Gating of connexin 43 gap junctions by a cytoplasmic loop calmodulin binding domain

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Xu Q, Kopp RF, Chen Y, Yang JJ, Roe MW, Veenstra RD. Gating of connexin 43 gap junctions by a cytoplasmic loop calmodulin binding domain. Am J Physiol Cell Physiol 302:C1548–C1556, 2012.First published March 14, 2012; doi:10.1152/ajpcell.00319.2011.—Calmodulin (CaM) binding sites were recently identified on the cytoplasmic loop (CL) of at least three α-subfamily connexins (Cx43, Cx44, Cx50), while Cx40 does not have this putative CaM binding domain. The purpose of this study was to examine the functional relevance of the predicted CaM binding site on the Ca2+-dependent regulation of gap junction proteins formed by Cx43 and Cx40. Dual whole cell patch-clamp experiments were performed on stable murine Neuro-2a cells expressing Cx43 or Cx40. Addition of ionomycin to increase external Ca2+ influx reduced Cx43 gap junction conductance (Gj) by 95%, while increasing cytosolic Ca2+ concentration threefold. By contrast, Cx40 Gj declined by <20%. The Ca2+-induced decline in Cx43 Gj was prevented by pretreatment with calmidazolium or reversed by the addition of 10 mM EGTA to Ca2+-free extracellular solution, if Ca2+ chelation was commenced before complete uncoupling, after which gj was only 60% recoverable. The Cx43 CL136–155 mimetic peptide, but not the scrambled control peptide, or Ca2+/CaM-dependent kinase II 290–309 inhibitory peptide also prevented the Ca2+/CaM-dependent decline of Cx43 Gj. Cx43 gap junction channel open probability decreased to zero without reductions in the current amplitudes during external Ca2+/ionomycin perfusion. We conclude that Cx43 gap junctions are gated closed by a Ca2+/CaM-dependent mechanism involving the carboxyl-terminal quarter of the connexin CL domain. This study provides the first evidence of intrinsic differences in the Ca2+ regulatory properties of Cx43 and Cx40.

Connexins are integral membrane proteins that form gap junctions, which mediate the intercellular transfer of ions and small molecules between coupled cells. There are at least three α-subfamily connexins (Cx43, Cx44, and Cx50), while Cx40 does not have this putative CaM binding domain. The purpose of this study was to examine the functional relevance of the predicted CaM binding site on the Ca2+-dependent regulation of gap junction proteins formed by Cx43 and Cx40. Dual whole cell patch-clamp experiments were performed on stable murine Neuro-2a cells expressing Cx43 or Cx40. Addition of ionomycin to increase external Ca2+ influx reduced Cx43 gap junction conductance (Gj) by 95%, while increasing cytosolic Ca2+ concentration threefold. By contrast, Cx40 Gj declined by <20%. The Ca2+-induced decline in Cx43 Gj was prevented by pretreatment with calmidazolium or reversed by the addition of 10 mM EGTA to Ca2+-free extracellular solution, if Ca2+ chelation was commenced before complete uncoupling, after which qj was only 60% recoverable. The Cx43 CL136–155 mimetic peptide, but not the scrambled control peptide, or Ca2+/CaM-dependent kinase II 290–309 inhibitory peptide also prevented the Ca2+/CaM-dependent decline of Cx43 Gj. Cx43 gap junction channel open probability decreased to zero without reductions in the current amplitudes during external Ca2+/ionomycin perfusion. We conclude that Cx43 gap junctions are gated closed by a Ca2+/CaM-dependent mechanism involving the carboxyl-terminal quarter of the connexin CL domain. This study provides the first evidence of intrinsic differences in the Ca2+ regulatory properties of Cx43 and Cx40.
lular Ca$^{2+}$ is sufficient to uncouple Cx43, but not Cx40, gap junctions. This process is dependent on CaM and the predicted Cx43 CL CaM binding site, since acute treatment with the CaM inhibitor calmidazolium (CDZ), intracellular application of Ca$^{2+}$/CaM-dependent kinase II (CaMKII) 290–309 inhibitory or Ac-C136KYGIEEHGKVKMRGGLLRTYIIS158-NH$_2$ Cx43–3 peptides inhibited the Ca$^{2+}$-mediated uncoupling response. Our findings suggest that the CaM binding site of Cx43$^{136–158}$ is critical for the Ca$^{2+}$-induced closure of Cx43 gap junctions.

**MATERIALS AND METHODS**

Prediction of CaMBDs. The topology and orientation of the transmembrane regions of the human Cx43 (Fig. 1A) were drawn based on predictions using four different programs, including SOSUI (34), TMHMM (23), MEMSAT (21), and HMMTOP (56). Sequence alignments of the gap junction proteins Cx43 (accession ID: NP_001516; human), Cx40 (accession ID: NP_005257; human), and CaMKII (accession ID: AAH40457; rat) were carried out by using the ClustalW2 algorithm (53). The probability of CaM binding regions in these proteins was predicted by CaM Target Database (64).

Cell cultures. Mouse N2a neuroblastoma cells stably expressing rat Cx43 or rat Cx40 have been described previously (27, 29). N2a cells were cultured in minimum essential media, supplemented with 10% fetal bovine serum, 0.25 g/l G418 sulfate (Invitrogen).

Cardiomyocyte cultures. Neonatal mouse atrial and ventricular myocyte primary cultures were prepared according to previously published methods (28, 30). Newborn C57Bl/6 mice were anesthetized with isoflurane, and the hearts excised in accordance with procedures approved by State University of New York Upstate Medical University Committee for the Humane Use of Animals. The atria and ventricles were dissociated separately in a Ca$^{2+}$- and Mg$^{2+}$-free collagenase type II ($\approx$1 mg/ml, Worthington Biochemicals) dissociation solution containing the following (in mM): 116 NaCl, 5.4 KCl, 1.0 NaH$_2$PO$_4$, and 5.5 dextrose. The supernatant was collected after each of four 10-min dissociation cycles, passed through a 70-μm cell strainer (Falcon), and centrifuged at low speed, and the cell pellet was resuspended in M199 media supplemented with 10% fetal bovine serum. The primary cell cultures were enriched for cardiomyocytes by differential cell adhesion for 30 min and plated onto 35-mm culture dishes.

Solutions. The standard external solution (SES) contained the following (in mM): 142 NaCl, 1.3 KCl, 4 CsCl, 2 tetraethylammonium chloride, 0.8 MgSO$_4$, 0.9 NaH$_2$PO$_4$, 1.8 CaCl$_2$, 5.5 dextrose, 10 HEPES-NaOH (pH 7.4). A nominally Ca$^{2+}$-free SES was prepared in the same manner without 1.8 mM CaCl$_2$. To remove trace amounts of Ca$^{2+}$, 10 mM EGTA were added. Ionomycin (calcium salt, Sigma) and CDZ chloride (Calbiochem) were added to SES. Perfusion of the 35-mm cell culture dish was gravity fed at a rate of 1 ml/min (3 ml bath volume) via an infow line positioned <300 μm from the recording cell pair. Patch pipettes were filled with a KCl internal pipette solution (IPS KCl, in mM): 140 KCl, 1.0 MgCl$_2$, 3.0 CaCl$_2$, 5.0 BAPTA, 25 HEPES; pH titrated to 7.4 (using 1 N KOH). BAPTA (5 mM) titrated with 3 mM CaCl$_2$ sets the solution [Ca$^{2+}$] to ~5 mM (MAXCHELATOR software). The [Ca$^{2+}$] of IPS KCl was verified by calibrated in vitro fluorescence measurements using conventional fura 2 fluorescence measurement methods (19). The calibrated $K_D$ of fura 2 was 78 ± 8 nM, and the measured free [Ca$^{2+}$] of the pipette solution was 137 ± 2 nM, based on the in vitro measurement. In select experiments, the Cx43–3, Cx43-scrambled (-scr), or the calmodulin-dependent protein kinase II 290–309 (Axxora) peptides were added to both patch pipette solutions (67). To test for the effects of whole cell dialysis and Ca$^{2+}$-BAPTA buffering during conventional ruptured patch recordings, some experiments were performed using perforated patch methods by adding 50 μM β-escin to

**Fig. 1.** A: the connexins (Cx) are composed of four transmembrane (TM) segments, two extracellular loops, a short NH$_2$-terminus (region 1), one cytoplasmic loop linking TM2 and TM3 (region 2 and region 3), and a longer COOH-terminal tail (region 4). The numerical score (1–9) represents the prediction probability of each amino acid in a potential calmodulin (CaM) binding site. PCsB is the overall probability for the entire potential CaM binding domain (CaMBD) predicted by the CaM Target Database. The predicted CaMBD of Cx43 is located in the distal portion of the Cx43 intracellular loop (region 3). Similar to the CaMBD of Ca$^{2+}$/CaM-dependent kinase II (CaMKII), the Cx43 CaMBD conforms to the 1–5–10 CaM-binding mode subclass with hydrophobic residues at positions 1, 5, and 10. In addition, they have conserved positive residues (underlined). On the other hand, there are major amino acid differences between Cx40 and Cx43/CaMKII (highlighted in gray). The corresponding Cx40 sequence does not have conserved positive residues such as K298 and K300 bracketing the hydrophobic L299 residue in CaMKII. PCsB for the corresponding region of Cx40 is 0 instead of 13 for Cx43 and 9 for CaMKII. Potential CaM binding regions corresponding to the four connexin intracellular domains are listed in the table: h, human; m, mouse; B, basic residues; Δ, hydrophobic residues; CaMBD, CaM binding domain; (Max), maximum score of CaM binding prediction for whole peptide fragment; PCsB, probability of CaM binding predicted by the CaM Target Database; * for score 1–3; ** for score 4–6; *** for score 7–9; N.B., no prediction for CaM binding. B: the decline in normalized Cx43 gap junction conductance (Gj) was temporally correlated with a threefold increase in cytosolic Ca$^{2+}$ concentration ([Ca$^{2+}$]c) induced by 1 μM ionomycin + 1.8 mM CaCl$_2$ saline superfusion. The Cx43-N2a cell [Ca$^{2+}$], measurements were obtained by ratiometric fura 2 imaging in independent experiments from the dual whole cell patch Gj measurements were obtained by ratiometric fura 2 imaging in independent experiments from the dual whole cell patch Gj measurements.
the IPS KCl (16). All external and internal solutions were adjusted to a final osmolarity of 310 mosM.

**Dual whole cell patch-clamp recording.** Gap junction currents (Ij) were recorded in the dual whole cell configuration, according to previously published methods (60). Both cells in the dual whole cell patch mode were held at −40 mV. Ij was measured in response to a +20 mV Vj pulse applied to one cell for 5 s every 15 s (4 pulses/min). To better resolve unitary Cx43 gap junction channel currents for the purpose of calculating the single-channel conductance (gj), the command Vj pulse was increased to +45 mV. The Ij values recorded during these Vj steps were recorded, and the macroscopic junctional conductance (gj) was calculated by the following equation: 

\[ g_j = -\Delta I_j / (V_j - (I_1-R_{11}) - V_2 + (I_2-R_{22})) \]

where I1 and I2 are whole cell currents, V1 and V2 are command voltages, and R11 and R22 are patch electrode resistances. In all experiments, Cx43 gj was normalized to the initial baseline control gj value before ionomycin perfusion (gj = gj/Bj,control). Table 1. All whole cell current recordings were low-pass filtered at 500 Hz and digitized at 2 kHz using pClamp8.2, and graphical analysis was performed using Origin 7.5 software. The Student’s paired t-test was performed to test for statistically significant (P value < 0.05) alterations in gj during ionomycin perfusion for each experimental group. One-way ANOVA analysis and the Bonferroni means comparison test was performed to test for statistical significance between the means of different experimental groups.

**[Ca2+]i measurements.** N2a-Cx43 cells were cultured on 25-mm-diameter poly-l-lysine-coated glass coverslips for 48 h, rinsed with PBS, and loaded with 1 μM fura 2-AM (Invitrogen) for 15 min at 37°C. The coverslips were transferred to a microperfusion chamber mounted on a Nikon TE2000U inverted microscope equipped with ×40 SuperFluor oil immersion objective (48). Images of fura 2-loaded cells were obtained with a Roper Scientific Cascade 650 charge-coupled device digital camera using Metamorph software. The cells were perfused (5 ml/min, 1-ml bath volume within the microperfusion chamber) with SES until equilibrated at 22°C, after which 1 μM ionomycin perfusion commenced. [Ca2+]i was calculated as described (19, 48).

**RESULTS**

**Bioinformatic analysis of CaM binding to the cytosolic loop of Cx40 and Cx43.** The Ca2+ regulatory properties of Cx40, the second most abundant myocardial gap junction protein, have not been specifically investigated, so we performed a CaM binding site analysis for Cx40 analogous to that used for Cx43 (67). The bioinformatic analysis did not reveal a connexin CL CaM binding site for Cx40. This is contrary to the presence of predicted CaM binding sites in Cx43, Cx44 (the sheep ortholog of human Cx46), or Cx50. Alternate CaM sites of lower probability were predicted for both Cx43 and Cx40 (Fig. 1A) (7, 66, 67). When aligned with the reported 1–5–10 subclass CxMKIIα CaM binding site (33), the conserved positively charged residues bordering the hydrophobic residue at position 1, such as K298/K300 in CaMKIIα (pdb code 1CDM) and K146/R148 in Cx43, are replace by A144/Q146 in Cx40. In the structure of CaM-CaMKIIα, the K298/K300 residues interact with the CaMKII target peptide. Intrinsic sequence differences between Cx40 139–156, Cx43 141–158, and CaMKII 293–310 sequences suggest that Cx40 does not bind CaM in the cytosolic loop region and is less susceptible to regulation by Ca2+. Differential Ca2+ dependence of Cx43 and Cx40 gap junction uncoupling. To test whether extracellular Ca2+ influx can induce uncoupling between N2a cell stably expressing Cx43, we perfused N2a-Cx43 cell pairs with SES or Ca2+-free SES containing 1 μM ionomycin. After a 2-min control recording period, Cx43 Gj declined by 95 ± 4% within 15 min following application of ionomycin in Ca2+-containing SES (Fig. 1B, top). We also performed dual whole cell perfused patch experiments with 50 μM β-escin to determine whether whole cell Cx43 buffering with BAPTA modifies the response (16). Two β-escin perforated patch experiments proceeded to uncouple completely with a slightly delayed onset and slower time course of uncoupling, the opposite of what might be expected if excessive Ca2+ buffering and dilution of endogenous CaM were limiting factors in the response (data not shown). In separate imaging experiments, N2a-Cx43 cell [Ca2+]i was observed to gradually increase threefold from a resting level of 80 ± 3 to 250 ± 10 nM, parallel to the change in gj (Fig. 1B, bottom). Both panels in Fig. 1B present a similar temporal response to 1 μM ionomycin perfusion, although the N2a-Cx43 cell gj and [Ca2+]i responses are not directly correlated. Assuming the same percentage increase in [Ca2+]i, in whole cell patch-clamped N2a-Cx43 cell pairs, the [Ca2+]i levels are estimated to increase from 140 to 360 nM during the ionomycin perfusion, Gj measurement experiments.

To examine the Ca2+ dependence of the uncoupling response, N2a-Cx43 cell pairs were also perfused with 1 μM ionomycin in Ca2+-free SES (Fig. 2A). Cx43 Gj declined by 20 ± 4% within 10 min and achieved a minimum Gj of 72 ± 5% after 20 min in the absence of external CaCl2. The Cx43 Gj response in the presence of 1.8 mM Ca2+-SES (Fig. 1B) is contrasted with the Ca2+-free SES results in this panel. The response of N2a-Cx40 gap junctions was also examined. There was no difference in Cx40 Gj following application of ionomycin in SES or Ca2+-free SES; the maximum decrease in Cx40 Gj under these conditions was ≤20%. Patch electrode series resistance errors, which increase with higher gj values (60), did not influence these measurements, since our stable N2a-Cx40 cell clones exhibited lower gj values, on average, than our N2a-Cx43 cells (Table 1). These observations provide the first evidence for an intrinsic difference in the Ca2+ regulatory properties of Cx43 and Cx40 and are consistent with the bioinformatic CaM binding site predictions. To determine whether the differential sensitivity of Cx40 and Cx43 gap
junctions to Ca\(^{2+}\)-induced uncoupling translated into functional differences between atrial and ventricular myocardial gap junctions, neonatal mouse atrial and ventricular myocyte cell pairs were superfused with SES containing 1 \(\mu\)M ionomycin (Fig. 2C). The decline in ventricular myocyte \(G_{j}\) resembled the ionomycin-induced changes in N2a-Cx43 \(G_{j}\). In contrast, atrial \(G_{j}\) declined 20% less than ventricular \(G_{j}\) \((P < 0.0002,\) one-way ANOVA). This is consistent with a partial contribution of less Ca\(^{2+}\)-sensitive Cx40 gap junctions to the incomplete atrial uncoupling response.

Reversibility of Ca\(^{2+}\)-dependent Cx43 gap junction uncoupling. The above data suggest that Ca\(^{2+}\) influx is responsible for the uncoupling of Cx43 gap junctions. Therefore, we hypothesize that this process should be reversible if the external Ca\(^{2+}\) is removed during ionomycin perfusion. To test this hypothesis, the 1 \(\mu\)M ionomycin perfusion solution was switched from 1.8 mM CaCl\(_2\) to nominally 0 CaCl\(_2\) + 10 mM EGTA during the uncoupling phase. When the external bath perfusion was switched to 0 Ca\(^{2+}\)/EGTA saline when the decline in Cx43 \(G_{j}\) reached \(\approx 50\%\) (\(\approx 5\) min), \(G_{j}\) increased to \(\approx 90\%\) of its initial value (Fig. 3A). Application of 1.8 mM CaCl\(_2\) saline demonstrated that the recoverable Cx43 \(G_{j}\) remained responsive to Ca\(^{2+}\)-induced uncoupling since reexposure of cells to SES containing Ca\(^{2+}\) decreased \(G_{j}\) by 98%. The level of recovery induced by removal of extracellular Ca\(^{2+}\) was dependent on the magnitude of the Ca\(^{2+}\)-induced reduction in Cx43 \(G_{j}\). If the decline in \(G_{j}\) was \(>90\%\), then only 60% of the initial Cx43 \(G_{j}\) was restored by applying 0 Ca\(^{2+}\)/EGTA Ca\(^{2+}\) SES (Fig. 3B).

![Fig. 2. A and B: Neuro-2a (N2a)-Cx43 cell pairs (A) or N2a-Cx40 cell pairs (B) were perfused with 1 \(\mu\)M ionomycin in the presence or absence of added 1.8 mM CaCl\(_2\) to the bath saline solution. In nominally zero CaCl\(_2\) saline, Cx43 \(G_{j}\) declined by \(<30\%\) over 20 min \((P < 0.05)\) compared with a 95% decline within 15 min in the presence of 1.8 mM external CaCl\(_2\) \((P < 10^{-5})\). In contrast, Cx40 \(G_{j}\) declined by \(<20\%\), whether in nominally zero or normal external CaCl\(_2\) conditions \((P < 0.05)\). C: neonatal mouse atrial or ventricular cardiomyocyte \(G_{j}\) decreased by 73 \(\pm\) 11 or 93 \(\pm\) 8%, respectively, when perfused with 1 \(\mu\)M ionomycin + 1.8 mM CaCl\(_2\) \((P < 10^{-5})\). Values are means \(\pm\) SE.](#)

![Fig. 3. A: the reversibility of the calcium-induced uncoupling of Cx43 gap junctions was examined by alternating 1 \(\mu\)M ionomycin perfusion with 1.8 mM CaCl\(_2\) or 0 mM added calcium plus 10 mM EGTA to chelate the trace amounts of calcium in the deionized water used to prepare the saline solutions. If the perfusion solution was switched to the chelated calcium saline when \(G_{j}\) had declined by \(\approx 50\%\), Cx43 \(G_{j}\) recovered to \(92 \pm 3\%\) of its initial value \((P < 0.05)\). This Cx43 \(G_{j}\) was responsive to external calcium, since reexposure to 1.8 mM CaCl\(_2\) saline produced complete uncoupling. Since the perfusion times for the 5 experiments in each data set were not identical, the time base was shifted to align with the onset of the 1.8 mM CaCl\(_2\) and 0 mM + 10 mM EGTA chelated calcium saline perfusions (+ = no. of experiments at each time point). Values are means \(\pm\) SE.](#)
This suggests that nearly one-half of the Cx43 gap junctions become irreversibly closed after Ca\(^{2+}\)-dependent uncoupling attains maximum levels.

The Ca\(^{2+}\) regulation of Cx43 gap junctions is blocked by CaM inhibitors. To determine whether CaM was involved in Ca\(^{2+}\)-induced uncoupling of Cx43 gap junctions, N2a-Cx43 cells were pretreated with 2 μM CDZ for 15 min before patch-clamp recording. In the presence of CDZ, the steady-state Cx43 G\(_i\) of 83 ± 7% was not significantly different from initial values after 20-min exposure to Ca\(^{2+}\)-containing SES (Fig. 4A). To address whether the Cx43 CL-136–158 CaMBD was involved in the Ca\(^{2+}\)/CaM-dependent uncoupling of Cx43, we added amino acid sequence-specific (Cx43–3) or scrambled (Cx43-scr) peptides to both whole cell patch pipettes. Since the apparent K\(_d\) of the Cx43–3 peptide for CaM in the presence of 100 mM KCl and 1 mM CaCl\(_2\) is 860 nM (67), 1 μM of the Cx43 CL peptides was added to each of the whole cell patch pipettes for these experiments. The Cx43–3 peptide, which mimics the Cx43 CL CaMBD sequence, limited the Ca\(^{2+}\)-induced reduction of Cx43 G\(_i\) to 14 ± 5% of initial values, although this G\(_i\) decline was statistically significant at the P < 0.05 value. Cx43-scr was without significant effect: Cx43 G\(_i\) decreased by 93 ± 3% following the application of ionomycin (Fig. 4B). The canonical CaMKII CaMBD 290–309 inhibitory peptide (K\(_d\) = 50 nM; see Fig. 1A) prevented any significant Ca\(^{2+}\)-induced decrease in Cx43 G\(_i\) (Fig. 4C).

Mechanism for Cx43 gap junction closure by Ca\(^{2+}\). Channel blockade can occur by two basic mechanisms: pore block, where a poorly permeable or impermeable ion (e.g., Ca\(^{2+}\)) inhibits the ion conduction pathway, or by gating between the open and closed configurations of the channel. A gating mechanism of channel closure is characterized by reductions in the cumulative product of the number of observed open channels (N) and the open probability (P\(_o\)), whereas ionic block typically results in rapid flickering channel currents and an overall reduction in single-channel conductance (γ). In an attempt to distinguish between these two mechanisms, Cx43 gap junction channel currents were resolved in one low G\(_i\) N2a-Cx43 cell pair during perfusion with ionomycin in SES. The average value of N·P\(_o\) for the observed channels was determined for each V\(_j\) pulse during this 1 μM ionomycin SES perfusion experiment. On average, 1.83 channels were open during four baseline control V\(_j\) pulses (Fig. 5A). This N·P\(_o\) value declined to zero following a stochastic but gradual trend downward during the 6-min recording period. The unitary Cx43 gap junction channel current fluctuations from three time points (T1–T3: baseline control, declining, and final uncoupled phases) illustrate the decline in the product of N·P\(_o\) without any decrease in the single-channel current (Fig. 5B). During the 5-min recording period, Cx43 γ\(_j\) averaged 114 ± 1 pS (SE).

**DISCUSSION**

The ability of Ca\(^{2+}\) to induce closure of gap junctions has been proposed since the early observations that cut cardiac muscle “heals-over” in the presence, but not absence, of external Ca\(^{2+}\) (10, 12). The ability of elevated intracellular Ca\(^{2+}\) to close gap junctions is considered to be a protective mechanism by isolating healthy cells from their injured neighbors. The direct modulation of intercellular gap junction com-
communication by intracellular Ca\(^{2+}\) or CaM has been controversial since the publication of contradictory reports claiming that pH\(_{i}\) or [Ca\(^{2+}\)]\(_{i}\) directly modulate junctional communication (26, 40, 41, 50, 52, 55). Using a bioinformatics approach, we identified putative CaM binding sites on the carboxyl-terminal portion of the connexin CL of three α-subfamily connexins: Cx43, sheep Cx44 (Cx46), and Cx50 (7, 66, 67). While CaM has previously been demonstrated to bind to at least two connexins, Cx32 and Cx50 (54, 65), the mechanisms by which Ca\(^{2+}\) or CaM modulate connexin gap junctions remain poorly defined.

In this study, we predicted that Cx40 does not contain a CaM binding site in the CL domain. In addition, we directly tested the involvement of Ca\(^{2+}\), CaM, and the CL CaMBD in the regulation of Cx43 and Cx40 gap junctions (Figs. 1–4). We have shown that Ca\(^{2+}\) influx uncoupled N2a-Cx43 cell pairs, while Cx40-N2a cell pairs were not uncoupled by Ca\(^{2+}\). Atrial gap junctions, which contain equal amounts of Cx40 and Cx43, uncoupled by 20% less than ventricular gap junctions comprised only of Cx43 (28). The relative insensitivity of Cx40 gap junctions to the Ca\(^{2+}\)/CaM gating mechanism suggests that atrial gap junctions may not close as completely in response to Ca\(^{2+}\) overload conditions, which may be relevant to atrial fibrillation. The apparent insensitivity of Cx40 gap junctions to uncoupling by hundreds of nanomolar cytosolic [Ca\(^{2+}\)] in this study does not preclude blockade by submillimolar or millimolar [Ca\(^{2+}\)] as evidenced by the closing of Cx40 hemichannels in the presence of 3.6 mM Ca\(^{2+}\) (3). The decline in Cx43 \(G_i\) was inhibited by the omission of external CaCl\(_2\) or pretreatment with the CaM inhibitor CDZ (Figs. 2 and 4A). The addition of 1 µM ionomycin in the presence of normal extracellular [Ca\(^{2+}\)] produced a threefold increase in N2a-Cx43 cytosolic [Ca\(^{2+}\)] of ~200 nM, within the physiological range of changes in systolic [Ca\(^{2+}\)]. Whole cell dialysis by the ruptured patch electrode recordings may alter the time course of the response due to internal Ca\(^{2+}\) buffering and dilution of endogenous CaM, but complete uncoupling was still observed (e.g., β-escin perforated patch experiment, data not shown). These results are consistent with previous findings in HeLa cells that the extracellular addition of Ca\(^{2+}\) in the presence of ionomycin could inhibit Cx43 cell-to-cell dye transfer (31). In those experiments, HeLa-Cx43 cell intracellular Ca\(^{2+}\) was elevated by 200–500 nM, with the addition of 1.8 or 21.8 mM CaCl\(_2\) in the presence of ionomycin, and the decrease in intercellular dye transfer was inhibited by CDZ, but not by chemical inhibitors of CaMKII or protein kinase C. By our measurements of the differential initial [Ca\(^{2+}\)]\(_{i}\) levels in fura 2 loaded or BAPTA/CaCl\(_2\)-buffered patch clamped N2a-Cx43 cells before 1 µM ionomycin perfusion with 1.8 mM CaCl\(_2\) SES, the [Ca\(^{2+}\)]\(_{i}\) level should increase within the estimated range of 400–800 nM [Ca\(^{2+}\)]\(_{i}\) in N2a-Cx43 cell pairs. This estimated range for Cx43 \(G_i\) uncoupling is consistent with previous estimations of a threshold [Ca\(^{2+}\)]\(_{i}\), for ventricular \(G_i\) uncoupling of 685 nM (9). Directly correlated [Ca\(^{2+}\)] and \(G_i\) measurements have not yet been performed in paired cardiomyocytes; these experiments are planned for the next phase of this project using wild-type and available cardiac connexin-modified (e.g., Cx40 knockout) mice (28).

Chelation of all external Ca\(^{2+}\) with 10 mM EGTA inhibited Ca\(^{2+}\)/ionomycin-induced uncoupling in our N2a-Cx43 cells. Interestingly, the magnitude of \(G_i\) recovery was dependent on the extent of uncoupling at the time the Ca\(^{2+}\)-free SES was administered. \(G_i\) recovery achieved >90%, if Ca\(^{2+}\) chelation began before complete uncoupling, but was only partially reversible if complete uncoupling was allowed to develop (Fig. 3). Previous Ca\(^{2+}\) ionophore experiments have indicated that the induced elevation in [Ca\(^{2+}\)]\(_{i}\) is paralleled by a crystallization and decrease in particle spacing in gap junction plaques (43). Our data are consistent with the interpretation that prolonged exposure to elevated [Ca\(^{2+}\)]\(_{i}\) induces an irreversible gap
junction uncoupling that may be correlated with plaque crystallization.

We directly tested for the involvement of the Cx43 CaMBD in Ca\(^{2+}\)/CaM-dependent regulation of Cx43 gap junctions using Cx43 CL\(_{136-158}\) mimetic or scrambled peptides. Zhou et al. (67) described a Cx43–3 peptide corresponding to the 136–158 amino acid CL domain of Cx43 that bound CaM in the presence of Ca\(^{2+}\) with a \(K_d\) around 860 ± 20 nM. Intracellular addition of this peptide prevented the decline in Cx43 Gl \(G_t\) presumably by binding Ca\(^{2+}\)/CaM and preventing its association with functioning Cx43 gap junctions (Fig. 4B). The Cx43-scr control peptide does not bind CaM and had no effect on the Ca\(^{2+}\)-induced uncoupling. These results also suggest that the other regions of Cx43 are not likely to be involved in the Ca\(^{2+}\)/CaM regulation (Fig. 1A).

Based on our results, we hypothesize that Ca\(^{2+}\)/CaM binding to the Cx43 CL\(_{136-158}\) domain induces a gating response that closes the Cx43 gap junctions. The gating mechanism of closure is favored over the alternative direct divalent cation block of Cx43 gap junction channels, since a decline in \(N/P_o\) was observed without significant alteration of single-channel current amplitudes upon exposure to ionomycin and extracellular Ca\(^{2+}\) (Fig. 5). We have similarly repeatedly observed an all-or-none channel closure response with Cx50 gap junctions (7), thus substantiating the gap junction channel-gating mechanism by Ca\(^{2+}\)/CaM. Our data, however, cannot distinguish between alternative connexin domain gating conformations for the Ca\(^{2+}\)/CaM hypothesis, only that individual Cx43 gap junction channels close in an all-or-none manner in the presence of elevated cytosolic Ca\(^{2+}\). For instance, liver gap junctions, composed predominantly of Cx32, may possess connexin CaM binding amino and carboxyl terminal domains instead of the Cx43 CL site (14, 54). Holo CaM may act directly as a “cork” or induce a conformational change in the CL domain that occludes the cytoplasmic vestibule (mouth) of the gap junction channel, thereby restricting the passage of current-carrying ions (14, 45). There are several possible Ca\(^{2+}\)/CaM gating mechanisms for connexin gap junctions that could be considered (Fig. 6). An alternative “iris” gating hypothesis based on the Unwin and Ennis (57) model suggests that the Ca\(^{2+}\)-induced closure of liver gap junctions occurs by a decrease in the tilt angle of the six connexins comprising one connexon of a gap junction channel.

One longstanding criticism of the Ca\(^{2+}\)-gating hypothesis of gap junctions is that it requires millimolar external Ca\(^{2+}\) and elevations in [Ca\(^{2+}\)]\(_i\) that cause permanent hypercontracture of cardiomyocytes to induce intercellular uncoupling (32, 59, 63). Whether or not physiological [Ca\(^{2+}\)]\(_i\) and levels were sufficient to modulate Cx43 gap junctions was not entirely understood (8, 36, 62). Our new data are consistent with previous observations in ventricular myocytes or Novikoff hepatoma cells that dynamic changes within the cardiac [Ca\(^{2+}\)]\(_i\) range of 200 to 500 nM are sufficient to produce effects on ventricular Gl, although the involvement of CaM was not tested in these prior studies (26, 36). Human disease causing connexin mutations may also lead to pathophysiologial conditions by producing dysregulation of gap junction gating mechanisms like Ca\(^{2+}\)/CaM or pH. Although yet to be proven, several Cx43 oculodentodigital dysplasia mutations occur within these CL regulatory domains, including I130T, K134E/N, G138S/R, G143D/S, K144E, V145G, M147T, R148G/Q, and T154A/N (37), that could possibly disrupt Ca\(^{2+}\)/H\(^+\) chemical gating mechanisms, in addition to their loss-of-function or nonfunctional consequences. We were unable to test the functional relevance of the Cx43 CaMBD site K146E+R148E and M147Q+L151E+I156E mutations because they failed to form detectable gap junctions or induce functional electrical coupling (67), perhaps because connexin interactions with CaM are required for gap junction formation or the mutations cause protein misfoldings that are incompatible with gap junction assembly (2, 24).

Fig. 6: A: a hypothetical model for the (gating) occlusion of the Cx43 gap junction channel opening by the direct blockade of Ca\(^{2+}\)-bound CaM (holo-CaM) (45), the induction of a conformational change in the cytoplasmic loop of Cx43 (67), or a combination of both steric hindrance mechanisms. Inhibition of the CaM-Cx43 cytoplasmic loop interaction by addition of the CaMKII or Cx43–3 peptides or a CaM inhibitor (e.g., CDZ) prevents the open (M\(_o\)) to closed (M\(_c\)) transition. B: an alternative gating hypothesis, based on the dual conformational model proposed by Unwin and Ennis (57), whereby a \(<10^\circ\) tilting of the connexin subunits within a connexon translates into narrowing of the TM pore that effectively occludes the channel. The gating process is also reversible upon Ca\(^{2+}\) removal (by EGTA).
Steady-state measurements of cardiac gj have suggested a synergistic action between H+ and Ca2+-ions (6, 63), although time course experiments have indicated that Cx43 or cardiac gj is insensitive to pH or declines only when pH drops below 6.1–6.5, provided that [Ca2+]i remains < 500 nM (26, 36). As shown in Figs. 1B and 2A, parallel to the increase of [Ca2+]i, from a resting level of 80 ± 3 nM to a maximum value of 250 ± 10 nM, Cx43 gj decreased to near zero after ~12 min of ionomycin perfusion. These findings suggest that a threefold increase in [Ca2+]i uncouples Cx43. Although we did not measure pH in conjunction with Ca2+-imaging, it is unlikely that pH is involved under the experimental conditions used in our experiments, since both SES and patch electrode solutions were buffered to pH 7.4 with 10 or 25 mM HEPES, respectively. Our data, however, do not exclude interplay between Ca2+ and pH in cells under more physiological settings. The CaMBD is located adjacent to a putative pH gating domain on the C-terminal portion of the Cx43 CL domain. Cx40 gap junctions lack this sub-micromolar Ca2+-sensitivity at pH 6.1–6.5, provided that [Ca2+]i remains ~500 nM (26, 36). Additional studies are required to determine whether a causal interrelationship between these domains would modify gap junction conductance. Experiments to further define the α-subfamily connexin Ca2+/CaM gating mechanism, including possible connexin trans-domain interactions with CaM, as proposed for Cx32 (14, 54), are also planned.

Our data strongly suggest that Ca2+/CaM-dependent regulation of Cx43 gap junction conductance requires the carboxyl-terminal portion of the Cx43 CL domain. Cx40 gap junctions lack this sub-micromolar Ca2+/CaM-dependent regulatory mechanism. Cx43 gap junctions are closed by a gating mechanism characterized by a reduction in channel Po without irreversible gap junction closure induced by prolonged elevated cytosolic Ca2+. The intracellular Ca2+ affinity and Cx43/ CaM conformational changes necessary to induce this chemical gating mechanism will require additional experimentation.

REFERENCES
