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

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1Department of Pharmacology, State University of New York, Upstate Medical University, Syracuse, New York; 2Department of Medicine, State University of New York, Upstate Medical University, Syracuse, New York; 3Department of Cell and Developmental Biology, State University of New York, Upstate Medical University, Syracuse, New York; and 4Department of Chemistry, Georgia State University, Atlanta, Georgia

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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 putative Cx43 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 CL136–158 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.

GAP JUNCTIONS, FORMED BY DOCKING of two gap junction hemichannels (or connexons) between neighboring cells, allow the transfer of ions and small molecules between coupled cells (15, 20, 58). The connexin gap junction proteins have four transmembrane domains, linked by one intracellular and two extracellular loops, with the NH2- and COOH-termini both located in the cytosol (15, 20). Gap junctions play an important role in maintaining cell and tissue function and homeostasis. For example, the low intercellular resistance pathway formed by connexin 43 (Cx43) and connexin 40 (Cx40) gap junctions in the working myocardium is critical for propagating electrical signals to allow rapid, synchronized contraction. Loss of function connexin mutations has been linked to several serious human diseases, including ocudolentodigital dysplasia for Cx43, the most abundantly expressed human connexin (17, 22, 24, 25, 37, 47, 49, 51). Identification of factors that regulate gap junction communication and their underlying mechanisms are physiologically relevant and significant to understanding the causes of gap junction-related diseases. Multiple factors, including transjunctional voltage (Vj), intracellular pH (pHi), intracellular Ca2+ concentration ([Ca2+]i), and protein phosphorylation, modulate gap junction intercellular communication (20, 41).

Increases in [Ca2+]i were proposed to downregulate gap junction-mediated electrical coupling since the early observations of the “healing-over” of cardiac muscle and were later confirmed by direct observation of electrical coupling and [Ca2+]i in cardiac and nonelectrically excitable cells (9–13, 50). Electrical uncoupling of cells paired by gap junctions with increased [Ca2+]i has since been observed in several cell types, including cardiac myocytes, astrocytes, Henson cells, Novikoff hepatoma cells, cultured lens cells, rat liver cells, and crayfish septate axons (1, 4, 5, 18, 26, 41, 61). Gap junction uncoupling is also mediated by intracellular acidification, although the independent role of protons (H+), or Ca2+, is questioned by numerous studies (6, 26, 40, 52, 55, 63). Calmodulin (CaM), a cytosolic Ca2+ binding protein, was proposed to have a role in the Ca2+-induced uncoupling of gap junctions, since CaM inhibitors were observed to prevent the response (38, 39, 42, 44). CaM colocalizes to the junctional plaques of Cx32 and Cx50 in transfected cells (44, 65). Török and coworkers (54) reported that the first 21 amino acids located on the NH2 terminus of Cx32 and amino acids 216–230 located on the COOH terminus bound CaM in a Ca2+-dependent manner with dissociation constants (Kd) of 27 nM and 1.2 μM. Recently, a CaM binding site was assigned to the second half of the cytoplasmic loop (CL) domain of Cx43, Cx44, and Cx50 and confirmed using surface plasmon resonance, circular dichroism, fluorescence spectroscopy, and nuclear magnetic resonance (7, 66, 67). Zhou et al. (67) indicated that the 136–158 amino acids of Cx43 bind with CaM with 1:1 stoichiometry in a Ca2+-dependent manner. However, the functional consequences of the connexin-CaM protein-protein interactions, other than Cx32 and Cx50, are unknown.

To examine the functional relevance of the predicted Cx43 CaM binding domain (CaMBD), we utilized the dual whole cell patch-clamp technique to directly evaluate the functional regulation of Cx43 and Cx40 gap junctions by Ca2+ and CaM. In contrast to Cx43, there is no putative CaM binding site in the CL region of Cx40, which led us to hypothesize a differential Ca2+-dependent regulation of Cx43 and Cx40. We demonstrate that elevation of intracellular Ca2+ of Neuro-2a (N2a)-Cx43 cells by ionomycin in the presence of normal extracellular...
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 (CaMKKII) 290–309 inhibitory or Ac-136KYGIEHGVKMRGGLLRTYIIS158-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 CaM(BDs).* 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_000156; human), Cx40 (accession ID: NP_005257; human), and CaM KKII (accession ID: AAK40457; 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. The primary cell cultures were enriched for cardiomyocytes by dissociating newborn C57Bl/6 mice using perforated patch methods by adding 50 M 5-bromo-2’-deoxyuridine (BrdU; Sigma). The supernatant was collected after each of four 10-min dissociation cycles, passed through a 70-μm 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): 116 NaCl, 5.4 KCl, 2.5 CaCl\(_2\), 1.0 NaH\(_2\)PO\(_4\), and 5.5 dextrose (pH 7.4). A nominally Ca\(^{2+}\)-free solution 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. The supernatant was collected after each of four 10-min dissociation cycles, passed through a 70-μm 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.

**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. PCBS 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 (CaM KKII), 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 CaM KKII. PCBS for the corresponding region of Cx40 is 0 instead of 13 for Cx43 and 9 for CaM KKII. Potential CaM binding regions corresponding to the four connexin intracellular domains are listed in the table: h, human; m, mouse; B, basic residues; H, hydrophobic residues; CaMBD, CaM binding domain; (Max), maximum score of CaM binding prediction for whole peptide fragment; PCBS, 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 (\(G_j\)) was temporally correlated with a threefold increase in cytosolic Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_{cyt}\)) induced by 1 mM ionomycin + 1.8 mM CaCl\(_2\) saline superfusion. The Cx43-N2a cell [Ca\(^{2+}\)]\(_{cyt}\) measurements were obtained by ratiometric fura 2 imaging in independent experiments from the dual whole cell patch \(G_j\) measurements. [Ca\(^{2+}\)]\(_{cyt}\), extracellular Ca\(^{2+}\) concentration. Values are means ± SE.
Dialysis of poly-L-lysine-coated glass coverslips for 48 h, rinsed with 
To better resolve unitary Cx43 gap junction channel currents for the 
not been specifically investigated, so we performed a CaM 
second most abundant myocardial gap junction protein, have

1/H9262

perifusion chamber) with SES until equilibrated at 22°C, after which 
2-loaded cells were obtained with a Roper Scientific Cascade 650 
37°C. The coverslips were transferred to a microperifusion chamber 
Rel 2 are patch electrode resistances. In all experiments, Cx43 gj was 
during these 
V
mand 
gj, junctional conductance; Cx, connexin; scr, scrambled; 

Cx 1.8 mM CaCl
2
0 mM CaCl
0 mM EGTA, partial uncoupling 
10 mM EGTA, complete uncoupling 
2 µM calmidazolium 
1 µM Cx43-3 peptide 
1 µM Cx43-scr peptide 
100 nM CaMKII peptide 

N2a-Cx43 cell clones exhibited lower gj values, on average, 
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 perforated patch experiments with 50 µM β-escin to determine whether whole cell Ca2+ 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 

Table 1. Control macroscopic junctional conductance values

<table>
<thead>
<tr>
<th>Connexin</th>
<th>Initial gj, nS</th>
<th>Experiment</th>
<th>Means</th>
<th>SE</th>
<th>N</th>
</tr>
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</table>
| Cx43     | 1.8 mM CaCl
0 mM CaCl | 22.3 ± 5.2 | 6 |
| Cx43     | 10 mM EGTA, partial uncoupling | 9.2 ± 3.3 | 6 |
| Cx43     | 10 mM EGTA, complete uncoupling | 19.2 ± 7.7 | 5 |
| Cx43     | 2 µM calmidazolium | 19.1 ± 5.9 | 5 |
| Cx43     | 1 µM Cx43-3 peptide | 30.5 ± 5.6 | 6 |
| Cx43     | 1 µM Cx43-scr peptide | 14.2 ± 4.5 | 5 |
| Cx43     | 100 nM CaMKII peptide | 10.9 ± 3.4 | 6 |
| Cx40     | 1.8 mM CaCl | 18.5 ± 8.8 | 6 |
| Cx40     | 0 mM CaCl | 6.3 ± 1.9 | 6 |

Cardiomycocytes

Atrial 1.8 mM CaCl | 42.9 ± 5.6 | 7 |

Ventricular 1.8 mM CaCl | 47.1 ± 6.4 | 6 |

N, no. of experiments. All experiments included bath salin perfusion with 
1 µM ionomycin. gj, junctional conductance; Cx, connexin; scr, scrambled; 
CaMKII, Ca2+/calmodulin-dependent kinase II.

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 
CaMKIα CaM binding site (33), the conserved positively 
charged residues bordering the hydrophobic residue at position 1, such as K298/K300 in CaMKIα (pdb code 1CDM) 
and K146/R148 in Cx43, are replace by A144/Q146 in Cx40. In the structure of CaM-CaMKIα, the K298/K300 residues directly interact with the CaMKII target peptide. Intrinsic sequence differences between Cx40 139–156, Cx43 141–158, and CxMKII 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 
duce uncoupling between N2a cells 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 perforated patch 
experiments with 50 µM β-escin to determine whether whole 
cell Ca2+ 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 endoge-
nous CaM were limiting factors in the response (data not 
shown). In separate imaging experiments, N2a-Cx43 cell 

[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 microperifusion 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 micro-
perifusion chamber) with SES until equilibrated at 22°C, after which 
1 µM ionomycin perfusion commenced. [Ca2+]i, was calculated as 
described (19, 48).
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 declined by 20% less than ventricular G_j (\(P < 0.002\), 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 \(-50\%\) (\(\leq 5\) min), G_j increased to \(-90\%\) 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 \(-50\%\), 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 a 98 \(\pm\) 2% decline in G_j. B: 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). 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 Gj 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 CL136–158 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 Kd of the Cx43–3 peptide for CaM is 50 nM (see Fig. 1A) prevented any significant Ca$^{2+}$-induced Cx43 Gj decline. We also determined the effects of K146E+R148E and M147Q+L151E+I156E mutations to the Cx43 CaMBD sequence that eliminates CaM binding to this CL domain (67). These combinatorial hydrophilic or hydrophobic site mutations failed to induce any functional N2a cell coupling and inefficiently formed gap junction plaques in HeLa cells (data not shown).

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 product of the number of observed open channels (N) and the open probability (Po), 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 Gj N2a-Cx43 cell pair during perfusion with ionomycin in SES. The average value of N·Po for the observed channels was determined for each Vj pulse during this 1 μM ionomycin SES perfusion experiment. On average, 1.83 channels were open during four baseline control Vj pulses (Fig. 5A). This N·Po 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·Po, without any decrease in the single-channel current (Fig. 5B). During the 5-min recording period, Cx43 γj 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-

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_**Fig. 4.** (A) pretreatment with 2 μM calmidazolium (CDZ) prevented the calcium-induced decrease in Cx43 Gj, limiting the decline to <20% after 20 min of 1 μM ionomycin, 1.8 mM CaCl2 exposure (P > 0.05). All Gj values were normalized to the initial junctional conductance (gj) value recorded over a 2-min period before ionomycin bath perfusion (mean ± SE, N = 6). B: the involvement of the Cx43 CL136–158 CaMBD in the Ca$^{2+}$/CaM-dependent uncoupling response of Cx43 was examined by including 1 μM of the Cx43 mimetic (Cx43–3: Ac-KYGIEEHGKVKMRGGLLRTYIIS-NH2) or scrambled control (Cx43-scr; Ac-LGGEYLVTMESKIHIKGKRIGYR-NH2) in both whole cell pipettes during ionomycin/CaCl2 perfusion. The scrambled peptide (B) failed to prevent the calcium-induced decline in Cx43 Gj (P < 0.05), whereas the Cx43–3 mimetic peptide (C) limited the decrease in Gj to <15% (P < 0.05). C: inclusion of 100 nM CaMKII 290–309 peptide in both patch pipettes also preserved Cx43 Gj relative to control experiments (from Fig. 1B, P > 0.05), further demonstrating the involvement of CaMBD in the Ca$^{2+}$-induced rundown of Cx43 Gj. Values are means ± SE._
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 \(\alpha\)-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\(^{2+}\)]\(_i\) in this study does not preclude blockade by submillimolar or millimolar [Ca\(^{2+}\)]\(_i\), 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 \(\mu\)M ionomycin in the presence of normal extracellular [Ca\(^{2+}\)]\(_e\) produced a threefold increase in N2a-Cx43 cytosolic [Ca\(^{2+}\)]\(_i\) of \(\approx\)200 nM, within the physiological range of changes in systolic [Ca\(^{2+}\)]\(_i\). 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., \(\beta\)-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 \(\mu\)M 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\(^{2+}\)]\(_i\), 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 G_i, 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 \(N\cdotPo\) 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 CaM binding amino and carboxyl terminal domains instead of the Cx43 CL site (14, 54). Holo CaM may act directly as a “cork” and 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 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 G_i, although the involvement of CaM was not tested in these prior studies (26, 36). Human disease causing connexin mutations may also lead to pathophysiological conditions by producing dysregulation of gap junction gating mechanisms like Ca\(^{2+}\)/CaM or pH_i. 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+L156E 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).

![Diagram](https://example.com/diagram.png)

**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_0) to closed (M_1) transition. B: an alternative gating hypothesis, based on the dual conformational model proposed by Unwin and Ennis (57), whereby a \(\alpha \times 10^{-8}\) 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+] remains < 500 nM (26, 36). As shown in Figs. 1B and 2A, parallel to the increase of [Ca2+], 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+] 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 CL of Cx43 (35). Additional studies are required to determine whether causal interrelationships between these domains 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 accompanying reductions in channel current amplitude. The Ca2+/CaM-dependent uncoupling is fully reversible, but only if the rise in [Ca2+], is blocked before complete uncoupling; only one-half of the Cx43 gap junctional conductance can be recovered after complete uncoupling occurs, suggestive of 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


Mitaku S, Hirokawa T, Tsuji T.


