confirm t-tubule localization of CaVβ2α-paGFP. F, scatter plot showing the mean CaV1.2 and CaVβ2α-paGFP cluster sizes, in control (black) and ISO-stimulated (blue) ventricular myocytes. G and H, (i) representative western blots illustrating the detection of CaV1.2 αC and insulin receptor β in plasma membrane.
of the non-unity (~0.8 based on previous studies) probability that any single GFP molecule is fluorescent (Ulbrich & Isacoff, 2007). The second factor is the presence of endogenous Cavβ subunits that, as discussed above, are probably associated with the majority of channels and thus with this approach, we can only make a reasonable estimation of the minimum number of channels per cluster. With that said, we did find a significantly altered distribution of the channel occupancy in sarcolemmal clusters after application of 100 nM ISO (Fig. 5B). The mean number of Cavβ2–paGFP molecules per cluster was 6.62 ± 0.16 in unstimulated control myocytes (calculated from 983 spots; n = 14, N = 3). The frequency distribution of all clusters examined revealed a dominant population at 5.17 ± 0.07 Cavβ2–paGFP per cluster (Fig. 5B and C). With ISO stimulation, the mean number of Cavβ2–paGFP per cluster was 8.75 ± 0.22 (calculated from 1,162 spots, n = 15, N = 3), representing a 32% increase compared to controls. Frequency distribution analysis revealed two dominant populations of clusters. The first was a similar size to control with 4.02 ± 0.05 Cavβ2–paGFP molecules per cluster, whereas the second had 8.33 ± 0.23 (Fig. 5B and C). These results provide further validation of our dynamic imaging data and suggest that βAR stimulation triggers enhanced Cavβ2–paGFP clustering and potentially increases Cav1.2 channel abundance at the sarcolemma.

### ISO increases sarcolemmal expression of the pore-forming α1c subunit in heart lysates

Biochemical techniques were employed to more directly test the postulate that ISO increases sarcolemmal Cav1.2 channel expression. Using differential centrifugation, we isolated plasma membrane and internal membrane fractions from whole heart lysates extracted from mice killed 20 min post-I.P. injection of saline (control) or 10 mg kg⁻¹ ISO. Detection of insulin receptor β was used to confirm the identity of the plasma membrane fraction, and the presence of endosomal membranes in the internal membrane fraction was corroborated by detection of Rab4A. Consistent with the hypothesis that a PKA-dependent, readily-insertable pool of Cav1.2 channels forms sarcolemmal super-clusters, western blots revealed that ISO induced a 28.1 ± 0.1% increase in Cav1.2 channel expression in the plasma membrane (control: N = 6, ISO: N = 7, P = 0.03) (Fig. 4G) and an almost equivalent, reciprocal ~21% decrease in the amount of Cav1.2 in the internal membrane fraction (control: N = 7, ISO: N = 6, P = 0.12) (Fig. 4H). Collectively, these western blots results, along with our super-resolution and dynamic imaging experiments, confirm that Cav1.2 channel expression in the sarcolemma is augmented in response to βAR stimulation with ISO.

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**Figure 5.** ISO treatment increases stepwise photobleaching assessed Cav1.2 channel cluster size

A, frequency distribution of photobleaching step sizes measured experiments performed on Cavβ2–paGFP transduced myocytes. The data were well-fit (r² value shown) by a two-component Gaussian function with peaks at ~23 and 46 representing the fluorescence intensity change attributable to one and two-GFPs bleaching, respectively. B, frequency distribution of bleaching steps obtained from histogram Cavβ2–paGFP transduced myocytes under control unstimulated conditions (grey; fit with a single Gaussian) or after application of 100 nM ISO (blue; fit with a two-component Gaussian). C, examples of bleaching steps for Cavβ2–paGFP associated with Cav1.2 channels.
ISO induced super-clustering of CaV1.2 promotes enhanced co-operativity and amplifies Ca\(^{2+}\) influx in tsA-201 cells

We next aimed to determine whether the ISO-stimulated nanostructural rearrangement of CaV1.2 channels has any appreciable consequences for channel function. We predicted that the increased proximity of channels within larger super-clusters could favour co-operative interactions of the channels. We tested this prediction initially in transiently transfected tsA-201 cells by examining the single CaV1.2 channel activity using electrophysiological (cell-attached patch clamp) approaches (Fig. 6). The utility of heterologous expressions systems in the study of βAR of CaV1.2 channels is debatable. Several studies have noted that current augmentation in response to β-agonists is unreliable and less vigorous in these models than in cardiomyocytes (Perez-Reyes et al., 1994; Charnet et al., 1995; Zong et al., 1995; Gao et al., 1997; Weiss et al., 2013). Indeed, we also had variable success in reproducing βAR regulation of CaV1.2 in tsA-201 cells, although we were able to do so in three separate cells where we observed augmented calcium currents and the characteristic leftward shift in current–voltage relationship (Fig. 7A–C). However, many tens of cells failed to show the response. In the cells that did respond, the fold-increase in peak current for each individual cell was 1.65, 1.49 and 1.42. These values are similar to the 1.6- to 2.8-fold increase in activity observed routinely in cardiomyocytes in the present study (1.63 ± 0.10-fold increase, N = 3, n = 8) (Fig. 8A–C) and in previous studies (Muth et al., 1999; Lemke et al., 2008; Nichols et al., 2010; Brandmayr et al., 2012) and suggest that a reasonable, albeit unreliable, βAR-mediated augmentation of current is achievable in these cells. Intriguingly, in the cell-attached patch configuration, we noted that augmentations in channel activity occurred much more reliably such that 55.6% of sampled patches displayed increased activity in response to ISO. Therefore, to be included in our analysis regimes, we set the criteria that, cells would have to display an increase in \(N_{Po}\) in response to ISO and exhibit multichannel

![Co-operative gating behaviour of heterologously expressed CaV1.2 channels is promoted by ISO](image_url)

**Figure 6.** Co-operative gating behaviour of heterologously expressed CaV1.2 channels is promoted by ISO

_A and B_, representative \(i_{Ca}\) traces and accompanying amplitude histograms from tsA-201 cells expressing CaV1.2 during depolarization steps from –80 mV to –30 mV before (A) and during the application of 100 nM ISO (B). Note that traces in (B) are from the same cell as those in (A). Grey boxes highlight overt co-operative gating episodes. Amplitude histograms for control and ISO were fit with multicomponent Gaussian functions (red lines). _C_, current averaged over multiple sweeps performed on the cell shown in (A) and (B). _D–F_, paired symbol and line plots showing the peak ensemble current (_D_), \(P_o\) (_E_) and apparent \(N_{f}\) (_F_) for five cells before and during ISO application.

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openings. Accordingly, using Ca\textsuperscript{2+} as the charge carrier, voltage steps from –80 mV to –30 mV were used to elicit CaV1.2 channel openings with a unitary amplitude of -0.47 ± 0.03 pA (Fig. 6A). Application of 100 nM ISO resulted in increased activity of the channels in the patch (Fig. 6B) but did not alter the unitary amplitude of the single channel current, which remained at 0.51 ± 0.02 pA (P = 0.27). Ensemble currents constructed by averaging single channel events before or in the presence of ISO, had activation and inactivation time courses commensurate with macroscopic currents (Fig. 6C). On average, a 1.34-fold increase in the ensemble current amplitude was observed after ISO (n = 5, P = 0.03) (Fig. 6D).

Macroscopic current (I_{Ca}) is the product of unitary current amplitude (i_{Ca}), open probability (P_o) and the number of functional channels (N_f). Because i_{Ca} did not change, we examined the other two variables. Application of ISO produced a 4-fold increase in P_o (0.01 ± 0.005 to 0.04 ± 0.008; n = 5, P = 0.01) (Fig. 6E) and a 1.5-fold increase in N_f (3 ± 1 to 5 ± 1, n = 5, P = 0.03) (Fig. 6F). Intriguingly, in many instances, we noted that depolarization in the presence of ISO revealed the concerted opening and closing of multiple channels (Fig. 6B, grey boxes). It is highly improbable that such temporally correlated multichannel openings could occur if these channels were only capable of acting as single, independently gating units. Instead, this behaviour suggests communication between the channels in the patch such that the opening of one channel can influence and co-ordinate the gating behaviour of its neighbors, leading to co-operative channel interactions.

The increase in N_f was further investigated by examining gating currents in CaV1.2-expressing tsA-201 cells before and after 100 nM ISO (Fig. 7D–F). Because ionic current was not blocked, we recorded gating currents at the reversal potential where ion flux is nil. Depolarizing pulses to E_{rev} should move the maximal gating charge, which is proportional to the total number of CaV1.2 channels expressed in the membrane of the cell according to the relationship Q_{ON} = N \times q (where Q_{ON} is the time integral of the ‘ON’gating charge and N is the number of channels in the membrane). A 16.7% increase in the number of channels in the membrane was found to occur upon application of ISO (n = 5) (Fig. 7F). These data are consistent with our live cell imaging observations.

![Figure 7. β-AR stimulation increases the number of functional channels in tsA-201 cell membranes](image-url)
and suggest that activation of βARs is associated with an increase in the number of CaV1.2 channels in the plasma membrane.

Next, we recorded Ca^{2+} sparklets from transiently transfected tsA-201 cells. Using this optical approach, we can visualize channel openings over the entire TIRF-footprint of the cell (Navedo et al., 2005; Nystoriak et al., 2013). This presents a broader view of the single channel activity in the cell than the limited one we obtain within the confines of cell-attached patches. As shown in Supporting information (Movie S2), the utility of this approach is validated because many sites can identified throughout the cell footprint where there are no apparent functional channels, whereas other sites are quiescent under control conditions but become active after application of ISO. Consistent with our electrophysiology data, application of ISO increased CaV1.2 channel activity (nP) 3-fold (from 0.10 ± 0.03 to 0.30 ± 0.32, n = 6 and 4, respectively) (Fig. 9A–C). We observed an increase in activity in all patches exposed to ISO. The number of active sparklet sites per μm^2 (i.e. sparklet density) increased 2.5-fold with ISO, from 0.02 ± 0.005 to 0.05 ± 0.01 sites μm^-2 (P = 0.009) (Fig. 9D). We analysed these sparklet traces using a coupled Markov chain model to obtain a measure of the co-operative of the channel gating (e.g. coupling coefficient, κ) underlying these discrete calcium influx events. κ values lie within a 0–1 range, where a 0 value indicates channels that always gate independently and 1 indicates channels that always gate together in multichannel groups (Navedo et al., 2010). Thus, any κ > 0 indicates some degree of co-operativity. The mean κ value of sparklet events was significantly higher after the application of ISO suggesting increased levels of co-operative gating behaviour (P = 0.03). This conclusion is also supported by the overtly co-operative behaviour, with several channels opening and/or closing in unison as seen in the representative traces in Fig. 9B. Collectively, these data suggest that application of ISO promotes increased activity and co-operativity between groups of CaV1.2 channels in tsA-201 cells. This creates localized regions of very high activity channels, which permits the influx of relatively large amounts of Ca^{2+} compared to that seen at the same site in the unstimulated cell.

**ISO promotes co-operative gating behaviour and amplified Ca^{2+} influx in ventricular myocytes**

Although we were able to occasionally observe reconstituted PKA modulation of recombinant CaV1.2 channels in tsA-201 cells, the lack of robust and reproducible increase in I_{Ca} upon application of ISO (except for the three cells mentioned above) is in agreement with the results of previous studies (Perez-Reyes et al., 1994; Zong et al., 1995; Gao et al., 1997; Weiss et al., 2013) and invites the criticism that any mechanistic insight gleaned from experiments in heterologous expression systems may not necessarily align with the mechanism at play in ventricular myocytes. We therefore turned our attention to freshly isolated adult mouse ventricular myocytes. If these same phenomena were to occur in ventricular myocytes during fight-or-flight, this could provide a means to tune Ca^{2+} influx to meet increased haemodynamic and metabolic demands. Therefore, we next measured elementary CaV1.2 channel currents from ventricular myocytes, before and after application of 100 nM ISO during a step depolarization to −30 mV (Fig. 10A and B). Unitary current amplitude was −0.44 ± 0.10 pA under control conditions and remained at a similar level (−0.49 ± 0.01 pA) after application of ISO (P = 0.4376, n = 6, N = 5) (Fig. 10A and B). As in tsA-201 cells, application of ISO augmented channel activity, as reflected by a 2-fold increase in the peak amplitude of the average ensemble current from −0.42 ± 0.09 pA in control to −0.82 ± 0.08 pA in ISO (P = 0.0076) (Fig. 10C and D). Channel P_o increased from 0.01 ± 0.008 in control

![Image of Figure 8](image-url)
ISO promotes co-operative interactions between CaV1.2 channels in ventricular myocytes. These data strongly support the hypothesis that ISO stimulates CaV1.2 clustering and co-operativity (Clatot et al., 2017). These data strongly support the hypothesis that ISO promotes co-operative interactions between CaV1.2 channels in ventricular myocytes.

**Discussion**

Our data add a new level of understanding of βAR-mediated regulation of CaV1.2 channels and provide three novel insights into this phenomenon. We find that acute stimulation of βARs with ISO promotes: (i) enhanced physical, allosteric interactions between CaV1.2 channel C-terminal tails; (ii) a PKA-dependent, dynamic nanoscale redistribution of CaV1.2 channels, manifesting in super-clustering of the channels on the plasma membrane of cardiomyocytes and transfected tsA-201 cells; and (iii) an enhanced proximity between channels that facilitates co-operative interactions, leading to amplification of Ca2+ influx during fight-or-flight. We propose that this dynamic clustering and co-operativity response to ISO represents a newly appreciated means to tune EC-coupling in ventricular myocytes to meet acute increases in haemodynamic and metabolic demands during the fight-or-flight response.

**A subsarcolemmal, presynthesized pool of channels to tune excitability?**

In dynamic imaging experiments, we report that the response to ISO (increased CaV1.2β2a-paGFP F/F0) could be detected immediately as the drug washed into the bath at room temperature (Fig. 4C), ruling out de novo protein synthesis and transport through the secretory pathway which takes more on the order of several minutes to hours (Lippincott-Schwartz et al., 2000) and, instead, favouring a model in which a nearby sub-sarcolemmal pool of presynthesized CaV1.2 channels exists in cardiomyocytes. Dynamic insertion of β-subunit-associated channels into the sarcolemma at defined hubs could explain the super-clustering response to ISO seen in Fig. 2. An additional implication is that βAR activation triggers enhanced trafficking not only of the α1c-pore forming subunit to the membrane, but also a possible co-transport of CaV1.2β2a-auxiliary subunits. We cannot rule out the possibility that ISO increases the rate of dynamic association and dissociation of CaV1.2β2a with the pore-forming α1c. However, western blots showing enhanced α1c expression at the sarcolemma post-ISO and super-resolution imaging data showing super-clustered α1c post-ISO suggest that it is not only β-subunit mobilization that is altered in response to ISO.

β-subunits can themselves enhance CaV1.2 channel P0 via the formation of a rigid helix between the alpha interaction domain (AID) on the I–II loop of the α1c and domain IS6 (Kanevsky & Dascal, 2006; Vitko et al., 2008; Findeisen & Minor, 2009). In addition, β-subunits are known to influence CaV1.2 channel expression. How β-subunits exert this effect has been studied extensively. Initially, binding of β-subunits to the I–II loop of CaV1.2 was proposed to shield an endoplasmic reticulum.
Figure 10. Elementary Cav1.2 channel currents are increased by ISO in adult ventricular myocytes

A and B, representative \(i_{\text{Ca}}\) traces elicited by a step depolarization from \(-80\) mV to \(-30\) mV before (control) (A) and during application of 100 nM ISO (B) each with amplitude histograms were fit with multicomponent Gaussian functions (red lines). C, average ensemble currents from the same cell before (black) and during ISO application (blue).

D–F, paired symbol and line plots of the peak ensemble current (D), \(P_o\) (E) and apparent \(N_f\) (F) in \(n = 6\), \(N = 5\). G and H, \(i_{\text{Ca}}\) traces and accompanying amplitude histograms from a cell that displayed a preference for dimeric channel openings in the presence of ISO.
retention signal on the channel, thus promoting enhanced expression (Bichet et al., 2000). However, the lack of an obvious endoplasmic reticulum retention motif on the I–II loop and data from subsequent studies led to the rejection of this idea and, instead, favoured the postulate that β-subunits protect CaV1.2 channels against proteasomal degradation and thus promote enhanced total channel protein and surface expression (Altier et al., 2011; Waite et al., 2011). When the current manuscript was under consideration, an interesting study was published reporting that AID mutant CaV1.2 channels, unable to bind CaVβ subunits, are insensitive to βAR stimulation with ISO or the adenylyl cyclase activator forskolin. (Yang et al., 2019). The results of the present study showing dynamic augmentation of CaV1.2 and CaVβ2a sarcolemmal expression upon stimulation with ISO (Figs 2 and 4) have relevance to this work, and shed more light on the importance of the CaVβ subunit in adrenergic regulation of CaV1.2. Although the exact element in the pathway that responds to PKA phosphorylation to initiate this response remains unclear, it would be interesting to investigate whether phosphorylation of intracellular α1C and/or CaVβ, promotes an enhanced association between the two subunits, protecting α1C from degradation, sustaining the sub-sarcolemmal pool, and promoting forward trafficking to the sarcolemma. If so, then this could be another previously unappreciated facet of βAR-mediated regulation of CaV1.2 channels.

In our model, channels within the readily insertable pool can be dispatched to the membrane in times of increased metabolic or haemodynamic demand. The volume of the pool therefore has direct implications for the magnitude of functional reserve that can be called upon to increase channel activity and inotropy in response to PKA-mediated channel phosphorylation. Additional questions that have not been explored in this initial study include: (i) can the size of this pre-synthesized pool be increased by exercise training regimens to contribute to the larger βAR stimulated inotropic response seen in athletes and (ii) does the pool become depleted during chronic βAR stimulation that is known to occur in heart failure and/or myocardial ageing?

There is precedent for such a readily insertable pool of ion channels. In cardiomyocytes, an intracellular compartment of KCNQ1 channels is assumed to lie close to the Z-lines and can be mobilized to the sarcolemmal crest in response to elevations in [Ca^{2+}], or stress, where they co-assemble with KCNE1 and form functional I_{KS} channels, providing a repolarization reserve (Wang et al., 2013; Jiang et al., 2017). Additionally, in neurons, [Ca^{2+}], and more specifically, influx of Ca^{2+} through CaV1.2 channels, is considered to regulate CaV1.2 channel trafficking and endocytosis to tune excitability there (Green et al., 2007; Hall et al., 2013). Furthermore, phosphorylation of the AMPAR subunit GluA1 at Ser^{845} by PKA and/or calcium/calmodulin-dependent protein kinase II, or PKC at Ser^{831}, promotes plasma membrane insertion of the receptors, facilitating long-term potentiation by increasing channel P_o and homeostatic scaling up (Makino et al., 2011; Diering et al., 2016; Olivito et al., 2016). Conversely, dephosphorylation by calcineurin leads to receptor internalization, long-term depression and homeostatic scaling down (Lee et al., 1998). A pool of intracellular GluA1-containing AMPARs is assumed to transition to a readily insertable state upon phosphorylation (Makino et al., 2011). In a striking parallel, we find that PKA-mediated phosphorylation of CaV1.2 channels, downstream of βAR activation, leads to increased insertion of these channels into the cardiomyocyte sarcolemma. In the presence of PKA inhibitors (H-89 or PKI), the significant super-clustering response to ISO was abolished, implying that PKA activity is needed for this dynamic augmentation of CaV1.2 cluster size. Although PKA appears to be necessary and sufficient for the initiation of the super-clustering response to ISO, it remains to be determined whether other kinases, such as calmodulin-dependent protein kinase II or PKC, can generate a similar nanoscale redistribution of channels and super-clustering response.

**Size of the functional reserve pool of channels**

In many of our datasets, we observed a ‘reserve’ of ~17–45% of channels that could be mobilized to the membrane in response to ISO: (i) in super-resolution experiments, a 17% increase in mean CaV1.2 cluster area was observed in ISO-treated cardiomyocytes; (ii) in dynamic imaging experiments, ISO increased CaVβ2a-paGFP intensity in the sarcolemma by ~45%; (iii) in western experiments, ISO produced a 28% increase in the expression of CaV1.2 in the plasma membrane; and (iv) in stepwise photobleaching experiments, ISO increased the mean number of CaVβ2a-paGFP per cluster by 32%. Could this be the size of the functional reserve? The size of this putative channel reserve pool is not directly proportional to the 1.6- to 2.8-fold βAR-mediated increase in CaV1.2 macroscopic current routinely observed in ventricular myocytes (Muth et al., 1999; Lemke et al., 2008; Nichols et al., 2010; Brandmayr et al., 2012). However, as noted earlier, a small number of high P_o phosphorylated channels may have a disproportionately large effect on I_{Ca} if they interact with other non-phosphorylated channels in channel clusters (Dixon et al., 2012). In an interesting parallel, a previous study performed on cortical neurons reported that 76% of CaV1.2 channels are on the cell surface (Green et al., 2007), whereas the remaining 24% occupied intracellular locations. Do neurons then have a similarly sized functional reserve? Phosphorylation...
of neuronal CaV1.2 channels is considered to play a role in CaV1.2 trafficking and stabilization at the membrane (Folci et al., 2018), although, to our knowledge, the effect on channel cluster size remains to be clarified.

**CaV1.2 channel trafficking: single channels or groups?**

In support of our model, we recently reported that FP-tagged CaV1.2 channels are trafficked in vesicles within tsA-201 cells where they exhibit robust intracellular and submembrane movement (Ghosh et al., 2018). In that study, we found that the diverse trafficking patterns of CaV1.2 required both microtubules (MTs) and an intact actin cytoskeleton. In cardiomyocytes, both of these scaffolding and transport elements have been postulated to play a role in CaV1.2 trafficking. There, a ‘targeted delivery’ model has been proposed for two channels: connexin 43 and CaV1.2 (Shaw et al., 2007; Hong et al., 2010; Chkourko et al., 2012; Kong et al., 2012; Xiao & Shaw, 2015). In this model, vesicular channel cargo enters the negative-end of the MT at the Golgi and is transported to defined delivery hubs at the plasma membrane where the MT plus-end binds to an anchor complex (Xiao & Shaw, 2015). In the case of CaV1.2 channels, BIN1 is the anchor/hub which docks the MT plus-end, targeting the channels to the t-tubule membrane (Hong et al., 2010; Hong et al., 2012). BIN1 interacts with non-sarcomeric actin (F-actin) to bind to α-actinin at the Z-lines, forming the anchor complex (Hong et al., 2014). α-actinin itself is known to play a role in promoting CaV1.2 channel expression and function at the membrane (Hall et al., 2013; Tseng et al., 2017). Although, in the present study, our methodology did not permit imaging of the pore-forming subunit of the channels directly in cardiomyocytes, we did observe dynamic insertion of FP-tagged CaV1.2 into the membrane in response to ISO (Fig. 4; see also the Supporting information, Movie S1). Notably, we observe that these insertions are not limited to single channels but frequently involve larger clusters of multiple channels. An interesting finding in the present study that lends credence to this idea arises when we consider the apparently counterintuitive kinetics of the BiFC experiments (Fig. 1). It might be predicted that, because ISO increases the likelihood of channel interactions, it should then increase the kinetics of venus reconstitution in the BiFC assayed response. However, we saw quite the opposite. ISO-sensitive CaV1.2–CaV1.2 channel interactions, although larger in magnitude, occurred on a notably slower time scale (τ = 35.76 min) than those occurring spontaneously in unstimulated cells (τ = 18.57 min). One possible explanation for this slower rate is that the more complex signalling pathway required to stimulate the ISO-sensitive interactions simply requires more time (i.e. the τ reflects the time taken for β2-AR activation, signalling, channel phosphorylation, subsequent physical interactions and maturation of the newly constituted venus fluorescence). Because we performed these experiments in TIRF mode, any readout is probably in or very close to the plasma membrane of the cells. Thus, an alternative explanation is that the longer timescale for the ISO-sensitive interactions reflects the time taken for CaV1.2 channels, interacting with one another in intracellular vesicles, to be trafficked to the surface where they would then be evident in our TIRF footprint. This second explanation is supported by dynamic imaging results where we captured ‘new’ clusters of CaV1.2–CaV1.2 appearing in myocyte TIRF footprints in response to ISO. Our biochemical expression studies lend further credence to this model, revealing an ISO-stimulated augmentation of a plasma membrane pool of cardiac CaV1.2 channels with a coincident, and almost equal magnitude depletion of an intracellular membrane pool. At least two open questions remain; first does PKA phosphorylate sarcoplasmal and/or vesicular CaV1.2? Our model supports phosphorylation of an internal pool of channels which results in enhanced targeting and insertion of those channels to the sarcolemma. The second question considers the internalized pool of functionally viable β1 and β2-ARs that has been reported in recent years (Irannejad et al., 2013; Irannejad et al., 2017). Can these internalized βARs signal to phosphorylate intracellular CaV1.2, promoting their membrane targeting? Future studies should pursue answers to these questions.

**Impact of super-clustering on EC-coupling**

Although we do not explicitly investigate the effect of super-clustering on EC-coupling in the present study, considering the indispensable role played by CaV1.2 channels in this vital process, it merits some discussion. In a previous study, we utilized a light activated dimerization strategy to force channels to physically interact, and found that CaV1.2–CaV1.2 contact resulted in increased co-operative gating and amplification of whole-cell I_{CaL}, resulting in larger [Ca^{2+}]_i transients in adult rat ventricular myocytes (Dixon et al., 2012). We have also confirmed and recapitulated these effects of channel co-operativity in a recent in silico study (Sato et al., 2018). Thus, CaV1.2 clustering and co-operative gating can increase Ca^{2+} influx to the point where it has a tangible effect on excitability. One would predict that the most impactful effect on EC-coupling and inotropy would be produced by preferentially localizing CaV1.2 channel super-clusters at positions opposite type 2 ryanodine receptor clusters on the junctional sarcoplasmic reticulum. Phosphorylated type 2 ryanodine receptors have been reported by others to display enhanced dyad-localized clustering in response to ISO (Fu et al., 2016). Future studies should investigate the juxta-positioning of these
two augmented cluster populations and the effect on EC-coupling.

In conclusion, the present study reveals a newly appreciated facet of the mechanism underlying βAR mediated regulation of Cav1.2 channels. We present a revised model in which βAR activation initiates a PKA-dependent dynamic augmentation of Cav1.2 channel expression on the sarcolemma of ventricular myocytes, facilitating their co-operative interactions, and amplifying Ca\(^{2+}\) influx. This adds a new layer of complexity to our understanding of adrenergic regulation of these channels, and could provide a means to fine tune EC-coupling to meet acutely increased haemodynamic and metabolic demands during fight-or-flight.

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### Additional information

#### Competing interests

The authors declare that they have no competing interests.

#### Author contributions

RED conceived and designed the experiments. RED, DI, KIH, BX, DG and SGV executed the experiments. RED, DI, KIH BX and DG collected data. RED, DI, KIH and BX analysed the data. RED, YKK, EJD and MFN interpreted data. RED, DI, KIH and BX wrote the manuscript. RED, DI, KIH, BX, DG, SGV, YKK, EJD and MFN revised the manuscript. All authors approve the
final version of the manuscript for publication and agree to be accountable for all aspects of the work. All listed authors meet the requirements for authorship, and all those who qualify for authorship are listed.

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Supporting information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Movie S1. ISO stimulates dynamic augmentation of sarcolemmal CaVβ2a-paGFP expression in transduced ventricular myocytes

Movie S2. Ca2+ sparklet activity, site density and co-operativity are augmented by ISO