Book Chapter

Calmodulin-Mediated Regulation of Gap Junction Channels

Camillo Peracchia*

Department of Pharmacology and Physiology, School of Medicine and Dentistry, University of Rochester, USA

***Corresponding Author:** Camillo Peracchia, Department of Pharmacology and Physiology, School of Medicine and Dentistry, University of Rochester, Rochester, NY, 14642 USA

Published **June 15, 2020**

This Book Chapter is a republication of an article published by Camillo Peracchia, et al. at International Journal of Molecular Sciences in January 2020. (Peracchia, C. Calmodulin-Mediated Regulation of Gap Junction Channels. Int. J. Mol. Sci. 2020, 21, 485.)

How to cite this book chapter: Camillo Peracchia. Calmodulin-Mediated Regulation of Gap Junction Channels. In: Song Guo Zheng, editor. Prime Archives in Molecular Biology. Hyderabad, India: Vide Leaf. 2020.

© The Author(s) 2020. This article is distributed under the terms of the Creative Commons Attribution 4.0 International License(<http://creativecommons.org/licenses/by/4.0/>), which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

Funding: This research received no external funding.

Conflicts of Interest: The authors declare no conflict of interest.

Abstract

Evidence that neighboring cells uncouple from each other as one dies surfaced in the late 19th century, but it took almost a century for scientists to start understanding the uncoupling mechanism (chemical gating). The role of cytosolic free calcium (Ca^{2+}_i) in cell–cell channel gating was first reported in the mid-sixties. In these studies, only micromolar $[\text{Ca}^{2+}]_i$ were believed to affect gating—concentrations reachable only in cell death, which would discard Ca^{2+}_i as a fine modulator of cell coupling. More recently, however, numerous researchers, including us, have reported the effectiveness of nanomolar $[\text{Ca}^{2+}]_i$. Since connexins do not have high-affinity calcium sites, the effectiveness of nanomolar $[\text{Ca}^{2+}]_i$ suggests the role of Ca-modulated proteins, with calmodulin (CaM) being most obvious. Indeed, in 1981 we first reported that a CaM-inhibitor prevents chemical gating. Since then, the CaM role in gating has been confirmed by studies that tested it with a variety of approaches such as treatments with CaM-inhibitors, inhibition of CaM expression, expression of CaM mutants, immunofluorescent co-localization of CaM and gap junctions, and binding of CaM to peptides mimicking connexin domains identified as CaM targets. Our gating model envisions Ca^{2+} -CaM to directly gate the channels by acting as a plug (“Cork” gating model), and probably also by affecting connexin conformation.

Keywords

Gap Junctions; Connexins; Innexins; Calmodulin; Membrane Channels; Channel Gating; Calcium; pH; Chemical Gating; Voltage Gating

Direct Cell-To-Cell Communication

The neighboring cells of most tissues freely exchange small cytosolic molecules via cell-to-cell channels clustered at gap junctions. This form of direct cell–cell communication (cell coupling) provides a fundamental mechanism for coordinating and regulating a host of cellular activities in mature and

developing organs [1–7]. Conversely, abnormal cell communication causes several diseases [8,9].

Each cell-to-cell channel is formed by the extracellular interaction of two hemichannels (connexons/innexons) that create a hydrophilic pathway spanning two apposed plasma membranes and a narrow extracellular space (gap). In turn, each connexon/innexon is an oligomer of six proteins (connexins/innexins) that span the membrane thickness and insulate the hydrophilic pore from the lipid bilayer and the extracellular medium. Gap junction channels are regulated by a gating mechanism sensitive to the cytosolic calcium concentration $[Ca^{2+}]_i$ [2,3,6,9,10].

Cell-To-Cell Uncoupling

In 1877, T.W. Engelmann made the startling discovery that cardiac cells become independent from adjacent injured cells [11], as he noticed that, in damaged cardiac muscle, unlike skeletal muscle, the injury potential soon vanishes, such that the electrical communication between healthy and damaged cardiac fibers ceases. This phenomenon, known as “healing over”, made him realize that there is a fundamental difference between cardiac and skeletal muscle cells. In his words: “*herzmuskelzellen leben zusammen und sterben einzeln* (cardiomyocytes live together and die alone)” [11]. Healing over, now known as “cell-to-cell uncoupling”, is present in all tissues with cells coupled by gap junction channels, and is mediated by the chemical gating mechanism [2–4,6,9,10,12–14].

Cytosolic Free-Calcium and Gap Junction Channel Gating

In 1965, Jean Déléze reported that cut cardiac fibers do not heal in the absence of extracellular calcium [12], suggesting for the first time a Ca^{2+} -role in gap junction channel-gating. This observation was soon confirmed by evidence that electrical and dye couplings are lost with a $[Ca^{2+}]_i$ rise [13]. The Ca^{2+}_i role in gating was proven by evidence that cell-to-cell uncoupling coincides with an increase in $[Ca^{2+}]_i$, monitored at the cell–cell

contacts by aequorin luminescence [14]. The Ca^{2+}_i role in gating was soon confirmed by many studies in both vertebrates and invertebrates [2,3,15–17].

$[\text{Ca}^{2+}]_i$ Effective on Channel Gating

Two early studies reported that only $[\text{Ca}^{2+}]_i$ in the high μM range causes cell-to-cell uncoupling [18,19]. However, numerous more recent reports have demonstrated that significantly lower $[\text{Ca}^{2+}]_i$, in the range of ~ 100 nM to low μM , are effective for channel gating. The effectiveness of low $[\text{Ca}^{2+}]_i$ was first published in studies on *Chironomus* salivary gland cells [20–22] and mammalian cardiac fibers [23,24].

In 1986, Noma and Tsuboi reported the effectiveness of $[\text{Ca}^{2+}]_i$ as low as 251 nM in cardiac cell-pairs [25,26]. Ten years later, Dekker and coworkers reported that the application of ionomycin and gramicidin to rabbit papillary muscle uncoupled the cells at $[\text{Ca}^{2+}]_i = \sim 685$ nM or greater [27], and the same $[\text{Ca}^{2+}]_i$ uncoupled cells subjected to ischemia followed by reperfusion [27]. Low $[\text{Ca}^{2+}]_i$ were also effective in crayfish axons [28,29], rat lacrimal epithelial cells [30], Novikoff hepatoma cells [31,32], astrocytes [33–35], lens cultured cells [36], human fibroblasts [37], cultured cells expressing Cx43 [38] and pancreatic cells [39–44], among others.

In 1990, we studied the relationship between junctional electrical resistance (R_j), $[\text{Ca}^{2+}]_i$ and pH_i in crayfish septate axons uncoupled by intracellular acidification caused by superfusion with Na^+ -acetate (pH 6.3) [28]. With acetate, a $[\text{Ca}^{2+}]_i$ rise of approximately one order of magnitude from basal values of 100–300 nM greatly increased R_j [28]. The $[\text{Ca}^{2+}]_i$ and R_j time-courses coincided, while those of pH_i and R_j did not [28] (see in the following).

In 1993, we determined more precisely the $[\text{Ca}^{2+}]_i$ effective on gating in Novikoff hepatoma cell pairs studied by double whole-cell patch-clamp [31,32]; these cell express connexin43 (Cx43). Ca^{2+} -sensitivity was tested by monitoring the decay of junctional conductance (G_j) at different $[\text{Ca}^{2+}]$ at $\text{pH}_i = 7.2$ or 6.1. Gating

was activated by $[Ca^{2+}]_i$ ranging from 500 nM to 1 μ M, regardless of pH_i [31] (Figure 1A), proving that Cx43 channels are sensitive to $[Ca^{2+}]_i$ in the nM range and are insensitive to pH_i as low as 6.1, as long as $[Ca^{2+}]_i$ is kept at resting level with BAPTA in the patch pipettes [31].

Figure 1: Junctional conductance (G_j) of Novikoff hepatoma cell-pairs expressing Cx43. (A). Cells dialyzed with patch-pipette solutions buffered for pH and Ca^{2+} . With $[Ca^{2+}]_i = 0.12 \mu M$ or lower, G_j decreases to 40%–50% with τ 's of 35.2 and 22.3 min, at $pH_i = 6.1$ and 7.2, respectively—note that this is the normal G_j decay of whole-cell-clamped cells. With $[Ca^{2+}]_i = 0.5$ – $1.0 \mu M$, G_j decreases to ~25%, with τ 's of 5.9 and 6.2 min, at $pH_i = 6.1$ and 7.2, respectively. (B). In cell-pairs treated for 20 s with 20 μM arachidonic acid (AA), the rapid and reversible G_j drop is prevented by the buffering of Ca^{2+}_i with low concentrations of BAPTA. Note that even a $[BAPTA]_i$ as low as 0.1 mM has some inhibitory effect. (A,B) are adapted from Ref. [31] and [32], respectively.

The effectiveness of nM $[Ca^{2+}]_i$ was also demonstrated in Novikoff cells during brief (20 s) exposure to 20 μM arachidonic acid (AA) [32] (Figure 1B). AA caused rapid and reversible uncoupling that was completely prevented by Ca^{2+}_i -buffering with BAPTA in the patch pipette solutions (Figure 1B). Significantly, similar concentrations of EGTA, a less efficient Ca^{2+} -buffer, were ten times less effective than BAPTA. AA (20 s exposure) had no effect on coupling in cells superfused with no-added- Ca^{2+} solutions (SES-no-Ca), suggesting that uncoupling resulted from Ca^{2+} entry [32]. In parallel experiments, $[Ca^{2+}]_i$ monitored with fura-2 increased with AA to 0.7–1.5 μM in cells with normal extracellular Ca^{2+} concentration ($[Ca^{2+}]_o$) [32]. However, extensive AA treatments slowly uncouple Novikoff cells, even in Ca^{2+} -free media, indicating that AA has also a

slow, Ca^{2+} -independent effect on gating [32], probably similar to that caused by anesthetics [45].

In cultured embryonic cells of chicken's lens exposed to Ca-ionophores A23187 or ionomycin, $[\text{Ca}^{2+}]_i$ increased from ~110 to ~400 nM and cell-cell transfer of Lucifer Yellow was drastically reduced [36]. With the subsequent superfusion of salines containing EGTA with no added Ca^{2+} , $[\text{Ca}^{2+}]_i$ dropped to ~260 nM and dye transfer resumed [36]. Similar nM $[\text{Ca}^{2+}]_i$ drastically decreased G_j in pancreatic β -cells, in which $[\text{Ca}^{2+}]_i$ was increased by lowering the temperature from 37 to 30 °C and increasing $[\text{Ca}^{2+}]$ from 2.56 to 7.56 mM [39].

$[\text{Ca}^{2+}]_i$ in the nM range was also effective in astrocytes co-injected with Lucifer Yellow and Ca^{2+} , as the dye-transfer blockage was linearly related to $[\text{Ca}^{2+}]_i$ ranging from 150 to 600 nM [33]. Consistent with this is evidence that 20 mM BAPTA added to patch pipette solutions significantly increases coupling between astrocytes [34], suggesting that gating may even be sensitive to resting $[\text{Ca}^{2+}]_i$. Similarly, dye coupling was blocked in ionomycin-treated astrocytes by $[\text{Ca}^{2+}]_i = 500$ nM [35], and comparable data were reported in lens-cultured cells [46]. In a recent study on murine Neuro-2a cells (N2a) expressing Cx43, exposure to ionomycin increased Ca^{2+} influx and reduced G_j by 95% [38]. $[\text{Ca}^{2+}]_i$ increased from ~80 to ~250 nM [38].

Based on these data, it is reasonable to believe that Ca^{2+}_i is a fine modulator of cell communication via gap junction channels. Furthermore, evidence for the effectiveness of nanomolar $[\text{Ca}^{2+}]_i$ suggests the role of a Ca^{2+} -modulated protein, calmodulin (CaM), as being the most likely candidate (see the following).

Intracellular pH and Channel Gating

In 1977, Turin and Warner reported that cytosolic acidification rapidly and reversibly uncouples *Xenopus laevis* embryonic cells [47,48], suggesting a pH_i role in channel gating. Soon after, uncoupling by lowered pH_i was confirmed in various vertebrate and invertebrate cells [19,49–53]. Curiously, however, pH_i has the opposite effect in cells expressing Cx36, as G_j increases with

acidification and decreases with alkalinization [54]. Similarly, the alkalinization of insect cells to $\text{pH}_i > 7.8$ decreased G_j to a complete uncoupling, attributed to a $[\text{Ca}^{2+}]_i$ rise [49].

It is generally accepted that, in most cells, acidification causes uncoupling. However, pH_i -sensitivity varies among cell types, and in most cases is related to the type of connexin expressed. In *Xenopus* oocyte pairs, for example, the rat liver connexin (Cx32) is much less sensitive than Cx38, the native oocyte connexin [55]. Delmar and coworkers tested the G_j sensitivity to pH_i in oocyte pairs expressing different connexins [56,57]. They demonstrated that Cx32 is the least sensitive ($\text{pK}_a \sim 6.5$) and Cx50 the most sensitive ($\text{pK}_a \sim 7.2$) connexin of this group; the other six connexins tested displayed pH-sensitivity in the following, decreasing order: Cx50, Cx46, Cx45, Cx26, Cx37, Cx43, Cx40 and Cx32 [58]. Other factors are also involved in determining pH_i sensitivity, because the same pH_i drops, attained by different procedures, have different effects on gating [28]. Furthermore, different cells expressing the same connexin have different pH_i gating sensitivities.

Does pH_i have a Direct Effect on Channel Gating?

Early data seemed to support a direct pH_i effect on connexin channels, although there were several inconsistencies [4,9,10,59]. Turin and Warner reported changes in coupling ratio (α) and pH_i in embryonic cells *Xenopus laevis*, but the pH_i - α relationship was only plotted for the recoupling phase; thus, potential hysteresis could not be revealed [48]. Spray and coworkers, using recessed-tip pH-sensitive microelectrodes, reported a hysteresis in the pH_i - α relationship, but interpreted it as an artifact caused by the CO_2 effect on non-junctional membrane conductance (G_m); thus, they concluded that protons act directly on connexins ($\text{pK}_a = 7.3$) [19]. However, subsequent work from the same group, using neutral-carrier, pH-sensitive microelectrodes, reported both a more pronounced hysteresis and a sensitivity to $[\text{H}^+]_i$ almost one order of magnitude lower ($\text{pK}_a = 6.5$ versus 7.3) [60].

Campos de Carvalho and coworkers found that in crayfish axons, G_j , measured when pH_i drops, follows the same Hill relationship as G_j measured during pH_i recovery, but only with short acetate applications [61]. With longer acetate exposures, G_j recovered slowly and incompletely, in spite of the fact that pH_i recovered at normal rate [61]. Significant hysteresis in the R_j - $[H^+]_i$ relationship [28] and several other inconsistencies were also reported in sheep cardiac fibers [62] and crayfish axons [28].

Channels made of Cx43 displayed a pK_a of 6.7 in *Xenopus* oocyte pairs [58], while $pH_i = 6.6$ had only a small effect on coupling in Cx43-expressing mammalian heart fibers [53], and H^+_i affected healing over in the heart only at $pH_i < 5$ [63]. In addition, internally perfused Cx43-expressing oocyte pairs were insensitive to pH_i [64], and Cx43-expressing Novikoff hepatoma cells were insensitive to $pH_i = 6.1$, as long as $[Ca^{2+}]_i$ was kept to resting levels with BAPTA in the patch pipettes (Figure 1) [31]. In crayfish axons, $pH_i = 6.3$ achieved by superfusion with acetate greatly increased R_j [28], while $pH_i = 6.0$ did not change G_j in internally perfused crayfish axons [65,66]. Note that, in crayfish axons, the R_j and $[H^+]_i$ time-courses markedly differ in shape, and $[H^+]_i$ maxima precede R_j maxima by 40–90 s (Figure 2A) [28]. In addition, media with pH as low as 5 do not affect the permeability of Cx32 hemichannels in liposomes [67], and several inconsistencies in the pH_i /electrical-coupling relationship were described in insect gland cells [49].

In several studies, similar pH_i values attained by different procedures had different effects on coupling. In crayfish axons, R_j maxima obtained with slow acidification rates were three times greater than those with faster acidifications, although the same pH_i minima were reached [28]. The acidification of amphibian blastomeres to pH_i 6, induced by 100% CO_2 exposure, brought G_j to zero, whereas a much greater degree of acidification was required to decrease G_j by the same amount when a low pH_i was achieved by HCl-injection, as pK_a shifted from ~6.4 to ~5.7 [60]. Dunina-Barkowska and coworkers reported a similar discrepancy in uncoupling efficiency between cytosolic acidification induced by weak diffusible acids (CO_2 ,

acetate, etc.) and by cytosolic dialysis with a strong acid (HCl) [68].

Although most data suggest an indirect $[H^+]_i$ effect, in one study on hemichannels it appears that direct protonation may affect gating [69]. In this study, Cx46 hemichannels expressed in *Xenopus* oocytes and studied by patch clamp in excised patches were sensitive to pH_i . Cx46 hemichannels closed rapidly and reversibly with short applications of low pH solutions, but channel gating was poorly reversible with longer treatments. This is unusual, because in intact cells uncoupling by acidification is always 100% reversible. A more recent study found that low pH may also act directly on Cx26 hemichannels [70]. This, however, contradicts earlier evidence for the role of aminosulfonates in the low-pH gating of Cx26 hemichannels [67,71,72].

Does Ca_i Mediate the Effect of Acidification on Channel Gating?

The $[H^+]_i$ -Rj and $[Ca^{2+}]_i$ -Rj relationships were first studied in crayfish axons uncoupled by acetate superfusion [28]. Plots of the time-courses of Rj and $[H^+]_i$ markedly differ in shape, and $[H^+]_i$ maxima preceded Rj maxima by 40–90s (Figure 2A), resulting in significant curve hysteresis in the $[H^+]_i$ -Rj relationship (Figure 2B). In contrast, the $[Ca^{2+}]_i$ -Rj time-courses matched extremely well (Figure 2C), with no hysteresis (Figure 2D) [28], and different rates of acidification caused comparable increases in $[Ca^{2+}]_i$ and Rj [28]. These data clearly indicate that acidification-induced uncoupling is more closely related to $[Ca^{2+}]_i$ than $[H^+]_i$ [28].

Figure 2: Percent change in junctional resistance (R_j) and $[H^+]_i$ in septal junctions of crayfish axons during cytosolic acidification with acetate solutions (Ac). (A). Plots of R_j - $[H^+]_i$ markedly differ in shape, and $[H^+]_i$ maxima precede R_j maxima by 40–90 s. (B). This results in marked curve hysteresis in the R_j - $[H^+]_i$ relationship, demonstrated in two different acetate superfusions (B: 1st and 2nd Ac treatments). In contrast, the R_j - $[Ca^{2+}]_i$ time courses (C) match extremely well in shape, peak time and magnitude. This results in negligible curve hysteresis in the R_j - $[Ca^{2+}]_i$ relationship (D). From Reference [28].

Similar results were obtained in *Xenopus* oocytes (Cx38) by monitoring G_j, with a double voltage clamp, as well as pH_i and pCa_i [73]. A drastic difference in time-course between G_j and pH_i was observed, as pH_i minima preceded G_j minima by ~4 min and pH_i recovered much faster than G_j (Figure 3A). In contrast, G_j minima and pCa_i minima matched well (Figure 3B) [73]. Plots of the time course of G_j and $[H^+]_i$ in *Xenopus* oocytes expressing Cx32 also showed marked differences [74].

Figure 3: Relationship between junctional conductance (Gj) and either pH_i (A) or pCa_i (B) in *Xenopus* oocyte pairs (Cx38) superfused with salines gassed with 100% CO₂. pH_i minima precede Gj minima by ~4 min (A), while Gj minima and pCa_i coincide (B). From Reference [73].

Our data on *Xenopus* oocytes (Cx38) intracellularly buffered with BAPTA are in agreement with evidence that a low pH_i uncouples cells by increasing [Ca²⁺]_i, as they greatly inhibited the effect of acidification on Gj [73]. A similar inhibition occurred in oocytes previously injected with ruthenium red [73]. Since ruthenium red blocks the mitochondrial Ca²⁺-uniporter [75], acidification is likely to increase [Ca²⁺]_i by releasing it, at least partially, from the mitochondria. A low-pH_i-induced Ca²⁺-release from the endoplasmic reticulum (ER) is also likely to be involved, as indicated by our study on crayfish axons uncoupled by a low pH_i in the presence of caffeine or ryanodine [59] (see the following).

To understand the mechanism by which low pH_i causes a [Ca²⁺]_i rise, we tested drugs that affect Ca²⁺ release from internal stores (caffeine and ryanodine) on crayfish axons, as well as treatments that cause Ca²⁺ entry [29]. A large increase in R_j and [Ca²⁺]_i maxima resulted from the addition of caffeine to acetate solutions, whereas a substantial drop in R_j and [Ca²⁺]_i maxima was seen when the acetate-caffeine treatment was preceded by caffeine pretreatment, suggesting that the caffeine pretreatment depleted the Ca²⁺-stores. In contrast, ryanodine always caused a significant decrease in R_j and [Ca²⁺]_i maxima [29]. Acetate-induced increase in Ca²⁺ entry was excluded because Ca²⁺-channel blockers were ineffective [59]. Acetate solutions

containing $[Ca^{2+}]_i$ as high 40 mM were also ineffective [59], further confirming that Ca^{2+} entry does not play a role in acetate-induced $[Ca^{2+}]_i$ increase.

Therefore, we have concluded that the effect of low pH_i on gating is mediated by Ca^{2+}_i [9,10]. Indeed, most compelling in this regard are our data on Novikoff cells, in which acidification to $pH_i = 6.1$ had no effect on gating as long as $[Ca^{2+}]_i$ was carefully buffered to resting levels by BAPTA in the patch pipettes [4,31] (see previous).

Calmodulin Role in Cell-To-Cell Channel Gating

In 1981, Johnston and Ramón reported that internally perfused crayfish lateral giant axons do not uncouple with either a high $[Ca^{2+}]_i$ or low pH_i solution [65]. Based on these data, confirmed by Arellano and coworkers [66], they suggested that a soluble intermediate mediates the Ca^{2+}/H^+ -induced channel gating. In the same year, we proposed the role of calmodulin (CaM) in gap junction channel gating [76,77]. The CaM role was also supported by evidence for CaM binding to Cx32 and gap junction fragments from crayfish hepatopancreas [78,79]. Over the years, the CaM role in gating has been confirmed by data generated from various approaches such as the application of CaM blockers, inhibition of CaM expression, overexpression of CaM mutants, co-localization of CaM and gap junctions, and in vitro evidence for CaM binding to connexins or synthetic connexin peptides mimicking CaM-binding sites, among others [9,10,80].

In addition to gap junction channels, CaM-regulated channels include voltage-gated Ca^{2+} channels (VGCC, CaV), Na^+ channels (VGSC, NaV), K^+ channels (VGPC, KV), small conductance Ca^{2+} -activated K^+ channels (SK), inwardly rectifying K^+ channels (Kir, IRK), cyclic nucleotide-gated channels (CNG), ryanodine receptors (RyR) and transient receptor potential channels (TRP), among others [81–84]. CaM also gates the water channel aquaporin-0 (AQP0), also known as the eye lens protein MIP26 [85–92].

CaM Inhibitors Prevent Cell-To-Cell Uncoupling

In 1981, we first reported the CaM role in chemical gating, based on the ability of trifluoperazine (TFP), a CaM blocker, to prevent CO₂-induced uncoupling of *Xenopus* embryonic cells (Figure 4A) [76,77]—indeed, this was the first example of CaM participation in the gating mechanism of membrane channels. In this study, we monitored the electrical coupling of *Xenopus* embryonic cells (morula stage) by measuring the coupling ratio (V_2/V_1), whose drop reflects electrical uncoupling. In controls, exposure to 100% CO₂ always reduced V_2/V_1 to nearly zero, while after 45–60 min treatment with 5 μ M TFP, CO₂ had a minimal effect on V_2/V_1 (Figure 4A). The TFP effect was reversible, but full recovery of gating efficiency was very slow (Figure 4A).

Figure 4: Electrical coupling of neighboring *Xenopus* embryonic cells at morula stage (A and B), monitored by measuring the coupling ratio (V_2/V_1). In the absence of the CaM-inhibitor trifluoperazine (TFP), 100% CO₂ reduces V_2/V_1 to nearly zero, while, with 5 μ M TFP, CO₂ has a minimal effect (A). The effect of TFP is slowly reversible (A). The most specific CaM-blocker, calmidazolium (CDZ), also inhibits the effect of CO₂ (B). With 100 nM [CDZ], 100% CO₂ causes V_2/V_1 to only drop to 0.5–0.7 (B). Another CaM inhibitor (W7, 100 μ M) also effectively inhibits Ac-induced Rj rise in crayfish axons (C), while its control (W5) is totally ineffective (C). A, B and C, from References [76,93,94], respectively.

In a subsequent study, calmidazolium (CDZ), the most specific CaM blocker, also prevented uncoupling in *Xenopus* embryonic cells [93]. With 100 nM CDZ, 5 min exposures to 100% CO₂ caused V_2/V_1 to drop to 0.5–0.7 (Figure 4B)—note that, in the absence of CDZ, CO₂ caused V_2/V_1 to drop to nearly zero (Figure 4B). CDZ also improved coupling, as V_2/V_1 reversibly increased at rest from ~0.6 to ~0.8 (Figure 4B) [93].

The CaM blocker W7 also inhibited uncoupling of crayfish axons superfused with acetate-containing salines (pH = 6.3) [94]. W7 (100 μ M) strongly inhibited the R_j rise within 15–20 min of treatment (Figure 4C), while its control (W5) was totally ineffective (Figure 4C)—recovery of normal uncoupling efficiency was slow and usually incomplete.

Studies on cardiac [95,96] and lens [46,97] cells reported similar results. In pairs of guinea pig ventricular myocytes, one of which was voltage clamped, where G_j was monitored after perforation of the partner-cell's non-junctional membrane, the gating sensitivity to Ca²⁺ increased from pCa 5.7 to pCa 7 when the cells were perfused with 10 μ M CaM. Significantly, W7 (but not W5) prevented uncoupling [98]. In some cases, CaM inhibitors have also been reported to increase coupling (Figure 4B) [93,99].

Lurtz and Louis tested CDZ in HeLa cells stably transfected with Cx43 [46]. Exposure to 1 μ M ionomycin in the presence of increased [Ca²⁺]_o increased [Ca²⁺]_i from ~110 to ~620 nM and blocked the cell-to-cell diffusion of AlexaFluor594. The block was prevented by pre-incubation with 10 μ M CDZ. Recently, CDZ was also effective in murine Neuro-2a cells (N2a) expressing Cx43 [38]—exposure to 1 μ M ionomycin increased [Ca²⁺]_i from ~80 to 250 nM and reduced G_j by ~95% in 15 min; the G_j drop was prevented by bathing the cells in Ca²⁺-free solutions or pretreating them with 2 μ M CDZ [38].

Paradoxically, CaM inhibitors uncoupled insect epidermal cells [100] and Hansen cells of the guinea pig cochlea [101], which express Cx26 and Cx30 [102]. Perhaps, in these cells, inhibition of the CaM–connexin interaction, or CaM release from connexins, causes channel closing rather than opening.

Significantly, this also occurs in Cx32 or Cx43 hemichannels, as W7 prevents the Ca^{2+} -induced hemichannel-opening [103,104].

Inhibition of CaM Expression Prevents Cell-To-Cell Uncoupling

The CaM hypothesis was also tested by monitoring the effect of exposing to 100% CO_2 *Xenopus* oocyte pair (Cx38) on G_j, in which CaM expression was inhibited by the previous injection of oligonucleotides antisense into the two CaM mRNAs expressed in oocytes [73]. Antisense oligonucleotides caused a progressive loss of uncoupling efficiency, starting 24 h post-injection (Figure 5A) [73]. Gating sensitivity to CO_2 partially recovered with CaM injection [73].

Antisense oligonucleotides had the same effect in oocytes expressing heterotypic mutant-Cx32 [105] or Cx45 (Figure 5B) channels [106]. Homotypic Cx32 channels (32–32) have low CO_2 sensitivity (Figure 8) [55]. In contrast, certain heterotypic mutant-32 channels are significantly more sensitive to CO_2 (Figure 9). Following inhibition of CaM expression, in mutant-32 channels CO_2 had a minimal effect on G_j [105]. In oocytes expressing Cx45, G_j drops to nearly zero with 15 min CO_2 application (Figure 5B) [106]. In contrast, with the inhibition of CaM expression, 15 min CO_2 applications caused G_j to drop by only 17.3% (Figure 5B) [106]. Note that with Cx45 the G_j course is biphasic. We propose that the initial G_j rise is caused by moderate $[\text{Ca}^{2+}]_i$ rise may activate the CaM Kinase II cascade, which, directly or indirectly, may open dormant channels likely to be gated by the CaM-Cork mechanism (see in the following).

A CaM Mutant with Higher Ca^{2+} -Sensitivity Greatly Enhances Gating Sensitivity

The effect of overexpressing the CaM mutant CaMCC on chemical- and V_j-gating was tested on oocytes expressing Cx32 channels [107,108]. In CaMCC, the NH_2 -terminal EF-hand pair (res. 9–76) of CaM is replaced by a duplication of the COOH-terminal pair (res. 82–148). Since the Ca^{2+} affinity constant of the COOH-terminal EF-hand pair is almost one order of

magnitude greater than that of the NH₂-terminal pair [109], we felt that expression of CaMCC might enhance chemical gating sensitivity. Indeed, in oocytes expressing CaMCC and Cx32 G_j was very low, but dramatically and reversibly increased (Figure 6A) when [Ca²⁺]_i, monitored with Calcium Green-1, was lowered by 180 μM BAPTA superfusion—note the reversible drop in F/F₀ (Figure 6A). This suggests that CaMCC increases the Ca²⁺ sensitivity of gating to such an extent that even resting [Ca²⁺]_i affect gating. This was confirmed by testing the effect of CO₂. With 3 min exposure to 100% CO₂, G_j rapidly dropped to zero, whereas in controls it decreased by only ~15% (Figure 6B). G_j remained nearly at zero indefinitely, but started recovering (reversibly) with a superfusion of 180 μM BAPTA (Figure 6B). Significantly, CaMCC was effective only when it was expressed before Cx32. This indicates that CaMCC, and by extension native CaM, binds to Cx32 before connexon assembly, further supporting the idea that CaM is an integral subunit of the connexon. The intimate CaMCC–Cx32 relationship was confirmed by a large reduction in V_j sensitivity [107].

CaM mutants lacking one or more of the four high-affinity Ca²⁺-binding sites were also tested [10]. In these mutants, glutamates (E) important for Ca²⁺ binding were replaced by alanines (A) in CaM's EF-hand domains—these mutations greatly decreased the Ca²⁺ affinity of the Ca²⁺-binding EF-hand loops [110]. The expression of CaM_{1,2,3,4} (E32A, E68A, E105A, E141A) or CaM_{1,2} (E32A, E68A), preceding Cx32 expression, strongly inhibited the formation of functional Cx32 channels, whereas the expression of CaM_{3,4} (E105A, E141A) had no effect [10]. Evidence that CaM_{3,4}, unlike CaM_{1,2}, enables an almost normal gap junction expression, suggests that N-lobe's Ca²⁺-activation is needed for gap junction formation. The effectiveness of CaM_{1,2,3,4} in competing against CaM wild-type confirms Ca²⁺-independent CaM-binding to connexins [111,112]. Furthermore, the observation that CaM_{1,2}, but not CaM_{3,4}, prevents channel formation, further indicates that normal Ca²⁺-affinity of CaM's N-lobe is needed. Indeed, the CaM–connexin interaction was reported to be relevant to connexin oligomerization into connexons, as in an in vitro cell-free synthesis system the formation of Cx32's hexameric hemichannels was reversibly

inhibited by a CaM-binding synthetic peptide and W7 [113]. Removal of the CaM-binding site at the COOH-terminus domain (CT1) impaired connexon formation and caused an accumulation of intermediate connexin oligomers [113]. Recently, these data were confirmed for Cx36 [114]. However, these data contradict our evidence for normal gap junction formation in oocytes expressing CT-truncated Cx32 [74,115,116] or Cx40 [117] channels.

Figure 5: A. Junctional conductance (G_j), monitored in *Xenopus* oocyte pairs (Cx38), exposed to 100% CO₂. Oocytes in which CaM expression is inhibited by the injection of antisense oligonucleotides progressively lose uncoupling efficiency (A). The CO₂ effect on G_j is reduced by ~60%, ~76%, ~93% in 24, 48 and 72 h, respectively (A). (B). G_j monitored in oocyte pairs expressing Cx45 during CO₂ exposure. The inhibition of CaM expression greatly reduces the CO₂ sensitivity of Cx45 channels—with 15 min application of CO₂, G_j reversibly drops by only ~17%, while in controls it drops to 0% (B). The G_j rise that precedes the G_j drop is absent with the inhibition of CaM expression (B). (A,B) from References [73,106], respectively.

Figure 6: Junctional conductance (G_j) and Ca²⁺_i (A) monitored in *Xenopus* oocyte pairs expressing Cx32. In oocytes expressing CaMCC, G_j is very low, but it dramatically and reversibly increases when [Ca²⁺]_i is lowered with 180 μM BAPTA superfusion (A). This indicates that CaMCC greatly increases Ca²⁺-gating sensitivity, such that even basal [Ca²⁺]_i affect gating. This was confirmed by testing the effect of CO₂ (B). With 3 min exposure to 100% CO₂, G_j rapidly drops to zero, whereas in controls it decreases by only ~15% (B); G_j remains at 0 indefinitely, but recovers (reversibly) with 180 μM BAPTA application (B). Adapted from Reference [107].

Do CaM-Activated Enzymes Play a Role in Gating?

Ca²⁺/CaM kinase II has been shown to phosphorylate Cx32, but only in isolated junctions as intact hepatocytes exposed to the Ca²⁺-ionophore ionomycin did not become phosphorylated [118]. The activation of Ca²⁺/CaM kinase II increased G_j in mouse astrocytes [119] and goldfish Mauthner cells [120]; we believe that the G_j rise may result from opening of dormant channels that were closed by the CaM-Cork mechanism (see in the following).

We have tested the potential role of inhibitors and/or activators of a number of enzymes [10]. None of them, however, significantly changed G_j or CO₂-induced channel gating [10]. The potential role of Ca²⁺-activated proteases is unlikely because proteolysis would be irreversible and the recovery rate from Ca²⁺-induced uncoupling is much more rapid than the connexin's turnover time (half-life = ~3 h) [121].

The possibility that gating by raised [Ca²⁺]_i results from the activation of Protein kinase C (PKC) or CaM-kinase II was tested in lens epithelial cells by raising [Ca²⁺]_i via Ca²⁺-

ionophore or ATP treatment [46,122]. Cell-to-cell transfer of AlexaFluor594 decreased with a rise in $[Ca^{2+}]_i$ and was prevented by CDZ treatment before ionomycin addition, but not by inhibitors of PKC or CaM-kinase II. Significantly, in HeLa cells transfected with a CT-truncated Cx43 mutant (Cx43-D257), the drop in coupling caused by a $[Ca^{2+}]_i$ rise to ~300 nM was similarly prevented by CDZ [122]. This not only confirms that Ca^{2+}_i regulates Cx43 channel gating in a CaM-dependent manner, but also proves that gating does not require the Cx43's CT domain. Evidence that CT does not play a role in Cx43 channel gating is also supported by recent data reporting that CT-deletion at res. 257 does not affect the gating sensitivity of Cx43 channels to a rise in ionomycin-induced $[Ca^{2+}]_i$ [123].

Co-Localization of Cam and Connexins

In 2000, we tested the direct CaM–connexin interaction by immunofluorescence microscopy [107,108]. In HeLa cells expressing Cx32, CaM and Cx32 co-localized in punctated or linear areas of cell–cell contact (Figure 7, overlay's arrow) as well as in few cytoplasmic spots [107,108]. CaM-Cx32 co-localization was also confirmed in cryosectioned mouse liver [108]. Similar results were obtained with Cx43 and Cx37 (Sotkis and Peracchia, unpublished data), as well as with Cx50 [124,125] and Cx36 [114]. These results confirm immunoelectron-microscopy data that demonstrated CaM binding to gap junctions of myocardial cells stained in frozen thin sections with colloidal gold-labeled CaM [126].

Figure 7: Immunofluorescence images of HeLa cells stably transfected with Cx32. CaM and Cx32 co-localize at the cell–cell contact area (overlay). Adapted from Reference [108].

The direct CaM–Cx32 interaction was also demonstrated by confocal fluorescence microscopy in HeLa cells, expressing Cx32 bound to the green fluorescent protein (Cx32-GFP) and CaM bound to the red fluorescent protein (CaM-RFP) [9,10,108]. In these samples, however, CaM and Cx32 only co-localized at cytoplasmic spots, as these cells did not form junctional plaques. The absence of junctional plaques may be due to steric hindrance, as the large size of the two fusion proteins may hinder connexin oligomerization into connexons. Similar results were obtained with Cx32 linked to the cyan fluorescent protein (Cx32-CFP) and CaM linked to the yellow fluorescent protein (CaM-YFP) [10,108].

Recently, the CaM–Cx45 interaction was directly visualized in a remarkable study that monitored it in living cells by

Bioluminescence Resonance Energy Transfer (BRET) [127]; the interaction was Ca^{2+} -dependent and blocked by W7. The role of CL2's CaM-binding site (res. 164–186; Figure 11) was confirmed by its high-affinity interaction ($K_d = \sim 5 \text{ nM}$) with a peptide mimicking the CL2 domain, monitored by fluorescence-labeled CaM [127]. Note, however, that there is evidence for both Ca^{2+} -dependent and Ca^{2+} -independent CaM binding to the CL2 domain of Cx32, Cx35, Cx45, and Cx57 [111,112]. The Ca^{2+} -independent CaM–CL2 interaction confirms previous evidence that CaM is anchored to connexins at resting $[\text{Ca}^{2+}]_i$ ($\sim 50 \text{ nM}$) [9,10,16,107,108,127].

Connexin Domains Potentially Relevant to Chemical Gating

In several studies, we have attempted to identify connexin domains relevant to chemical gating [55,74,116,128–130]. In 1996, we tested the CO_2 sensitivity of channels made of Cx32 and Cx38 chimeras and mutants in oocytes. As previously mentioned, Cx32 and Cx38 make channels that are at the opposite end of the spectrum in terms of CO_2 sensitivity (Figure 8) [55,128].

We focused first on the cytoplasmic loop (CL) of Cx32 and Cx38 (Figure 8A). Channels made of Cx32/38CL (Cx38's CL replacing that of Cx32) reproduced the chemical gating efficiency of Cx38 channels in uncoupling magnitude and both uncoupling and recoupling rates almost exactly (Figure 8B). In contrast, channels made of Cx32/38NT (Cx38's NT replacing that of Cx32) behaved closer to Cx32 channels [55]. Cx38 channels are more sensitive to fast- V_j gating than those of Cx32 [55]. Of the two chimeric channels, Cx32/38CL channels displayed fast- V_j sensitivity, similar to that of Cx38 channels, whereas Cx32/38NT channels displayed very low V_j sensitivity. The data suggest that CL plays a major role in both chemical gating and fast- V_j gating sensitivities [55].

To further identify the most relevant CL domains, we tested Cx32/Cx38 chimeras in which either the first half (CL1) or the second half (CL2) (Figure 8A) of Cx38's CL replaced those of

Cx32 [128]. The Cx32/Cx38CL2 channels (Cx32 with CL2 of Cx38) were like Cx38 in CO₂ sensitivity, although G_j recovered faster than in Cx38 channels (Figure 8C), but similar to Cx32 in fast-V_j gating sensitivity. Cx32/Cx38CL1 (Cx32 with CL1 of Cx38) did not express channels. These data indicate that CL1 and CL2 contain domains that are relevant for fast-V_j gating and chemical gating, respectively [128]. In fact, CL2's relevance to chemical gating is consistent with evidence for the presence of a CaM binding-site at CL2 (Figure 11) of Cx32, Cx35, Cx45 and Cx57 [111,112] (Table 1), as well as that of Cx43, Cx50 and Cx44 [80]. CaM binding sites at CL2 are also present in Cx26, Cx31, Cx33, Cx36, Cx37, Cx40 and Cx46 (Figure 11).

Figure 8: (A) Cytoplasmic loop (CL) sequences of rat Cx32 and *Xenopus* Cx38. (B) and (C) Junctional conductance (G_j) monitored in *Xenopus* oocyte pairs expressing Cx32, Cx38 or Cx32/38 chimeras. Channels made of Cx32/38CL (Cx32's CL replaced by that of Cx38) reproduce almost exactly the gating efficiency of Cx38 channels in magnitude and rate (B). Similar results were obtained with channels made of Cx32/38CL2 (Cx32's CL2 replaced by that of Cx38, C), although G_j recovered faster. Note that CL2 contains the CaM binding site (A). The postulated CL1–CT1 interaction (see text) could explain the faster recovery of Cx32/38CL2 channels (C). B and C adapted from References [55,128], respectively.

The faster recovery of Cx32/Cx38CL2 channels (Figure 8C) might result from a potential CL1-CT1 interaction, the idea being that in open channels CL1 might be bound to CT1 (see in the following). If so, the presence of the Cx32's CL1 domain in Cx32/Cx38CL2 channels may enable a fast channel reopening due to the rapid recovery of the CL1–CT1 interaction, following the unbinding of the CaM's lobe from the gating site (see in the following: Ca–CaM–Cork gating).

The potential relevance of Cx32's CT was tested by constructing a chimera in which Cx32's CT was replaced with that of Cx38 (Cx32/Cx38CT). However, this chimera did not express functional channels. Therefore, we focused instead on the potential role of a domain at the NH₂-end of CT (CT1), which has been identified as a CaM binding site in Cx32 [131,132], and indeed binds CaM [132,133]. Various mutations at Cx32's CT1, and CT deletions, yielded interesting yet puzzling results [74,116]. Although much of Cx32's CT is irrelevant to chemical gating, as 84% CT deletion (Cx32–D219) does not affect CO₂ sensitivity [74,134], basic CT1 residues appear to contribute to the low CO₂ sensitivity of Cx32 channels. This is suggested by the functional behavior of mutants in which five CT1 arginines (R215, R219, R220, R223 and R224) were replaced by neutral-polar residues (asparagine, N or threonine, T), another positively charged residue (lysine, K), histidine (H) (Figure 9) or the acidic residue glutamate (E) [74,116]. 5R/N and 5R/T channels were much more sensitive to CO₂ than Cx32 channels (Figure 9) with both full-length Cx32 (5R/N) and Cx32 with CT deleted at residue 225 (D225–5R/N). In contrast, 5R/K and 5R/H channels were as sensitive to CO₂ as Cx32 wild-type (Figure 9) [116]. CO₂ sensitivity is strongly inhibited by R215 and mildly by R219, whereas R220, R223 and R224, may slightly increase, rather than inhibit, CO₂ sensitivity (Figure 9) [116]; thus, R215 (and to a lesser extent R219) are most relevant in inhibiting CO₂ gating sensitivity [74,116]. The mutation of the five R residues to glutamate (5R/E), tested in heterotypic 5R/E-32 channels, increases the CO₂ gating sensitivity even more [105,135]. Therefore, the mutants ranked as follows in terms of CO₂ sensitivity: 5R/E > 5R/N > Cx32wt.

Figure 9: Normalized junctional conductance ($G_j/G_{j_{max}}$) monitored in *Xenopus* oocyte pairs expressing Cx32 or Cx32's CT1 mutants. Channels made of Cx32 mutants in which two or more CT1-arginines (R215, R219, R220, R223 and R224) are replaced by neutral-polar residues (asparagine, N or threonine, T) are much more sensitive to CO_2 than controls. In contrast, channels in which the R-residues are replaced with another basic residue (lysine, K) or histidine (H), as well as those with preserved R215 (4R/N#1), behave like Cx32 wild-type channels. From Reference [116].

These data were puzzling because CT1 (res. 208–227) was reported to be a CaM binding domain [132,133], and so its basic residues are expected to be relevant for CaM binding. The greater CO_2 sensitivity of 5R/N and 5R/E mutants seems to indicate that the greater the reduction in CT1's CaM-binding affinity, the greater the CO_2 channel gating sensitivity. Indeed, presently we doubt that the CT1's CaM binding is relevant to chemical gating, because CT-deleted Cx32 mutants (Cx32–D219) generated channels indistinguishable from Cx32 wild-type in CO_2 gating sensitivity [74].

A possible role of CT1 in channel regulation is its potential binding to CL1 (Figure 10)—this binding, if present, may need to break to allow CaM to bind to CL2. Perhaps, in the absence of CT1's R residues, this domain does not bind to CL1. This is further suggested by data showing that heterotypic channels made of Cx32 and the mutants 5R/E or 3R/N–ML/NN (R215, 219 and 220 replaced by N, and M105 and L106 replaced by N) are very sensitive to CO_2 [105,135]. Therefore, it could be that

the postulated CL1–CT1 binding requires both electrostatic and hydrophobic links—note that the hydrophobic residues, M105 and L106, and the acidic residues E102, E109 and D113, which are expected to interact with CT1’s R residues, are on the same side of the alpha-helix (Figure 10) [9].

Figure 10: Alpha-helical structure of Cx32’s CL1 and CT1 domains. CL1 is the only cytoplasmic domain rich in negative charges—it also contains positive charges, but they are on opposite sides of the helix. Our hypothesis is that, in coupled conditions, CL1 interacts with CT1. In CL1, acidic residues (E102, E109 and D113) and hydrophobic residues (M105 and L106) are on the same side of the helix. The idea is that, with uncouplers, a CaM lobe accesses the gating site by breaking the CL1–CT1 interaction (Ca–CaM–Cork model).

While the Cx32’s CT domain is unlikely to play a role in chemical gating [74,130], it seems to be relevant to gap junction assembly. Indeed, in human pancreatic and prostatic cancer cells, CT-deleted Cx32 (at res. 220) assembles into smaller gap junctions [136].

CaM Binding to Connexins and Connexin Peptides—Relevance to Channel Gating

Hertzberg and Gilula first demonstrated the ability of CaM to bind to Cx32 in gel overlays [78]. CaM-binding to Cx32 and

Cx32 fragments was soon confirmed by several teams [79,132,137]. CaM–Cx32 interaction was also demonstrated by evidence that CaM prevents both Cx32 proteolysis by m-calpain [138] and Cx32 phosphorylation by EGF receptor tyrosine kinase [139]. CaM–connexin interaction is also indirectly suggested by evidence for its participation in Cx32 oligomerization into connexons in vitro [113].

In 1988, we first identified two CaM binding sites in Cx32: one at NT (res. 15–27; RHSTAIGRVWLSV) and one at CT1 (res. 209–221; EVVYLIIRACARR) [131]. Török and coworkers tested the CaM-binding to peptides matching the sequences of Cx32's NT and CT1 domains by the fluorescent CaM derivative TA-CaM (2-chloro-(t-amino-Lys7s)-[6-[4-(7V,7V-diethylamino)-phenyl]-1,3,5-triazin-4-yl]calmodulin) [140] and equilibrium fluorescence methods [132]. Both peptides bound TA-calmodulin in Ca^{2+} -dependent manner. The dissociation constants (K_d) of TA-CaM binding to the NT and CT1 peptides were 27 nM and 1.2 μM , respectively. In a subsequent study, Dodd and coworkers tested the lobe-specific interactions of CaM with Cx32 peptides by stopped flow kinetics, using Ca^{2+} -binding-deficient CaM mutants [133]. Peptides matching the NT domain Cx32 (res. 1–22) bound to both NH_2 - and COOH -terminal CaM lobes (N- and C-lobes), but bound with higher affinity to the C-lobe. In contrast, peptides corresponding to the CT1 domain (res. 208–227) interacted with either CaM lobe, but with only one lobe at a time [133].

CaM binding to Cx32's CT1 was confirmed by testing the interaction with Isothermal Titration Calorimetry (ITC) and Nuclear Magnetic Resonance (NMR) [141]. However, in this study, which used a longer amino acid chain (res. 217–283), both CaM lobes bound to the peptide. Recently, the Cx43's CT res. K264–T290 has also been found to bind CaM [142]. However, none of these Cx43's CT CaM-binding sites are relevant for chemical gating because Cx43's CT-deletion at res. 257 does not affect the gating sensitivity to the ionomycin-induced increase in $[\text{Ca}^{2+}]_i$ [123].

CaM also binds to the CT1 domain of mouse Cx34.7 [114,143]. The interaction of CaM with Cx36's CT1 was further tested by NMR; this study demonstrated that the CaM binds to a synthetic peptide mimicking the CT1 site in its typical compact state to an eight-residue domain (mostly hydrophobic) spanning residues W277–V284 [114]. The Cx36–CaM complex preceded the formation of Cx36 gap junction plaques and enabled dye coupling [114]. Significantly, CaM inhibitors, or the mutation of the residue W277, relevant for CaM binding to Cx36, inhibited dye coupling [114]. Evidence for CaM–Cx36 interaction before plaque formation confirms the role of CaM in gap junction formation [113].

The original study of Burr and coworkers reported that the dissociation constants (kDa's) of the high affinity sites range from 11 to 72 nM, and $K_{1/2}$'s for Ca^{2+} range from 3 to 5 μM [143]. Ca^{2+} –CaM sensitivity in the μM range is higher than expected, but consistent perhaps with evidence that Cx36 channels are insensitive to pH_i as low as ~ 6.5 [54]. In these Cx36-expressing cells, uncoupling occurred with alkalinization; this may result from a high- pH_i -induced $[\text{Ca}^{2+}]_i$ rise [144]. In fact, the cytosolic alkalinization of insect cells increased $[\text{Ca}^{2+}]_i$ and caused uncoupling at $\text{pH}_i > 7.8$ [49].

Our data, suggesting that CL2 is most relevant to chemical gating [55,128], agree with evidence of a CaM binding site in Cx43's CL2 (res. 136–158; Figure 11) [145]. To test the chemical gating efficiency of Cx43 mutants lacking the CaM binding site at CL2, two mutants bound to EYFP (a fluorescent protein) were expressed in HeLa cells. The absence of the site eliminated Ca^{2+} -dependent gating, confirming that the CL2 domain (res. 136–158) contains the CaM-binding site relevant to Cx43's Ca^{2+} gating [145]. The relevance of CL2's CaM-binding site (Figure 11) was further confirmed with channels made of Cx43 [38], Cx50 [146] or Cx44 [80,147].

A remarkable study [148] used a synthetic peptide matching the CL2's CaM-binding domain of Cx43 (res. 144–158; Figure 11) for testing, by small angle X-ray scattering, Ca^{2+} -induced conformational changes. Upon peptide interaction, CaM adopted

a more globular conformation, indicating that CaM interacts with the peptide in a typical “collapsed” conformation [148].

Xu and coworkers [38] studied whole-cell, patch-clamp N2a cells expressing human Cx43 or Cx40. Ionomycin application to N2a cells expressing Cx43 resulted in a threefold increase in $[Ca^{2+}]_i$ and caused Gj to drop by 95%; in contrast, ionomycin did not significantly affect Gj in N2a cells expressing human-Cx40 channels. The chemical gating incompetence of the human-Cx40 [38] at first seems inconsistent with our evidence for the great chemical gating sensitivity of rat-Cx40 channels expressed in oocytes [117], but is consistent with the Ca^{2+} -CaM role in gating. In fact, a computer analysis of CL2’s potential CaM binding sites shows that the absence of two residues (V38 and V43) in rat-Cx40, replaced by G39 and A44 in the human-Cx40 (Figure 11), is the likely reason for the predicted inability of the CL2 domain of human-Cx40 to bind CaM [9]. The Ca^{2+} -induced drop in Cx43’s Gj was prevented by pretreatment with CDZ, and was reversed by the addition of 10 mM EGTA to Ca^{2+} -free salines [38]. The addition of a peptide matching the Cx43’s CL2 CaM-binding domain (res.136–158) to patch-pipette solutions also prevented gating, while neither a scrambled control peptide nor the Ca^{2+} /CaM-dependent kinase II inhibitory peptide (res. 290–309) did so [38]. These data indicate that CL2’s CaM-binding domain plays a key role in Cx43’s channel gating.

We have analyzed this CL2 domain as potential CaM-binding site in 13 mammalian connexins by a computer program developed at the University of Toronto (<http://calcium.uhnres.utoronto.ca/ctdb/ctdb/sequence.html>;

Copyright © 2002 Ikura Lab, Ontario Cancer Institute. All Rights Reserved). Significantly, in all of the connexins tested, but human-Cx40, the CL2 domain displays a potential CaM-binding site (Figure 11). The CaM-CL2 interaction has been confirmed experimentally by Jenny Yang’s team for Cx43 [145], Cx44 [147] and Cx50 [146], and by Katalin Török’s team for Cx32, Cx35, Cx45 and Cx57 [111,112].

sensitive CaM mutant, drastically reduces the Vj sensitivity of Cx32 channels [107]. Similarly, the Vj sensitivity of Cx45 channels is greatly reduced by inhibition of CaM expression [106]—note that channels made of Cx45 are very sensitive to Vj, and are unique among connexin channels because they close with Vj preferentially by means of the chemical/slow gate [149]. The behavior of heterotypic mutant/Cx32 channels also suggests that CaM is anchored to connexins even at resting $[Ca^{2+}]_i$ (~50 nM) because the inhibition of CaM expression in mutant/Cx32 channels drastically reduces the effect of Vj on Gj [105].

Evidence that CaM is anchored to connexins at resting $[Ca^{2+}]_i$ has recently been confirmed by in vitro experiments testing CaM-binding to peptides matching the CL2's CaM site of Cx32, Cx35, Cx45 and Cx57, in the presence and absence of Ca^{2+} [111,112] (Table 1). In this study, changes in the fluorescence of the double-labelled FRET-probe and Ca^{2+} -sensitive TA–CaM were revealed by fluorescence spectroscopy and stopped-flow fluorimetry [140] at physiological ionic strength (pH 7.5, 20 °C). Ca^{2+} -dependent and -independent bindings were monitored and the following kD values were obtained (Table 1)

Table 1. Ca-Dependent and -Independent CaM-Binding to CL2 Domains.

Connexins	kD (with Ca^{2+})	kD (without Ca^{2+})
Cx32	40 ± 4 nM	280 ± 10 nM
Cx35	31 ± 2 nM	2.67 ± 0.09 μM
Cx45	75 ± 4 nM	78 ± 1 nM
Cx57	60 ± 6 nM	52 ± 14 nM

FRET measurements demonstrated partial compaction (54%–70% quenching with Ca^{2+} and 33%–62% quenching without Ca^{2+}) of DA–CaM (DDP–maleimide and AEDANS substituted T34C, T110C-calmodulin). Kinetic data showed a two-step process of rapid interaction followed by isomerization, indicating that CaM is anchored to connexins and becomes totally bound upon stimulation [111,112].

Calmodulin Role in Hemichannel Gating

The existence of connexin hemichannels was first proven in cultured cells expressing Cx43 by evidence of 5(6)-carboxyfluorescein influx with lowered $[Ca^{2+}]$ [150]. Hemichannel permeability proved similar to that of gap junction channels and, similarly, hemichannels were sensitive to octanol and heptanol [150]. We further confirmed the presence of hemichannels by demonstrating that the membrane resistance (R_m) of Novikoff hepatoma cells drastically drops with no-added- Ca^{2+} solutions [150]. In control cells, R_m was lower than in cells transfected with anti-sense Cx43 (~ 800 and ~ 4000 M Ω , respectively), proving that the number of open hemichannels in low Ca^{2+} saline is much lower than in controls [150].

While external Ca^{2+} clearly plays a major role in keeping hemichannels closed [151,152], an increase in $[Ca^{2+}]_i$ actually causes hemichannel opening [103,104,153]. In Cx32-expressing cells, a $[Ca^{2+}]_i$ rise to ~ 500 nM, caused by treatment with 2 μ M A23187 (a Ca^{2+} ionophore), triggered ATP release and dye uptake that was blocked by a Cx32 mimetic peptide [104]. Significantly, this peptide ("32gap 24"; GHGDPLHLEEVK, res. 110–121) mimics a CL sequence that just precedes the CaM binding site (Figure 11). Hemichannel opening was prevented by W7 [104], suggesting a CaM role in hemichannel gating that is opposite its role in cell-to-cell channels. A subsequent study confirmed these data on Cx43 hemichannels expressed in glioma cells and primary glial cells [103]. Surprisingly, however, while a $[Ca^{2+}]_i$ rise to ~ 500 nM opened hemichannels, this phenomenon vanished with a greater $[Ca^{2+}]_i$ rise. Note, however, that the hemichannel closure at high $[Ca^{2+}]_i$ is likely to be CaM-independent [154]. CaM's role in hemichannel gating has been also reported for Cx50 hemichannels expressed in HeLa cells [125].

The gating mechanism of hemichannels and the role of CaM in hemichannel opening and closure are still poorly understood. A recent study [153] reported that a CT-deleted Cx32 mutant (Cx32–D220) renders hemichannels less sensitive to $[Ca^{2+}]_i$; significantly, Ca^{2+} -sensitivity is restored by application of the

peptide “32gap 24”. These Authors suggested that the interaction of “32gap 24” with the Cx32–D220 hemichannel stabilizes CL fluctuations, and proposed that CL fluctuations may prevent the exposure of CL residues to a target domain relevant to gating [153]. In agreement with our “CaM–Cork” gating model [9,10,16], they believe that Cx32 hemichannels are kept closed at resting $[Ca^{2+}]_i$ by a plugging molecule likely to be a CaM lobe [153]. Also consistent with the idea that a CaM lobe plugs the hemichannels (cork gating), is evidence that the hemichannels are opened by positive (depolarizing) voltage pulses [153,155]. Significantly, Castro and coworkers directly measured the opening and closure of Cx32 hemichannels by patch-clamp in response to a $[Ca^{2+}]_i$ rise, hence the kinetics of the hemichannel’s “cork” unplugging [155]. Indeed, based on the CaM–Cork, model the negatively charged CaM lobe is expected to be displaced out of the positively charged hemichannel’s mouth (vestibule) by membrane depolarization caused by positive voltage pulses [9].

A recent study on a Cx46 mutant (G143R) confirmed the direct CaM role in hemichannel gating [156]. The G143R mutation in the CaM-binding site (Figure 11), which increases hemichannel permeability [157], affected CaM binding to CL2 [156]. As predicted, both CaM binding to Cx46’s G143R mutant and increased hemichannel permeability were inhibited by CDZ. Significantly, G143R substitution greatly increases the CaM–Cx46 interaction in the presence and absence of Ca^{2+} [156], confirming that that CaM is anchored to connexins at normal $[Ca^{2+}]_i$ [111,112]. Perhaps, the enhanced Ca^{2+} –CaM affinity of the G143R mutant is caused by a slight shift in the site toward the COOH-terminus end [9]. The CaM–Cx46 interaction was also confirmed by immunofluorescent CaM–Cx46 co-localization [156].

Recently, Garcia and coworkers reported that the mutation G12R in Cx26’s NT increases the kinetics speed of slow-gate closure and, although the hemichannels still close completely at a very negative V_m , they are not affected by Ca^{2+} , even though Ca^{2+} still binds [158]. While it might be irrelevant to this phenomenon, it is noteworthy to realize that the G12R mutation

reduces the extent of the NT's CaM-binding site [9]. Another mutation (N14K) [159] decreased the extent of the CaM binding site even more [9]. The N14K mutation raises the energy barrier between open and closed hemichannel states and shifts calcium sensitivity, voltage sensitivity and deactivation time constants [159].

Chemical Gate, Slow Vj-Gate and Calmodulin

Gap junction channels are thought to have five types of gate: fast Vj-gate, slow Vj-gate, chemical gate, Vm-sensitive gate and extracellular gate (sensitive to Ca^{2+} in hemichannels). The slow Vj-gate and the Vm-sensitive gate behave like the chemical gate in terms of kinetics and efficiency [160,161]; therefore, we believe that they are the same gate. Consequently, this gate will be here named the “chemical/slow gate”.

Bukauskas and I have monitored single channel's gating behavior in rat fibroblasts and HeLa cells transfected with Cx43 during exposure to 100% CO_2 [161]. Junctional current (I_j), single channel conductance (γ_j) and I_j -kinetics were recorded during uncoupling and recoupling at different Vj-gradients, in order to distinguish chemical/slow gate and fast Vj-gate behaviors. At Vj = 55 mV, both gates are active: the fast Vj-gate displays fast I_j flickering between open γ_j (main state) and residual γ_j (residual) states, while the chemical/slow gate shows slow I_j transitions between open and closed states (Figure 12, left arrows and inset a) [161]. During recoupling, each channel reopens by a slow transition from closed to open state, followed by fast I_j flickering between open and residual state (Figure 12, right arrow and inset b). Significantly, the transitions from open to closed state, and vice versa, often display fluctuations (Figure 12c,d) [161]. Therefore, the CO_2 -induced chemical gating of Cx43 channels exclusively involves the chemical/slow gating mechanism [161]. These data are consistent with earlier findings on insect cells [162] and mammalian cells expressing Cx40 [163].

Figure 12: Junctional current (I_j) and single channel conductance (γ_j) monitored by a double, whole-cell clamp in HeLa cells stably transfected with Cx43, during uncoupling by 100% CO_2 . At $V_j = 55$ mV, the fast V_j -gating of the last five open channels (left side) is manifested by quick (~ 2 ms) I_j flickering between open (γ_j main state; top yellow line), and residual (γ_j residual; pink dashed-line), states, with a γ_j (residual)/ γ_j (main state) ratio of 20%–25%. In contrast, the chemical/slow gate closes the channel completely (bottom yellow line) by a slow I_j transition (~ 10 ms; insets a and c). Each channel reopens by a slow transition (~ 10 ms) from closed to open state (right side and insets b and d). Sampling points (green dots in a and b, and circles in c and d) were recorded at 1 ms intervals. The slow transitions often display fluctuations during channel closing (inset c) or opening (inset d), suggesting that a large particle, like a CaM lobe, flickers in and out of the channel's mouth (or competes with other CaM lobes) before settling in its final position. Adapted from Reference [161].

While in most connexin channels the chemical/slow gates are preferentially in open state, in Cx45 expressing cells, many of them are spontaneously closed at resting $[\text{Ca}^{2+}]_i$ and pH_i [106,149], most likely by the CaM-Cork gating mechanism (see in the following). Similarly, the chemical/slow gate is spontaneously closed in a variety of heterotypic mutant Cx32 and mutant Cx26 channels [105,135].

We have tested several Cx32 mutants and a Cx26 mutant that generate heterotypic channels that are spontaneously closed by the chemical/slow gate at $V_j = 0$ and are opened by positive V_j -

gradients at the mutant side [105,135]. The Cx32 mutants tested were: tandem, 5R/E, 5R/N, ML/NN, ML/CC, ML/EE, 3R/N and ML/NN+3R/N. In tandems, two Cx32 monomers are bound NT-to-CT. In 5R/E and 5R/N, five arginines of CT (R215, R219, R220, R223 and R224) are replaced by glutamates (E) or asparagines (N), respectively. In ML/NN, ML/CC and ML/EE, two CL residues, methionine (M105) and leucine (L106), are replaced by N, cysteines (C) or E, respectively. In 3R/N, R215, R219 and R220 are replaced by N. In ML/NN+3R/N, two previously listed mutations are combined.

In view of the fact that the chemical/slow gating behavior of these heterotypic channels is qualitatively the same, here we will focus on heterotypic tandem–Cx32wt channels (tandem-32). While homotypic Cx32 junctions (32–32) display a typical I_j – V_j sensitivity (Figure 13A), tandem-32 channels show a unique I_j – V_j behavior [135]. With a negative mutant side, as V_j is increased stepwise from -20 to -120 mV, I_j gradually decreases to very low values, and V_j sensitivity is manifested even at the lowest V_j . In contrast, with a positive mutant side, I_j gradually increases to high values, as lots of closed channels become operational. This results in great asymmetry in the relation between V_j and normalized G_j (G_{jss}/G_{jmax}) (Figure 13A). This I_j/V_j behavior indicates that V_j -positive or -negative at the mutant side opens or closes, respectively, a greater number of channels [105,135]. Significantly, the V_j - G_{jss}/G_{jmax} asymmetry (Figure 13A) vanishes with the inhibition of CaM expression (Figure 13B) [105].

Figure 13: V_j sensitivity of homotypic Cx32 (32–32) or heterotypic tandem–Cx32 (tandem–32) channels monitored in *Xenopus* oocyte pairs. With the application of V_j in steps (20–120 mV), 32–32 channels display a typical V_j sensitivity, as I_j decays exponentially for $V_j > \pm 20$ –40 mV (A and B). In contrast, tandem–32 channels display a unique I_j – V_j behavior: with a negative mutant side, initial and final I_j progressively decrease to very low values, while with a positive mutant side, I_j progressively increases to high values. The large asymmetry in the relationship between V_j and normalized G_j (G_{jss}/G_{jmax} ; A) is eliminated by the inhibition of CaM expression (B). From Reference [105].

The tandem-32 channel behavior was also tested by applying trains of long, 60 mV V_j -pulses that were positive at the tandem side (Figure 14) [135]. Three distinct I_j behaviors were seen: a monophasic I_j increase (pulses #1–3), a biphasic I_j time-course (pulses #4–9), characterized by an initial progressive I_j rise followed by exponential decay, and a conventional I_j behavior (pulses #10–18), represented by an initial I_j peak followed by exponential decay to a steady-state level (Figure 14). This indicates that the repeated application of V_j pulses positive at the mutant side gradually opens (renders operational) all of the “dormant” channels, eventually allowing the fast V_j -gate behavior of the adjoined Cx32wt hemichannels to be revealed (Figure 14), after the inhibition of CaM expression (Figure 13B) [105]. In fact, the application of conventional V_j -protocols immediately following the train of 60 mV-positive pulses briefly results in a normal behavior, similar to that of 32–32 channels [135]. The reason for this phenomenon is that V_j pulses positive at the tandem side made most or all of the available channels momentarily operational; thus, with all or most of the chemical/slow gates momentarily in open state, the normal activity of the fast V_j -gates of both tandem and wild-type Cx32 is manifested [135].

Figure 14: Junctional current (I_j) and V_j sensitivity monitored in *Xenopus* oocyte pairs expressing heterotypic tandem-Cx32 channels. With trains of 60 mV V_j pulses positive at tandem side, three I_j behaviors are observed: monophasic I_j increase (pulses #1–3); biphasic I_j time-course (pulses #4–9) and conventional I_j behavior (pulses #10–18). This suggests that repeated application of V_j pulses, positive at the tandem side, renders all of the available channels (from pulse #10 onwards) progressively operational. Indeed, the application of the conventional V_j protocol to either oocyte immediately after the trains of 60 mV pulses results in a behavior very similar to that of 32–32 channels (pulses #19–27). Adapted from Reference [135].

The slow change in G_j was interpreted as a gating phenomenon based on the activity of the chemical/slow gate, clearly distinct from that of the fast V_j -gate. There are several reasons for making this distinction [135], one being that, in all of the connexins tested, the chemical/slow gate always closes at the negative side of V_j [105,135], while the fast V_j -gate closes at the negative (Cx32) or positive (Cx26) side of V_j , depending on the type of connexin expressed. Indeed, heterotypic channels between Cx26 and a Cx26 mutant (4pos/E), in which the four basic residues of CT were mutated to E (4pos/E-26), behaved qualitatively as a heterotypic tandem-32, 5R/E-32 and other mutant-32 channels [105,135] (see previously) when exposed to steady-state V_j gradients [9,16] (Table 2).

Table 2: Changes in G_j Caused by Steady-State V_j-Gradients (±40 mV).

Heterotypic Channels	+40 mV at Mutant Side	-40 mV at Mutant Side
Tandem-32	↑262% ± 64%	↓84% ± 11%
5R/E-32	↑182% ± 50%	↓85.2% ± 3.3%
4pos/E-26	↑65% ± 10%	↓35% ± 10%

This is significant because the fast V_j-gates of Cx26 and Cx32 are sensitive to opposite voltage polarities—Cx32 is a “negative gater” while Cx26 is a “positive gater” [164]. This obviously indicates that in both Cx32 and Cx26 mutant channels, this gating behavior is a manifestation of the activity of the negatively charged chemical/slow gate (CaM’s N-lobe, see the following).

One may question why most diverse mutations unmask a similar slow gating behavior. We feel that, while without uncouplers the chemical/slow gate of most connexins, perhaps except Cx45 channels [106,149], is open, certain mutations unbalance the gating state of the mutant hemichannel, favoring the closed state to different degrees. This would allow the negatively charged chemical/slow gate, likely to be a CaM lobe, to gain access to the channel’s mouth (vestibule) and plug it (CaM–Cork gating; see in the following). Closed and open states could be interconverted by V_j, with V_j-positive and -negative at the mutant side, opening and closing, respectively, the mutant hemichannel.

Data generated by experiments in which mutant–Cx32 or Cx32–Cx32 channels were subjected to V_j-gradients of different polarity during CO₂ exposure (Figure 15) suggest that the chemical/slow gate may close the channels by two different mechanisms [105,135]. With 32–32 channels, V_j gradients of either polarity always resulted in a significant G_j drop (Figure 15A). In contrast, with mutant-32 channels G_j, progressively reduced to lower values by CO₂ at V_j = 0 mV, dramatically and reversibly increased with V_j gradients positive at the mutant side (Figure 15B) [105,135]. Significantly, with all of the heterotypic mutant-32 channels, the effect of CO₂ on G_j was minimal following the inhibition of CaM expression [105].

Figure 15: Junctional conductance (G_j) and V_j sensitivity monitored in *Xenopus* oocyte pairs expressing homotypic Cx32 (32–32; A) or tandem–Cx32 (tandem–32; B) channels, exposed to 100% CO_2 . With 32–32 channels, 40 mV V_j -gradients of either polarity always cause a G_j drop (A). In contrast, with tandem–32 channels, G_j increases with 40 mV V_j -gradients positive at the mutant side, and decreases with negative V_j (B). The effect of positive V_j progressively decreases as uncoupling progresses (B). This confirms evidence that the chemical/slow gate is V_j -sensitive, and suggests that there are two gating states: V_j -reversible and V_j -irreversible. Adapted from Reference [135].

V_j -positive at the mutant side was gradually less efficient in raising G_j as uncoupling developed, and gradually more efficient during recovery (Figure 15B) [107,135]. This suggests that we are dealing with two populations of gated channels: one in *closed state 1* and the other in *closed state 2* [135]. In *closed state 1*, the gates of the mutant hemichannels can be opened by positive V_j , while in *closed state 2* they cannot. We previously named the two gating types “Cx-driven” and “CaM-driven”, respectively [10]. We have now renamed them *CaM-Cork* gating and *Ca–CaM-Cork* gating, respectively [9] (see the following).

While we previously felt that, with most connexins (aside from Cx45), the chemical/slow gate is inactive in the absence of uncouplers or Cx-mutations, our 2007 data indicate that this gate

can also be activated by applying a series of large V_j gradients (Figure 16) [115]. With Cx32 channels, the application of a series of -100 mV V_j -pulses caused both peak (I_{jpeak}) and steady-state (I_{jss}) I_j to progressively and exponentially drop by 50%–60% (Figure 16) [115]. G_j , measured during recovery by applying small V_j -pulses slowly recovered, often reaching values greater than initial ones. Similar, but even more drastic, results were obtained with the mutant Cx32–D225 [115] that lacks fast V_j -gating [165], as CT-deleted Cx43 [165,166] and Cx40 [167].

These data clearly confirm the idea that the gate responsible for this phenomenon is the chemical/slow gate, rather than the fast V_j -gate. The manifestation of the activity of the chemical/slow gate in wild-type Cx32 indicates that this negatively charged gate, likely to be the CaM's N-lobe, can be rendered operational even without chemical uncouplers and/or connexin mutations. Indeed, our data [115] confirm previous evidence of sporadic slow-gating events to zero conductance state in cells expressing Cx32 channels subjected to V_j pulses [168].

Figure 16: Junctional current (I_j) and V_j sensitivity monitored in *Xenopus* oocyte pairs expressing Cx32 channels. A series of long (12 s, 30 s intervals) 100 mV V_j pulses progressively decrease peak and steady-state I_j by 50%–60% ($\tau \sim 1.2$ min). I_{jpeak} drops more dramatically, such that I_{jss}/I_{jpeak} increases from 0.4 to 0.6 (see inset). From Reference [115].

The phenomena described above are the manifestation of a direct CaM role in chemical/slow gating, because all of them are virtually eliminated by the inhibition of CaM expression [9,105,106]. Furthermore, the inhibition of CaM expression drastically reduced the V_j sensitivity of Cx45's chemical/slow

gate; while, with normal CaM expression, I_j decayed with time for V_j values greater than ± 5 mV, following the inhibition of CaM expression, I_j did not decay with V_j values lower than ± 40 mV [106]. The inhibition of CaM expression also significantly reduced the CO_2 sensitivity of Cx45 channels; indeed, with CO_2 , G_j reversibly decreased by only $\sim 17\%$, while, in controls, it rapidly dropped to zero (Figure 5B) [106].

Chemical Gating Model—Direct Calmodulin Role

Gating models not involving CaM have been named: “iris” [169–171]; ball-and-chain [57,172,173]; amino–sulfonate [67,71]; **light-switch** [130]; **electrostatic Ca^{2+} -mediated** [174] and integrated [175]. Since they are not CaM-related, these models will not be presented here; for their description and discussion, see [9].

The “Cork-Gating” Model

In 2000, we proposed a CaM-based “cork-type” gating model. This model envisions the physical obstruction of the channel’s mouth (vestibule) by a CaM lobe (Figure 17A) [10,16], probably combined with conformational changes in connexins, brought about by Ca^{2+} –CaM binding to connexin sites. This model is based on numerous findings suggesting a direct CaM role in chemical gating [9,10,16,80]. As previously mentioned, experimental evidence suggests that the chemical/slow gate is a sizable, negatively charged particle, likely to be a CaM lobe [105,135].

Figure 17: Ca–CaM–Cork gating model (**A**). Gating is believed to involve the physical obstruction of the channel's by a CaM lobe (**A**). The negatively charged CaM lobe would bind to the positively charged channel's vestibule hydrophobically and electrostatically, and probably cause conformational changes in connexins as well. Most connexins have three CaM-binding sites (**B**): NH₂-terminus (NT), the second half of the cytoplasmic loop (CL2), and the initial domain of the COOH-terminus (CT1). These sites (**B**) were proven to bind CaM (see text).

There are many reasons why CaM is the most likely gating candidate. In summary:

- Chemical gating is sensitive to $[Ca^{2+}]_i$ in the nM range [4,9,10,80]. Since connexins do not have sequences capable of binding Ca^{2+} in the nM range, gating must be mediated by a CaM-like protein—CaM being the most obvious;
- CaM binds to connexins [78,79,107,108,127,156];
- Most connexins have a CaM binding site at NT, CL2 and CT1 domains (Figure 17B) [9,10,80,133]. Most relevant for gating are likely to be the CL2 (Figure 11) and NT sites [9,55,80,111,112,127,128,133];
- Peptides mimicking the CaM-binding sites of various connexins bind CaM with high affinity [9,55,80,111,112,127,128,133]. Recent data show

that in several connexins CaM binds to the CL2 site both in Ca^{2+} -dependent and -independent ways [111,112], suggesting that CaM is anchored to connexins at resting $[\text{Ca}^{2+}]_i$;

- CaM and connexins co-localize at gap junctions and intracellular spots [107,108,114,126,127];
- Each of the two *negatively* charged CaM's lobes is $\sim 25 \times 35 \text{ \AA}$ in size [176], which is the same size as the *positively* charged channel's mouth (vestibule; Figure 18) [177–179];
- Chemical gating is eliminated by the inhibition of CaM expression [73,105,106].
- CaM blockers (inhibitors) prevent uncoupling by acidification and/or increased $[\text{Ca}^{2+}]_i$ [4,38,46,76,77,93,94,96–98,122];
- Overexpression of CaMCC, a CaM mutant with a higher Ca^{2+} -affinity, greatly increases the chemical gating sensitivity of Cx32 channels [107,108];
- At the single channel level, the chemical/slow gate opens and closes completely and very slowly, and most often displays fluctuations (Figure 12) [161], consistent with the idea that a large particle, likely to be a CaM lobe, flickers in and out of the channel's mouth before settling in the final position;
- Channels made of human-Cx40, a connexin that lacks the CL2's CaM-binding site (Figure 11), are not gated by increased $[\text{Ca}^{2+}]_i$ [38]. In contrast, in channels made of rat-Cx40, which has the CL2's CaM-binding site (Figure 11), chemical gating is fully functional [117];
- An increase in $[\text{Ca}^{2+}]_i$ opens Cx32 and Cx43 hemichannels [103,104,153]; this is prevented by W7 [104], suggesting a CaM role in hemichannel gating opposite to that in cell-to-cell channels. The direct CaM role in hemichannel gating was also reported for Cx50 [125] and Cx46 [156] channels.

Figure 18: Each negatively charged CaM lobe is $\sim 25 \times 35 \text{ \AA}$ in size (right panel), which is similar to the positively charged channel's mouth. Thus, a CaM lobe could interact with the connexon's mouth. In the right panel, the channel is split lengthwise so that the actual pore diameter (light blue area) is visible along the entire channel. CaM and connexons images (right panel) were generously provided by Drs. Francesco Zonta and Mario Bortolozzi (Venetian Institute of Molecular Medicine, VIMM, University of Padua, Italy).

The cork-gating model envisions two types of CaM-mediated gating: “Ca–CaM–Cork” and “CaM–Cork”. In the former, gating is initiated by Ca^{2+} -induced CaM activation. In the latter, gating occurs without a $[\text{Ca}^{2+}]_i$ rise and in most connexins, except in Cx45 [106], would require either a connexin mutation [105,135] or the application of large Vj gradients [115].

Ca–CaM–Cork Gating Mechanism

The Ca–CaM–Cork model proposes that a $[\text{Ca}^{2+}]_i$ rise above resting levels ($> \sim 50 \text{ nM}$) activates CaM and enables a CaM lobe (probably the N-lobe) to plug the channel's mouth (Figures 17A and 18) [9,10,16]. At resting $[\text{Ca}^{2+}]_i$, CaM is believed to be anchored to each connexin of the connexon by one of its lobes (most likely the C-lobe) at the CL2 site (Figure 19, a) [111,112]. The other lobe is likely free, but unable to gain access to the channel's mouth without CaM's activation by $[\text{Ca}^{2+}]_i$ higher than

resting values. In Cx32 channels, the inaccessibility of the channel mouth may be caused by a postulated CL1–CT1 interaction (Figure 10) [9,74].

The possibility that CaM only binds to connexins when the $[Ca^{2+}]_i$ increases above basal levels is unlikely because there is evidence for a CaM–connexin co-localization at resting $[Ca^{2+}]_i$ before and after gap junction formation in cells expressing Cx32 (Figure 7) [107,108], Cx50 [124,125] or Cx36 [114]. This is also consistent with evidence for CaM-dependent gating at resting $[Ca^{2+}]_i$ in special conditions (CaM–Cork gating; see the following) [9,105,106,115,135]. Evidence that expression of the CaM mutant CaM_{1,2,3,4}, which lacks Ca^{2+} -binding sites (see previously), prevents the expression of functional gap junctions is also consistent with this idea [10]. In addition, evidence for Ca^{2+} -independent CaM-binding to the CL2 site of α , β and γ connexins (Cx32, Cx35, Cx45 and Cx57; Table 1) [111,112] supports the idea that CaM is anchored to connexins at the CL2 site even at resting $[Ca^{2+}]_i$, and gates with an increase in $[Ca^{2+}]_i$ above resting values [10,16].

Figure 19: CaM is likely to be anchored to the connexin's CL2 site by its C-lobe (a). With $[Ca^{2+}]_i > \sim 50$ nM, the N-lobe would gate the channel by binding hydrophobically and electrostatically to the CL2 (b) or NT (c) domain of the same connexin (trans-domain interaction) or another connexin of the same connexon (trans-subunit interaction).

The Ca^{2+} -affinity constant of the C-lobe's EF-hand pair is greater than that of the N-lobe's pair by almost one order of magnitude

($K_{d(app)} = 5.6$ and $32 \mu\text{M}$ for C-lobe and N-lobe, respectively) [109,180]. Therefore, it is likely that the N-lobe interacts with the gating site (CL2 or NT; Figure 19, b or c, respectively) only when $[\text{Ca}^{2+}]_i$ increases above resting levels. This model agrees with evidence for the separate functions of CaM's N- and C-lobes in interacting with Cx32 [133].

Although the fine details of the CaM-mediated gating mechanism are still unclear, present data suggest that gating results from the plugging of the connexon's mouth (vestibule) by a Ca^{2+} -activated CaM lobe. The Ca–CaM–Cork gating model proposes that at resting $[\text{Ca}^{2+}]_i$ ($\sim 50 \text{ nM}$) CaM is anchored to each connexin by its C-lobe to the CL2 site (Figure 20A, white-colored connexins).

With a $[\text{Ca}^{2+}]_i$ rise, potential scenarios may be as follows:

1. Each of the six CaM's N-lobes is activated, binds to the NT or CL2 site of the same connexin (*trans-domain interaction*) and changes connexin conformation (Figure 20B, yellow-colored connexins). The connexins' conformational change allows an N-lobe (negatively charged) to access the channel's mouth and plug the pore by binding to the NT or CL2 site of the opposite connexin (Figure 20B, *trans-subunit interaction*) and interacting electrostatically and hydrophobically with the positively charged channel's mouth ("cork gating"; Figure 18);
2. All of the N-lobes are activated, but only one binds to a site (NT or CL2) of the opposite connexin (Figure 20C, *trans-subunit interaction*) and plugs the pore (cork gating) by interacting with the positively charged channel's mouth electrostatically and hydrophobically ("cork gating"; Figure 18).

Figure 20: The Ca-CaM-Cork gating model proposes that at normal $[Ca^{2+}]_i$ ($\sim 50 \text{ nM}$) CaM is anchored to each connexin by its C-lobe to the CL2 site (A, white-colored connexins). With a $[Ca^{2+}]_i$ rise, one scenario could be that each N-lobe binds to the NT or CL2 site of the same connexin (*trans-domain interaction*) and change the connexin conformation (B, yellow-colored connexins); this would allow an N-lobe to access the channel's mouth and plug the pore by binding to the NT or CL2 site of the opposite connexin (B, *trans-subunit interaction* – “cork gating”). Another scenario could be that with a $[Ca^{2+}]_i$ rise all of the N-lobes are activated, but only one binds to a site of the opposite connexin and plugs the pore (C, “cork gating”). If this were the case, the first Ca^{2+} -activated N-lobe would win the competition (*first come, first served*), preventing other N-lobes from accessing the channel's mouth.

In both cases, one N-lobe would win the competition (*first come, first serve*) by plugging the channel's mouth and preventing other N-lobes from accessing the channel's mouth.

We have reported that heteromeric 5R/N-Cx32 channels behave as if Cx32 were dominant in determining gating sensitivity to CO_2 , because even one wild-type Cx32 monomer among the six connexins of the 5R/N hemichannel was sufficient to maintain the low sensitivity to CO_2 typical of wild-type Cx32 channels [129]. Based on our hypothesis that the high CO_2 -sensitivity of the 5R/N-Cx32 channels may result from 5R/N's inability to establish CL1-CT1 interactions (Figure 10), it would seem possible that this interaction, present just in one Cx32 monomer

of each connexon, is enough to partially impair a CaM's N-lobe's access to the channel's mouth [129].

CaM–Cork Gating Mechanism

The CaM–Cork gating mechanism is exemplified by the behavior of heterotypic mutant Cx32 channels [105,135], homotypic Cx45 channels [106] and Cx32 channels subjected to large V_j gradients [115]. The idea is that, at the mutant hemichannel side of heterotypic mutant Cx32 (mutant Cx26) channels, the N-lobe of CaM is able to access the channel's mouth even at resting $[Ca^{2+}]_i$, perhaps because the mutations render the channel's mouth unprotected. The *negatively* charged CaM's N-lobe would interact electrostatically with the *positively* charged channel's mouth, such that it could be displaced by V_j-positive at the mutant hemichannel's side [9,105,135].

As previously mentioned, the CaM–Cork gating phenomenon unquestionably involves the chemical-slow gate (N-lobe) rather than the fast V_j-gate because the channel is opened by positive V_j at the mutant side both with “negative fast-V_j-gaters” like Cx32 and “positive fast-V_j-gaters” like Cx26 (Table 2) [9,16]. If the fast V_j-gate were involved, V_j positive at the mutant side would close rather than open heterotypic mutant Cx26 channels (4pos/E-26; Table 2).

Cytosolic connexin domains have a high ratio of basic versus acidic residues. For example, in Cx32, if we neglect most of CT, whose deletion by over 80% does not affect chemical gating sensitivity [74], and assign charge values of 1 for R, K, D and E residues and 1/2 for H residues, we find 18 basic and six acidic residues per connexin—108 and 36 residues, respectively, per hemichannel (connexon). Based on the short range of electric field's effectiveness, the V_j-sensitive slow gating behavior of heterotypic mutant Cx32 channels could only manifest itself if the gating CaM lobe were always very close to the channel's mouth.

One may question why the channel's mouth (vestibule) would be freely accessible to the gating CaM's N-lobe in Cx32 mutants

only. Possibly, in wild-type connexins the inaccessibility of the channel's mouth may result from the postulated CL1–CT1 electrostatic/hydrophobic interaction (Figure 10). The absence of this interaction in mutant channels that lack CT1's positive charges (5R/N, 5R/T) or in mutant channels in which the charges were converted to negative (5R/E) [74,116,135], and CL1's hydrophobic residues M105 L106 are mutated to N or E (ML/NN, ML/EE, ML/NN+3R/N) [105], would enable the N-lobe to freely access the channel's mouth. Similarly, in Cx32-tandem channels the linkage of three NT chains to three CT chains might hinder the postulated CL1–CT1 interaction [105,135].

In view of the effect of large V_j -gradients applied to homotypic Cx32 channels (Figure 19) on G_j [115], it seems likely that the gating particle (CaM's N-lobe) could be forced to plug the mouth of the hemichannel subjected to negative V_j in the absence of Cx mutations or Ca^{2+} -activation. Therefore, the hemichannel's mouth (vestibule) may not be totally inaccessible, so that the N-lobe could be forced to access it by large negative V_j -gradients (100 mV) (Figure 16) [115].

In several wild-type connexin channels, large V_j -gradients may not be needed to force the gating element (CaM's N-lobe) into the channel's mouth. Indeed, in cells expressing Cx45, a number of channels are closed by the chemical/slow gate, even at $V_j = 0$ and without uncouplers [106,149]. Possibly, the postulated CL1–CT1 interaction (Figure 10) is weaker or absent in Cx45 channels. In view of the fact that inhibition of CaM expression eliminates the spontaneous gating of Cx45 channels [106], the gating element is likely to be a CaM lobe.

Locked-Gate Model—Irreversible Channel Gating

In 2012, Xu and coworkers reported that in some cases chemical gating is irreversible [38]. This study tested the Ca^{2+} -dependent gating of Cx43 channels in N2a cell pairs exposed to 1 μM ionomycin and 1.8 mM Ca^{2+} ; competitive peptides or CDZ prevented the G_j drop, confirming the CaM role in gating [38]. To test the uncoupling reversibility, the Ca^{2+} -containing solution

was switched to a no- Ca^{2+} -added solution containing 10 mM EGTA. Significantly, if the switch to no- Ca^{2+} -EGTA solutions was made when Gj had dropped by just 50%, Gj recovered almost fully; in contrast, if the solution-switch was done when Gj had dropped to 0% (full uncoupling), Gj recovered to only ~60% [38]. This suggests that there might be two Ca^{2+} -induced gating states: one reversible and the other irreversible (locked-gate).

The possibility of a “locked-gate” state [38] brought to mind our early data that demonstrated changes in gap junction channel-packing in freeze-fracture replicas from loose (Figure 21A) to tight-crystalline (Figure 21B) in cells in which $[\text{Ca}^{2+}]_i$ was increased by exposure to uncouplers [2,3,181–185]. We proposed that gap junction crystallization might reflect channel conversion from open to closed state. Based on the observation of Xu and coworkers [38], it is possible that crystallization reflects an irreversible gating state, leading to gap junction internalization, the formation of annular gap junctions, and eventual degradation. Indeed, a study reported the apparent irreversibility of junction crystallization with prolonged exposure to uncouplers, as well as an increased number of annular (internalized) gap junctions [186].

As a hypothesis, we propose that, while in coupled condition CaM is linked to each of the connexins (Figure 21A, inset), with prolonged exposure to high $[\text{Ca}^{2+}]_i$, all of the CaM molecules but the gating one, might become detached from connexins. This would allow the channels to tightly pack into crystalline (hexagonal) arrays (Figure 21B, inset), by interacting hydrophobically with each other. This might explain why isolated, negatively stained gap junctions are crystalline (hexagonal particle arrays), and the channels remain tightly connected to each other even though the junctions are subjected to harsh isolation procedures [2,9,187].

Figure 21: Freeze fracture images of gap junctions from rat stomach epithelium. In controls, most gap junctions display particles (connexons) and pits irregularly packed at center-to-center spacing of 103-105 Å (A). In cells uncoupled by 1 h treatment with 2,4-dinitrophenol (DNP) particles and pits aggregate into crystalline, hexagonal, arrays with an average center-to-center spacing of ~85 Å (B). We propose that while in coupled conditions CaM is linked to each of the 6 connexins (A, inset), with high $[Ca^{2+}]_i$ all of the CaM molecules, but the gating one, might detached from connexins, allowing the channels to tightly pack into crystalline (hexagonal) arrays (B, inset). P and E: Protoplasmic and Exoplasmic faces, respectively. Adapted from Ref. [182].

If so, what would cause CaM molecules to be released from connexins at high $[Ca^{2+}]_i$? In 2004, Black and coworkers developed a genetically encoded fluorescent biosensor to determine the intracellular concentration of both Ca^{2+} -free (apo) and Ca^{2+} -activated (holo) CaM [188]. This study reported that, in a human kidney cell line stably expressing the biosensor, the $[CaM]_i$ at rest is $8.8 \pm 2.2 \mu M$. A $[Ca^{2+}]_i$ rise induced by agonist, store-operated Ca^{2+} -entry, or ionophores caused a Ca^{2+} -dependent consumption of available CaM, resulting in a $[CaM]_i$ drop to ≤ 200 nM. This is caused by the drastic buffering of free CaM due to excess availability of CaM binding sites. The same results were obtained in cardiac myocytes of adult rabbits. In fact, in both permeabilized and intact myocytes, with a $[Ca^{2+}]_i$ rise, CaM reversibly moved into the nucleus [189].

The same phenomenon might apply to gap junctions. A potential scenario, therefore, could be that, with a great/prolonged $[Ca^{2+}]_i$ rise, the five non-gating CaM molecules become detached from connexins and bind to other CaM binding sites with a higher affinity to Ca^{2+} -CaM, which would successfully compete against connexins for CaM binding.

Conclusion

We have proposed a two-facet CaM-mediated gating mechanism: Ca-CaM-Cork and CaM-cork. In summary:

- At resting $[Ca^{2+}]_i$, ($<50\text{nM}$) some channels are spontaneously closed by the CaM-Cork gating mechanism.
- With moderate $[Ca^{2+}]_i$ rise ($50\text{-}100\text{ nM?}$), the CaM kinase II cascade may be activated and directly or indirectly open channels closed by the CaM-Cork mechanism.
- With greater $[Ca^{2+}]_i$ rise ($>100\text{ nM?}$), the channels start closing by the Ca-CaM-Cork mechanism. Channel-mouth plugging by a CaM-lobe is likely to include connexin conformational changes.
- CaM-Cork gated channels could be reopened by Vj positive at gated side, but since they would close at the negative side no Gj change would occur. This is not the case with heterotypic channels between wild-type connexins paired with more gating-sensitive mutants.
- Ca-CaM-Cork gated channels only reopen with a drop in $[Ca^{2+}]_i$ to resting values ($<50\text{ nM}$).

While much still needs to be investigated to understand the gating mechanism of gap junction channels in detail, most relevant, thus far, is evidence that direct cell-to-cell communication is finely modulated by the direct action of Ca^{2+} –CaM at nanomolar $[Ca^{2+}]_i$. This is important not only because this finding brings together the vast knowledge of two major fields of cell biology, but also because it proves that the function of chemical gating is much greater than just protecting healthy cells from damaged or dead neighboring cells (healing over).

Further knowledge of the CaM-role in modulating the permeability of channels and hemichannels made of connexins/innexins may also pave the way for understanding the behavior of intracellular connexin/innexin channels. Indeed, evidence accumulated over several decades indicates that these proteins form hexameric connexons/innexons in non-junctional

plasma membranes, the Golgi apparatus, mitochondria and, in some cases, also in the endoplasmic reticulum [9]. Many questions need to be answered, such as: are intracellular connexons/innexons capable forming functional intracellular hemichannels? Do they interact with each other to form “intracellular” junctions? Do they interact with gap junctions? Indeed, some intriguing findings, published over the last four decades and before, have raised the possibility that connexin/innexin-mediated communication might also occur intracellularly between organelles, as well as between organelles and gap junctions, possibly via “inverted” intracellular gap junctions [9].

References

1. Evans WH, Martin PE. Gap junctions: Structure and function (Review). *Mol. Membr. Biol.* 2002; 19: 121–136.
2. Peracchia C. Structural correlates of gap junction permeation. *Int. Rev. Cytol.* 1980; 66: 81–146.
3. Peracchia C. Cell coupling. In: Martonosi AN, editor. *Membrane Structure and Dynamics*. New York: Plenum Press. 1985; 81–130.
4. Peracchia C, Lazrak A, Peracchia LL. Molecular models of channel interaction and gating in gap junctions. In: Peracchia C, editor. *Handbook of Membrane Channels. Molecular and Cellular Physiology*. San Diego: Academic Press. 1994; 361–377.
5. Peracchia C. Gap Junctions—Molecular Basis of Cell Communication in Health and Disease. San Diego: Academic Press. 2000; 49.
6. Loewenstein WR. Permeable junctions. *Cold Spring Harbor Symp. Quant. Biol.* 1975; 40: 49–63.
7. Evans WH. Cell communication across gap junctions: A historical perspective and current developments. *Biochem. Soc. Trans.* 2015; 43: 450–459.
8. Srinivas M, Verselis VK, White TW. Human diseases associated with connexin mutations. *Biochim. Biophys. Acta.* 2017; 1860: 192–201.
9. Peracchia C. Gap Junction Structure and Chemical Regulation. Direct Calmodulin Role in Cell-to-Cell Channel

- Gating. London: Academic Press. An imprint of Elsevier. 2019.
10. Peracchia C. Chemical gating of gap junction channels, roles of calcium, pH and calmodulin. *Biochim. Biophys. Acta.* 2004; 1662: 61–80.
 11. Engelmann TW. Vergleichende Untersuchungen zur Lehre von der Muskel und Nerven elektricität. *Pflügers Arch.* 1877; 116-148.
 12. Déléze J. Calcium ions and the healing over of heart fibers. In: Taccardi B, Marchetti C, editors. *Electrophysiology of the Heart.* Oxford: Pergamon Press. 1965; 147–148.
 13. Loewenstein WR, Nakas M, Socolar SJ. Junctional membrane uncoupling. Permeability transformations at a cell membrane junction. *J. Gen. Physiol.* 1967; 50: 1865–1891.
 14. Rose B, Loewenstein WR. Permeability of cell junction depends on local cytoplasmic calcium activity. *Nature.* 1975; 254: 250–252.
 15. Peracchia C. *Handbook of Membrane Channels. Molecular and Cellular Physiology.* San Diego: Academic Press Inc.1994.
 16. Peracchia C, Wang XC, Peracchia LL. Behavior of chemical and slow voltage-gates of connexin channels. The cork gating hypothesis. In: Peracchia C, editor. *Gap Junctions—Molecular Basis of Cell Comunication in Health and Disease.* San Diego: Academic Press. 2000; 271–295.
 17. Loewenstein WR. Junctional intercellular communication: the cell-to-cell membrane channel. *Physiol. Rev.* 1981; 61: 829–913.
 18. Oliveira-Castro GM, Loewenstein WR. Junctional membrane permeability: Effects of divalent cations. *J. Membr. Biol.* 1971; 5: 51–77.
 19. Spray DC, Harris AL, Bennett MV. Gap junctional conductance is a simple and sensitive function of intracellular pH. *Science.* 1981; 211: 712–715.
 20. Rose B, Loewenstein WR. Permeability of a cell junction and the local cytoplasmic free ionized calcium concentration: A study with aequorin. *J. Membr. Biol.* 1976; 28: 87–119.

21. Délèze J, Loewenstein WR. Permeability of a cell junction during intracellular injection of divalent cations. *J. Membr. Biol.* 1976; 28: 71–86.
22. Rose B, Simpson I, Loewenstein WR. Calcium ion produces graded changes in permeability of membrane channels in cell junction. *Nature.* 1977; 267: 625–627.
23. Weingart R. The actions of ouabain on intercellular coupling and conduction velocity in mammalian ventricular muscle. *J. Physiol.* 1977; 264: 341–365.
24. Dahl G, Isenberg G. Decoupling of heart muscle cells: Correlation with increased cytoplasmic calcium activity and with changes of nexus ultrastructure. *J. Membr. Biol.* 1980; 53: 63–75.
25. Noma A, Tsuboi N. Dependence of junctional conductance on proton, calcium and magnesium ions in cardiac paired cells of guinea-pig. *J. Physiol.* 1987; 382: 193–211.
26. Noma A, Tsuboi N. Direct measurement of the gap junctional conductance under the influence of Ca^{2+} in dissociated paired myocytes of guinea-pig. *Jpn. Heart J.* 1986; 27: 161–166.
27. Dekker LR, Fiolet JW, VanBavel E, Coronel R, Opthof T, et al. Intracellular Ca^{2+} , intercellular electrical coupling, and mechanical activity in ischemic rabbit papillary muscle. Effects of preconditioning and metabolic blockade. *Circ. Res.* 1996; 79: 237–246.
28. Peracchia C. Increase in gap junction resistance with acidification in crayfish septate axons is closely related to changes in intracellular calcium but not hydrogen ion concentration. *J. Membr. Biol.* 1990; 113: 75–92.
29. Peracchia C. Effects of caffeine and ryanodine on low pH_i -induced changes in gap junction conductance and calcium concentration in crayfish septate axons. *J. Membr. Biol.* 1990; 117: 79–89.
30. Neyton J, Trautmann A. Single-channel currents of an intercellular junction. *Nature.* 1985; 317: 331–335.
31. Lazrak A, Peracchia C. Gap junction gating sensitivity to physiological internal calcium regardless of pH in Novikoff hepatoma cells. *Biophys. J.* 1993; 65: 2002–2012.
32. Lazrak A, Peres A, Giovannardi S, Peracchia C. Ca-mediated and independent effects of arachidonic acid on gap

- junctions and Ca-independent effects of oleic acid and halothane. *Biophys. J.* 1994; 67: 1052–1059.
33. Enkvist MO, McCarthy KD. Astroglial gap junction communication is increased by treatment with either glutamate or high K⁺ concentration. *J. Neurochem.* 1994; 62: 489–495.
34. Cotrina ML, Kang J, Lin JH, Bueno E, Hansen TW, et al. Astrocytic gap junctions remain open during ischemic conditions. *J. Neurosci.* 1998; 18: 2520–2537.
35. Giaume C, Venance L. Characterization and regulation of gap junction channels in cultured astrocytes. In: Spray DC, Dermietzel R, editors. *Gap Junctions in the Nervous System*. Austin: R.G. Landes Medical Pub. 1996; 135–157.
36. Crow JM, Atkinson MM, Johnson RG. Micromolar levels of intracellular calcium reduce gap junctional permeability in lens cultures. *Invest. Ophthalmol. Vis. Sci.* 1994; 35: 3332–3341.
37. Dakin K, Zhao Y, Li WH. LAMP, a new imaging assay of gap junctional communication unveils that Ca²⁺ influx inhibits cell coupling. *Nat. Methods.* 2005; 2: 55–62.
38. Xu Q, Kopp RF, Chen Y, Yang JJ, Roe MW, et al. Gating of connexin 43 gap junctions by a cytoplasmic loop calmodulin binding domain. *Am. J. Physiol. Cell Physiol.* 2012; 302: C1548–C1556.
39. Mears D, Sheppard NF, Atwater I, Rojas E. Magnitude and modulation of pancreatic beta-cell gap junction electrical conductance in situ. *J. Membr. Biol.* 1995; 146: 163–176.
40. Iwatsuki N, Petersen OH. Membrane potential, resistance, and intercellular communication in the lacrimal gland: Effects of acetylcholine and adrenaline. *J. Physiol.* 1978; 275: 507–520.
41. Iwatsuki N, Petersen OH. Pancreatic acinar cells: Acetylcholine-evoked electrical uncoupling and its ionic dependency. *J. Physiol.* 1978; 274: 81–106.
42. Iwatsuki N, Petersen OH. Electrical coupling and uncoupling of exocrine acinar cells. *J. Cell Biol.* 1978; 79: 533–545.
43. Matthews EK, Petersen OH. Pancreatic acinar cells: Ionic dependence of the membrane potential and acetylcholine-induced depolarization. *J. Physiol.* 1973; 231: 283–295.

44. Scheele GA, Palade GE. Studies on the guinea pig pancreas. Parallel discharge of exocrine enzyme activities. *J. Biol. Chem.* 1975; 250: 2660–2670.
45. Peracchia C. Effects of the anesthetics heptanol, halothane and isoflurane on gap junction conductance in crayfish septate axons: A calcium- and hydrogen-independent phenomenon potentiated by caffeine and theophylline, and inhibited by 4-aminopyridine. *J. Membr. Biol.* 1991; 121: 67–78.
46. Lurtz MM, Louis CF. Calmodulin and protein kinase C regulate gap junctional coupling in lens epithelial cells. *Am. J. Physiol. Cell Physiol.* 2003; 285: C1475–C1482.
47. Turin L, Warner A. Carbon dioxide reversibly abolishes ionic communication between cells of early amphibian embryo. *Nature.* 1977; 270: 56–57.
48. Turin L, Warner AE. Intracellular pH in early *Xenopus* embryos: Its effect on current flow between blastomeres. *J. Physiol.* 1980; 300: 489–504.
49. Rose B, Rick R. Intracellular pH, intracellular free Ca, and junctional cell-cell coupling. *J. Membr. Biol.* 1978; 44: 377–415.
50. Giaume C, Spira ME, Korn H. Uncoupling of invertebrate electrotonic synapses by carbon dioxide. *Neurosci. Lett.* 1980; 17: 197–202.
51. Giaume C, Korn H. Ammonium sulfate induced uncouplings of crayfish septate axons with and without increased junctional resistance. *Neuroscience.* 1982; 7: 1723–1730.
52. Schuetze SM, Goodenough DA. Dye transfer between cells of the embryonic chick lens becomes less sensitive to CO₂ treatment with development. *J. Cell Biol.* 1982; 92: 694–705.
53. Reber WR, Weingart R. Ungulate cardiac purkinje fibres: The influence of intracellular pH on the electrical cell-to-cell coupling. *J. Physiol.* 1982; 328: 87–104.
54. Gonzalez-Nieto D, Gomez-Hernandez JM, Larrosa B, Gutierrez C, Munoz MD, et al. Regulation of neuronal connexin-36 channels by pH. *Proc. Natl. Acad. Sci. USA.* 2008; 105: 17169–17174.

55. Wang X, Li L, Peracchia LL, Peracchia C. Chimeric evidence for a role of the connexin cytoplasmic loop in gap junction channel gating. *Pflugers Arch.* 1996; 431: 844–852.
56. Liu S, Taffet S, Stoner L, Delmar M, Vallano ML, et al. A structural basis for the unequal sensitivity of the major cardiac and liver gap junctions to intracellular acidification: The carboxyl tail length. *Biophys. J.* 1993; 64: 1422–1433.
57. Morley GE, Taffet SM, Delmar M. Intramolecular interactions mediate pH regulation of connexin43 channels. *Biophys. J.* 1996; 70: 1294–1302.
58. Stergiopoulos K, Alvarado JL, Mastroianni M, Ek-Vitorin JF, Taffet SM, et al. Hetero-domain interactions as a mechanism for the regulation of connexin channels. *Circ. Res.* 1999; 84: 1144–1155.
59. Peracchia C. Possible involvement of caffeine- and ryanodine-sensitive calcium stores in low pH-induced regulation of gap junction channels. In: Peracchia C, editor. *Biophysics of Gap Junction Channels*. Boca Raton: CRC Press. 1990; 13–28.
60. Bennett MVL, Verselis VK, White RL, Spray DC. Gap junction conductance: Gating. In: Hertzberg E, Johnson RG, Alan R, editors. *Gap Junctions*. New York: Liss. 1988; 207–304.
61. Campos de Carvalho A, Spray DC, Bennett MV. pH dependence of transmission at electrotonic synapses of the crayfish septate axon. *Brain Res.* 1984; 321: 279–286.
62. Pressler ML. Intracellular pH and cell-to-cell transmission in sheep Purkinje fibers. *Biophys. J.* 1989; 55: 53–65.
63. De Mello WC. The influence of pH on the healing-over of mammalian cardiac muscle. *J. Physiol.* 1983; 339: 299–307.
64. Nicholson BJ, Zhou L, Cao F, Zhou H, Chen Y. Diverse molecular mechanisms of gap junction channel gating. In: Werner R, editor. *Gap Junctions*. Amsterdam: IOS Press. 1998; 3–8.
65. Johnston MF, Ramon F. Electrotonic coupling in internally perfused crayfish segmented axons. *J. Physiol.* 1981; 317: 509–518.
66. Arellano RO, Ramon F, Rivera A, Zampighi GA. Lowering of pH does not directly affect the junctional resistance of crayfish lateral axons. *J. Membr. Biol.* 1986; 94: 293–299.

67. Bevens CG, Harris AL. Regulation of connexin channels by pH. Direct action of the protonated form of taurine and other aminosulfonates. *J. Biol. Chem.* 1999; 274: 3711–3719.
68. Dunina-Barkowskaia AY, Bujurina IM, Pivovarov VS, Frolov VA. Effects of transmembrane gradients of bicarbonate and ammonium on junctional and non-junctional conductances in bhk cells. *Biol. Membr.* 2000; 17: 637–652.
69. Trexler EB, Bukauskas FF, Bennett MV, Bargiello TA, Verselis VK. Rapid and direct effects of pH on connexins revealed by the connexin46 hemichannel preparation. *J. Gen. Physiol.* 1999; 113: 721–742.
70. Sanchez HA, Bienkowski R, Slavi N, Srinivas M, Verselis VK. Altered inhibition of Cx26 hemichannels by pH and Zn^{2+} in the A40V mutation associated with keratitis-ichthyosis-deafness syndrome. *J. Biol. Chem.* 2014; 289: 21519–21532.
71. Locke D, Kieken F, Tao L, Sorgen PL, Harris AL. Mechanism for modulation of gating of connexin26-containing channels by taurine. *J. Gen. Physiol.* 2011; 138: 321–339.
72. Tao L, Harris AL. Biochemical requirements for inhibition of Connexin26-containing channels by natural and synthetic taurine analogs. *J. Biol. Chem.* 2004; 279: 38544–38554.
73. Peracchia C, Wang X, Li L, Peracchia LL. Inhibition of calmodulin expression prevents low-pH-induced gap junction uncoupling in *Xenopus* oocytes. *Pflugers Arch.* 1996; 431: 379–387.
74. Wang XG, Peracchia C. Positive charges of the initial C-terminus domain of Cx32 inhibit gap junction gating sensitivity to CO_2 . *Biophys. J.* 1997; 73: 798–806.
75. Gunter TE, Gunter KK, Sheu SS, Gavin CE. Mitochondrial calcium transport: physiological and pathological relevance. *Am. J. Physiol.* 1994; 267: C313–C339.
76. Peracchia C, Bernardini G, Peracchia LL. Is calmodulin involved in the regulation of gap junction permeability? *Pflugers Arch.* 1983; 399: 152–154.
77. Peracchia C, Bernardini G, Peracchia LL. A calmodulin inhibitor prevents gap junction crystallization and electrical uncoupling. *J. Cell Biol.* 1981; 91: 124a.

78. Hertzberg EL, Gilula NB. Liver gap junctions and lens fiber junctions: Comparative analysis and calmodulin interaction. Cold Spring Harbor Symp. Quant. Biol. 1981; 46: 639–645.
79. Van Eldik LJ, Hertzberg EL, Berdan RC, Gilula NB. Interaction of calmodulin and other calcium-modulated proteins with mammalian and arthropod junctional membrane proteins. Biochem. Biophys. Res. Commun. 1985; 126: 825–832.
80. Zou J, Salarian M, Chen Y, Veenstra R, Louis CF, et al. Gap junction regulation by calmodulin. FEBS Lett. 2014; 588: 1430–1438.
81. Saimi Y, Kung C. Calmodulin as an ion channel subunit. Annu. Rev. Physiol. 2002; 64: 289–311.
82. Kovalevskaya NV, van de Waterbeemd M, Bokhovchuk FM, Bate N, Bindels RJ, et al. Structural analysis of calmodulin binding to ion channels demonstrates the role of its plasticity in regulation. Pflugers Arch. 2013; 465: 1507–1519.
83. Adelman JP. SK channels and calmodulin. Channels. 2016; 10: 1–6.
84. Gabelli SB, Yoder JB, Tomaselli GF, Amzel LM. Calmodulin and Ca^{2+} control of voltage gated Na^+ channels. Channels. 2016; 10: 45–54.
85. Peracchia C, Girsch SJ. Permeability and gating of lens gap junction channels incorporated into liposomes. Curr. Eye Res. 1985; 4: 431–439.
86. Peracchia C, Girsch SJ. Calmodulin site at the C-terminus of the putative lens gap junction protein MIP26. Lens Eye Toxic. Res. 1989; 6: 613–621.
87. Girsch SJ, Peracchia C. Lens cell-to-cell channel protein: I. Self-assembly into liposomes and permeability regulation by calmodulin. J. Membr. Biol. 1985; 83: 217–225.
88. Nemeth-Cahalan KL, Hall JE. pH and calcium regulate the water permeability of aquaporin 0. J. Biol. Chem. 2000; 275: 6777–6782.
89. Nemeth-Cahalan KL, Kalman K, Hall JE. Molecular basis of pH and Ca^{2+} regulation of aquaporin water permeability. J. Gen. Physiol. 2004; 123: 573–580.
90. Reichow SL, Clemens DM, Freites JA, Nemeth-Cahalan KL, Heyden M, et al. Allosteric mechanism of water-channel

- gating by Ca^{2+} -calmodulin. *Nat. Struct. Mol. Biol.* 2013; 20: 1085–1092.
91. Reichow SL, Gonen T. Noncanonical binding of calmodulin to aquaporin-0: Implications for channel regulation. *Structure*. 2008; 16: 1389–1398.
92. Fields JB, Nemeth-Cahalan KL, Freites JA, Vorontsova I, Hall JE, et al. Calmodulin Gates Aquaporin 0 Permeability through a Positively Charged Cytoplasmic Loop. *J. Biol. Chem.* 2016; 292: 185–195.
93. Peracchia C. Communicating junctions and calmodulin: Inhibition of electrical uncoupling in *Xenopus* embryo by calmidazolium. *J. Membr. Biol.* 1984; 81: 49–58.
94. Peracchia C. Calmodulin-like proteins and communicating junctions. Electrical uncoupling of crayfish septate axons is inhibited by the calmodulin inhibitor W7 and is not affected by cyclic nucleotides. *Pflugers Arch.* 1987; 408: 379–385.
95. Wojtczak JA. Electrical uncoupling induced by general anesthetics: A calcium-independent process? In: Bennett MVL, Spray DC, editors. *Gap Junctions*. Cold Spring Harbor: Cold Spring Harbor Laboratory. 1985; 167–175.
96. Tuganowski W, Korczynska I, Wasik K, Piatek G. Effects of calmidazolium and dibutyryl cyclic AMP on the longitudinal internal resistance in sinus node strips. *Pflugers Arch.* 1989; 414: 351–353.
97. Gandolfi SA, Duncan G, Tomlinson J, Maraini G. Mammalian lens inter-fiber resistance is modulated by calcium and calmodulin. *Curr. Eye Res.* 1990; 9: 533–541.
98. Toyama J, Sugiura H, Kamiya K, Kodama I, Terasawa M, et al. Ca^{2+} -calmodulin mediated modulation of the electrical coupling of ventricular myocytes isolated from guinea pig heart. *J. Mol. Cell Cardiol.* 1994; 26: 1007–1015.
99. Verselis V, Campos de Carvalho A, White RL, Bennett MVL. Calmodulin and gap junction regulation. *J. Cell Biol.* 1986; 91: 73a.
100. Lees-Miller JP, Caveney S. Drugs that block calmodulin activity inhibit cell-to-cell coupling in the epidermis of *Tenebrio molitor*. *J. Membr. Biol.* 1982; 69: 233–245.
101. Blodow A, Ngezahayo A, Ernst A, Kolb HA. Calmodulin antagonists suppress gap junction coupling in isolated

- Hensen cells of the guinea pig cochlea. *Pflugers Arch.* 2003; 446: 36–41.
102. Zhao HB, Yu N. Distinct and gradient distributions of connexin26 and connexin30 in the cochlear sensory epithelium of guinea pigs. *J. Comp. Neurol.* 2006; 499: 506–518.
 103. De Vuyst E, Wang N, Decrock E, De BM, Vinken M, et al. Ca^{2+} regulation of connexin 43 hemichannels in C6 glioma and glial cells. *Cell Calcium.* 2009; 46: 176–187.
 104. De Vuyst E, Decrock E, Cabooter L, Dubyak GR, Naus CC, et al. Intracellular calcium changes trigger connexin 32 hemichannel opening. *EMBO J.* 2006; 25: 34–44.
 105. Peracchia C, Wang XG, Peracchia LL. Slow gating of gap junction channels and calmodulin. *J. Membr. Biol.* 2000; 178: 55–70.
 106. Peracchia C, Young KC, Wang XG, Peracchia LL. Is the voltage gate of connexins CO_2 -sensitive? Cx45 channels and inhibition of calmodulin expression. *J. Membr. Biol.* 2003; 195: 53–62.
 107. Peracchia C, Sotkis A, Wang XG, Peracchia LL, Persechini A. Calmodulin directly gates gap junction channels. *J. Biol. Chem.* 2000; 275: 26220–26224.
 108. Sotkis A, Wang XG, Yasumura T, Peracchia LL, Persechini A, et al. Calmodulin colocalizes with connexins and plays a direct role in gap junction channel gating. *Cell Commun. Adhes.* 2001; 8: 277–281.
 109. Persechini A, Gansz KJ, Paresi RJ. Activation of myosin light chain kinase and nitric oxide synthase activities by engineered calmodulins with duplicated or exchanged EF hand pairs. *Biochemistry.* 1996; 35: 224–228.
 110. Geiser JR, van TD, Brockerhoff SE, Neff MM, Davis TN. Can calmodulin function without binding calcium? *Cell.* 1991; 65: 949–959.
 111. Kerruth S, Coates C, Rezavi SA, Peracchia C, Torok K. Calmodulin interaction with gap junction intracellular loop peptides. *Biophys. J.* 2018; 114: 468a.
 112. Kerruth S, Coates C, Rezavi SA, Peracchia C, Torok K. Ca^{2+} -dependent and -independent interaction of calmodulin with gap junction cytoplasmic loop peptides. *Biochem. J.* 2020, submitted.

113. Ahmad S, Martin PE, Evans WH. Assembly of gap junction channels: Mechanism, effects of calmodulin antagonists and identification of connexin oligomerization determinants. *Eur. J. Biochem.* 2001; 268: 4544–4552.
114. Siu RC, Smirnova E, Brown CA, Zoidl C, Spray DC, et al. Structural and functional consequences of connexin 36 (Cx36) interaction with calmodulin. *Front. Mol. Neurosci.* 2016; 9: 120.
115. Peracchia C, Salim M, Peracchia LL. Unusual slow gating of gap junction channels in oocytes expressing connexin32 or its COOH-terminus truncated mutant. *J. Membr. Biol.* 2007; 215: 161–168.
116. Wang XG, Peracchia C. Molecular dissection of a basic COOH-terminal domain of Cx32 that inhibits gap junction gating sensitivity. *Am. J. Physiol.* 1998; 275: C1384–C1390.
117. Peracchia C, Chen JT, Peracchia LL. CO₂ sensitivity of voltage gating and gating polarity of gap junction channels--connexin40 and its COOH-terminus-truncated mutant. *J. Membr. Biol.* 2004; 200: 105–113.
118. Saez JC, Nairn AC, Czernik AJ, Spray DC, Hertzberg EL, et al. Phosphorylation of connexin 32, a hepatocyte gap-junction protein, by cAMP-dependent protein kinase, protein kinase C and Ca²⁺/calmodulin-dependent protein kinase II. *Eur. J. Biochem.* 1990; 192: 263–273.
119. De Pina-Benabou MH, Srinivas M, Spray DC, Scemes E. Calmodulin kinase pathway mediates the K⁺-induced increase in Gap junctional communication between mouse spinal cord astrocytes. *J. Neurosci.* 2001; 21: 6635–6643.
120. Pereda AE, Bell TD, Chang BH, Czernik AJ, Nairn AC, et al. Ca²⁺/calmodulin-dependent kinase II mediates simultaneous enhancement of gap-junctional conductance and glutamatergic transmission. *Proc. Natl. Acad. Sci. USA.* 1998; 95: 13272–13277.
121. Laird DW, Puranam KL, Revel JP. Turnover and phosphorylation dynamics of connexin43 gap junction protein in cultured cardiac myocytes. *Biochem. J.* 1991; 273: 67–72.
122. Lurtz MM, Louis CF. Intracellular calcium regulation of connexin43. *Am. J. Physiol. Cell Physiol.* 2007; 293: C1806–C1813.

123. Wei S, Cassara C, Lin X, Veenstra RD. Calcium-calmodulin gating of a pH-insensitive isoform of connexin43 gap junctions. *Biochem. J.* 2019; 476: 1137–1148.
124. Zhang X, Qi Y. Role of intramolecular interaction in connexin50: mediating the Ca^{2+} -dependent binding of calmodulin to gap junction. *Arch. Biochem. Biophys.* 2005; 440: 111–117.
125. Zhang X, Zou T, Liu Y, Qi Y. The gating effect of calmodulin and calcium on the connexin50 hemichannel. *Biol. Chem.* 2006; 387: 595–601.
126. Fujimoto K, Araki N, Ogawa KS, Kondo S, Kitaoka T, et al. Ultracytochemistry of calmodulin binding sites in myocardial cells by staining of frozen thin sections with colloidal gold-labeled calmodulin. *J. Histochem. Cytochem.* 1989; 37: 249–256.
127. Zou J, Salarian M, Chen Y, Zhuo Y, Brown NE, et al. Direct Visualization of Interaction between Calmodulin and Connexin45. *Biochem. J.* 2017; 474: 4035–4051.
128. Wang XG, Peracchia C. Connexin 32/38 chimeras suggest a role for the second half of inner loop in gap junction gating by low pH. *Am. J. Physiol.* 1996; 271: C1743–C1749.
129. Wang XG, Peracchia C. Chemical gating of heteromeric and heterotypic gap junction channels. *J. Membr. Biol.* 1998; 162: 169–176.
130. Peracchia C, Wang XG. Connexin domains relevant to the chemical gating of gap junction channels. *Braz. J. Med. Biol. Res.* 1997; 30: 577–590.
131. Peracchia C. The calmodulin hypothesis for gap junction regulation six years later. In: Hertzberg E, Johnson RG, Alan R, editors. *Gap Junctions*. New York: Liss. 1988; 267–282.
132. Torok K, Stauffer K, Evans WH. Connexin 32 of gap junctions contains two cytoplasmic calmodulin-binding domains. *Biochem. J.* 1997; 326: 479–483.
133. Dodd R, Peracchia C, Stolady D, Torok K. Calmodulin association with connexin32-derived peptides suggests trans-domain interaction in chemical gating of gap junction channels. *J. Biol. Chem.* 2008; 283: 26911–26920.
134. Werner R, Levine E, Rabadan-Diehl C, Dahl G. Gating properties of connexin32 cell-cell channels and their mutants

- expressed in *Xenopus* oocytes. *Proc. R. Soc. London B. Biol. Sci.* 1991; 243: 5–11.
135. Peracchia C, Wang XG, Peracchia LL. Is the chemical gate of connexins voltage sensitive? Behavior of Cx32 wild-type and mutant channels. *Am. J. Physiol.* 1999; 276: C1361–C1373.
136. Katoch P, Mitra S, Ray A, Kelsey L, Roberts BJ, et al. The carboxyl tail of connexin32 regulates gap junction assembly in human prostate and pancreatic cancer cells. *J. Biol. Chem.* 2015; 290: 4647–4662.
137. Zimmer DB, Green CR, Evans WH, Gilula NB. Topological analysis of the major protein in isolated intact rat liver gap junctions and gap junction-derived single membrane structures. *J. Biol. Chem.* 1987; 262: 7751–7763.
138. Elvira M, Villalobo A. Calmodulin prevents the proteolysis of connexin32 by calpain. *Bioelectrochem. Bioenerg.* 1997; 42: 207–211.
139. Diez JA, Elvira M, Villalobo A. The epidermal growth factor receptor tyrosine kinase phosphorylates connexin32. *Mol. Cell. Biochem.* 1998; 187: 201–210.
140. Torok K, Trentham DR. Mechanism of 2-chloro-(epsilon-amino-Lys75)-[6-[4-(N,N-diethylamino)phenyl]-1,3,5-triazin-4-yl]calmodulin interactions with smooth muscle myosin light chain kinase and derived peptides. *Biochemistry.* 1994; 33: 12807–12820.
141. Stauch K, Kieken F, Sorgen P. Characterization of the structure and intermolecular interactions between the connexin 32 carboxyl-terminal domain and the protein partners synapse-associated protein 97 and calmodulin. *J. Biol. Chem.* 2012; 287: 27771–27788.
142. Sorgen PL, Trease AJ, Spagnol G, Delmar M, Nielsen MS. Protein-Protein Interactions with Connexin 43: Regulation and Function. *Int. J. Mol. Sci.* 2018; 19: 1428–1449.
143. Burr GS, Mitchell CK, Keflemariam YJ, Heidelberger R, O'Brien J. Calcium-dependent binding of calmodulin to neuronal gap junction proteins. *Biochem. Biophys. Res. Commun.* 2005; 335: 1191–1198.
144. Li S, Hao B, Lu Y, Yu P, Lee HC, et al. Intracellular alkalinization induces cytosolic Ca^{2+} increases by inhibiting

- sarco/endoplasmic reticulum Ca^{2+} -ATPase (SERCA). PLoS ONE. 2012; 7: e31905.
145. Zhou Y, Yang W, Lurtz MM, Ye Y, Huang Y, et al. Identification of the calmodulin binding domain of connexin 43. *J. Biol. Chem.* 2007; 282: 35005–35017.
 146. Chen Y, Zhou Y, Lin X, Wong HC, Xu Q, et al. Molecular interaction and functional regulation of connexin50 gap junctions by calmodulin. *Biochem. J.* 2011; 435: 711–722.
 147. Zhou Y, Yang W, Lurtz MM, Chen Y, Jiang J, et al. Calmodulin mediates the Ca^{2+} -dependent regulation of Cx44 gap junctions. *Biophys. J.* 2009; 96: 2832–2848.
 148. Myllykoski M, Kuczera K, Kursula P. Complex formation between calmodulin and a peptide from the intracellular loop of the gap junction protein connexin43: Molecular conformation and energetics of binding. *Biophys. Chem.* 2009; 144: 130–135.
 149. Bukauskas FF, Angele AB, Verselis VK, Bennett MV. Coupling asymmetry of heterotypic connexin 45/ connexin 43-EGFP gap junctions: Properties of fast and slow gating mechanisms. *Proc. Natl. Acad. Sci. USA.* 2002; 99: 7113–7118.
 150. Li H, Liu TF, Lazrak A, Peracchia C, Goldberg GS, et al. Properties and regulation of gap junctional hemichannels in the plasma membranes of cultured cells. *J. Cell Biol.* 1996; 134: 1019–1030.
 151. Gomez-Hernandez JM, MdM, Larrosa B, Gonzalez D, Barrio LC. Molecular basis of calcium regulation in connexin-32 hemichannels. *Proc. Natl. Acad. Sci. USA.* 2003; 100: 16030–16035.
 152. Fasciani I, Temperan A, Perez-Atencio LF, Escudero A, Martinez-Montero P, et al. Regulation of connexin hemichannel activity by membrane potential and the extracellular calcium in health and disease. *Neuropharmacology.* 2013; 75: 479–490.
 153. Carrer A, Leparulo A, Crispino G, Ciubotaru CD, Marin O, et al. Cx32 hemichannel opening by cytosolic Ca^{2+} is inhibited by the R220X mutation that causes Charcot-Marie-Tooth disease. *Hum. Mol. Genet.* 2018; 27: 80–94.

154. Ponsaerts R, Wang N, Himpens B, Leybaert L, Bultynck G. The contractile system as a negative regulator of the connexin 43 hemichannel. *Biol. Cell.* 2012; 104: 367–377.
155. Castro C, Gomez-Hernandez JM, Silander K, Barrio LC. Altered formation of hemichannels and gap junction channels caused by C-terminal connexin-32 mutations. *J. Neurosci.* 1999; 19: 3752–3760.
156. Hu Z, Riquelme MA, Wang B, Bugay V, Brenner R, et al. Cataract-associated Connexin 46 Mutation Alters Its Interaction with Calmodulin and Function of Hemichannels. *J. Biol. Chem.* 2018; 293: 2573–2585.
157. Ren Q, Riquelme MA, Xu J, Yan X, Nicholson BJ, et al. Cataract-causing mutation of human connexin 46 impairs gap junction, but increases hemichannel function and cell death. *PLoS ONE.* 2013; 8: e74732.
158. Garcia IE, Villanelo F, Contreras GF, Pupo A, Pinto BI, et al. The syndromic deafness mutation G12R impairs fast and slow gating in Cx26 hemichannels. *J. Gen. Physiol.* 2018; 150: 697–711.
159. Chatterjee P, Garcia I, Harris AL, Luo Y, Contreras JE. Insights of gating functions of cytosolic domains of connexin 26 hemichannels revealed by a human pathogenic mutation (N14K). *Biophys. J.* 2018; 114.
160. Bukauskas FF, Verselis VK. Gap junction channel gating. *Biochim. Biophys. Acta.* 2004; 1662: 42–60.
161. Bukauskas FF, Peracchia C. Two distinct gating mechanisms in gap junction channels: CO₂-sensitive and voltage-sensitive. *Biophys. J.* 1997; 72: 2137–2142.
162. Bukauskas FF, Weingart R. Voltage-dependent gating of single gap junction channels in an insect cell line. *Biophys. J.* 1994; 67: 613–625.
163. Bukauskas FF, Elfgang C, Willecke K, Weingart R. Biophysical properties of gap junction channels formed by mouse connexin40 in induced pairs of transfected human HeLa cells. *Biophys. J.* 1995; 68: 2289–2298.
164. Verselis VK, Ginter CS, Bargiello TA. Opposite voltage gating polarities of two closely related connexins. *Nature.* 1994; 368: 348–351.

165. Revilla A, Castro C, Barrio LC. Molecular dissection of transjunctional voltage dependence in the connexin-32 and connexin-43 junctions. *Biophys. J.* 1999; 77: 1374–1383.
166. Moreno AP, Chanson M, Elenes S, Anumonwo J, Scerri I, et al. Role of the carboxyl terminal of connexin43 in transjunctional fast voltage gating. *Circ. Res.* 2002; 90: 450–457.
167. Anumonwo JM, Taffet SM, Gu H, Chanson M, Moreno AP, et al. The carboxyl terminal domain regulates the unitary conductance and voltage dependence of connexin40 gap junction channels. *Circ. Res.* 2001; 88: 666–673.
168. Oh S, Ri Y, Bennett MV, Trexler EB, Verselis VK, et al. Changes in permeability caused by connexin 32 mutations underlie X-linked Charcot-Marie-Tooth disease. *Neuron.* 1997; 19: 927–938.
169. Zampighi G, Unwin PN. Two forms of isolated gap junctions. *J. Mol. Biol.* 1979; 135: 451–464.
170. Unwin PN, Zampighi G. Structure of the junction between communicating cells. *Nature.* 1980; 283: 545–549.
171. Unwin PN, Ennis PD. Two configurations of a channel-forming membrane protein. *Nature.* 1984; 307: 609–613.
172. Delmar M, Stergiopoulos K, Homma N, Calero G, Morley G, et al. A molecular model for the chemical regulation of the connexin43 channels: the ball-and-chain hypothesis. In: Peracchia C, editor. *Gap Junctions. Molecular Basis of Cell Communication in Health and Disease.* San Diego: Academic Press. 2000; 223–248.
173. Delmar M, Stergiopoulos K, Homma N, Ek-Vitorin JF, Taffet SM. A ball-and-chain model for chemical regulation of connexin 43. In: Werner R, editor. *Gap Junctions.* Amsterdam: IOS Press. 1998; 8–12.
174. Bennett BC, Purdy MD, Baker KA, Acharya C, McIntire WE, et al. An electrostatic mechanism for Ca^{2+} -mediated regulation of gap junction channels. *Nat. Commun.* 2016; 7: 8770.
175. Ek Vitorin JF, Pontifex TK, Burt JM. Determinants of Cx43 Channel Gating and Permeation: The Amino Terminus. *Biophys. J.* 2016; 110: 127–140.

176. Babu YS, Bugg CE, Cook WJ. Structure of calmodulin refined at 2.2 Å resolution. *J. Mol. Biol.* 1988; 204: 191–204.
177. Fleishman SJ, Unger VM, Yeager M, Ben-Tal N. A Calpha model for the transmembrane alpha helices of gap junction intercellular channels. *Mol. Cell.* 2004; 15: 879–888.
178. Perkins G, Goodenough D, Sosinsky G. Three-dimensional structure of the gap junction connexon. *Biophys. J.* 1997; 72: 533–544.
179. Maeda S, Nakagawa S, Suga M, Yamashita E, Oshima A, et al. Structure of the connexin 26 gap junction channel at 3.5 Å resolution. *Nature.* 2009; 458: 597–602.
180. Astegno A, La VV, Marino V, Dell'Orco D, Dominici P. Biochemical and biophysical characterization of a plant calmodulin: Role of the N- and C-lobes in calcium binding, conformational change, and target interaction. *Biochim. Biophys. Acta.* 2016; 1864: 297–307.
181. Peracchia C, Dulhunty AF. Low resistance junctions in crayfish. Structural changes with functional uncoupling. *J. Cell Biol.* 1976; 70: 419–439.
182. Peracchia C. Gap junctions. Structural changes after uncoupling procedures. *J. Cell Biol.* 1977; 72: 628–641.
183. Peracchia C. Calcium effects on gap junction structure and cell coupling. *Nature.* 1978; 271: 669–671.
184. Peracchia C, Peracchia LL. Gap junction dynamics: Reversible effects of divalent cations. *J. Cell Biol.* 1980; 87: 708–718.
185. Peracchia C, Bernardini G. Gap junction structure and cell-to-cell coupling regulation: Is there a calmodulin involvement? *Fed. Proc.* 1984; 43: 2681–2691.
186. Raviola E, Goodenough DA, Raviola G. Structure of rapidly frozen gap junctions. *J. Cell Biol.* 1980; 87: 273–279.
187. Peracchia C. Low resistance junctions in crayfish. II. Structural details and further evidence for intercellular channels by freeze-fracture and negative staining. *J. Cell Biol.* 1973; 57: 54–65.
188. Black DJ, Tran QK, Persechini A. Monitoring the total available calmodulin concentration in intact cells over the physiological range in free Ca^{2+} . *Cell Calcium.* 2004; 35: 415–425.

189. Wu X, Bers DM. Free and bound intracellular calmodulin measurements in cardiac myocytes. *Cell Calcium*. 2007; 41: 353–364.