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Gap Junction Regulation by Calmodulin

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Abstract

Intracellular Ca^{2+} activated calmodulin (CaM) inhibits gap junction channels in the low nM to high μM range of $[Ca^{2+}]_i$. This regulation plays an essential role in numerous cellular processes that include hearing, lens transparency, and synchronized contractions of the heart. Previous studies have indicated that gap junction mediated cell-to-cell communication was inhibited by CaM antagonists. More recent evidence indicates a direct role of CaM in regulating several members of the connexin family. Since the intracellular loop and carboxyl termini of connexins are largely "invisible" in electron microscopy and X-ray crystallographic structures due to disorder in these domains, peptide models encompassing the putative CaM binding sites of several intracellular domains of connexins have been used to identify the Ca^{2+}-dependent CaM binding sites of these proteins. This approach has been used to determine the CaM binding affinities of peptides derived from a number of different connexin-subfamilies.

Keywords

Ca^{2+}; connexin; gap junction regulation; Calmodulin binding

1. Introduction

Modulation of the cytoplasmic Ca^{2+} concentration ($[Ca^{2+}]_i$) is a ubiquitous mechanism by which cells transduce external signals into biological responses. The signaling cascade initiated by the rise in $[Ca^{2+}]_i$ is often mediated via Ca^{2+}-binding proteins such as calmodulin (CaM) [1, 2]. CaM, a key multifunctional transducer of Ca^{2+} signals in eukaryotes, has four EF-hand Ca^{2+} binding motifs in two globular N- and C-domains that

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are separated by a flexible linker. Upon Ca^{2+} binding, CaM undergoes a large conformational change, exposing hydrophobic patches that are important in its binding to more than 300 target proteins in multiple cellular processes [3–5]. CaM uses either its N- or C-domains with different Ca^{2+} binding affinities to differentiate between local and global $[Ca^{2+}]_i$ changes, and to regulate a diverse group of membrane channels/pumps that include cyclic nucleotide-gated (CNG) channels [6], N-methyl-D-aspartate receptor (NMDA receptor) [7], ryanodine receptors (RyR) [8], Ca^{2+}-activated K^+ channels of small or intermediate conductance (SK or IK) [9, 10], Trp family channels [11], Ca^{2+} channels, and gap junction channels [12–16].

Gap junctions are formed from the docking of paired hexameric connexons, which are also called hemichannels with each connexon comprised of six monomeric connexins. Approximately 21 connexin isoforms have been identified in a multitude of human cell types [17]. According to their gene structure, homology and specific sequence motifs, connexins have been grouped into three major categories, termed the α, β and γ connexin subfamilies [18]. The different connexin subfamily members all share the same structural topology with four α-helical transmembrane domains connected by two extracellular loops and a cytoplasmic loop; both N- and C-terminal face the cytosol. Three highly conserved Cys residues residing in each extracellular loop form intraconnexin disulfide bonds. The transmembrane domains and the extracellular loops are highly conserved among different connexin families. Although the N-terminus is also relatively conserved, the cytoplasmic loop and C-terminus exhibit significant variation among different connexins.

Connexins are almost universally expressed in vertebrate tissues with the exception of several highly differentiated cell types that include skeletal muscle, erythrocytes, and mature sperm cells [19]. Gap junctions facilitate the direct intercellular cytoplasmic connections that allow the exchange of ionic current, secondary messengers, and small metabolites up to 1 kDa between the cytoplasms of adjacent cells. Gap junctions play important roles in many biological processes [20–22], including development, differentiation, cell synchronization, neuronal activity, and the immune response. The functional loss of gap junction may result in embryonic lethality (e.g. Cx45) [23, 24], perinatal death due to congenital malformations (e.g. Cx43–/−) [25], and plays a major role in the pathogenesis of several other diseases (e.g. Charcot-Marie-Tooth disease, Cx32; Oculodentodigital dysplasia, Cx43; Non-syndromic deafness, Cx26, Cx30, Cx43; lens cataracts, C46, Cx50) [26, 27]. Connexin mutations may interfere with gene expression, affect protein or DNA stability, impede protein trafficking, translocation and assembly, or alter the regulation of gap junction.

Intercellular communication mediated by gap junctions can be regulated by multiple factors that include $[Ca^{2+}]_i$, H^+, CaM, voltage, and phosphorylation of the connexin subunits [28, 29]. The role of CaM in the regulation of gap junction channels has been previously reviewed by Peracchia [30]. In this paper, we review current understanding of the Ca^{2+}-CaM regulation of gap junctions comprised of different connexins that is effected by the direct binding of Ca^{2+}-CaM to specific sequences of these connexins (Fig. 1). We will first discuss the functional regulation of gap junctions by CaM, we will then discuss the molecular basis of such regulation including the CaM binding locations, relative binding

affinities, and binding modes for the different connexins, and finally, a gating model for the regulation of the α-subfamily of connexins by Ca^{2+}-CaM is proposed.

2. Functional Regulation of Gap Junction by CaM

Over the years, a significant body of data supports the involvement of CaM in the regulation of gap junctions comprised of the three connexin subfamilies. Délèze [31] first showed that Ca^{2+} is essential for the healing process in mammalian heart muscle by preventing longitudinal diffusion of molecules in cardiac fibers. Subsequently, Rose et al. [32] showed that in salivary gland cells, inhibition of cell-to-cell coupling occurs when the intracellular concentration of Ca^{2+} was increased from 0.1 μM to 50 μM.

Recognizing that most Ca^{2+}-induced phenomena are mediated by CaM [33], Peracchia and colleagues [34] first tested the hypothesis that gap junctions were regulated by CaM by examining the effect of the CaM inhibitor trifluoperazine (TFP) on the electrical coupling of amphibian embryonic cells exposed to CO_2 to lower intracellular pH. While the role of CaM in mediating the action of Ca^{2+} on enzyme activation was well known in the 1980s, gap junctions represented the first membrane channels shown to be modulated by CaM; only a decade later was the next CaM regulated channel identified [6]. Peracchia and colleagues demonstrated that TFP reversibly inhibits the CO_2-induced electrical uncoupling in amphibian embryo cells by interfering with the mechanism which closes the cell-to-cell channels. Subsequently, more specific CaM blockers (calmidazolium and W7) were shown to prevent uncoupling of Xenopus embryonic cells [35] and crayfish axons [36, 37], indicating the generalized nature of this role for CaM in regulating gap junctions. This hypothesis was strengthened by evidence that the gap junction protein Cx32 bound CaM in gel overlays [38–40]. The suppression of CaM expression in oocytes can also inhibit CO_2 induced electrical uncoupling and injection of CaM into oocytes can recover it [41]. Cx32 was also shown to colocalize with CaM using immunofluorescence microscopy [42]. Later, Blodow et al. reported that CaM antagonists suppress gap junction coupling of Cx26 in isolated Hensen cells of the guinea pig cochlea [43].

Louis and Lurtz first showed that the gap junction mediated cell-to-cell transfer of dye between lens epithelial cells was inhibited by Ca^{2+}-CaM [44]. The rapid onset of this inhibition (within seconds) suggested that this inhibition was mediated by the direct interaction of CaM with one or more of the lens connexins rather than by the action of a CaM-dependent protein kinase. They subsequently demonstrated in Cx43-transfected HeLa cells that cell-to-cell dye transfer was inhibited by Ca^{2+}-CaM [45]. Cell-to-cell communication was half-maximally inhibited at ~ 300 nM $[Ca^{2+}]_i$ [46], and this inhibition was prevented by pre-incubation of lens cultures with CaM antagonists [47]. In HeLa cells transiently expressing the CaM-binding-deficient mutants (Cx43$_{K146E,R148E}$-EYFP and Cx43$_{M147Q,L151E,I156E}$-EYFP), elevated $[Ca^{2+}]_i$ was unable to inhibit cell-cell dye transfer, confirming that residues 136–158 in the intracellular loop of Cx43 contain the CaM-binding site that mediates the Ca^{2+}-dependent regulation of Cx43 gap junctions [48]. Direct gap junction conductance (G_j) measurements confirmed that increases in $[Ca^{2+}]_i$ and decreases in Cx43 G_j were temporally correlated and inhibitable by CaM antagonists like calmidazolium, CaMKII CaMi peptide, or the Cx43p$_{136–158}$ peptide (Fig.2) [49]. We have

further shown that inhibition of gap junction conductance by intracellular Ca^{2+} and CaM can be reversed by 90% with the addition of 10 mM EGTA and removal of external $CaCl_2$ from the bath saline solution, but only if Ca^{2+}-chelation commenced prior to complete uncoupling [49]. On the other hand, Cx40-transfected N2a neuroblastoma cell pairs were not uncoupled by intracellular Ca^{2+}, such lack of intracellular Ca^{2+} regulation of Cx40 was consistent with the observation that Cx40 does not contain the putative CaM binding site in the same cytosolic loop region [49].

The inhibition of Cx44-mediated cell-to-cell dye transfer by Ca^{2+} was also shown to be regulated by CaM as this inhibition was prevented by prior incubation of Cx44-transfected cells with a CaM antagonist [50]. Cx50 was also shown to colocalize with CaM [51], and by measuring gap junctional conductance of Cx50-transfected N2a neuroblastoma cells, the reduction of Cx50-mediated junctional coupling was shown to be Ca^{2+}-dependent. Junctional coupling mediated by either Cx50 or Cx43 was prevented by pre-incubation of transfected cells with a CaM inhibitor, indicating that the $[Ca^{2+}]_i$-dependent inhibition of Cx50 and Cx43 was CaM mediated. Furthermore, the Ca^{2+}-dependent inhibition of gap junction permeability in Cx50-transfected cells was prevented by intracellular injection of a synthetic peptide encompassing the CaM binding domain of Cx50, while the scrambled peptide was without effect [52].

CaM regulation of the γ-connexin Cx45 was reported by Peracchia and coworkers by monitoring the sensitivity of Cx45 channels to CO_2, and inhibiting CaM expression in oocytes [53]. Mouse Cx36, perch Cx35 and Cx34.7 that form electrical synapses have also been reported to bind CaM in a Ca^{2+}-dependent manner using surface plasmon resonance assays and GST fusion proteins harboring the carboxyl-domain of these connexins [54, 55].

3. Molecular Basis of CaM Regulation of Connexins

There are several challenges in developing an understanding of the molecular mechanism by which CaM regulates the functional activity of connexins. Thus, the crystallization of these membrane proteins for determination of their high resolution atomic structure by X-ray crystallography is especially challenging. At this time, the structure of only one connexin, Cx26 in a presumed open state, has been resolved to 3.5 Å resolution [56, 57], although lower resolution electron microscopy crystallographic structures are available [58]. However, the intracellular loop and carboxyl terminus are largely "invisible" in these structures due to intrinsic disorder in these domains. In addition, in whole cell experiments, it is difficult to unambiguously distinguish between the effects of Ca^{2+} directly on the channel and the effects of Ca^{2+} mediated via CaM. CaM is a versatile and mobile trigger protein with many target proteins and is involved in both its direct and indirect regulations of its target proteins. To overcome these challenges, peptide models are often used to understand the molecular mechanism by which CaM interacts with its target proteins. Typically a number of questions are posed that include identification of the location(s) in the target proteins that bind CaM, whether the CaM binding to its target proteins is Ca^{2+}-dependent or -independent, the consequences of the Ca^{2+} sensitivity of CaM on its interaction with its target protein, and the CaM binding modes by which it interacts with its target proteins.

3.1 CaM binds to Cx32 in β-connexin subfamily

Equilibrium binding studies using a fluorescent CaM derivative identified two CaM-binding domains in Cx32, a site in the N-terminal tail, and a site in the C-terminal tail region[59]. A peptide encompassing the N-terminal domain of Cx32 was shown to bind a fluorescent CaM derivative in a Ca^{2+}-dependent manner with high affinity (K_d, 27 nM). A peptide encompassing a C-terminal region of Cx32 was shown to bind this fluorescent CaM derivative with a K_d of 1.2 uM [59]. The CaM binding domains of the N- and C-terminal regions of Cx32 were best defined as residues 1–21 (Cx32NT) and 216–227 (Cx32CT), respectively with the Cx32CT CaM binding region showing α-helical propensity indicating that CaM binding induced an α-helical structure in the Cx32CT and involved both the N- and C-lobes of this connexin. These data, showing separate functions of the N- and C-lobes of CaM in its interactions with Cx32, suggests trans-domain or trans-subunit bridging by CaM as a possible mechanism of gap junction gating [60]. Interestingly, both the N-terminal and the C-terminal CaM-binding domains of Cx32 are located close to the membrane and are contiguous with hydrophobic membrane-spanning sequences (Fig.1). Furthermore, although the C-terminal CaM binding domain in Cx32 represents only 15% of the C-terminal tail of Cx32, the removal of the other 85% had little effect on junctional permeability and chemical gating [61–63]. Recently, Stauch et al. further demonstrated that CaM binding could convert the intrinsically disordered C-terminal domain to an α-helical conformation that may enable Cx32 to interact with the protein partner synapse-associated protein 97 [64].

3.2 CaM binds to the γ-connexin subfamily

Using surface plasmon resonance with rapid kinetics, CaM was shown to bind in a concentration- and Ca^{2+}-dependent manner to peptide fragments corresponding to a 10–30 amino acid segment at the beginning of the C-terminal intracellular domain of mouse Cx36, Cx35 from perch, and the related perch Cx34.7 [55]. Dissociation was also very rapid; K_ds for CaM binding at a high-affinity site ranged from 11 to 72 nM (Table 1). No binding of CaM to the intracellular loops of these connexins was observed. The micromolar $K_{1/2}$s, and the rapid on and off rates suggest that this interaction may change dynamically in neurons, and may occur transiently when $[Ca^{2+}]_i$ is elevated to a level that would occur in the near vicinity of an activated synapse [54, 55].

3.3 CaM binds to the α-connexin subfamily

We have predicted that a CaM binding region resides in the central cytoplasmic loop of α-connexins that include Cx43 [48], Cx44 (the ovine analog of rodent Cx46) [50] and Cx50 [52, 65]. We used various biochemical approaches including circular dichroism (CD) nuclear magnetic resonance (NMR), isothermal titration calorimetry (ITC) and fluorescence spectroscopy to demonstrate that CaM binds to the cytoplasmic loop of Cx43 [48], Cx44 [50] and Cx50 [52] in a Ca^{2+}-dependent manner. Figure 3A shows the far UV CD spectra of CaM in the absence and presence of a Cx43 cytoplasmic loop peptide. The addition of this Cx43 peptide increased the α-helical content likely due to the conversion of unstructured free peptide to helical conformation upon complex formation with CaM. On the other hand, based on the use of peptide models, no direct interaction was observed for other regions in

this connexin such as the N-terminal region of Cx43 [59] and the C-terminal domain of Cx43 [66].

Studies in our laboratories identified CaM binding domains in Cx43 (residues 136–158) [48], Cx44 (residues 132–153) [50] and Cx50 (residues 141–166) [52]. NMR studies demonstrated all three Cx43-, Cx44- or Cx50-derived peptides (Cx43p$_{136-158}$, residues from 136 to 158; Cx44p$_{132-153}$, residues from 132 to 153; Cx50p$_{141-166}$, residues from 141 to 166) bind CaM with a 1:1 stoichiometry. Such interactions of CaM with Cx43- or Cx50-derived peptides can also be observed using surface plasmon resonance and florescence anisotropy. Dansylated CaM florescence and high-resolution NMR studies demonstrated that the binding of a Cx43-derived peptide Cx43p$_{136-158}$ to CaM in the presence but not the absence of Ca^{2+}. The apparent dissociation constant binding of this peptide to CaM in physiologic K^+ was 860 ± 20 nM [48]. On the other hand, the respective K_ds for the peptides derived from Cx44 (Cx44p$_{132-153}$) and Cx50 (Cx50p$_{141-166}$) are 49 ± 3.0 and 4.9 ± 0.6 nM respectively in the presence of Ca^{2+}. In the absence of Ca^{2+}, both peptides bound to CaM with significantly reduced affinities (K_ds greater than 5000 and 8000 nM, respectively). Thus, Cx50p$_{141-166}$ exhibited a 10-fold higher binding affinity for CaM than Cx44p$_{132-153}$, which was approximately 20-fold higher than Cx43p$_{136-158}$.

Since a "ball-and-chain" or "particle-receptor" hypothesis involving the pH-dependent intramolecular interaction between the cytoplasmic domain and part of the intracellular loop of Cx43 has been proposed to explain the low pH-induced closure of gap junction [66, 67], we have also determined the CaM binding affinity of the Cx44 and Cx50 peptides to CaM as a function of pH. Both Cx44p$_{132-153}$ and Cx50p$_{141-166}$ peptides behaved similarly when pH was varied with binding affinities decreasing at pH values lower than 5.5 and greater than 8.5 [50]. However, over the pH range 5.5–8.5, CaM exhibits affinities for both peptides at nanomolar or submicromolar range. CaM binding to the peptide Cx50p$_{141-166}$ was ~ 1 order of magnitude stronger than to the peptide Cx44p$_{132-153}$ likely due to difference in electrostatic interactions.

It is interesting to note that Ca^{2+}-CaM may directly or indirectly affect cytoplasmic loop/C tail interactions in a Cx43 hemichannel, either through its binding to the cytoplasmic loop of Cx43 which corresponds to amino acids 119–144 in the C-terminal portion of the cytoplasmic loop of Cx43, or through a Ca^{2+}-dependent phosphorylation/dephosphorylation processes [29, 68, 69]. It is also possible that phosphorylation or other regulatory mechanisms such as pH could modulate this action of Ca^{2+}-CaM. Our results provide direct evidence that CaM binds to a specific region of the intracellular loop region of the gap junction proteins Cx43, Cx44 and Cx50 in a Ca^{2+}-dependent manner. Our data suggest a common mechanism by which the Ca^{2+}-dependent inhibition of the α-class of gap junction proteins is mediated by the direct association of an intracellular loop region of these proteins with Ca^{2+}-CaM.

3.4 Key factors contributing to the CaM binding affinity of the α-subfamily of connexins

Our results have also revealed that the α-helicity of the CaM binding peptide derived from connexin is an important factor in predicting their CaM binding affinities (Table 1) although the binding affinity of the full length connexin is yet available. The difference in CaM

binding affinity of the Cx50 and Cx44 peptides may originate from intrinsic sequences encoded in the CaM binding regions of these different connexins especially at conserved residue positions 1 and 5 (Fig.1); Cx43 peptide contains two flexible Glys instead of the α-helical forming residues present in Cx44 and Cx50 (Fig.1). Such sequence variations, that affect the ability of these peptides to form α-helices, are likely important in determining their ability to interact with CaM [65]. In aqueous buffer, these peptides are largely unstructured in the absence of CaM. 2,2,2-Trifluoroethanol (TFE) is an organic solvent which when added to aqueous buffer can reveal the intrinsic helical propensity of a peptide [70]. We have shown that all these peptides form α-helical structures in the presence of TFE that follows the rank order $Cx50p_{141-166} > Cx44p_{132-153} \sim Cx43p_{136-158}$ (Fig.3B). The higher CaM binding affinity of Cx44 versus that of Cx43 may also be due in part to the relative helical content of these two peptides in solution indicating that the intrinsic helicity of the peptide may also contribute to this binding. The negatively charged E151 located close to R149 in Cx50 may stabilize the α-helical conformation of this peptide, while Cx43 with a positive charged R at the same location may destabilize the α-helix conformation of this connexin [48, 52].

There are several positively charged residues such as K147, R149 and R156 in Cx50 that are conserved in the α-connexin subfamily members Cx43, Cx44 and Cx50. Indeed, the observed pH dependence of CaM binding affinity to the cytosolic loop peptides of Cx44 and Cx50 suggests electrostatic interactions following the protonation of Asp and Glu, and the deprotonation of Lys, are important for the connexin-CaM complex formation [50, 52].

It is also noteworthy that the binding affinity of CaM to the connexin-derived CaM binding peptides derived from the cytosolic region of three α-subfamily connexins does not agree with their predicted CaM binding scores based on the CaM Target Database [65]. These predicted scores are based on parameters including average hydrophobicity, average hydrophobic moment, and average propensity for α-helix formation and are not intended to define the CaM binding affinity for the complex formation [65]. It is interesting to note that the reported CaM binding site in the N-terminal region of Cx32 (residue 1–21) is predicted to have a high score by the CaM target database. This region is likely α-helical since it is homologous to the NTH motif (N-terminal helix) in the X-ray-determined structure of Cx26 [57]; it is believed to form the channel "plug" in the β-subfamily of connexins [71]. However, the reported C-terminal CaM binding region of Cx32 is not predicted by the CaM target database and is largely undefined in the determined Cx26 X-ray-derived structure. Therefore it is important to perform detailed experimental studies to define the actual CaM binding sites in its target proteins especially for membrane channel proteins such as the connexins.

3.5 CaM binding modes to the α-connexin subfamily using a peptide model

One of the key aspects to understand the molecular mechanism by which CaM interacts with its target proteins is the CaM binding modes, by which it interacts with its target proteins. Currently, over 200 structures of CaM complexed with its receptors are reported in the protein data bank. CaM exhibits different target recognition modes that can be classified into two general binding styles, namely extended and collapsed [48]. In the extended mode, CaM

has different recognition modes when it binds to different target proteins such as the IQ motif of myosin V Ca^{2+} channels [48], anthrax toxin [50], SK channels (Small conductance Ca^{2+}-activated potassium channels) [52], Ca^{2+} pump [40], and glutamate decarboxylase [39]. In the collapsed binding style, CaM can be further divided into different binding modes depending on the spaces between conserved hydrophobic residues at the CaM binding regions. For example, CaM binds to N-methyl-D-aspartate receptors (NMDARs) in the C1 region of its NR1 subunit in a 1–7 binding mode [72]. A 1–10 binding mode has been reported for CaM binding to CaM Kinase II α [73] and the Ca^{2+} channel $Ca_V1.2$ [74]. A 1–5-10 CaM binding mode was observed for the CaM complex with myristoylated alanine-rich C-kinase substrate (MARCKS) and CaMKII [75]. A 1–14 binding mode was observed in the CaM complex with cyclic nucleotide-gated ion channels (CNG) [76], Endothelial nitric-oxide synthase (eNOS) [6], CaM kinase I [7], a myosin light chain kinase that is exclusively expressed in adult skeletal muscle (skMLCK) [8], and metabotropic glutamate receptors (mGluR7a) [9]. Ca^{2+}/CaM-dependent protein kinase kinase (CaMKK) [7] has a 1–16 binding mode. The ryanodine receptor 1 (RyR1) [10] has 1–17 binding mode. The CaM binding regions of such CaM target proteins generally have a helical conformation on complex formation with CaM.

We have also used peptide models to understand the structural mechanisms driving CaM regulation of connexins with the advantage of obtaining site specific molecular recognition information. Several of our studies using various spectroscopic methods suggest that CaM binds to $Cx50p_{141–166}$, $Cx44p_{132–153}$, or$Cx43p_{136–158}$ with a 1–5-10 collapsed CaM binding mode as observed in the CaM-CaMKII complex. All three peptides are largely unstructured in aqueous buffer in the absence of CaM. Far UV circular dichroism studies have indicated that these CaM binding peptides likely become more helical upon binding CaM. Fluorescence spectroscopy revealed conformational changes of both the peptide and CaM following formation of the CaM:connexin-derived-peptide complex. Pulse-field gradient NMR studies demonstrated the hydrodynamic radius of CaM (22.6 ± 0.6 Å) was decreased by 23% upon formation of the CaM-$Cx50p_{141–166}$ complexes. The determined size of the Ca^{2+}/CaM–$Cx50p_{141–166}$ complex of 17.3 ± 0.6 Å is comparable to the hydrodynamic radii of CaM in complex with CaM-CaMKIIα (PDB code 1CDM) [77]. Furthermore, chemical shift changes of ^{15}N-labeled CaM have been used to show that these three connexin peptides are able to induce structural changes in both the N- and C-terminal domains of CaM as well as in the linker region. Using small angle X-ray scattering, Myllykoski et al. also observed that on binding a Cx43 cytoplasmic loop peptide (residues 144–158), CaM adopted a more globular conformation and collapsed the target [78]. In summary, these studies suggest that CaM likely embraces the peptides of α-connexins in a collapsed structure that involves the unwinding of the CaM central helix.

Because of its abundance, the mammalian water-channel aquaporin-0 (AQP0, or MIP26), was long considered to be the gap junction protein in the mammalian lens [79]. Like the connexins, aquaporin-0 channels were shown to close when bound with Ca/CaM [80–86]. More recent biochemical and NMR studies suggest that one CaM binds two AQP0 CBD peptides in a stepwise manner and two CaM molecules bind to a single AQP0 tetramer [83]. The 3D computational reconstruction of full-length AQP0-CaM complex based on the electron microscopy map and the determined X-ray structure of the plant glutamate

decarboxylase (ptGAD)-CaM complex [87] suggest that two Ca^{2+}-CaM are located directly under two of the AQP0 subunits and bind to two adjacent antiparallel C-terminal helices from two subunits of AQP0. This CaM binding mode to the water channel aquaporin-0 (AQP0) with a ratio of 1:2 is different from our observed CaM binding mode of the α-connexin subfamily which using peptide models have a 1:1 binding ratio. It would be interesting to observe how CaM is able to regulate the full length connexins using the same action mode. However, in the fully assembled gap junction, these connexin cytoplasmic loop CaM binding domains likely form a six-sided barrel near the M3 (third transmembrane) interface that may sterically limit the number of CaM molecules that may bind to this motif. The cytoplasmic loop and C terminal domains are thought to dimerize to close Cx43 gap junctions by the pH-dependent chemical gating mechanism [88]. Conversely, the six NT domains that fold into the cytoplasmic pore of the gap junction channel that contribute to the formation of the ion permeation pathway and transjunctional voltage (V_j) sensor apparently require the "gating" of only one NT domain to induce partial closure of the channel via the fast V_j-gating mechanism [57, 89, 90]. The connexin-CaM stoichiometry of binding required for gap junction channel closure remains to be determined, but is likely to require only 1–2 connexin subunits, based on current knowledge of the fast V_j- and slow chemical gating mechanisms.

3.6 Expanding the $[Ca^{2+}]_i$ sensing range of CaM upon binding to connexins

We have shown that when connexin peptides bind to CaM, the apparent K_ds of Ca^{2+} for CaM decreased and the Hill Coefficients increased. Isothermal titration studies of the $Cx44p_{132-153}$ peptide suggest that its interaction with CaM is an exothermic event that is both enthalpically and entropically driven in which electrostatic interactions play an important role. The binding of $Cx44_{129-150}$ peptide to CaM increases the CaM intradomain cooperativity, and further enhances the Ca^{2+}-binding affinities of the C-domain of CaM by slowing the rate of Ca^{2+} release from the complex [50]. CaM is also able to decrease the Ca^{2+} affinity of the N-lobe of $Cx50p_{141-166}$-bound CaM 2-fold, whereas the Ca^{2+} affinity of the C-lobe increased by ~20%. CaM responds to subtle changes in $[Ca^{2+}]_i$ over a broader range of $[Ca^{2+}]_i$ concentrations as a result of the binding of $Cx50p_{141-166}$ [52].

4.0 Proposed Ca^{2+}-CaM gating model

Results from our studies on the changes in Ca^{2+}-binding kinetics, affinities, energies, dye-transfer and gap junction conductance between cells support the following working model of gap junction inhibition by Ca^{2+}-CaM (Fig.4). Initially, an increase in $[Ca^{2+}]_i$ is sensed by the C-domain of CaM which effects the interaction of CaM with the cytoplasmic loop of the α-connexin subfamily. This interaction enhances the efficiency and sensitivity of intracellular Ca^{2+} sensing by CaM because of an increase in both the Ca^{2+}-binding affinity and intradomain cooperativity of the C-domain of CaM. The partially saturated, connexin-bound CaM might serve as an intermediate state to prevent the free diffusion of CaM into the cytoplasm. Further increase in $[Ca^{2+}]_i$ to the near micromolar range allows the half-saturated CaM to rapidly respond to this further increase in $[Ca^{2+}]_i$ such that it adopts a fully open conformation which is now able to inhibit gap junction mediated intercellular communication [48, 50]. Inhibition of the interaction of the cytosolic loop region with CaM

by the addition of a CaM inhibitor (e.g. CDZ) or a Cx43 cytosolic loop peptide prevents the open (M_0) to close (M_1) state transition. The Ca^{2+}-CaM induced gating response that closes the Cx43 gap junction is likely effected via a conformational change in the cytosolic loop of Cx43 [48]. It is also possible that, as proposed earlier by Peracchia and colleagues [91], Ca^{2+}-CaM binds to the N and C-terminal domains of Cx32 to act as a "cork", or it induces a conformational change in the cytosolic domain that occludes the cytosolic mouth of the gap junction channel, resulting in the restriction of the passage of current-carry ions [60, 92]. Unwin and Ennis proposed an alternative "iris" gating hypothesis that the extracellular Ca^{2+}-induced closure of liver gap junctions is a result of a decrease in the tilt angle of six connexins within a connexon junction channel [93]. This model relied on the presence of the "bulky" phenylalanines in the third transmembrane domain (M3), the major channel lining domain. Unwin and Ennis' idea was that the phenylalanine residues would gate the channel by obstructing its lumen, as they would be brought into the lumen by the M3 rotation. However, combined mutations of Cx32's phenylalanine145 and phenylalanine149 residues (the most conserved phenylalanines among connexins) to valine (a much smaller hydrophobic residue) generated channels with gating sensitivities that were indistinguishable from Cx32 wild type channels [30].

In summary, due to the challenges associated with large oligomeric membrane proteins and the "invisibility" of the cytosolic loop and C-terminal domains within a X-ray-derived structures of these proteins [56, 57], the mechanism by which CaM regulates gap junction channels remains to be fully defined. However, several recent studies provide strong evidence for a direct role of CaM in the regulation of several classes of connexin gap junction channels. While CaM was reported to bind to the N and C–terminal regions of the β-subfamily Cx32, it interacts with the cytosolic loop region of three α-subfamily connexins Cx43, Cx44 and Cx50. Cx35 and Cx36, and Cx34.7 are regulated by CaM in a Ca^{2+}-dependent manner through binding to only their C-terminal domains [55]. Furthermore, all these different connexins are regulated by CaM in a Ca^{2+}-dependent manner. The gated closure of the α-subfamily of gap junction channels by Ca^{2+} is effected by Ca^{2+}-CaM binding to connexins via an embracing CaM-binding mode with hydrophobic residues in the CaM-binding region at positions 1, 5, and 10. Electrostatic and hydrophobic interactions play an indispensable role in the intrinsic α-helicity of the CaM binding domains of α-subfamily connexins. Future studies are needed to address the stoichiometry of CaM binding to the full length connexin proteins, the intracellular Ca^{2+} affinity, and the connexin/CaM induced conformational changes required to induce the chemical gating mechanism. Furthermore, innovative approaches are required to reveal the novel mode of CaM action at the membrane interface via its association with flexible intracellular connexin regions resulting in the large conformational changes required for regulating the function of these membrane channels [94]. Since several spontaneous mutations have been identified in the CaM binding site of the cytosolic loop of Cx43 (such as in oculodentodigital dysplasia), understanding the mechanisms by which intracellular Ca^{2+}-CaM regulates gap junctions is an essential step towards revealing the molecular bases of such connexin-linked diseases.
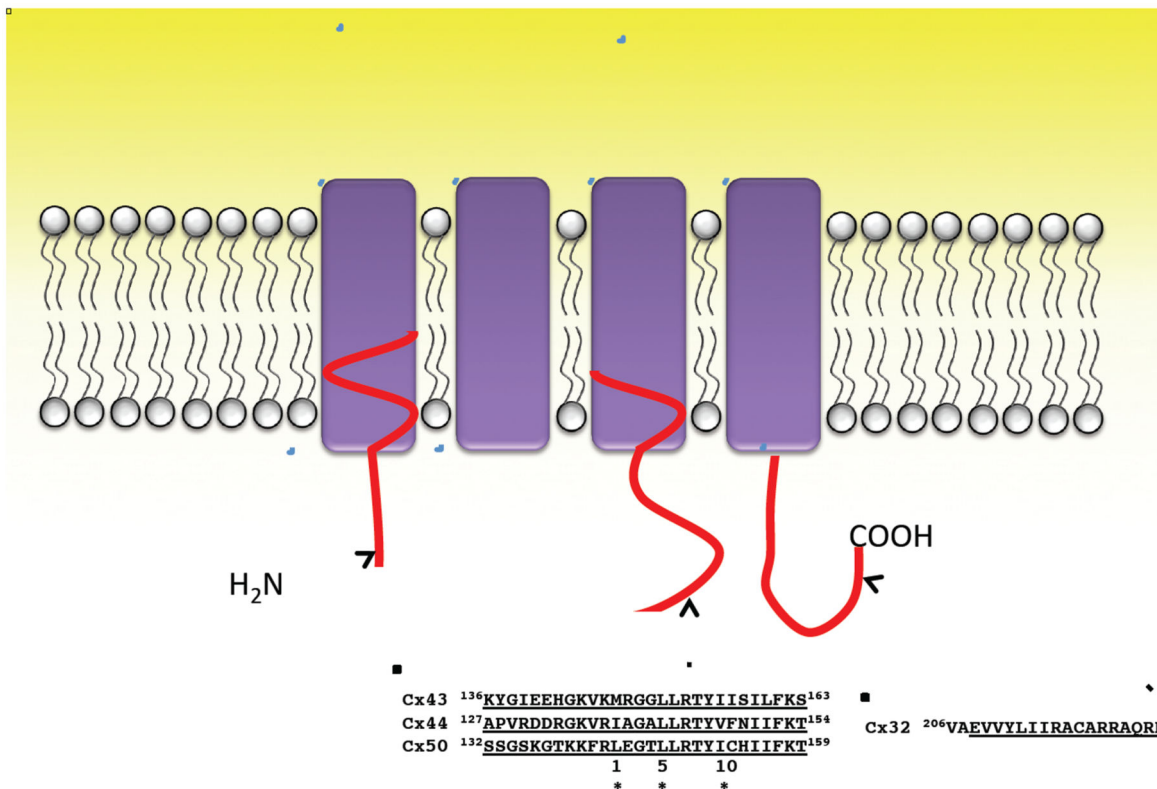
Acknowledgments

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Cx43 ^{136}KYGIEEHGKVKMRGGLLRTYIISILFKS^{163}
Cx44 ^{127}APVRDDRGKVRIAGALLRTYVFNIIFKT^{154}
Cx50 ^{132}SSGSKGTKKFRLEGTLLRTYICHIIFKT^{159}

Cx32 ^{206}VAEVVYLIIRACARRAQRRSNP^{227}

Cx32 ^{1}MNWTGLYTLLSGVNRHSTAIGRVWLSVIFIFRIM^{34}

Figure 1. Reported CaM-binding sites in rat Cx32, sheep Cx44, human Cx43 and 50
Two CaM-binding sites are located at the N- and C-terminus of Cx32. The identified CaM-binding sites in α-subfamily connexins are in the second half of the intracellular loop and fit the 1-5-10 subclass binding mode, where each number represents the presence of a hydrophobic residue. (*, Hydrophobic residues).
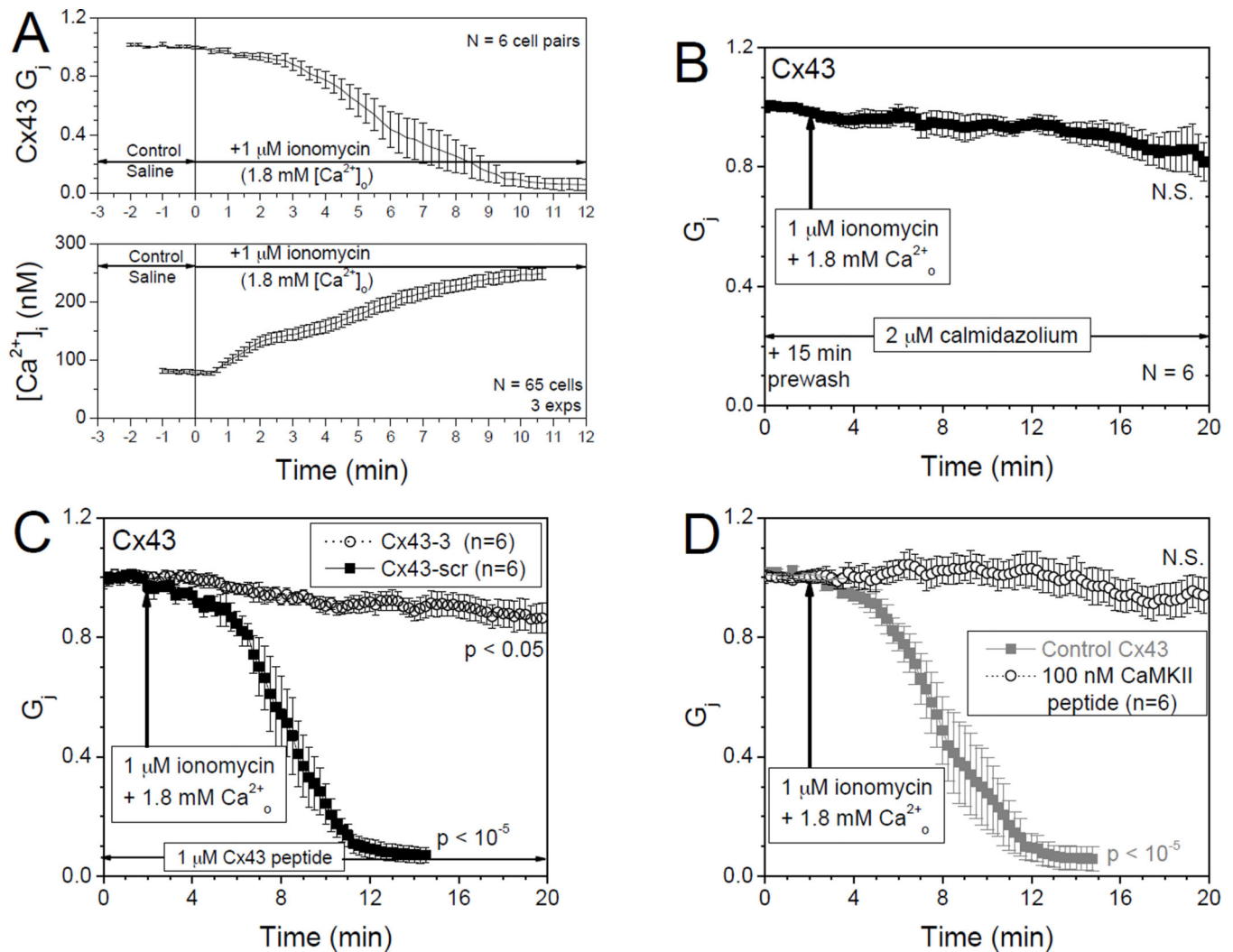
Figure 2. Ca^{2+}/CaM regulation of Cx43 gap junctions

A. Separate fura-2 and two whole cell patch clamp experimental measurements of [Ca^{2+}]$_i$ and normalized gap junction conductance (G$_j$) in Cx43-N2a cells illustrate the temporal decrease in G$_j$ (upper panel) and increase in [Ca^{2+}]$_i$ (lower panel) during 1 μM ionomycin perfusion (1 ml/min). B. Pretreatment with 2 μM calmidazolium prevented the Ca^{2+}-ionomycin induced reduction in Cx43 G$_j$. Inclusion of inhibitory CaM binding peptides Cx43p$_{136-158}$ (C) or CaMKII peptide (D) in both patch pipettes at the indicated concentrations (1–2 K$_d$ for CaM binding) also prevented the Ca^{2+}-ionomycin induced reduction in Cx43 G$_j$. Adapted from Xu et al. [51] with Author's permission from the American Physiological Society.
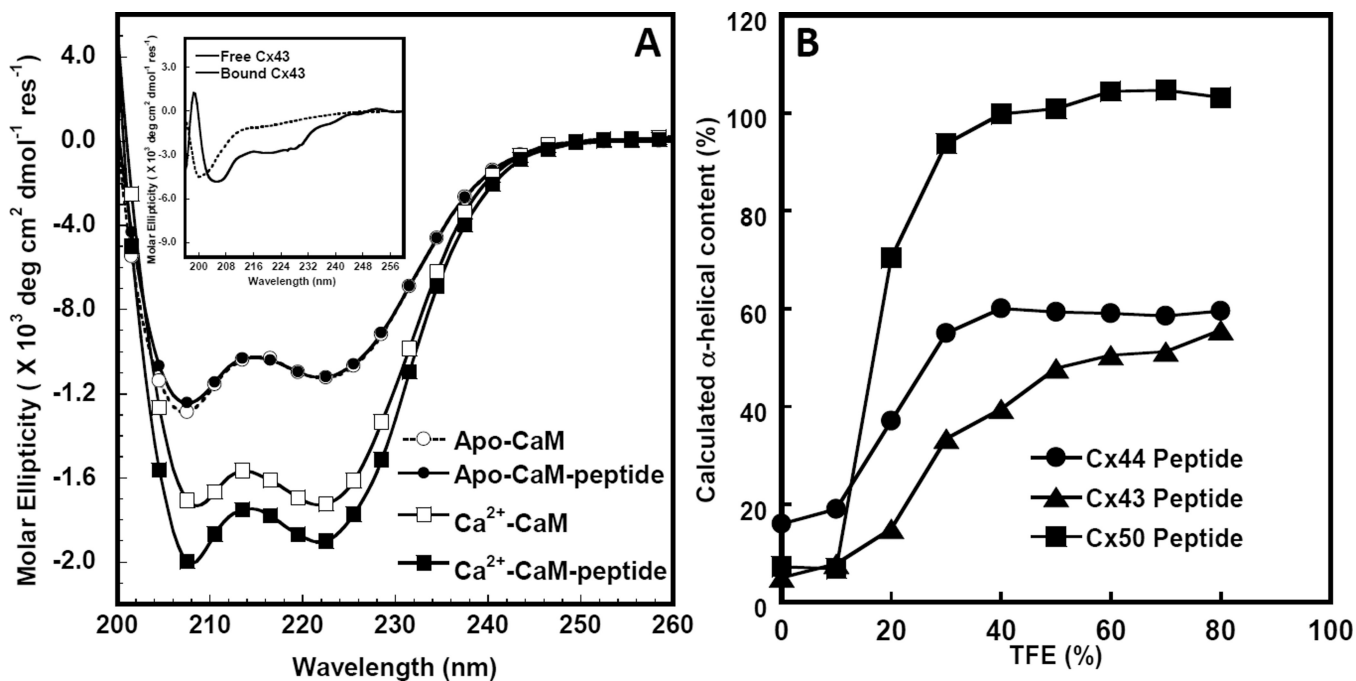
Figure 3. Circular dichroism studies of the interaction between Cx43p$_{136-158}$ and Apo/Holo CaM

A. Far UV circular dichroism spectrum of CaM in the presence of 1 mM EGTA (*white circle*) or 1 mM CaCl$_2$ (*white square*) and a 1:1 CaM/synthetic peptide mixture with 1 mM EGTA (*black circle*) or 1 mM CaCl$_2$ (*black square*) after subtracting the contribution from the buffer. Buffer conditions: 10 mM Tris, 100 mM KCl at pH=7.4 at room temperature. The insetdemonstratesthe far UVcircular dichroism spectra of Cx43p$_{136-158}$ (*dashed line*) and the calculated difference spectrum (*solid line*) by subtracting the spectrum of Ca^{2+}-CaM from that of the Ca^{2+}-CaM-Cx43p$_{136-158}$ mixture with 1 mM Ca^{2+} in a buffer consisting of 100 mM KCl, 10 mM Tris-HCl, at pH=7.4. B. TFE induced α-helical content increase of Cx43 (*triangle*), Cx44 (*circle*) and Cx50 (*square*) peptides corresponding to CaM-binding sites.
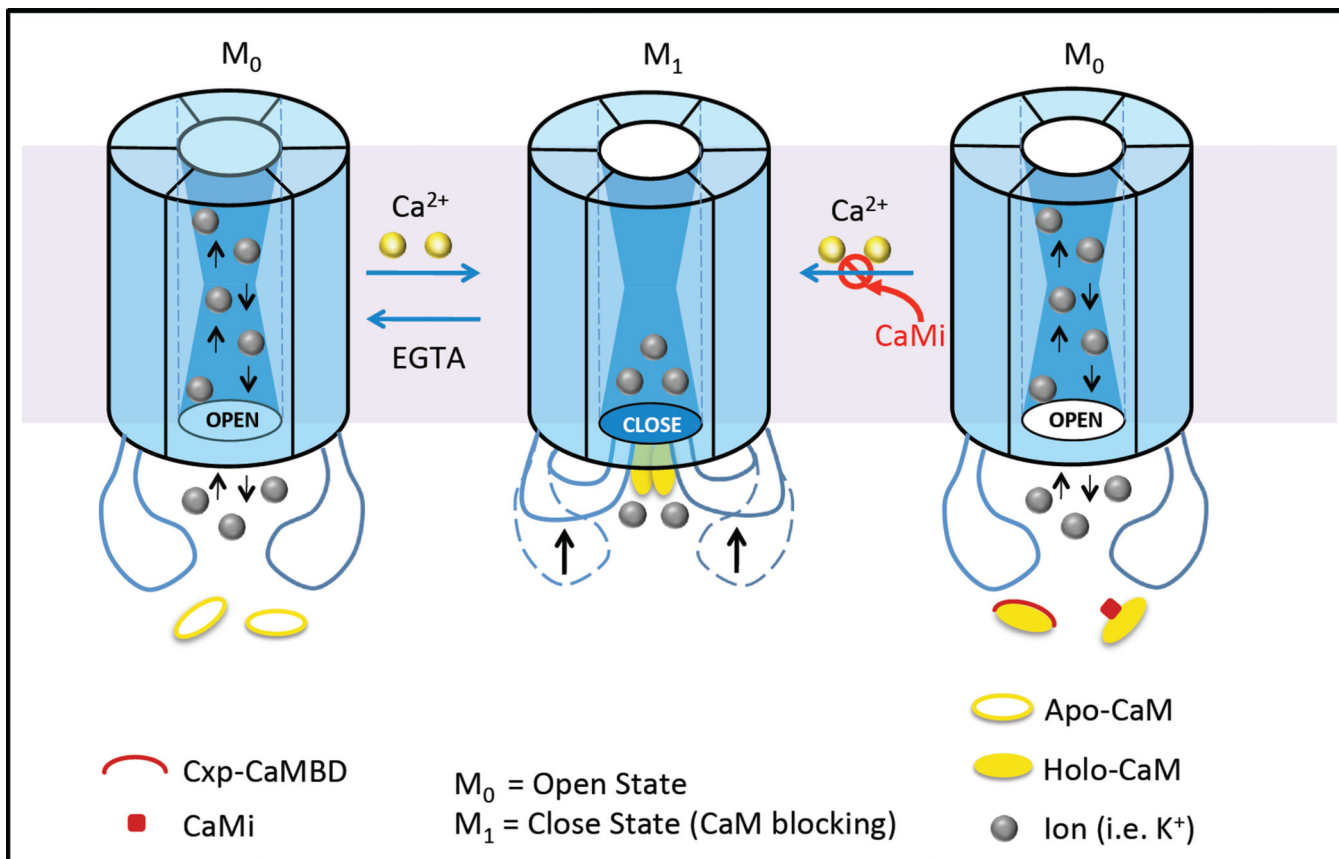
Figure 4. A proposed gating model for the mechanism of the closure of Cx43 gap junction channels by direct Ca^{2+}/CaM binding

In this model, connexin has one open (Mo) state and open closed state (M_1). The induction of a conformational change in the cytoplasmic loop (CL) of Cx43 through Ca^{2+}/CaM binding, steric hindrance of CaM binding near the cytoplasmic opening of the transmembrane pore, or a combination of both steric hindrance and conformational gating mechanisms causes the closure of the channel. Addition of the Cx43-CL-derived peptides or a CaMi (CaM inhibitor e.g., CDZ), prevents the Ca^{2+}/CaM-Cx43 cytoplasmic loop interaction which changes the status of the channel from closed (M_1) to the open (M_0) state.

Table 1

CaM-binding affinities of peptides derived from different subfamilies of connexins.

Peptide	Family	Position	Predicted Score[a]	Helicity[b]	K_d (nM) Ca²⁺	K_d (nM) EGTA	Reference
hCx43p$_{138-157}$	α	CL	16	55.6	860	nd	[48]
shCx44p$_{129-150}$	α	CL	6	59.5	49	>5000	[50]
hCx50p$_{141-166}$	α	CL	13	95.3	4.9	>8000	[52]
hCx32p$_{1-21}$	β	NT	9	nd	27	nd	[60]
rCx32p$_{216-230}$	β	CT	0	nd	2100	nd	[60]
mCx36p$_{269-321}$	γ	CT	14	nd	11	nd	[55]

[a]Predicted by CaM Target Database

[b]Peptide helicity induced by 90% TFE