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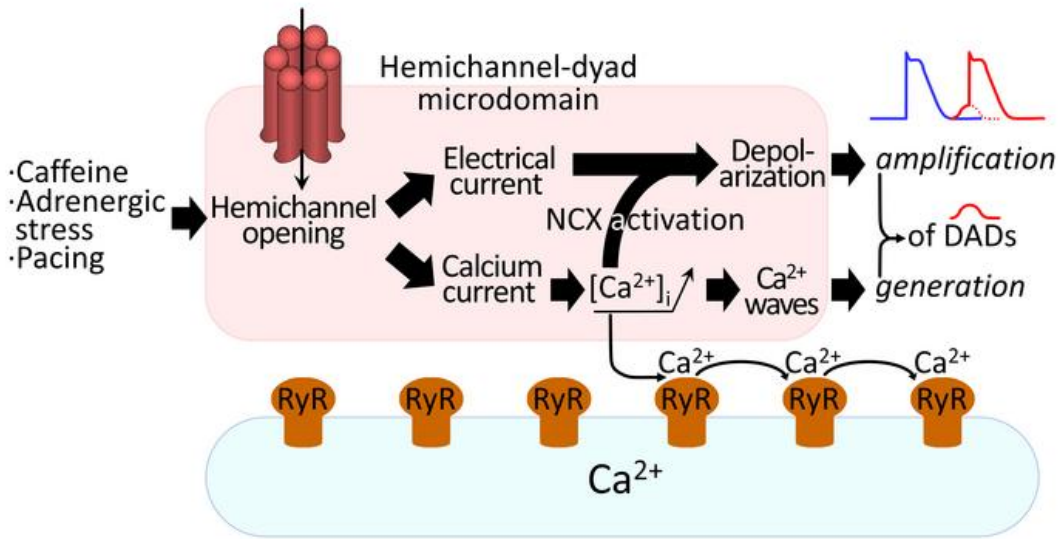
Graphical abstract



Cx43 hemichannel microdomain signaling at the intercalated disc enhances cardiac excitability

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Conflict of Interest

The authors have declared that no conflict of interest exists.

Abstract

Cx43, a major cardiac connexin, forms precursor hemichannels that accrue at the intercalated disc to assemble as gap junctions. While gap junctions are crucial for electrical conduction in the heart, little is known on potential roles of hemichannels. Recent evidence suggests that inhibiting Cx43 hemichannel opening with Gap19 has antiarrhythmic effects. Here, we used multiple electrophysiology, imaging and super-resolution techniques to understand and define the conditions underlying Cx43 hemichannel activation in ventricular cardiomyocytes, their contribution to diastolic Ca^{2+} release from the sarcoplasmic reticulum, and their impact on electrical stability. We showed that Cx43 hemichannels are activated during diastolic Ca^{2+} release in single ventricular cardiomyocytes and cardiomyocyte cell pairs from mouse and pig. This activation involved Cx43 hemichannel Ca^{2+} entry and coupling to Ca^{2+} release microdomains at the intercalated disc resulting in enhanced Ca^{2+} dynamics. Hemichannel opening furthermore contributed to delayed afterdepolarizations and triggered action potentials. In single cardiomyocytes, cardiomyocyte cell pairs and arterially perfused tissue wedges from failing human hearts, increased hemichannel activity contributed to electrical instability as compared to non-failing rejected donor hearts. We conclude that microdomain coupling between Cx43 hemichannels and Ca^{2+} release is a novel, targetable, mechanism of cardiac arrhythmogenesis in heart failure.

Introduction

The pumping function of the heart is coordinated by electrical waves of excitation propagating through the myocardium and initiating cardiac contraction. Propagation of action potentials (APs) between cardiomyocytes is coordinated by gap junctions (GJs) that accrete at the intercalated discs (IDs). Each GJ channel consists of two apposed hemichannels and each hemichannel is further composed of six connexin (Cx) subunits; Cx43 is the predominant Cx isotype in adult heart ventricles (1). GJs organize as channel plaques, called the 'nexus', at the IDs of adjacent cardiomyocytes, which are peripherally surrounded by perinexal zones where unapposed (free) hemichannels reside (2); at rest, GJs are open and hemichannels are closed. In ventricular cardiomyocytes, Cx43 hemichannels need strongly positive membrane voltages (V_m > +50 mV) to open; the activation threshold is lowered when the cytoplasmic Ca^{2+} concentration ($[Ca^{2+}]_i$) is moderately increased (200-500 nM range), bringing the threshold in the +30 mV range, which may be attained during the peak and plateau phase of the cardiac action potential (3, 4). When open, hemichannels form a large conductance (~220 pS for a single Cx43 hemichannel) and poorly selective ion channel and transmembrane conduit with a molecular weight cutoff of ~1.5 kDa (3-9) that allows the passage of various small molecules such as ATP, and ions such as Na^+, K^+ and Ca^{2+}.

Inspired by findings in non-cardiac cells where Cx43 hemichannels open in response to moderate $[Ca^{2+}]_i$ elevation at resting V_m (7, 9), we were interested whether Cx43 hemichannels in cardiomyocytes could open during sarcoplasmic reticulum (SR) Ca^{2+} release under conditions of Ca^{2+} overload and disease. Cardiomyocytes display synchronized transient $[Ca^{2+}]_i$ elevations during systole, sparked by Ca^{2+} entry through L-

type Ca^{2+} channels during the AP plateau, which triggers Ca^{2+}-induced SR Ca^{2+} release via ryanodine receptors (RyRs) (10). Under pathological conditions, spontaneous SR Ca^{2+} release may occur, leading to delayed afterdepolarizations (DADs) during diastole as a result of Ca^{2+} extrusion via the electrogenic Na^{+}/Ca^{2+} exchanger (NCX) that generates depolarizing inward current (11, 12). DADs in turn may lead to disturbances of heart rhythm (12, 13). We hypothesized that hemichannel-associated Ca^{2+} entry as well as hemichannel depolarizing current could enhance DADs. Recent work based on pharmacological inhibition of Cx43 hemichannel opening with Gap19, which renders hemichannels non-available for opening without inhibiting gap junctions (4, 14), has demonstrated antiarrhythmic effects in muscular dystrophy in mice (15, 16), prevention of atrial fibrillation in myosin light chain 4 (MYL4-)related atrial cardiomyopathy (17) and protective effects on disturbed intracellular Ca^{2+} homeostasis in plakophilin-2 (PKP2-) deficient mice, a model of arrhythmogenic right ventricular cardiomyopathy (18).

We mimicked pathological spontaneous Ca^{2+} release by challenging freshly isolated single ventricular cardiomyocytes and cardiomyocyte cell pairs from mice and pig with caffeine under controlled conditions. Activation of Cx43 hemichannels resulted from microdomain coupling of Cx43 hemichannels to RyRs and SR Ca^{2+} release. We further found that Cx43 hemichannels contribute to spontaneous Ca^{2+} release events and accompanying afterdepolarizations during adrenergic stimulation. In a next translational step, we studied single human ventricular cardiomyocytes and cardiomyocyte cell pairs obtained from heart failure patients, and found that Cx43 hemichannel opening, spontaneous Ca^{2+} release and afterdepolarizations were increased compared to cardiomyocytes from non-failing donor hearts. In arterially perfused tissue wedges,

adrenergic stimulation increased the occurrence of DADs and triggered APs, which was clearly more pronounced in wedges from heart failure patients than from non-failing control hearts, and was suppressed by TAT-Gap19 hemichannel inhibition. Collectively, these data show that Cx43 hemichannels become more active in end-stage heart failure and contribute to arrhythmogenic triggered activities in disease, which can be suppressed by TAT-Gap19.

Results

Ca^{2+} release from the sarcoplasmic reticulum at resting membrane potentials activates Cx43 hemichannels

Freshly isolated left ventricular cardiomyocytes from mouse and pig hearts were voltage clamped at -70 mV, under physiological extracellular Ca^{2+} concentrations and continuous $[Ca^{2+}]_i$ monitoring with fluo-4 (50 µmol/L), and subjected to a two-pulse caffeine stimulation protocol (Figure 1A). Caffeine stimulation (10 mmol/L) created controlled conditions of SR Ca^{2+} release, mimicking the spontaneous release observed in pathological conditions. Caffeine activates ryanodine receptors (19) and triggers transient SR Ca^{2+} release with resulting forward-mode inward NCX current (10, 20). In line with this, the first caffeine pulse triggered a $[Ca^{2+}]_i$ transient and resulting NCX current; by contrast, the second caffeine pulse was not accompanied by $[Ca^{2+}]_i$ elevation and NCX current, indicating Ca^{2+} store depletion (Figure 1B). Superimposed on the macroscopic NCX current during the first caffeine pulse, microscopic unitary current events appeared in a Ca^{2+} release-dependent manner (Supplemental Figure 2B). Such events were absent during the second caffeine pulse and were abolished by including the Ca^{2+} chelator BAPTA (10 mmol/L) in the pipette (Figure 1 C-E and Supplemental Figure 3). Additionally, imposing stably buffered $[Ca^{2+}]_i$ did not induce unitary currents, indicating that unitary current activity is dependent on SR Ca^{2+} release rather than on caffeine or $[Ca^{2+}]_i$ elevation itself (Supplemental Figure 4) as previously noted (21). Since Ca^{2+} release in cardiomyocytes is not homogeneous but characterized by differences in bulk cytoplasmic and subsarcolemmal $[Ca^{2+}]_i$ (22), we probed the involvement of $[Ca^{2+}]_i$ microdomains in unitary current activation by plotting ensemble current versus subsarcolemmal $[Ca^{2+}]_i$ as derived from the NCX current (23) (Supplemental Figure 2C). Ensemble currents showed no

hysteresis in a low $[Ca^{2+}]_i$ range of NCX-derived subsarcolemmal measurements, but hysteresis became apparent at higher concentrations resulting from hemichannel closure at high $[Ca^{2+}]_i$ (21).

Unitary events in response to SR Ca^{2+} release could be readily observed in ~96% of mouse myocytes and ~60% of pig myocytes with a representative event count of ~38 and ~6 respectively over the 8 s recording window (Supplemental Table 6). Most unitary current activity occurred within less than 100 ms after the start of the NCX current and had a ~220 pS unitary conductance in both species (Figure 1C-E), typical for Cx43 hemichannel opening (3, 4, 6). A ~110 pS substate could also be resolved, especially in pig. Occasionally, we observed 'stacked' opening events with a conductance of multiples of ~220 pS (Figure 1C-E). Stepping V_m to different voltages during caffeine application, under conditions of K^+-channel blockade, allowed us to construct IV plots with a slope conductance of ~220 pS and reversal potential ~0 mV for both species (Figure 1F-G). The zero reversal potential is a typical property of poorly selective Cx hemichannels. Increasing extracellular Ca^{2+} 5-fold significantly increased the slope conductance and shifted the reversal potential rightward in the direction of the increased Nernst potential for Ca^{2+}, indicating substantial Ca^{2+} flow through the channel (Figure 1G). When a caffeine challenge was given at positive V_m instead of -70 mV, the short ~8 ms unitary opening events attained a strongly prolonged character (100 – 120x increase in open time), resembling the long duration hemichannel openings observed previously (3, 4, 6) (Figure 1F and Supplemental Figure 2D). Unitary current properties and interspecies differences are summarized in Supplemental Table 6.

To confirm the Cx43 hemichannel origin of the Ca^{2+} release-induced unitary currents, we used a combination of pharmacological and genetic approaches targeting Cx43 (Figure 1H-I). Cx43 knockdown by ~80% (Supplemental Figure 5A-D) strongly and significantly reduced unitary current activities. We additionally tested a set of Cx43 targeting peptides that interfere with hemichannel function (Figure 1H-I and Supplemental Figure 6A; peptide administration and concentrations see Methods). Gap19 significantly reduced unitary current activities while the inactive mutant (4), $Gap19^{I130A}$, had no effect. Conversely, the hemichannel opening enhancer CT9 (5, 24) significantly increased unitary currents. We screened for other channels with a similar biophysical profile such as Panx1, TRPP2 and TRPP5 (25, 26). Firstly, ventricular Panx1 and TRPP2/5 protein levels were unchanged or undetected in $Cx43^{Cre\text{-}ER(T)/fl}$ ventricles (Supplemental Figure 5E-H). Additionally, the pannexin-1 targeting peptide ^{10}Panx1 did not affect unitary currents (Figure 1I). None of the peptides or experimental conditions affected SR Ca^{2+} content as determined by integrating the NCX current during caffeine superfusion (Supplemental Figure 6B).

Cx43 hemichannel opening is modulated by stimulation frequency and enhanced during β-adrenergic stimulation

It is well known that cardiomyocyte responses to repeated electrical stimulation or β-adrenergic stimulation occur in part through alterations in $[Ca^{2+}]_i$ signaling and SR Ca^{2+} content (20). We thus verified whether Cx43 hemichannel activity was influenced by increasing stimulation frequency and β-adrenergic activation. Ca^{2+} release-induced unitary current activity significantly and reversibly increased when increasing the stimulation frequency before caffeine application (0.5 to 4 Hz range in mouse and 0.5 to 2 Hz range in pig) (Figure 2A-D). The increase was proportional to changes in the

amplitude of the caffeine-induced SR Ca^{2+} release (Figure 2E-F). Moreover, β-adrenergic stimulation by superfusion of isoproterenol (ISO) during the 2 min train at 1 Hz significantly increased unitary current activities (Figure 3A-D), in line with concurrent changes in SR Ca^{2+} content (Figure 3E).

Cx43 associates with dyads at the intercalated disc where microdomain activation of Cx43 hemichannels occurs

Since Cx43 hemichannels are activated by a subsarcolemmal increase in Ca^{2+} during caffeine-induced SR Ca^{2+} discharge, we hypothesized that these channels would intimately associate with cardiac dyads at the ID. We used single-molecule light microscopy (SMLM) by performing stochastical optical reconstruction microscopy (STORM) to map Cx43 distribution to dyadic nanodomains in single cardiomyocytes and cardiomyocyte cell pairs from mouse left ventricle. We first used immunolabeling of Cx43 in combination with an SR marker (RyR2, Junctophilin-2 (JPH2) or total Phospholamban (tPLN)) or a sarcolemmal membrane marker (pore-forming subunit of the L-type Ca^{2+} channel (Cav1.2), NCX or Caveolin-3 (Cav3)). All markers showed the largest cluster density and cluster size at the cell ends of single cardiomyocytes and at the IDs of cardiomyocyte cell pairs as compared to the lateral surface or cell interior (Supplemental Figure 7A-C). By contrast, the Z-disc protein α-actinin - used as a negative control - did not follow such distribution and showed highest cluster density and size in the cell interior (Supplemental Figure 7C). Distance analysis revealed that 30-50 % of the studied markers occurred within ~20 nm of Cx43 at the cell ends of single cardiomyocytes or at the IDs of cardiomyocyte cell pairs (Supplemental Figure 7D). To exclude random association of Cx43 to these overtly abundant markers, we performed stochastic simulations on these

data to determine the interaction factor, which expresses the relation of the experimentally observed overlap versus the probability for random overlap (Interaction factor ImageJ plugin (27)). Cx43 co-localized to all markers with an interaction factor > 0.6, indicating that the observed overlap is deterministic rather than random (Supplemental Figure 7E-F).

We used a triple staining of Cav1.2, RyR2 and Cx43 to directly map the relation of Cx43 to dyads (based on Cav1.2 and RyR2 clusters occurring < 250 nm of each other (28, 29)) (Figure 4A-B). We found that ~80% of RyR2 associated in dyads and that dyadic RyR2 clusters were larger than their extradyadic counterparts. Both dyadic and extradyadic RyR2 cluster densities and number of molecules were largest at the cell ends of single cardiomyocytes and at the IDs of cardiomyocyte cell pairs as compared to the lateral surface or cell interior (Figure 4C). Only a small fraction of extradyadic RyR2 clusters occurred within ~20 nm of Cx43 while ~50% of dyadic RyR2 co-localized with Cx43 (Figure 4C). Recent work has shown that closely localized RyR clusters, < 100 nm edge-to-edge distance, may act cooperatively as superclusters to generate Ca^{2+} signals (30). These RyR superclusters preferentially organized in dyads and occurred more frequently at the cell ends of single cardiomyocytes and at the IDs of cardiomyocyte cell pairs as compared to the lateral surface or cell interior (Figure 4D). Superclusters at these sites contained more RyRs (Figure 4D). Overall, dyadic superclusters formed the majority of structures that co-localized with Cx43.

Next, we used a relative localization algorithm (31, 32) to categorize dyadic Cav1.2 clusters at the IDs of cardiomyocyte cell pairs as either located in the perinexus (based

on the signal overlapping with Cx43 and extending within 200 nm of Cx43 clusters) or distant from Cx43 (based on signal extending beyond 200 nm of Cx43 clusters). We found that ~80% of Cav1.2 clusters occurred in dyads; approximately 42% of these clusters overlapped with Cx43 and another ~28% occurred adjacent to Cx43, resulting in ~70% of dyads at the ID occurring in the perinexal nanodomain where Cx43 hemichannels are known to reside (Figure 4E and Supplemental Figure 8A). Cx43 and dyadic Cav1.2 clusters overlapped only partially, confirming association at the edge of Cx43 clusters (Supplemental Figure 8B). In line with these observations, electron microscopy of murine cardiac ventricular tissue revealed cleft formation of SR cisterns with the perinexal nanodomain where Cx43 hemichannels reside (Figure 4F, Supplemental Video 1 and 2) (33, 34). Based on the protein structure of Cx43 channels (35), the distance to nearest neighbor in cardiac GJ plaques (36) and the lower density of Cx43 in the perinexus (37), we calculated that perinexal dyads or dyads at the cell end may contain 1-2 Cx43 hemichannels per dyad (Supplemental Figure 9).

To provide a functional correlate to these structural observations, we used the macropatch technique (38) to map single Cx43 hemichannel activity at discrete sarcolemmal microdomains. During caffeine-induced SR Ca^{2+} release, we observed single-channel currents with a conductance of ~220 pS at the cell ends of single cardiomyocytes and IDs of cardiomyocyte cell pairs. One third of patches at these sites had currents, with currents being absent in lateral membranes (Figure 5A-D, Supplemental Table 7). Open probability was highest in cardiomyocyte cell pairs. Events were inhibited by TAT-Gap19, enhanced by TAT-CT9 and abolished in cells not expressing Cx43. ^{10}Panx1 and inactive TAT-Gap19^{I130A} had no effect (Figure 5D). We used scanning ion conductance microscopy

(SICM) to map membrane topology at the cell end and recorded ~220 pS caffeine-induced unitary current activity (including ~110 pS substate) in nanopatches (R_p ~30 MΩ, ~300 nm pipette internal diameter) just distally of t-tubules at the start of the ID (Figure 5E-G). Patches contained 1-2 active channels, in line with our predictions (Supplemental Figure 9 and Supplemental Table 7).

Cx43 hemichannels modulate diastolic Ca^{2+} release during adrenergic stimulation and associated arrhythmogenic afterdepolarizations

Since highly conductive and Ca^{2+} permeable hemichannels are localized near dyads at the ID, it is conceivable that they could contribute to diastolic SR Ca^{2+} release itself (11). To investigate this, we exposed the cardiomyocytes to fast pacing and β-adrenergic stimulation without caffeine, and recorded spontaneous SR Ca^{2+} release events and currents occurring after such stimulation (Figure 6A). Additionally, we monitored V_m changes after switching to current clamp conditions following the same stimulation protocol (Figure 6B). Compared to baseline, diastolic Ca^{2+} release, accompanying currents and afterdepolarizations became prominent following fast pacing and ISO exposure (Figure 6C). Gap19 and Cx43 knockdown reduced the number of diastolic Ca^{2+} release events and associated currents while CT9 enhanced these events, $Gap19^{I130A}$ had no effect (Figure 6D). Accordingly, DADs and triggered APs following a period of fast pacing and ISO stimulation (Figure 6B-C), were significantly reduced by TAT-Gap19, not affected by TAT-$Gap19^{I130A}$ and enhanced by TAT-CT9 (Figure 6D).

We verified whether the Cx43 targeting peptides or Cx43 knockdown would perhaps affect the properties of the global $[Ca^{2+}]_i$ transient. This was not the case, indicating absence of

gross effects of these interventions on cardiomyocyte global Ca^{2+} homeostasis (Supplemental Figure 10). In a next approach, we further scrutinized the occurrence of hemichannel activity following adrenergic stimulation in the time window of macroscopic NCX currents associated with spontaneous Ca^{2+} release. We found that ~220 pS unitary current events occurred not only during the NCX current (superimposed on it, see Figure 7A) but also preceding it (Figure 8A; corresponding conductance histograms are shown in Figure 7B and 8B respectively). Event probabilities before and during NCX were comparable (compare Figure 7C with 8C) and strongly increased by pacing and ISO. Unitary current activity during adrenergic stimulation was robustly associated with larger Ca^{2+} transients, larger SR Ca^{2+} content and increased spontaneous Ca^{2+} release (Supplemental Figure 11). Genetic ablation and pharmacological tools had effects as observed in the caffeine-triggered responses (Figure 7D and 8D; compare to Figure 1I). As expected, charge transfer associated with these unitary hemichannel events was significantly smaller compared to charge transfer linked to the NCX current during spontaneous Ca^{2+} release (Figure 7E).

As a control experiment, we tested whether Gap19, CT9 and their TAT-versions affected Ca^{2+} release via RyR2. To this end, we challenged RyR2 overexpressing HEK293 cells (39) with caffeine and quantified Ca^{2+} signals in the presence or absence of peptide. Overall, peptides did not influence caffeine-induced Ca^{2+} signals, except for acute TAT-CT9 exposure which significantly increased Ca^{2+} release at 5 mM caffeine but not at 0.4 mM; no effect was observed when TAT-CT9 was pre-incubated (Supplemental Figure 12).

Cx43 hemichannel depolarizing current, microdomain Ca^{2+} entry and RyR-coupling underlie hemichannel associated triggered activities

To better understand these observations, we performed order of magnitude calculations within a mathematical framework (see supplemental information) incorporating the present unitary current and STORM imaging data. We reasoned that open hemichannels pass inward current and facilitate Ca^{2+} entry into the cell. The direct contribution of depolarizing current through a single hemichannel was determined to be ~1.6 mV (at -70 mV and 37°C; Figure 9A). We further estimated the hemichannel Ca^{2+} current to be ~0.84 and ~1.46 pA for 1.0 (mouse) and 1.8 mM (pig and human) extracellular Ca^{2+} respectively, corresponding to a Ca^{2+} entry rate of ~4.35×10^{-18} and ~7.57×10^{-18} mol Ca^{2+} per second and per open hemichannel respectively. We calculated that such Ca^{2+} influxes do not impact global cytosolic Ca^{2+} (in line with experimental findings presented in Supplemental Figure 10 (40-44)), but result in a pronounced elevation of peak $[Ca^{2+}]_i$ in the hemichannel-dyad microdomain of ~0.81 and ~3.44 µmol/L respectively (Figure 9B, calculations based on a hemichannel open time τ of 8 ms derived from the open time distribution). Such Ca^{2+} elevation may activate significant NCX current in the order of 0.12 and 0.13 pA/pF respectively, producing ~1.28 and ~1.51 mV depolarization respectively (Figure 9A and C). Thus, during a DAD, where hemichannel opening probability is largest during peak NCX current (Figure 7F), membrane depolarization will amount to ~2.88 and ~3.11 mV per hemichannel (Figure 9A), which is in line with the experimental observation that TAT-Gap19 decreases DAD amplitude by 2.83 mV (Figure 6D). Conversely, TAT-CT9 increased DAD amplitude by 4.9 mV, which may be related to longer opening or occasional stacked hemichannel openings (Figure 6D). Thus, hemichannel opening will

increase the DAD peak amplitude, bringing it closer to the threshold for action potential firing.

We further included a 4-state Markov RyR gating model (45) in the hemichannel-dyad microdomain to estimate the effect of hemichannel Ca^{2+} entry on Ca^{2+}-induced Ca^{2+} release. This suggested that single hemichannel Ca^{2+} entry may activate a RyR supercluster at the cell end with a probability of ~0.72 and ~0.99 (for 1.0 and 1.8 mM extracellular Ca^{2+} respectively) thus providing a Ca^{2+} spark (Figure 9A). This Ca^{2+} spark at the intercalated disc has a ~0.99 probability of activating a neighboring RyR cluster; accordingly Ca^{2+} entry through a single hemichannel has a respective probability of ~0.71 and ~0.98 to induce a propagating Ca^{2+} wave (Figure 9A, D-E) (46). Experimentally, we analyzed coupling of Cx43 hemichannel opening activity to diastolic Ca^{2+} release as well as the relation to the site of origin, in particular at the cell end. This analysis revealed that hemichannel currents preceded ~50% of the spontaneous Ca^{2+} release events following adrenergic stimulation in mice, i.e. somewhat lower than the 71% predicted; this coupling was fast ($\tau \approx 10$ ms) and occurred almost exclusively for waves that started at the cell end (Supplemental Video 4, Figure 8A and E) as compared to the middle (Supplemental Video 3, Figure 7A and Figure 8E). Ca^{2+} waves originating from the cell end had a significantly higher amplitude and larger associated NCX currents as compared to those originating in the cell middle (Figure 8F).

Cx43 hemichannels affect cardiac excitability in human heart failure

In human heart failure, Cx43 expression and distribution change and we asked the question whether in those conditions, as compared to healthy hearts, Cx43 hemichannels affected cardiac excitability. We examined explanted hearts from patients with end-stage heart failure; non-failing rejected donor hearts were used as controls. Patient characteristics are summarized in Supplemental Table 8. Failing hearts were hypertrophic, dilated and showed severely reduced left ventricular ejection fraction (Supplemental Table 8, Supplemental Figure 13A-B). Concurrently, isolated left ventricular cardiomyocytes from failing hearts were hypertrophied compared to controls (Supplemental Figure 13C-D). In cardiomyocytes from non-failing human left ventricles, caffeine-induced SR Ca^{2+} release evoked ~220 pS unitary current activity superimposed on the NCX current (Figure 10A-C). Unitary events increased with stimulation at higher frequency and were ISO sensitive (Figure 10D-G), as observed in mice and pig (Figure 2). Interestingly, these unitary current activities were more frequent in cardiomyocytes from failing hearts, especially during fast pacing and adrenergic stimulation (Figure 10D-I) and were inhibited by Gap19 (Figure 10H-I). In non-failing human cardiomyocytes, ~220 pS unitary current activity could only be recorded at the cell ends of single cardiomyocytes or the ID of cardiomyocyte cell pairs, while in failing cardiomyocytes unitary current activity was also recorded at the lateral membrane of single cardiomyocytes and cardiomyocyte cell pairs (Figure 11A-D). In heart failure, patches contained more channels with higher open probability (Figure 11E-F). In non-failing and failing cardiomyocytes, these unitary currents were blocked by subsequent application of TAT-Gap19 (Figure 11D and F).

Similar to mouse, we observed unitary current activity with ~220 pS unitary conductance preceding and during spontaneous diastolic Ca^{2+} release following adrenergic stimulation

(Figure 12A-C), which was inhibited by Gap19. Additionally, we found that Gap19 significantly decreased spontaneous Ca^{2+} release and resulting NCX currents in cardiomyocytes from non-failing and failing hearts (Figure 12A, D-E). Current clamp recording revealed significantly increased frequency and amplitude of DADs and triggered APs in failing hearts as compared to non-failing hearts, and these events (induced by fast pacing and ISO stimulation) were significantly reduced by TAT-Gap19 (Figure 12F-G). Finally, monophasic action potential recordings on arterially perfused tissue wedges from failing human hearts showed the highest occurrence of DADs and triggered APs following 2 Hz and ISO stimulation as compared to non-failing wedges, which was significantly reduced by TAT-Gap19 (Figure 12H-I). Neither in non-failing nor in failing hearts, did we find gross effects of Gap19 on cardiomyocyte Ca^{2+} transients or SR Ca^{2+} content (Supplemental Figure 14A). Similar to mouse, we found that Cx43 hemichannel opening was related to diastolic Ca^{2+} release originating from the cell end in non-failing cardiomyocytes; most interestingly, in myocytes from failing hearts hemichannel opening occurred more frequently in relation to Ca^{2+} release originating from the cell middle as compared to those isolated from non-failing hearts (Supplemental Figure 14D-E). Gap19 attenuated both frequency and amplitude of Cx43 hemichannel-associated Ca^{2+} release and associated NCX currents (Supplemental Figure 14F).

Discussion

Our results demonstrate that Ca^{2+}-permeable Cx43 hemichannels can be activated by SR Ca^{2+} release at resting V_m. Microdomain coupling of Cx43 to distinct dyads at the ID facilitates this activation and underlies modulation of local Ca^{2+} release by Cx43 at these sites. Through both these mechanisms, Cx43 hemichannels contribute to triggered activity. Cx43 hemichannels are more active in human heart failure, especially during adrenergic stimulation, and thereby contribute to electrical instability and arrhythmogenesis in heart failure.

Molecular identity of the large conductance channel during SR Ca^{2+} release

In 1990, Pott and Mechmann (47) were the first to describe the large conductance channel activated by caffeine-induced or spontaneous SR Ca^{2+} release in guinea pig atrial cardiomyocytes. They argued that these channels were possibly related to “cardiac gap junction hemichannels” based on their biophysical profile and pharmacology. Here, we expand and validate these findings across species, including humans, and demonstrate that Cx43 hemichannels constitute the large conductance channel using genetic and pharmacological tools to complement the biophysical profiling. Over the years, other large-conductance channels expressed in cardiomyocytes have been proposed to underlie the spiking unitary current activities superimposed on the NCX current. Sarcolemmal RyRs have been suggested (48, 49) but our finding that unitary activity disappears upon SR depletion contradicts the possibility of sarcolemmal RyRs. Transient receptor potential (TRP) channel family members such as PKD2 (TRPP2) or PKD2L2 (TRPP5) as well as Panx1 channels have also been suggested (25, 26); however, Panx1 and TRPP2/5 expression were unchanged upon Cx43 knock down and [10]Panx1 did not

influence unitary activity. Instead, the biophysical profile described here, combined with Gap19 hemichannel inhibition and genetic interventions, strongly point to Cx43 hemichannels as the responsible large conductance channel.

Importantly, this is the first study demonstrating that Cx43 hemichannels may be activated by SR Ca^{2+} release in cardiomyocyte cell pairs, where gap junctions are present (50, 51), indicating that functional Cx43 hemichannels are present in ventricular myocardium and are not a byproduct of cell dissociation.

Cx43 hemichannels and microdomain Ca^{2+} signaling

Cx43 hemichannel activity was modulated by stimulation frequency and isoproterenol. High frequency and beta-adrenergic stimulation enhance microdomain Ca^{2+} signaling at the dyad (52, 53). Dyads are composed of junctional SR that is juxtaposed to L-type Ca^{2+} channels in T-tubules; EM-based evidence has indicated that junctional SR is also present at the ID (54, 55). Here, we provide structural and functional support for the existence of a Cx43 hemichannel-SR Ca^{2+} release signaling microdomain consisting of large dyadic RyR2 superclusters at the perinexus in healthy myocardium. Cx43 hemichannel opening had no effect on global cellular Ca^{2+} dynamics or SR Ca^{2+} content, indicating that hemichannels do not contribute to canonical excitation-contraction coupling. However, we show that Cx43 hemichannels impact cardiomyocyte electrical excitability by (i) providing direct depolarizing current and (ii) Ca^{2+} entry, increasing NCX current and consequent DAD amplitudes and triggered APs. A third level of impact relates to the finding that Ca^{2+}-permeable hemichannels facilitate and increase diastolic SR Ca^{2+} release, in particular at the ID. Numerical simulations corroborate this and indicate that single hemichannel Ca^{2+}

entry into dyads at the ID highly promotes Ca^{2+}-induced Ca^{2+} release from neighboring RyR superclusters followed by propagating Ca^{2+} waves resulting in DADs. Together these observations may explain the previously observed impact of Cx43 hemichannel inhibition on adrenergic arrhythmias in muscular dystrophy (15, 16, 56), ARVC (18) and MYL4 mutation-associated atrial fibrillation (17).

Cx43 hemichannels as an arrhythmogenic mechanism in human heart failure

In human heart failure, we observed a significant increase in hemichannel activity during SR Ca^{2+} release and adrenergic stimulation. Cx43 hemichannel inhibition significantly reduced adrenergically-mediated diastolic SR Ca^{2+} release and associated DADs and triggered APs at the cellular level as well as in arterially perfused tissue wedges from non-failing and failing human left ventricle. As in mouse, Cx43 hemichannel inhibition had no gross effects on cellular Ca^{2+} signaling or SR Ca^{2+} content in non-failing and failing cardiomyocytes. Yet, hemichannels enhance diastolic SR Ca^{2+} release presumably through microdomain Ca^{2+} signaling. Interestingly, Cx43 events and the coupling to Ca^{2+} were observed at the cell ends as well as in the middle, reflecting a shift in Cx43 location as well as in ryanodine receptor activity (54). Indeed, macropatch recording shows that – in heart failure – hemichannel activity may redistribute from the cell ends to the lateral membranes of single cardiomyocytes and cardiomyocyte cell pairs. Taken together, these data provide evidence that Cx43 hemichannels function as a ventricular arrhythmogenic mechanism that can be targeted by Gap19 without negative impact on inotropy.

A limitation of this study is that numerical simulations were based on (i) several assumptions and only give an order of magnitude of the effect, and (ii) the functional and

structural data were obtained in control cardiomyocytes, not in diseased cells. This may be the reason why the calculated probability of single hemichannel opening leading to Ca^{2+} waves was somewhat higher than observed in the experiments (0.71 *vs* 0.5). Second, in the human heart experiments, control non-failing cardiomyocytes were obtained from hearts that were not suitable for transplantation for various reasons (e.g. age, comorbidity), implying that these controls may differ from what is considered as healthy.

Methods

We utilized commercially available C57/BL6 mice and healthy control pigs. Additionally, we used an established inducible Cx43 knock-down mouse model ($Cx43^{Cre\text{-}ER(T)/fl}$ mice (57-63)). Daily intraperitoneal injection of 3 mg 4-hydroxytamoxifen (4-OHT; Sigma-Aldrich, Bornem, Belgium) dissolved in sunflower oil, for 5 consecutive days, induces Cre-ER(T) activity which progressively deletes the floxed Cx43 allele in adult $Cx43^{Cre\text{-}ER(T)/fl}$ mice. Experimental work was performed on day 11 after the first injection. 4-OHT-treated $Cx43^{fl/fl}$ mice were used as controls. Animals were housed in a licensed facility and handled in accordance with European Directive 2010/63/EU. For human studies, we used rejected donor hearts as well as explanted failing hearts. Patient characteristics are summarized in Supplemental Table 8.

Procedures used for cardiomyocyte isolation, cellular electrophysiology and simultaneous Ca^{2+} imaging in single ventricular cardiomyocytes, DNA extraction, PCR, western blots, confocal and electron and single-molecule localization microscopy, cell culture, siRNA transfection, macropatch and super-resolution patch clamp, monophasic action potential recording in ventricular tissue wedges and numerical simulations follow previously published protocols and are detailed in the Online data supplement.

Synthetic peptides used in this study to interfere with Cx/Panx channel function were all obtained from Pepnome (Hong Kong, China). Gap19, $Gap19^{I130A}$ and CT9 were added to the pipette solution at a concentration of 100 µmol/L. TAT-Gap19, TAT-$Gap19^{I130A}$ and TAT-CT9 were added to the bath solution at 80 µmol/L. ^{10}Panx1, a blocker of Panx1 channels, was pre-incubated at 200 µmol/L for 30 minutes and present in the extracellular

solution during whole cell recording. For macropatch experiments, ^{10}Panx1 was included in the pipette solution at 200 µmol/L. The identity of the peptides was confirmed by mass spectrometry and purity was ≥ 90 %.

Statistics

Data are expressed as mean ± standard error of the mean (SEM) unless otherwise indicated, with 'n' denoting the number of cells and 'N' denoting the number of independent experiments. Following outlier analysis (mean ± 3xSD), normality of distribution was tested and appropriate statistical test was determined for comparative statistics. Data were compared using a nested design taking into account n cells and 'N' animals or human hearts. A two-tailed P value < 0.05 was considered as indicating statistical significance. In the graphs, the actual P values are provided. Statistical analysis and graphical data representation was done with Graphpad Prism (v.9).

Study approval

Experimental methods were approved by the local ethical committees of animal research at Ghent University, KU Leuven and New York University School of Medicine.

For human studies, experimental procedures were approved by the Ethical Committee of the University Hospital of Leuven with permit number S58824; as this is residual tissue no informed consent was necessary.

Author contributions

M.D.S. designed, conducted and analyzed most of the experiments, and wrote and revised the manuscript with input from all the authors. A.L. contributed to experimental design. T.N. designed the software for analysis of membrane currents and numerical simulations. N.W. contributed to the experimental design. E.D. contributed to cardiomyocyte cell isolation and cellular electrophysiology. M.P.H. contributed to the design and analysis of super-resolution and electron microscopy experiments. X.L. performed SICM-guided patch clamp experiments. M.A. contributed to cardiomyocyte cell isolation, cellular electrophysiology and monophasic action potential recording. T.V. performed and analyzed all experiments with tetracycline-inducible RyR2 overexpressing cells. K.W. contributed to single channel analysis. E.R. guided experimental design and provided computational tools for processing and analysis of super-resolution fluorescence microscopy. G.B. guided experiments in tetracycline-inducible RyR2 overexpressing cells. R.S. provided inducible Cx43 knock down mice. A.V.P. guided design of analysis software and numerical simulations. M.D., K.R.S. and L.L. conceived, initiated and supervised the project, gave scientific input and revised the manuscript.

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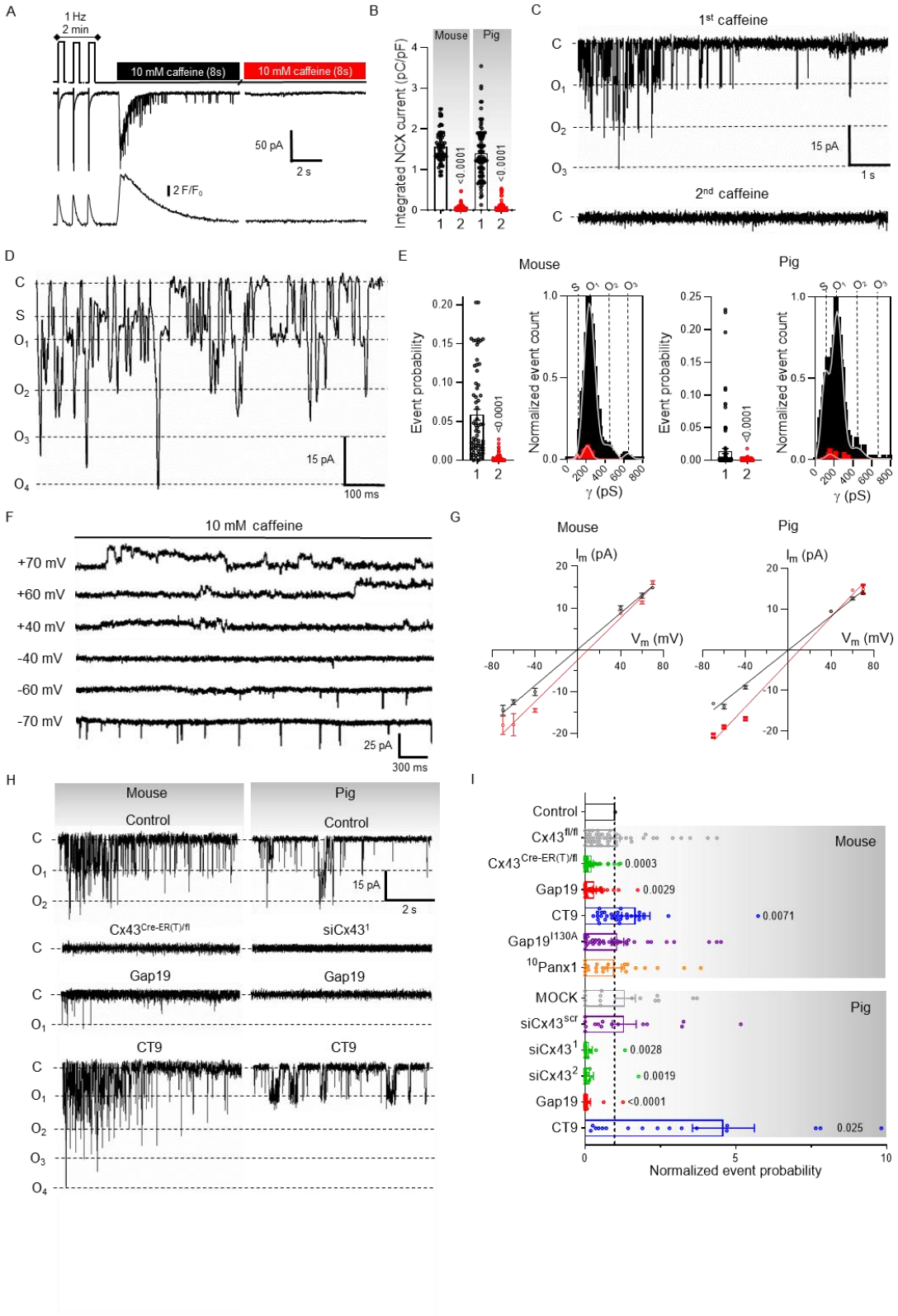
Figures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1 Hz
2 min
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10 mM caffeine (8s)
50 pA
2 s
2 F/F0
B
Mouse
Pig
Integrated NCX current (pC/pF)
<0.0001
<0.0001
C
1st caffeine
C
O1
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O3
15 pA
1 s
2nd caffeine
C
D
C
S
O1
O2
O3
O4
15 pA
100 ms
E
Mouse
Pig
Event probability
<0.0001
Normalized event count
γ (pS)
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F
10 mM caffeine
+70 mV
+60 mV
+40 mV
-40 mV
-60 mV
-70 mV
25 pA
300 ms
G
Mouse
Pig
Im (pA)
Vm (mV)
H
Mouse
Pig
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Control
C
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O2
15 pA
2 s
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siCx432
Gap19
CT9
Mouse
Pig
0.0003
0.0029
0.0071
0.0028
0.0019
<0.0001
0.025
Normalized event probability

Figure 1. Caffeine-induced Ca^{2+} release from the sarcoplasmic reticulum activates Cx43 hemichannels at resting membrane potential.

(A) Freshly isolated left ventricular cardiomyocytes were studied under voltage clamp with continuous $[Ca^{2+}]_i$ recording. Top trace shows experimental protocol. Middle and bottom traces depict current and $[Ca^{2+}]_i$ signals recorded in mouse.

(B) Summary data illustrating abolished NCX current during second caffeine pulse as compared to first pulse (nested t-test), indicating depleted SR Ca^{2+} stores (N/n_{mouse} = 90/281, N/n_{pig} = 20/55).

(C) Unitary current example traces during first and second caffeine applications with NCX current subtracted. 'C' indicates closed state; O_1 corresponds to fully open state while O_2 and O_3 indicate multiples of fully open state.

(D) Expanded trace of unitary current activity. 'S' indicates substate.

(E) Summary dot plots and transition histograms indicate significantly reduced unitary current event probability during the second caffeine pulse (red) as compared to the first application (black) (nested t-test; N/n_{mouse} = 90/281, N/n_{pig} = 20/55).

(F) Unitary current example traces during caffeine application at different membrane voltages. Recordings under conditions of K^+ channel blockade following 30 seconds of 1 Hz pacing.

(G) IV plots depicting linear current-voltage relationship with slope conductance ~220 pS and $E_{rev} \approx 0$ mV (black line; N/n_{mouse} = 5/20, N/n_{pig} = 5/15). A 5-fold elevation of $[Ca^{2+}]_e$ shifted E_{rev} from 0 to ~9.5 mV (red line; N/n_{mouse} = 5/20, N/n_{pig} = 5/15).

(H) Unitary current example traces under control conditions and following Cx43 knockdown or application of Gap19 or CT9.

(I) Summary data of Ca^{2+} release induced unitary current event probability under conditions of Cx43 knockdown or in the presence of Gap19, inactive $Gap19^{I130A}$, CT9 or $^{10}Panx1$ (N/n_{mouse} = 5-16/20-49 per condition, N/n_{pig} = 5-6/15-21 per condition). P-values indicate significance compared to control (nested one-way ANOVA).

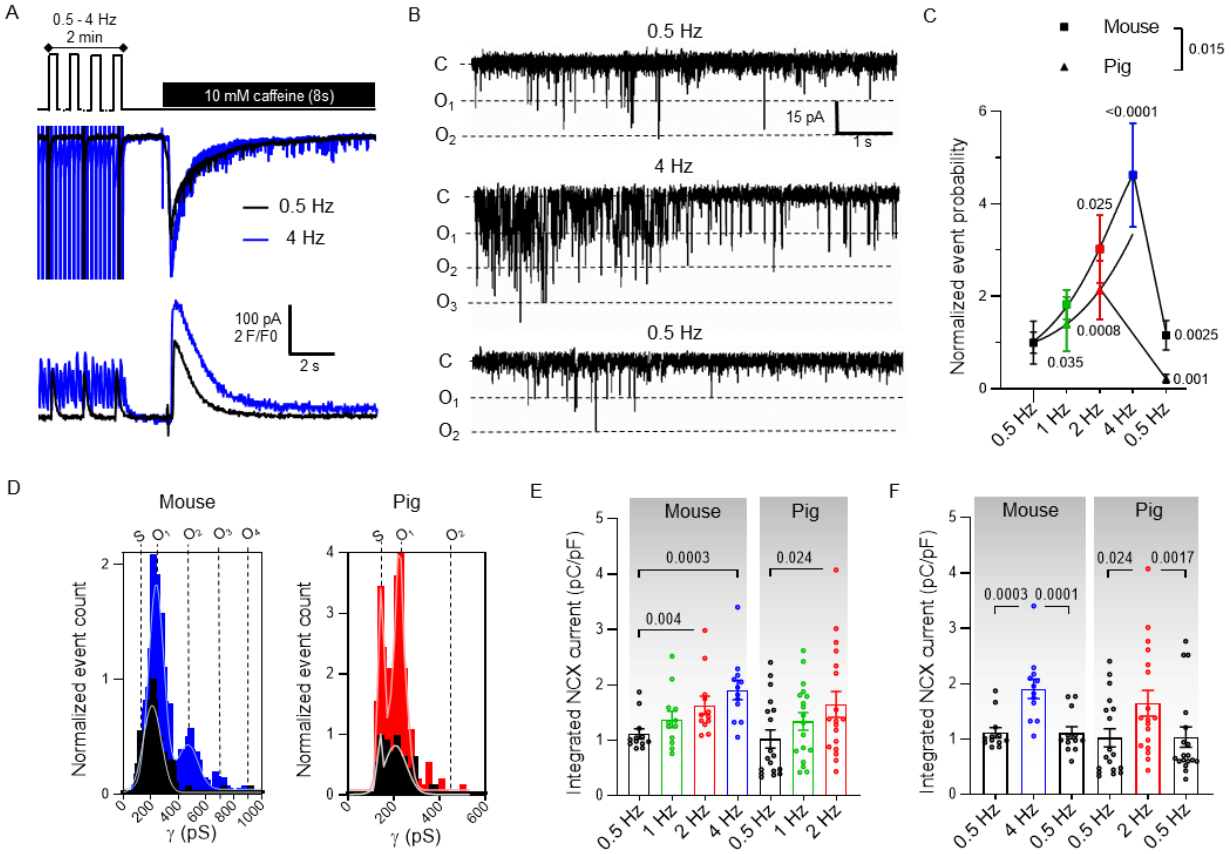


Figure 2. Cx43 hemichannel activation is frequency dependent

(A) Top trace shows experimental protocol: cells were paced for 2 min to steady-state (mouse 0.5, 1, 2 or 4 Hz; pig 0.5, 1 or 2 Hz) followed by caffeine superfusion. Middle and bottom traces depict current and $[Ca^{2+}]_i$ signals recorded in mouse following 0.5 (black) or 4 Hz (blue) pacing.

(B) Unitary current example traces following 0.5 and 4 Hz including reversibility (NCX currents subtracted).

(C) and (D) Summary graph and transition histograms indicate significant and reversible increase in unitary current event probability with increasing pacing frequency (N/n_{mouse} = 4/12, N/n_{pig} = 7/19). P-values compare to 0.5 Hz or to 2/4 Hz (nested one-way ANOVA).

(E) Summary dot plot depicting significant increase in SR Ca^{2+} content with increasing frequency, as determined by integrating NCX current during caffeine (nested one-way ANOVA; N/n_{mouse} = 4/12, N/n_{pig} = 7/19).

(F) Summary dot plot depicting reversible increase in SR Ca^{2+} content with increasing frequency (nested one-way ANOVA; N/n_{mouse} = 4/12, N/n_{pig} = 7/19).

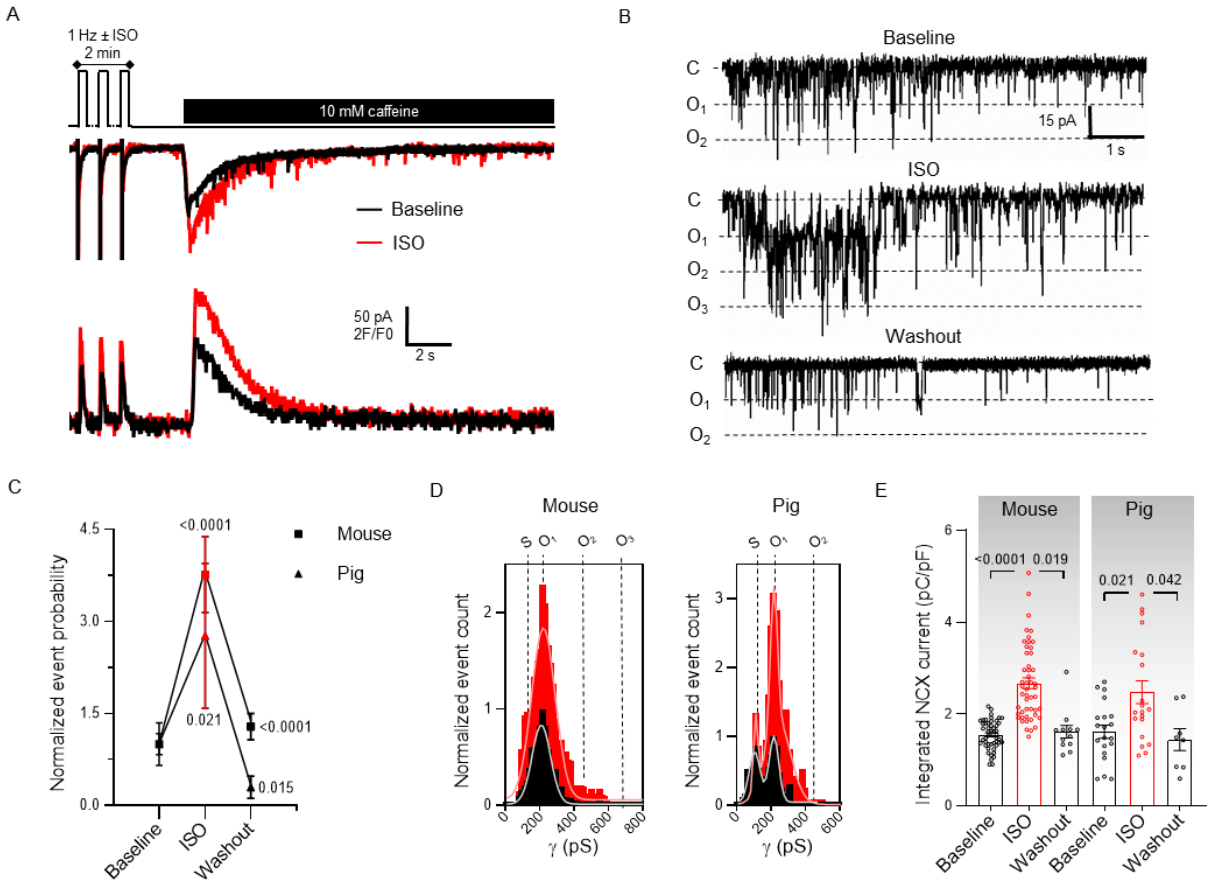
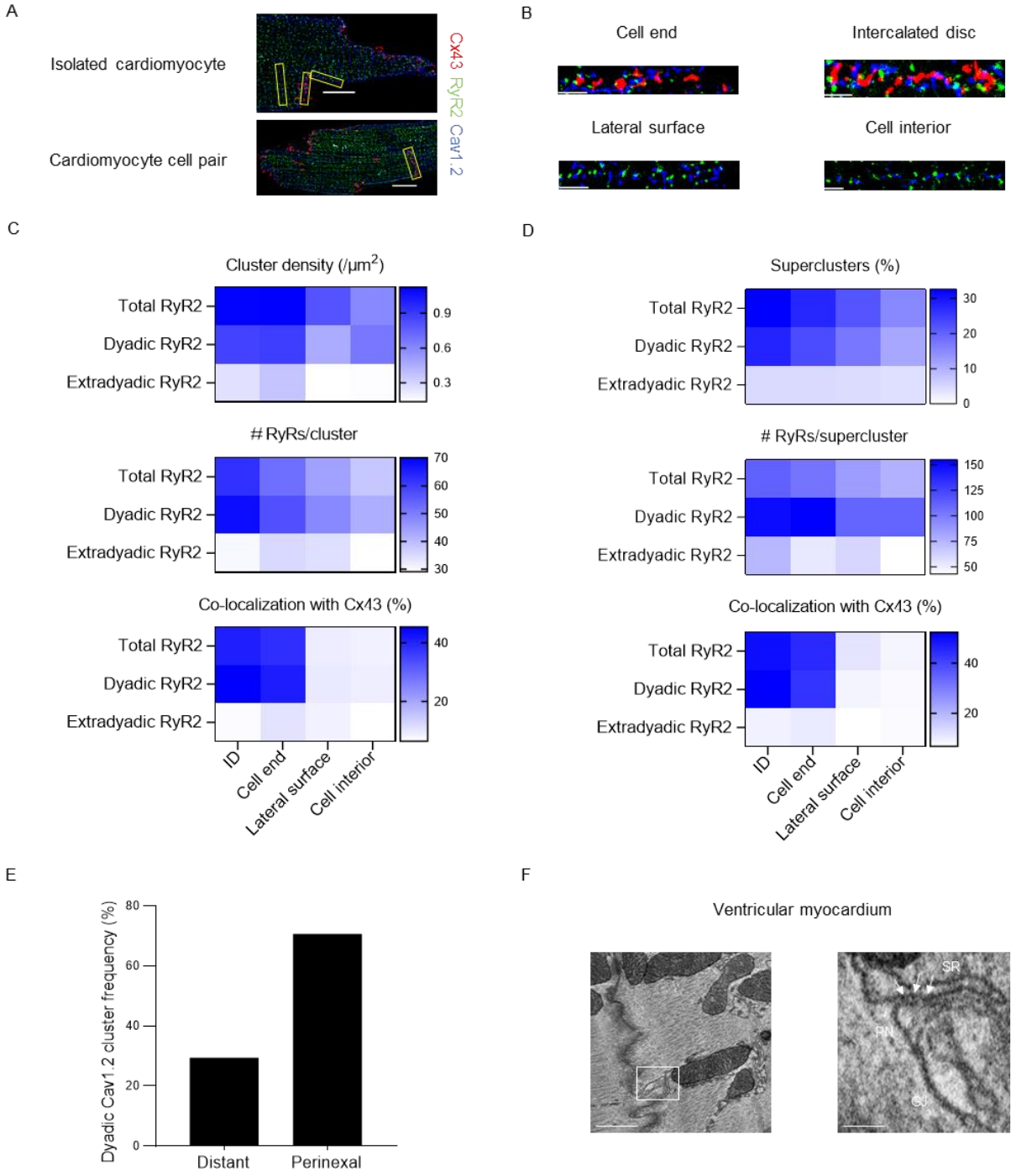


Figure 3. Cx43 hemichannel activation is modulated by β-adrenergic stimulation. (A) Top trace shows experimental protocol: β-adrenergic stimulation (1 µmol/L isoproterenol for mouse; 10 nmol/L pig) was applied when pacing frequency was 1 Hz. Middle and bottom traces depict current and $[Ca^{2+}]_i$ signals recorded in mouse following 1 Hz pacing without (black) or with isoproterenol (red).
(B) Unitary current example traces in the absence or presence of isoproterenol, including washout (NCX currents subtracted).
(C) and (D) Summary graph and transition histograms indicate significant and reversible increase in unitary current event probability with isoproterenol compared to baseline (nested one-way ANOVA; N/n_{mouse} = 20/49, N/n_{pig} = 8/20).
(D) Summary dot plot illustrating reversible increase in SR Ca^{2+} content with isoproterenol compared to baseline (nested one-way ANOVA; N/n_{mouse} = 20/49, N/n_{pig} = 8/20).


A
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Cardiomyocyte cell pair
Cx43 RyR2 Cav1.2
B
Cell end
Intercalated disc
Lateral surface
Cell interior
C
Cluster density (/μm2)
Total RyR2
Dyadic RyR2
Extradyadic RyR2
0.9
0.6
0.3
RyRs/cluster
70
60
50
40
30
Co-localization with Cx43 (%)
40
20
ID
Cell end
Lateral surface
Cell interior
D
Superclusters (%)
30
20
10
0
RyRs/supercluster
150
125
100
75
50
Co-localization with Cx43 (%)
E
Dyadic Cav1.2 cluster frequency (%)
80
60
40
20
0
Distant
Perinexal
F
Ventricular myocardium
SR
PN
G

762

763

Figure 4. Cx43 co-localizes with large dyadic RyR2 superclusters and forms microdomains at the perinexus.

(A) 2D SMLM images of a murine cardiomyocyte (top) and cardiomyocyte cell pair (bottom), triple stained for Cx43 (red), RyR2 (green) and Cav1.2 (blue). Scale bar = 10 µm.

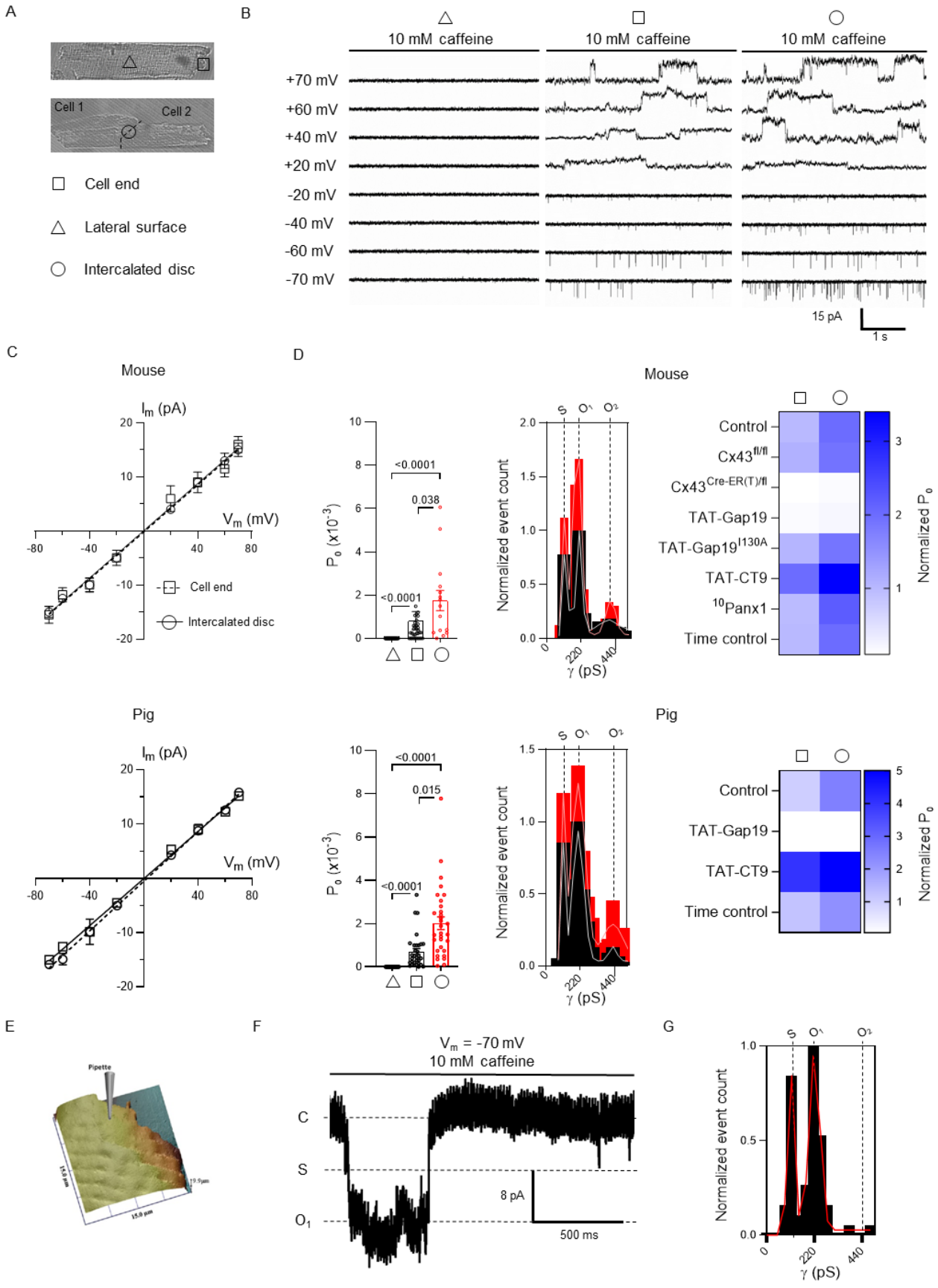
(B) Straightened region of interests (from yellow boxes in A) of Cx43, RyR2 and Cav1.2 at different subcellular domains. Scale bar = 2 µm.

(C) Heat map of RyR2 cluster density, number of molecules and co-localization with Cx43 at different subcellular domains (N = 5, n = 42 single cardiomyocytes, 16 cardiomyocyte cell pairs). RyR2 clusters were classified as dyadic or extradyadic based on the proximity of Cav1.2 clusters, RyR2 clusters occurring < 250 nm of a Cav1.2 clusters were categorized as dyadic.

(D) Heat map of RyR2 supercluster abundance, size and co-localization with Cx43 at different subcellular domains (N = 5, n = 42 single cardiomyocytes, 16 cardiomyocyte cell pairs).

(E) Relative localization overview in left ventricular mouse cardiomyocyte cell pairs. Dyadic Cav1.2 clusters were categorized as perinexal or distant based on edge distance ≤ or > 200 nm from edge of Cx43 cluster respectively (N = 5, n = 16 cardiomyocyte cell pairs).

(F) EM images of a SR cistern forming a dyadic cleft at the perinexus in mouse ventricular myocardium. Left image shows an EM overview of a murine ventricular intercalated disc. Scale bar = 500 nm. White box is enlarged on the right. Arrows indicate electron dense particles, likely ryanodine receptors. Scale bar = 100 nm. PN = perinexus.


A
Cell 1
Cell 2
Cell end
Lateral surface
Intercalated disc
B
10 mM caffeine
10 mM caffeine
10 mM caffeine
+70 mV
+60 mV
+40 mV
+20 mV
-20 mV
-40 mV
-60 mV
-70 mV
15 pA
1 s
C
Mouse
Im (pA)
Vm (mV)
Cell end
Intercalated disc
D
Mouse
<0.0001
0.038
<0.0001
Po (x10-3)
Normalized event count
γ (pS)
Control
Cx43fl/fl
Cx43Cre-ER(T)/fl
TAT-Gap19
TAT-Gap19I130A
TAT-CT9
10Panx1
Time control
Normalized Po
Pig
<0.0001
0.015
<0.0001
E
Pipette
F
Vm = -70 mV
10 mM caffeine
C
S
O1
8 pA
500 ms
G

788

Figure 5. Discrete sites of Cx43 hemichannel activation at the intercalated disc during Ca^{2+} release from the sarcoplasmic reticulum.

(A) Transmitted light images of a single cardiomyocyte (top) and cardiomyocyte cell pair (bottom). Triangle, square and circle symbols indicate cell-attached macropatch (R_p = ~2 MΩ, ~2 µm pipette inner diameter) recording positions at the lateral membrane and cell end of single cardiomyocytes and intercalated disc of cardiomyocyte cell pairs respectively.

(B) Example traces showing single channel currents recorded at the lateral membrane, cell end or intercalated disc. Traces recorded in mouse cardiomyocytes during caffeine superfusion (10 mM, 8 s) at indicated membrane potentials.

(C) IV plots depicting linear current-voltage relationship with slope conductance of ~220 pS and E_{rev} ≈ 0 mV (N/n_{mouse} = 5/10-18 patches per conditions, N/n_{pig} = 5/15-20 patches per condition).

(D) Summary dot plots and transition histograms indicate recording of ~220 pS single channel currents at the cell end of single cardiomyocytes, but not at the lateral membrane, with significantly increased open probability at the intercalated disc of cardiomyocyte cell pairs. Comparative statistics with nested one-way ANOVA. Heat map summarizes single channel open probability at the cell end or at the intercalated disc under conditions of Cx43 knockdown or in the presence of TAT-Gap19, inactive TAT-$Gap19^{I130A}$, TAT-CT9 or ^{10}Panx1 (N/n_{mouse} = 5/10-18 patches per conditions, N/n_{pig} = 5/15-20 patches per condition).

(E) SICM-generated membrane topology of the cell end of a mouse left ventricular cardiomyocyte. Pipette indicates the recording position distally of the last Z-line.

(F) Example trace recorded at -70 mV during caffeine superfusion.

(G) Transition histogram from all experiments (N = 5, n = 35) showing a fully open state at ~220 pS and a substate at ~110 pS.

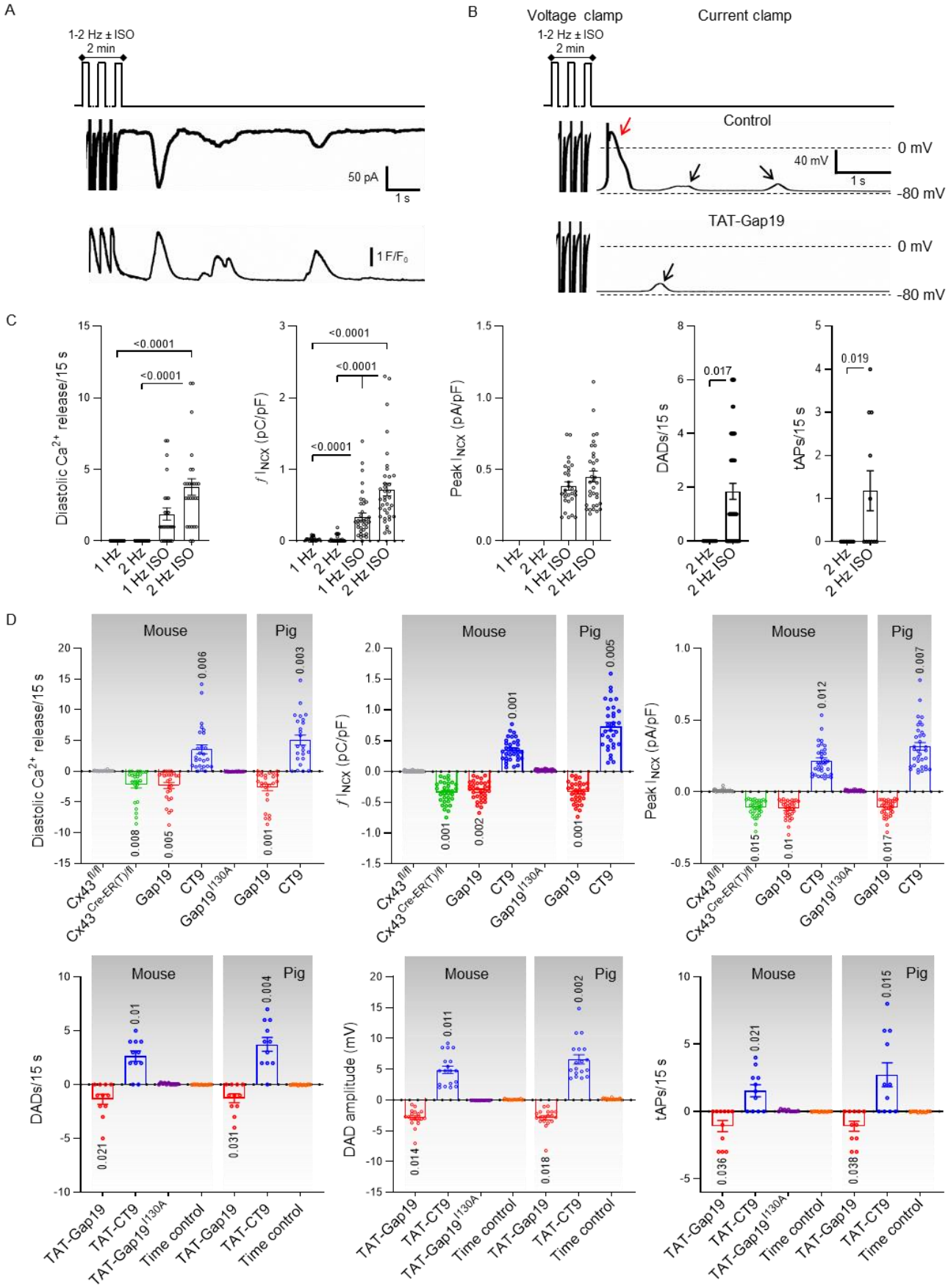

A
1-2 Hz ± ISO
2 min
50 pA
1 s
1 F/F0
B
Voltage clamp
Current clamp
1-2 Hz ± ISO
2 min
Control
0 mV
40 mV
1 s
-80 mV
TAT-Gap19
0 mV
-80 mV
C
Diastolic Ca2+ release/15 s
<0.0001
<0.0001
f INCX (pC/pF)
<0.0001
<0.0001
<0.0001
Peak INCX (pA/pF)
DADs/15 s
0.017
tAPs/15 s
0.019
1 Hz
2 Hz
1 Hz ISO
2 Hz ISO
D
Mouse
Pig
Diastolic Ca2+ release/15 s
0.006
0.003
0.008
0.005
0.001
f INCX (pC/pF)
0.001
0.005
0.001
0.002
0.001
Peak INCX (pA/pF)
0.012
0.007
0.015
0.01
0.017
Cx43fl/fl
Cx43Cre-ER(T)/fl
Gap19
CT9
Gap19I130A
DADs/15 s
0.01
0.004
0.021
0.031
DAD amplitude (mV)
0.011
0.002
0.014
0.018
tAPs/15 s
0.021
0.015
0.036
0.038
TAT-Gap19
TAT-CT9
TAT-Gap19I130A
Time control

Figure 6. Cx43 hemichannel opening during adrenergic stimulation modulates spontaneous Ca^{2+} release from the sarcoplasmic reticulum and arrhythmogenic afterdepolarizations.

(A) Freshly isolated mouse and pig left ventricular cardiomyocytes were subjected to voltage clamp experiments while $[Ca^{2+}]_i$ was simultaneously monitored. Top trace shows experimental protocol: cells were paced to steady-state for 2 min at 1 Hz and then clamped to -70 mV. Middle and bottom traces depict resulting current and $[Ca^{2+}]_i$ signals: final three paced Ca^{2+} transients and accompanying currents followed by 15 seconds rest period showing spontaneous diastolic Ca^{2+} release with resulting NCX current. Protocols were repeated at 2 Hz with and without isoproterenol. Example traces were recorded in pig.

(B) In a subset of experiments, we switched to current clamp mode following steady-state pacing in voltage clamp. Example traces, recorded in the same pig cardiomyocyte, without and with TAT-Gap19 recorded in current clamp mode following 2 min pacing to steady-state at 2 Hz with isoproterenol (in voltage clamp mode). Black arrows indicate DADs, red arrow indicates a triggered action potential.

(C) Summary dot plots (nested one-way ANOVA; N/n_{mouse} = 23/75 for voltage clamp experiments and N/n_{mouse} = 5/45 for current clamp experiments) illustrating increased frequency and amplitude of diastolic Ca^{2+} release with increased resulting NCX current and membrane depolarization during adrenergic stimulation (2 Hz + ISO) as compared to baseline. tAP = triggered action potential. Similar results were obtained in pig (not shown).

(D) Summary data showing the impact of different interventions on diastolic Ca^{2+} release and resulting NCX currents and membrane depolarization (nested one-way ANOVA; N/n_{mouse} = 5-11/15-24 per condition, N/n_{pig} = 5/15-20 per condition). Values reported as differences from the control condition.

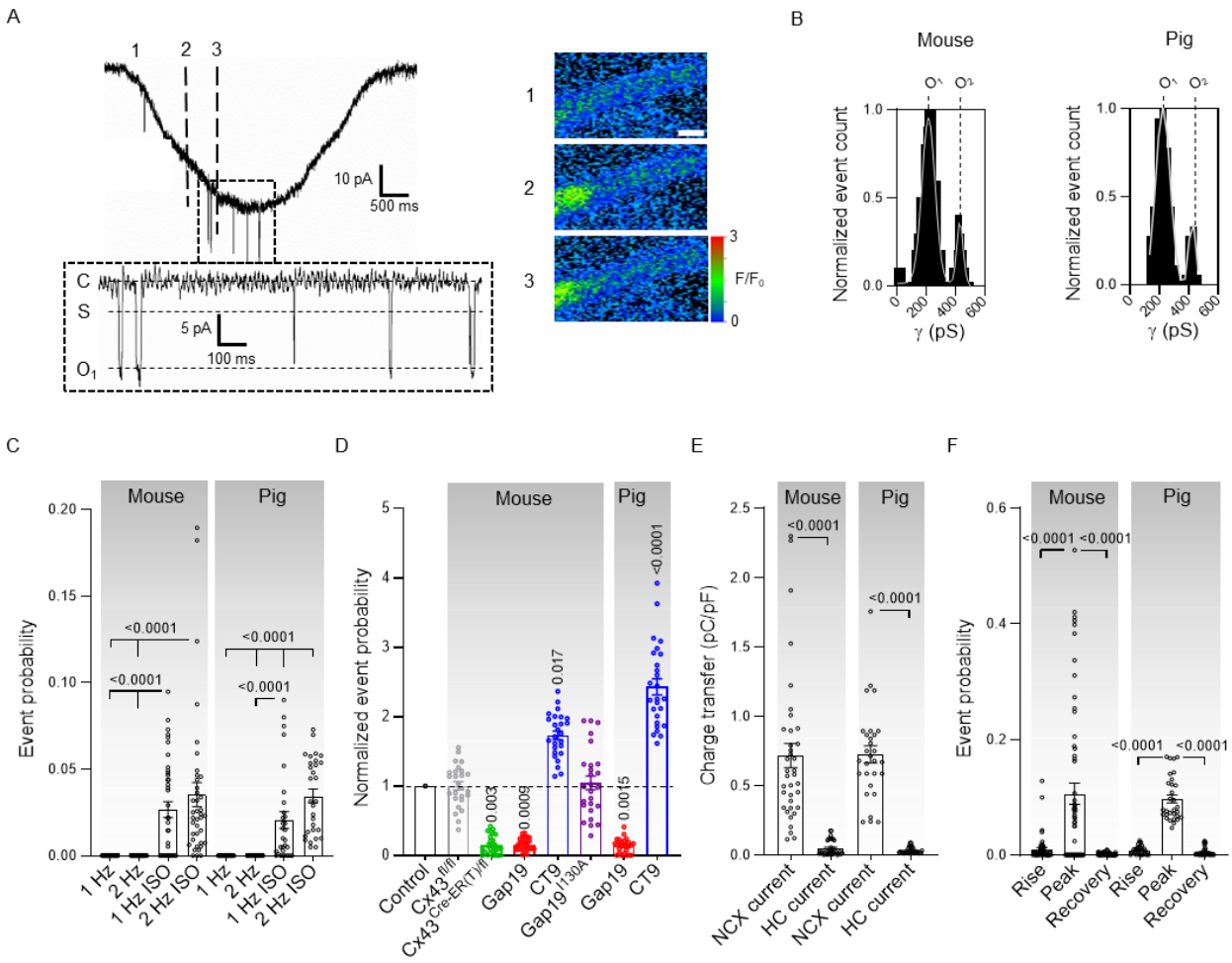


Figure 7. Spontaneous Cx43 hemichannel openings during Ca^{2+} waves

(A) Left, NCX current during spontaneous Ca^{2+} release with superimposed unitary currents. Inset shows detail of unitary current activity. Right, Ca^{2+} images corresponding to time points indicated by dashed lines in left trace. Scale bar = 10 µm.

(B) Transition histograms of superimposed unitary activity showing ~220 pS unitary conductance (N/n_{mouse} = 23/75, N/n_{pig} = 10/30).

(C) Summary dot plot illustrating increasing unitary current event probability with increasing pacing frequency and with isoproterenol (nested one-way ANOVA; N/n_{mouse} = 23/75, N/n_{pig} = 10/30).

(D) Summary data showing the effect of different interventions at 2 Hz + ISO (N/n_{mouse} = 5-11/15-24 per condition, N/n_{pig} = 5/15 per condition). P-values indicate significance compared to control (nested one-way ANOVA).

(E) Summary dot plot depicting relative integrals of NCX and unitary current at 2 Hz + ISO (nested t-test; N/n_{mouse} = 23/75, N/n_{pig} = 10/30).

(F) Summary dot plot depicting unitary current event probability during different phases of NCX current induced by spontaneous Ca^{2+} release (nested one-way ANOVA; N/n_{mouse} = 23/75, N/n_{pig} = 10/30). These phases include the rising phase ('rise', 10-90%), peak and recovery (90-10%).

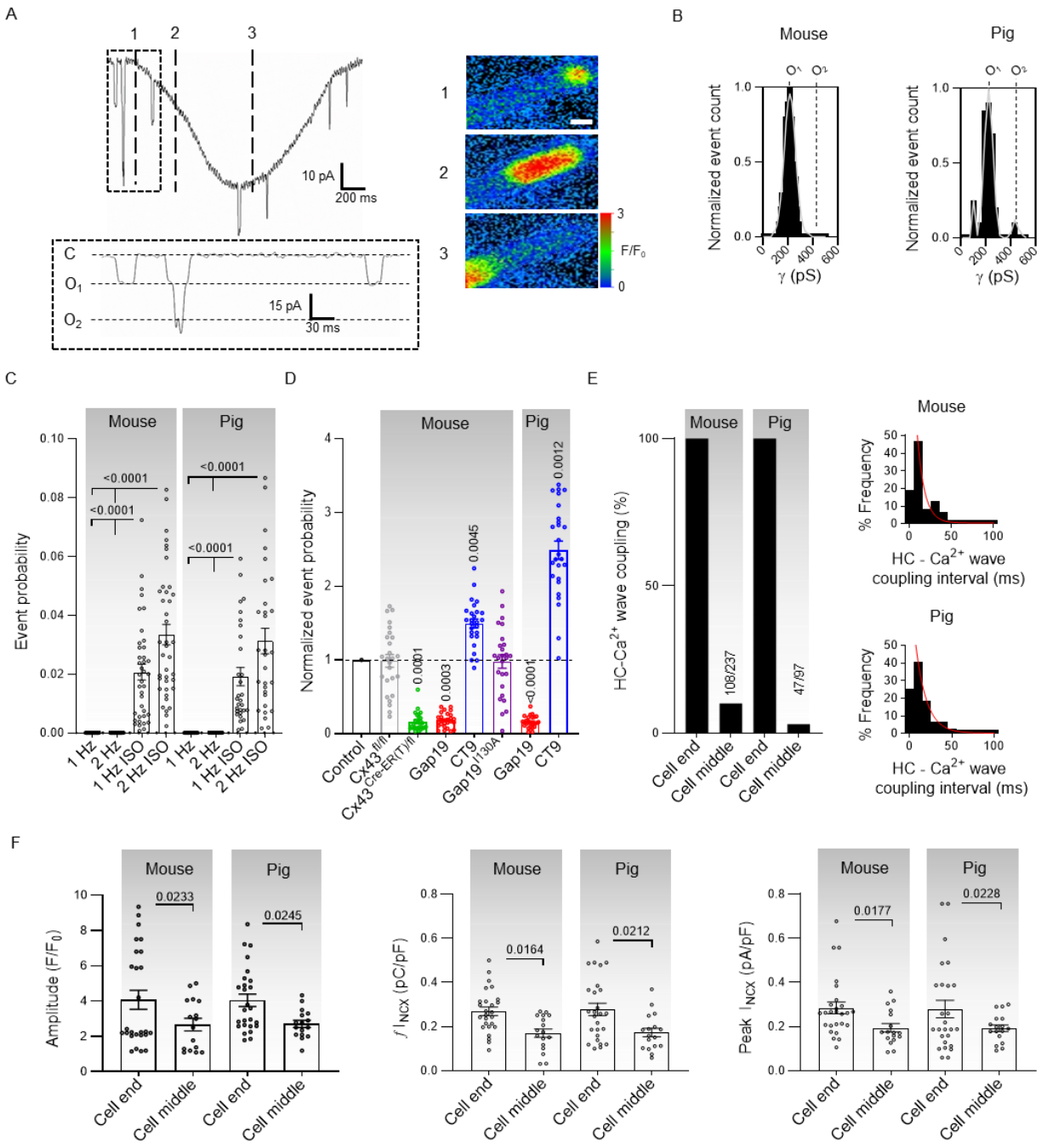
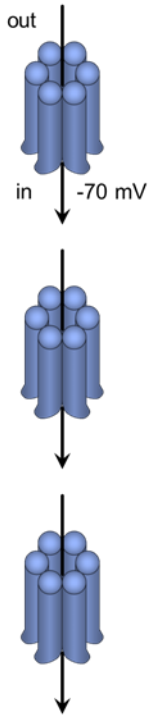


Figure 8. Spontaneous Cx43 hemichannel openings preceding Ca^{2+} waves promote arrhythmogenic Ca^{2+} release and resulting depolarizing current.
(A) Left, unitary currents preceding diastolic Ca^{2+} release. Inset shows detail of unitary current activity. Right, Ca^{2+} images corresponding to time points indicated by dashed lines in left trace. Scale bar = 10 µm.
(B) Transition histograms showing ~220 pS unitary conductance of preceding unitary activity (N/n_{mouse} = 23/75, N/n_{pig} = 10/30).

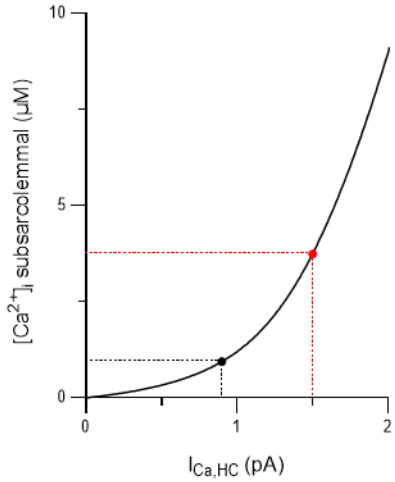
(C) Summary dot plot illustrating increasing unitary current event probability with increasing pacing frequency and with isoproterenol (nested one-way ANOVA; N/n_{mouse} = 23/75, N/n_{pig} = 10/30).
(D) Summary data showing the effect of different interventions at 2 Hz + ISO (N/n_{mouse} = 5-11/15-24 per condition, N/n_{pig} = 5/15 per condition). P-values indicate significance compared to control (nested one-way ANOVA).
(E) Fraction and coupling interval of Cx43 hemichannel (HC)-associated Ca^{2+} release (N/n_{mouse} = 23/75, N/n_{pig} = 10/30). Left graph indicates that HC-Ca^{2+} release coupling to Ca^{2+} waves occurs at the cell end. Numbers show absolute counts. Right histogram indicates time from hemichannel opening to Ca^{2+} release.
(F) Dot plots summarizing properties of diastolic Ca^{2+} release and resulting NCX currents categorized by origin (nested t-test; N/n_{mouse} = 23/75, N/n_{pig} = 10/30).

A

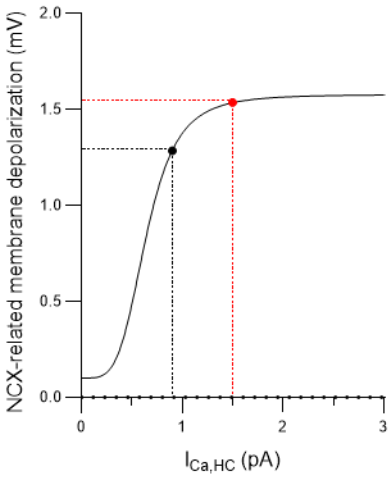


CURRENT	magnitude	EFFECT	magnitude
Electrical current	15.9 pA	Direct depolarizing effect	~1.6 mV
Calcium current	1.0 mM Ca^{2+}_{out} ~0.8 pA	NCX-related depolarization	~1.3 mV
	1.8 mM Ca^{2+}_{out} ~1.5 pA		~1.5 mV
		Summed depolarizing effect	~2.9 mV
			~3.1 mV
Calcium current	1.0 mM Ca^{2+}_{out} ~0.8 pA	RyR cluster activation	P = 0.72
		RyR-related Ca^{2+} waves	P = 0.71
	1.8 mM Ca^{2+}_{out} ~1.5 pA		P = 0.99
			P = 0.98

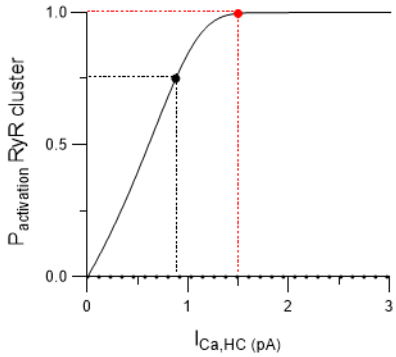
C



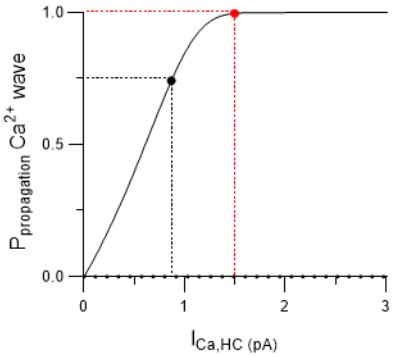
D



E



F



885

Figure 9. Modeling based estimations of electrical and Ca^{2+} consequences of single hemichannel opening.

(A) Schematic overview of electrical and Ca^{2+} consequences of single hemichannel opening. The 15.9 pA electrical current is a measured value while the 0.8 pA and 1.5 pA Ca^{2+} currents are calculated estimates using 1.0 and 1.8 mM of extracellular Ca^{2+} respectively. Panels B to E further explore the impact of a range of hemichannel Ca^{2+} currents. Note that the values given are only valid at -70 mV membrane potential and 37°C. Further modeling details can be found in supplemental information.

(B) Peak elevation of subsarcolemmal $[Ca^{2+}]_i$ as a function of single hemichannel Ca^{2+} current ($I_{Ca,HC}$).

(C) Membrane depolarization associated with Ca^{2+} entry associated NCX activation. Black and red points indicate hemichannel Ca^{2+} current estimates, which are close to or in the plateau phase of the curve.

(D) Probability of activation of RyR superclusters as a function of single hemichannel Ca^{2+} current.

(E) Probability of Ca^{2+} wave propagation as a function of single hemichannel Ca^{2+} current.

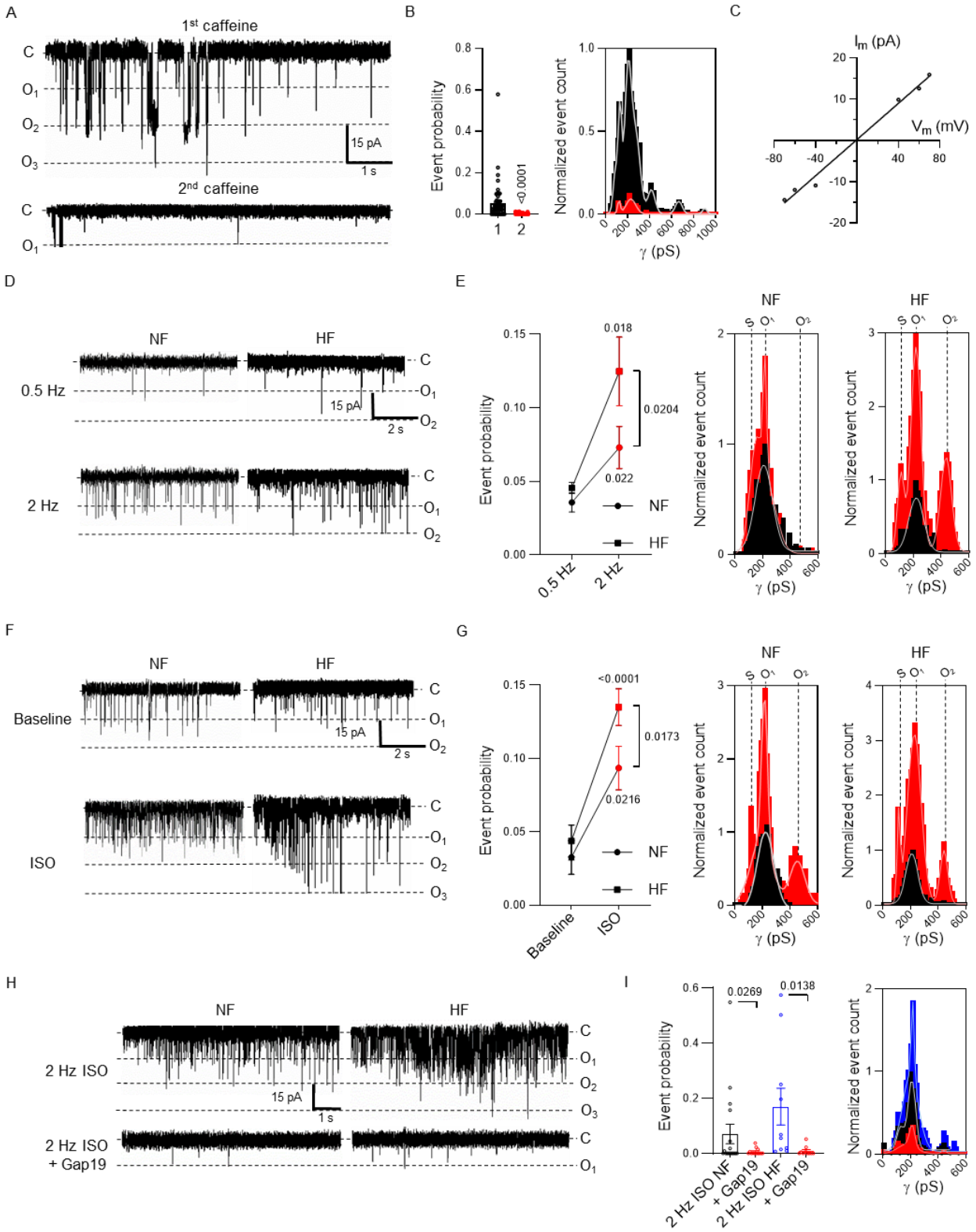

A
1st caffeine
C
O1
O2
O3
15 pA
1 s
2nd caffeine
B
Event probability
<0.0001
Normalized event count
γ (pS)
C
Im (pA)
Vm (mV)
D
NF
HF
0.5 Hz
2 Hz
15 pA
2 s
E
0.018
0.0204
0.022
0.5 Hz
2 Hz
F
Baseline
ISO
G
<0.0001
0.0173
0.0216
H
2 Hz ISO
2 Hz ISO + Gap19
I
0.0269
0.0138
2 Hz ISO NF
+ Gap19
2 Hz ISO HF
+ Gap19

Figure 10. Identification and regulation of Cx43 hemichannels in non-failing and failing human ventricular cardiomyocytes.

(A) Unitary current example traces during first and second caffeine applications, NCX current subtracted. Recorded in non-failing left ventricular human cardiomyocyte.

(B) Summary dot plot and transition histogram indicating significantly reduced unitary current event probability during the second caffeine pulse (red) as compared to the first (black) (nested t-test; N/n_{NF} = 20/64).

(C) IV plots depicting linear current-voltage relationship with slope conductance ~220 pS and $E_{rev} \approx 0$ mV (N_{NF}/n_{NF} = 4/14).

(D) Ca^{2+} release-induced unitary current example traces following 0.5 and 2 Hz pacing in non-failing and failing human cardiac myocytes (NCX currents subtracted).

(E) Summary graph and transition histograms indicate significant increase in unitary current event probability with increasing pacing frequency (nested t-test; N/n_{NF} = 5/25, N/n_{HF} = 5/25). This effect is significantly stronger at 2 Hz in failing as compared to non-failing cardiomyocytes (nested t-test).

(F) Ca^{2+} release-induced unitary current example traces in the absence and presence of isoproterenol (10 nmol/L) in non-failing and failing human cardiac myocytes (NCX currents subtracted).

(G) Summary graph and transition histograms indicate significant increase in unitary current event probability with isoproterenol as compared to baseline (nested t-test; N/n_{NF} = 5/13, N/n_{HF} = 5/14). The effect is significantly stronger with ISO in failing as compared to non-failing cardiomyocytes (nested t-test).

(H) Ca^{2+} release-induced unitary current example traces during fast pacing and adrenergic stimulation in non-failing and failing human cardiomyocytes. Including Gap19 in the pipette solution abolished unitary current activity (NCX currents subtracted).

(I) Summary dot plot and transition histogram illustrating increased event probability in failing as compared to non-failing myocytes. Gap19 significantly reduced event probability in non-failing and failing cardiomyocytes (nested one-way ANOVA; N/n_{NF} = 4/15, N/n_{HF} = 5/15).

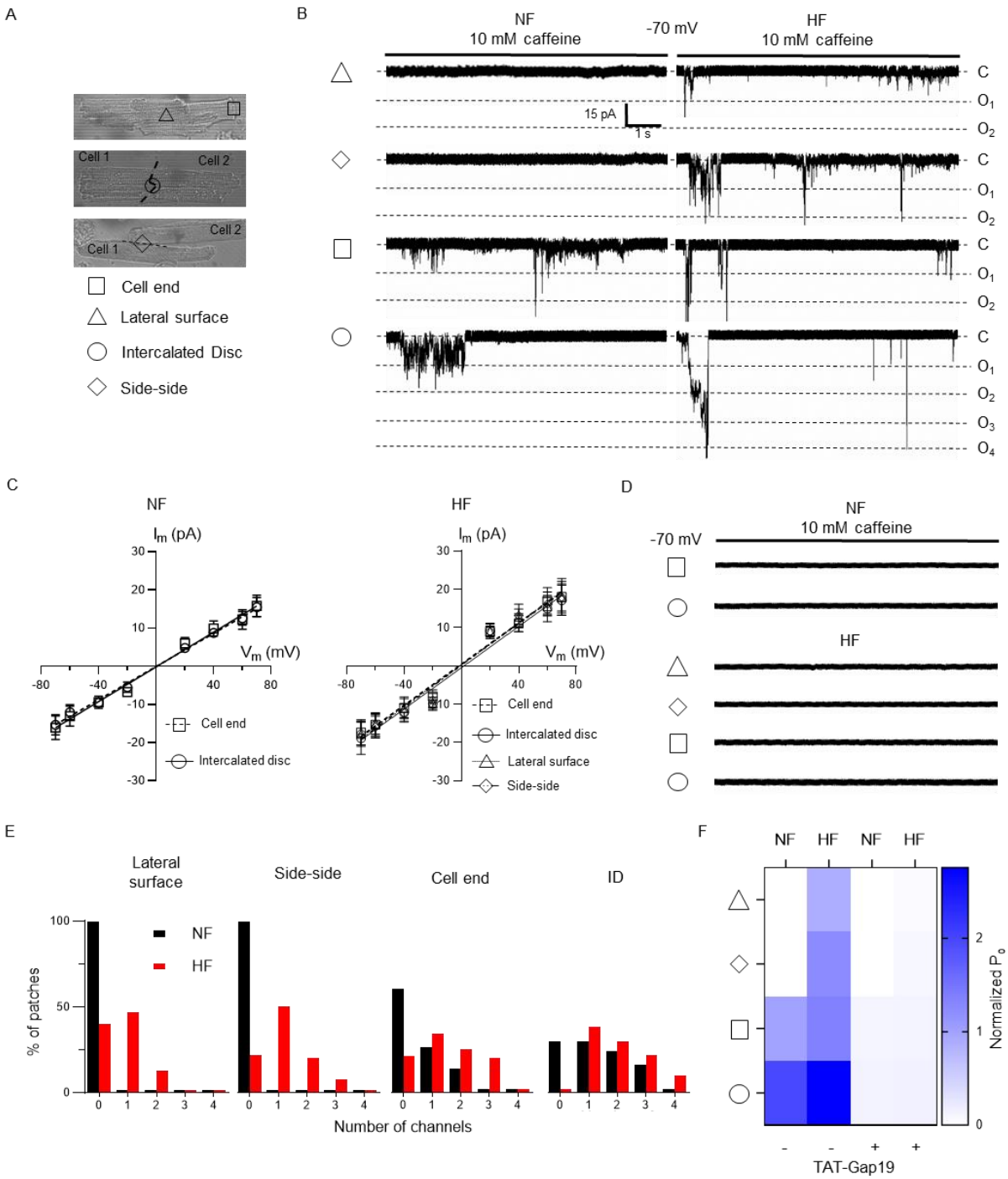
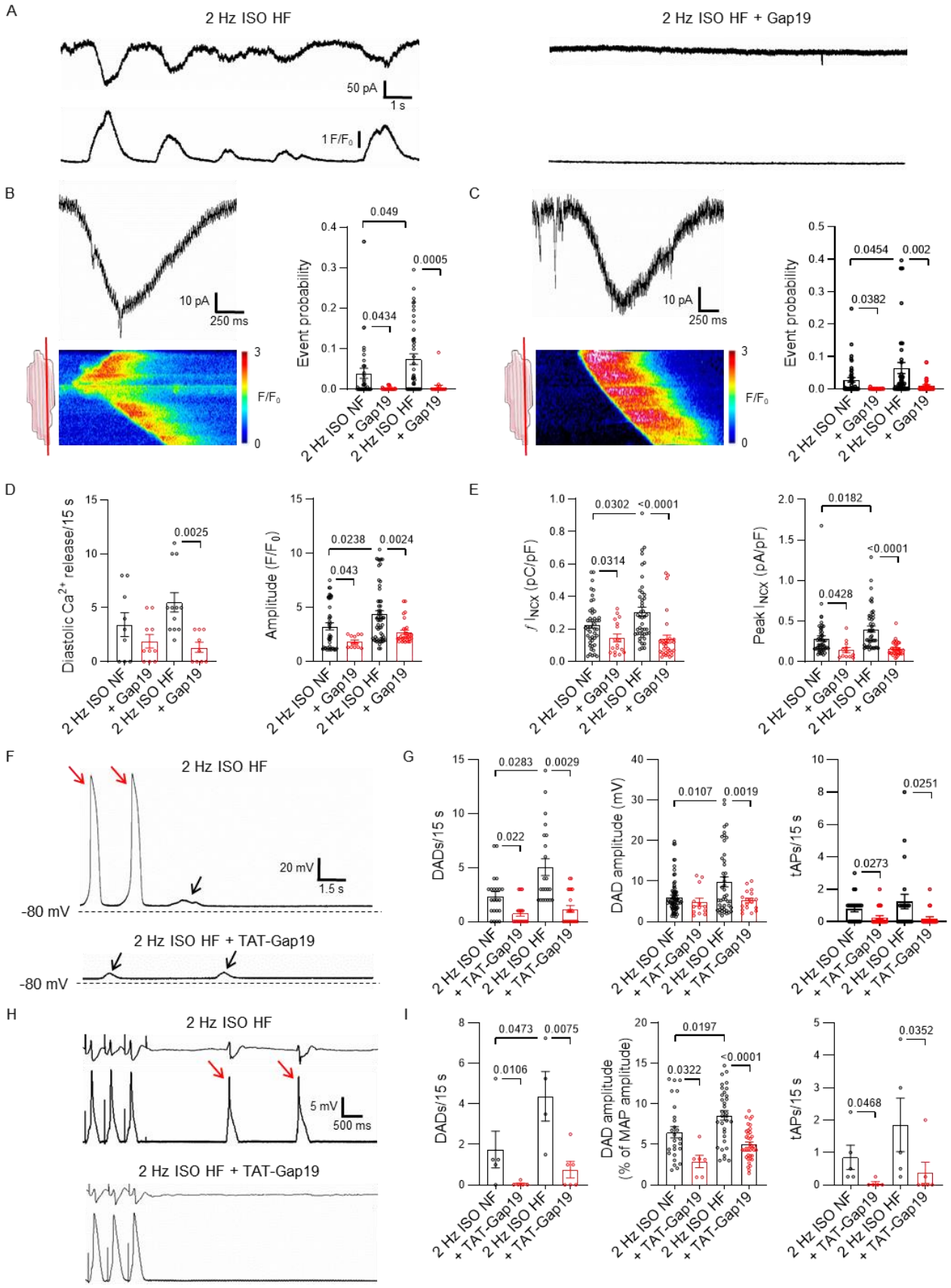


Figure 11. Microdomain specific activation of Cx43 hemichannels in non-failing and failing human cardiomyocytes.
(A) Transmitted light images of single cardiomyocyte (top) and cardiomyocyte cell pairs (middle, bottom). Triangle, square, circle and diamond symbols indicate cell-attached macropatch positions at the lateral membrane and cell end of single cardiomyocytes, and intercalated disc and side-side of cardiomyocyte cell pairs respectively.

(B) Example traces showing single channel currents recorded at the different macropatch recording positions. Traces recorded in non-failing and failing human cardiomyocytes during caffeine superfusion (10 mM, 8 s) at -70 mV.
(C) IV plots depicting linear current-voltage relationship with slope conductance of ~220 pS and $E_{rev} \approx 0$ mV (N/n_{NF} = 3/10-15 per recording position, N/n_{HF} = 3/10-15 per recording position).
(D) Example traces showing single channel currents recorded at the different macropatch recording positions following TAT-Gap19 superfusion.
(E) Summary histograms depicting number of channels per patch for the different macropatch recording positions (N/n_{NF} = 3/10-15 per recording position, N/n_{HF} = 3/10-15 per recording position). Black and red bars indicate recordings in non-failing and failing human cardiomyocytes respectively.
(F) Heat map summarizing single channel open probability at different macropatch recording positions with and without TAT-Gap19 in non-failing and failing human single cardiomyocytes and cardiomyocyte cell pairs (N/n_{NF} = 3/10-15 per recording position, N/n_{HF} = 3/10-15 per recording position).


A
2 Hz ISO HF
2 Hz ISO HF + Gap19
50 pA
1 s
1 F/F0
B
10 pA
250 ms
F/F0
Event probability
0.049
0.0434
0.0005
2 Hz ISO NF
+ Gap19
2 Hz ISO HF
+ Gap19
C
10 pA
250 ms
0.0454
0.002
0.0382
D
Diastolic Ca2+ release/15 s
0.0025
Amplitude (F/F0)
0.0238
0.0024
0.043
E
f INCX (pC/pF)
0.0302
<0.0001
0.0314
Peak INCX (pA/pF)
0.0182
<0.0001
0.0428
F
2 Hz ISO HF
20 mV
1.5 s
-80 mV
2 Hz ISO HF + TAT-Gap19
-80 mV
G
DADs/15 s
0.0283
0.0029
0.022
DAD amplitude (mV)
0.0107
0.0019
tAPs/15 s
0.0251
0.0273
2 Hz ISO NF
+ TAT-Gap19
2 Hz ISO HF
+ TAT-Gap19
H
2 Hz ISO HF
5 mV
500 ms
2 Hz ISO HF + TAT-Gap19
I
DADs/15 s
0.0473
0.0075
0.0106
DAD amplitude
(% of MAP amplitude)
0.0197
0.0322
<0.0001
tAPs/15 s
0.0352
0.0468

959

Figure 12. Contribution of Cx43 hemichannels to spontaneous Ca^{2+} release and afterdepolarizations in human heart failure.

(A) Example traces of spontaneous diastolic Ca^{2+} release and resulting NCX currents recorded in failing human left ventricular cardiomyocytes following fast pacing and adrenergic stimulation with or without Gap19.

(B) and (C) unitary current detail and associated confocal line scan images during and preceding spontaneous Ca^{2+} release respectively. Red line on cartoons indicate scan line position. Dot plots show significant increase in unitary current event probability in failing as compared to non-failing cardiomyocytes. Gap19 abolished unitary current activities (nested one-way ANOVA; N/n_{NF} = 5/13, N/n_{HF} = 5/14).

(D) and (E) Summary dot plots illustrating increased frequency and significantly increased amplitude of spontaneous Ca^{2+} release and associated NCX currents in human heart failure. Gap19 abolished Ca^{2+} release and NCX currents, especially in heart failure (nested one-way ANOVA; N/n_{NF} = 5/13, N/n_{HF} = 5/14).

(F) Example traces of delayed afterdepolarizations and triggered action potentials recorded in a failing human left ventricular cardiomyocytes at baseline and after TAT-Gap19 superfusion. Black arrows indicate DADs, red arrows indicate triggered action potentials.

(G) Summary dot plots showing significantly increased frequency and amplitude of DADs and triggered action potential frequency in failing as compared to non-failing cardiac myocytes. TAT-Gap19 abolished DADs and triggered action potentials, especially in human heart failure (nested one-way ANOVA; N/n_{NF} = 6/22, N/n_{HF} = 5/22).

(H) Example ECG (top traces) and monophasic action potential traces (lower traces) recorded during adrenergic stimulation (baseline and following TAT-Gap19) in an arterially perfused left ventricular tissue wedge prepared from failing human heart. Red arrows indicate triggered action potentials.

(I) Summary dot plots showing significantly increased frequency and amplitude of DADs and triggered action potential frequency in failing as compared to non-failing tissue wedges. TAT-Gap19 abolished DADs and triggered action potentials, especially in human heart failure (nested one-way ANOVA; N/n_{NF} = 5/10, N/n_{HF} = 4/9).