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Connexin domains relevant to the chemical gating of gap junction channels

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Abstract

Most cells exchange ions and small metabolites via gap junction channels. These channels are made of two hemichannels (connexons), each formed by the radial arrangement of six connexin (Cx) proteins. Connexins span the bilayer four times (M1-M4) and have both amino- and carboxy-termini (NT, CT) at the cytoplasmic side of the membrane, forming two extracellular loops (E1, E2) and one inner (IL) loop. The channels are regulated by gates that close with cytosolic acidification (e.g., CO_2 treatment) or increased calcium concentration, possibly via calmodulin activation. Although gap junction regulation is still unclear, connexin domains involved in gating are being defined. We have recently focused on the CO_2 gating sensitivity of Cx32, Cx38 and various mutants and chimeras expressed in *Xenopus* oocytes and studied by double voltage clamp. Cx32 is weakly sensitive to CO_2, whereas Cx38 is highly sensitive. A Cx32 chimera containing the second half of the inner loop (IL_2) of Cx38 was as sensitive to CO_2 as Cx38, indicating that this domain plays an important role. Deletion of CT by 84% did not affect CO_2 sensitivity, but replacement of 5 arginines (R) with asparagines (N) at the beginning of CT (C_1) greatly enhanced the CO_2 sensitivity of Cx32. This suggests that whereas most of CT is irrelevant, positive charges of C_1 maintain the CO_2 sensitivity of Cx32 low. As a hypothesis we have proposed a model that involves charge interaction between negative residues of the beginning of IL (IL_1) and positive residues of either C_1 or IL_2. Open and closed channels would result from IL_1-C_1 and IL_1-IL_2 interactions, respectively.

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Introduction

In most tissues, cells in contact with each other exchange charged and neutral cytosolic molecules lower than 1 kDa in molecular mass. This exchange involves ions as well as small metabolites such as amino acids, nucleotides, second messengers and high-energy compounds, and enables electrical and metabolic signals to spread widely among cell populations (reviewed in Ref. 1). This form of direct cell-to-cell communication (cell coupling) provides an important mechanism for coordinating and regulating a host of cellular activities in mature and developing organs. Conversely, abnormal cell-to-cell communication is believed to play a role in the pathogenesis of diseases such as cardiac arrhythmias and uterine malfunction at birth (2), X-linked Charcot-Marie-Tooth demyelinating disease (3-6), cardiac malformation and defects of laterality (7), epileptic sei-

zures (8), spreading depression (9), Chagas' disease (10), among others.

Cell coupling is mediated by channels clustered at cell-cell contact domains known as gap junctions. Each channel is formed by the extracellular interaction of two hemichannels (connexons), and creates a hydrophilic pathway that spans the two apposed plasma membranes and a narrow extracellular space (gap). In turn, each connexon is an oligomer formed by the radial arrangement of six identical proteins (connexins) that span the membrane thickness and insulate the hydrophilic pore from the lipid bilayer and the extracellular medium (reviewed in Ref. 11).

In recent years, structural studies have generated a portrait of gap junction architecture, channel framework and connexin topology. Studies on dye diffusion and metabolite exchange have defined the nature, size limit and charge characteristics of channel permeants, and the extent of metabolic cooperation among cells. Biochemistry and molecular genetics have provided the means for identifying connexin sequences and for mapping secondary and tertiary structure. Electrophysiology, particularly double whole-cell patch clamp recording, the development of reliable channel expression systems, and channel reconstitution in artificial membranes have paved the way for defining single channel attributes such as conductance, gating kinetics, voltage dependence, subconductance and residual conductance states, etc., and for clarifying mechanisms of channel regulation and modulation (reviewed in Ref. 12). Nonetheless, crucial aspects of channel structure and regulation are still hypothetical.

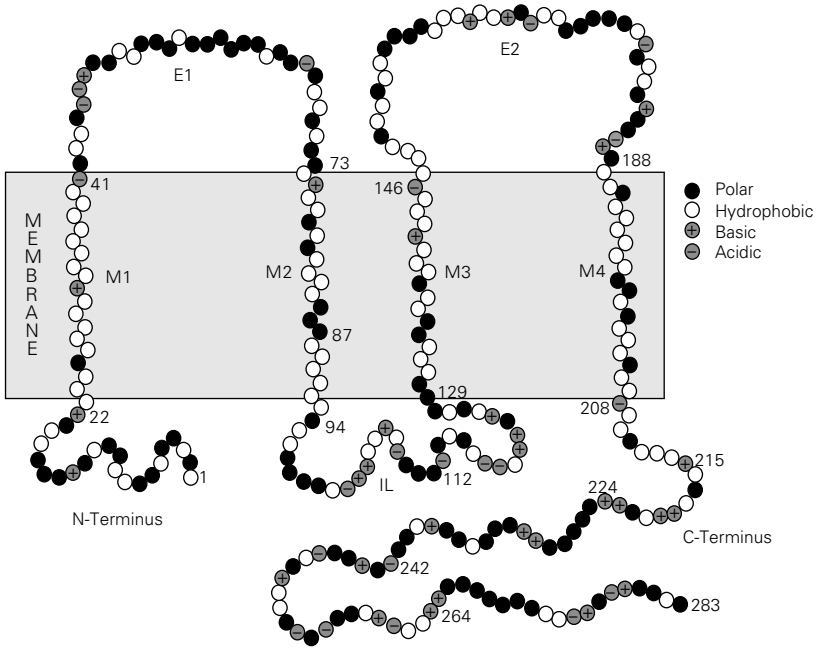


Figure 1 - Model of connexin (Cx32) topology. The molecule is believed to span the bilayer four times (M1-M4) and to have both N- and C-termini (NT, CT) at the cytoplasmic side of the membrane, forming two extracellular loops (E1, E2) and one inner loop (IL). Two connexin regions are conserved: one spans approximately the first 100 residues, comprising NT, E1, M1, M2 and the beginning of IL; the other contains M3, M4, E2 and the beginning of CT. The two remaining regions, most of IL and CT, vary in sequence and length.

In the absence of high resolution crystallographic information, the three-dimensional structure of connexins can only be guessed, and still unclear are the parameters that determine connexin-connexin interaction within and across junctional membranes, the structure of the channel lining, the molecular domains and the mechanisms involved in channel regulation and gating, the functional meaning of connexin diversity and differential expression, the physiological consequences of heterotypic junction formation (junctions between cells expressing different connexins), the reason for multiple connexin expression in the same cell, etc.

During the last decade at least 16 members of the connexin family have been cloned (reviewed in Ref. 12). Sequence analyses and studies using site-specific antibodies or selective proteolysis have defined connexin topology (13-22). Connexins span the bilayer four times (M1-M4) and have both amino- and carboxy-termini (NT, CT) at the cytoplasmic side of the membrane, forming two extracellular loops (E1, E2) and one inner loop (IL) (Figure 1). Two connexin regions are conserved: one spans approximately the first 100 residues, comprising NT (~23 residues), E1 (~35 residues), both M1 and M2 (~18 residues each), and the beginning of IL; the other contains M3 (18-20

residues), M4 (~20 residues), E2 (44-48 residues), and the beginning of CT. The two remaining regions, most of IL and CT, vary in sequence and length. IL ranges from less than 30 residues (Cx31.1) to over 70 residues (Cx45), and CT from 18 residues (Cx26) to 188 residues (Cx56). M3 is believed to provide the channel lining structure, as it is the most amphiphilic of the four transmembrane domains.

It is still unclear how connexins interact with each other within the membrane and across the gap. Recently, we have proposed a model that envisions a staggered (one-to-two) interaction between opposite connexins (12) (Figure 2). This model is based on the idea that each junctional membrane is unlikely to be a mirror-symmetrical image of the other, because connexins are believed to be identical rather than mirror-symmetrical images of their counterparts in a gap junction. Thus, if M3 lines the channel and both E1 and E2 interact with homologous domains across the gap, a likely model would place E1 and E2 in a radial arrangement around the channel with their axes at ~30° angle from each other (Figure 2). In this model, opposite connexins would not bind one-to-one but rather would be staggered with each other, such that each connexin of one membrane would interact with two connexins of the adjoined membrane. Indeed, a staggered (one-to-two) connexin interaction would provide a stronger junction than a matched (one-to-one) arrangement. There are two possible configurations of the staggered model: in one, both E1 and E2 would have the same N-to-C sequence orientation (Figure 2), centrifugal with respect to the channel, whereas in the other, only E2 would have this orientation (12).

Role of calcium and pH in gap junction channel gating

Functional gap junction channels are mostly in an open state, but can close in response to certain changes in the ionic composition of the cytosol. As a result of channel closure neighboring cells uncouple from each other electrically and metabolically. Although cell uncoupling is generally believed to be just a protective all-or-nothing mechanism, recent evidence for channel permeability regulation by nearly physiological changes in $[Ca^{2+}]_i$ (23-28) indicates that a fine modulation of cell communication may play a role in normal cellular functions. For understanding how cell communication is modulated physiologically and how cell coupling regulation is linked to specific cellular activities we need to define the nature of the uncoupling agents and the molecular basis of channel gating. The latter can only be defined once we fully understand which connexin domains participate in the gating mechanism.

Over the years a large body of evidence has emphasized the role of cytosolic calcium and hydrogen ions in cell coupling regulation. Evidence for gap junction channel sensitivity to internal calcium first surfaced in the mid-sixties through studies on insect gland cells (29), following an earlier observation

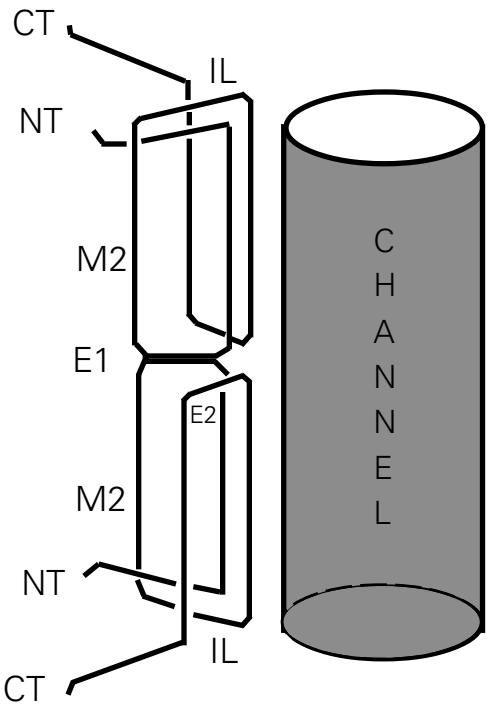


Figure 2 - Model of staggered (one-to-two) interaction between opposite connexins (12). Based on our present understanding that M3 lines the channel and both E1 and E2 interact with homologous domains across the gap, this model places E1 and E2 radially arranged around the channel with their axes at ~30° angle from each other. In this model, opposite connexins do not bind one-to-one but are staggered with each other, such that each connexin of one membrane interacts with two connexins of the adjoined membrane. There are two possible configurations of the staggered model: in one (shown here), both E's would have the same N-to-C sequence orientation, centrifugal with respect to the channel, and in the other (see Ref. 12), only E2 would have this orientation.

in cardiac myocytes (30). These findings were later confirmed in various cell systems (31-33). The role of H^+, first proposed by Turin and Warner (34,35) for amphibian embryonic cells, was later supported by Spray et al. (36) who proposed that the junctional conductance (G_j) of these cells is a simple function of pH_i. However, during the last two decades a number of conflicting data on the role of calcium and pH have been reported (23,24,28,37-41), such that it is still unclear whether H^+ and Ca^{2+} act independently from each other, and which of them regulates cell coupling under physiological circumstances.

Recently, we have tested in detail by double whole-cell patch clamp (DWCC) gap junction gating sensitivity to Ca^{2+} and H^+ in Novikoff hepatoma cell pairs internally buffered for Ca^{2+} with either EGTA or BAPTA, as well as the effects on G_j of internal solutions buffered to various pH values and $[Ca^{2+}]$ (24). Novikoff cells express Cx43. The effect of cytosolic acidification on G_j varied depending on the Ca^{2+} buffer used. With EGTA, CO_2 had a large effect on G_j, whereas with BAPTA it had virtually no effect. This observation suggested that Ca^{2+} mediates the effect of low pH_i on gap junctions, as previously shown in crayfish axons (23), because the Ca^{2+}-buffering efficiency of EGTA is severely weakened by low pH, whereas that of BAPTA is only minimally affected. The Ca^{2+}-EGTA affinity constant drops by two orders of magnitude with a decrease in pH from 7 to 6, whereas that of Ca^{2+}-BAPTA decreases only slightly with the same pH drop.

For further testing this idea, we have monitored the single exponential decay of G_j in cells buffered to different pCa_i and pH_i values. At pCa_i 6.9 or higher, G_j decreased with a time constant (τ) of 28 min, whereas at pCa_i 6-6.3 G_j decreased with a τ of ~5 min. A pCa_i of 5.5 resulted in fast uncoupling with a τ of ~20 s. The same results were obtained at pH_i 7.2 and at pH_i 6.1 (24). These data indicate that the channels of Novikoff cells are sensitive to nanomolar $[Ca^{2+}]_i$ and are insensitive to pH_i, at least in the range 7.2-6.1.

Recently, we have reevaluated the relationship among pH_i, pCa_i and G_j in *Xenopus* oocyte pairs expressing Cx38 (28). Exposure to 100% CO_2 for 3 min caused a rapid drop of G_j, pH_i and pCa_i (28). The time course of G_j was close to that of pCa_i, but contrasted sharply with that of pH_i (Figure 3). This finding, also supported by the inhibitory effect of intracellularly injected BAPTA (28), further confirmed the idea that junctional permeability is more closely related to $[Ca^{2+}]_i$ than to $[H^+]_i$. Low pH_i appears to increase $[Ca^{2+}]_i$ by releasing it from internal stores, such as endoplasmic reticulum and/or mitochondria, rather than by increasing Ca^{2+} entry (28,41). The lack of correspondence between G_j and pH_i is consistent with data obtained in other cells (23,24,39,40).

In crayfish axons (23,41), Novikoff cells (24,25) and oocytes (28), Ca^{2+} appears to affect G_j at nanomolar concentrations. Over the years, various $[Ca^{2+}]_i$ have been reported to induce uncoupling. Only $[Ca^{2+}]_i$ as high as 40-400 µM was reported to be effective in ruptured (42) or internally perfused (43) cells, whereas low micromolar to high nanomolar concentrations were shown to induce gating in intact cells (23,32,44-49). This was recently confirmed in pancreatic ß-cells (26), and in Novikoff cells studied by dye coupling (27). A channel gating sensitivity to nearly physiological $[Ca^{2+}]_i$ does not conflict with data for gap junction permeability to Ca^{2+} (50-52), because the gating mechanism is relatively slow at near physiological $[Ca^{2+}]_i$ (24), and because the $[Ca^{2+}]_i$ required to close all of the channels is in the high nM to low µM range (24), and thus above physiological values (70-200 nM).

Evidence for gap junction sensitivity to near physiological $[Ca^{2+}]_i$ (23-25,28) indicates that modulation of cell coupling may also play a role in Ca^{2+}-mediated phenom-

ena involving second messengers. Indeed, we have recently found that brief exposures to arachidonic acid uncouple Novikoff hepatoma cells in a Ca^{2+}-dependent manner, whereas long exposures affect coupling in both Ca^{2+}-dependent and Ca^{2+}-independent ways (25). Ca^{2+} participation was supported by the exquisite sensitivity of the arachidonic acid effect to $[Ca^{2+}]_i$ buffering (Figure 4). The absence of uncoupling in Ca^{2+}-free external solutions pointed to a role of Ca^{2+} entry in the uncoupling process (25).

Potential role of calmodulin in the uncoupling mechanism

In the early eighties, three independent observations suggested the existence of uncoupling intermediates. Johnston and Ramón (53) reported the inability of Ca^{2+} and H^+ to uncouple internally perfused crayfish axons. Peracchia et al. (54,55) suggested the participation of calmodulin (CaM) in the uncoupling mechanism, based on the ability of a CaM inhibitor (trifluoperazine) to prevent uncoupling in *Xenopus* embryonic cells. Hertzberg and Gilula (56) demonstrated the ability of CaM to bind to Cx32.

More recently, calmidazolium and W7, two more specific CaM blockers, inhibited uncoupling in various cells (57-61), and internally perfused crayfish axons uncoupled with Ca^{2+} only in the presence of CaM (62). CaM binding to Cx32 was further confirmed through gel overlay (13,63) and some evidence for CaM association with gap junction membranes was obtained by immunoelectron microscopy (64). In pairs of cardiac myocytes in which one cell was voltage clamped and G_j was measured after perforation of the

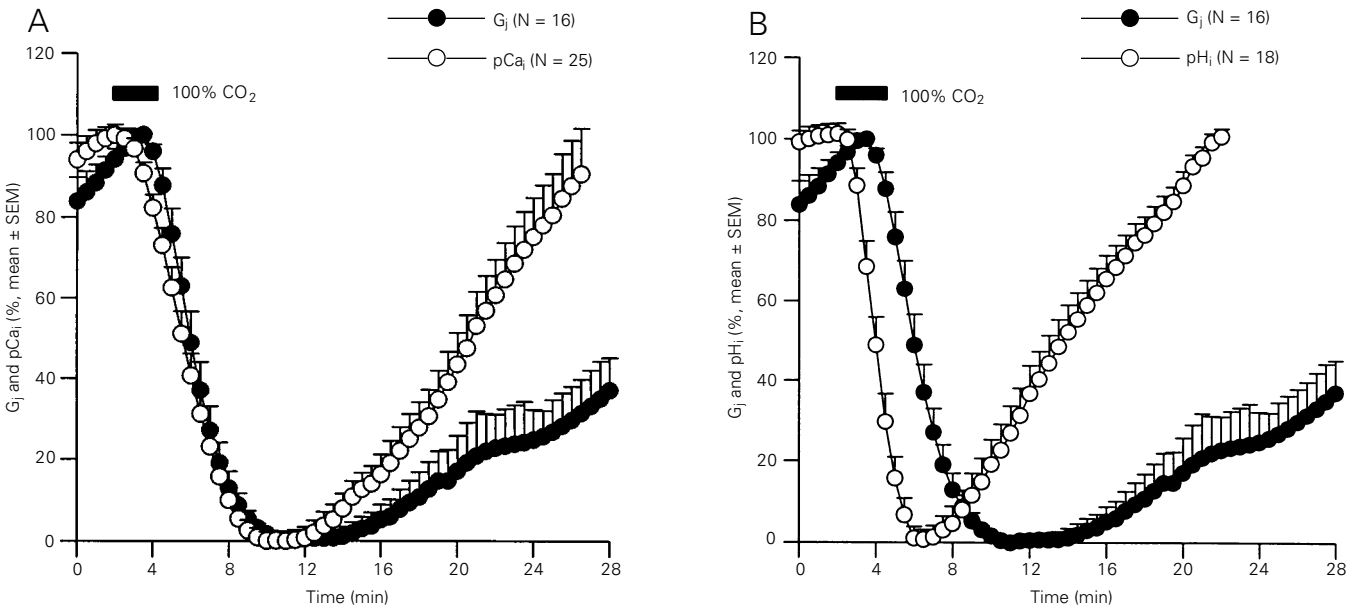


Figure 3 - Time course of changes in normalized pCa_i (A), pH_i (B) and junctional conductance (G_j, A and B) in *Xenopus* oocyte pairs exposed to 100% CO_2 for 3 min. pCa_i and pH_i were measured at the oocyte periphery with fura-C_{18} (a membrane-associated Ca^{2+} indicator) and BCECF, respectively. G_j was measured by double voltage clamp electrophysiology. Before CO_2 exposure, the oocytes had a pCa_i of 6.66 ± 0.17 (mean ± SD; N = 25) and pH_i of 7.63 ± 0.115 (N = 18). With CO_2, pCa_i dropped to 6.37 ± 0.263 (N = 25) at a maximum rate of ~23%/min (A). pCa_i minima were reached within 8-10 min and pCa_i recovered to normal or slightly higher than normal values within ~15 min. In contrast, pH_i dropped to 6.54 ± 0.113 (N = 18) at a maximum rate of ~34%/min (B). pH_i minima were reached within ~4 min and pH_i recovered to normal or slightly higher than normal values within ~10 min. The time course of pH_i contrasted sharply with that of G_j, which dropped at a maximum rate of ~25%/min and was lowest 8-10 min from the beginning of the CO_2 treatment (A and B), whereas the time course of G_j was very close to that of pCa_i during uncoupling. pCa_i minima preceded only slightly G_j minima, but pCa_i recovered at a faster rate (A). From Ref. 28, with permission.

partner cell, gap junction sensitivity to Ca^{2+} increased from pCa_i 5.7 to pCa_i 7 upon perfusion with 10 µM CaM, and W7 (but not W5) prevented uncoupling (65).

To test more directly the participation of CaM in gating, we have studied CO_2-induced uncoupling in *Xenopus* oocytes in which CaM gene expression was inhibited (28). In oocytes injected with oligonucleotides antisense to CaM mRNA, CaM mRNA was permanently degraded within 5 h, and the oocytes gradually lost junctional sensitivity to CO_2 within 72 h. Uncoupling competence recovered by ~35% following CaM injection. These data further confirm previous evidence for CaM participation in coupling regulation (reviewed in Ref. 12).

CaM could affect coupling by directly binding to connexins or by activating CaM-dependent enzymes. Phosphorylation of Cx32 by Ca^{2+}/CaM kinase II has been reported, but only in isolated junctions (66). Phosphatases could also play a role. There is evidence that connexins can be phosphorylated by various kinases (66,67) and that connexin phosphorylation decreases with Ca^{2+}-induced uncoupling (27). Furthermore, a difference in Cx43 phosphorylation has been observed between communication-competent and -deficient cell lines (67), suggesting that phosphorylation may convert impermeable hemichannels to permeable cell-cell channels.

Connexin domains relevant for pH/Ca^{2+} gating

The molecular mechanism of CO_2-induced gating is still unknown, but data on the potential involvement of certain connexin domains are accumulating. The C-terminus domain has been suggested to play a role in determining the difference in CO_2 gating sensitivity between Cx43 and Cx32, because a Cx43 mutant missing over 80% of it decreased in pH sensitivity to match that of Cx32 (40). Recently, the same group has proposed a ball-and-chain model for CO_2 gating of Cx43 in which the carboxy-terminus (CT) end (the ball) would close the channel by binding to a receptor domain located somewhere else in Cx43. This model, similar to that proposed for K^+ channels (68,69), is based on provocative data showing that the reduced CO_2 sensitivity of a Cx43 mutant deleted at the CT end is re-

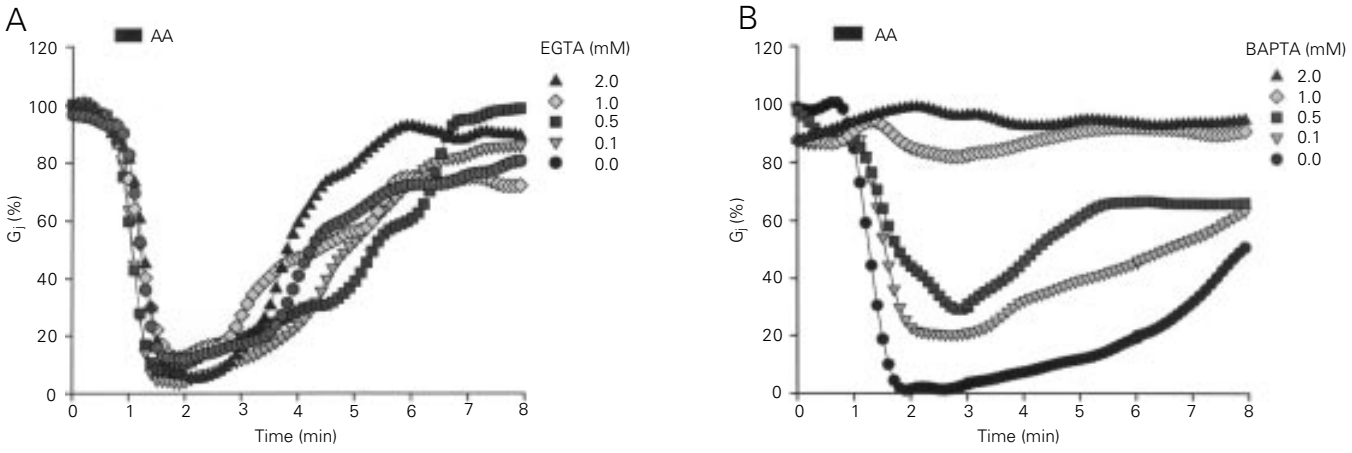


Figure 4 - Effect of arachidonic acid (AA) on electrical coupling studied in Novikoff cell pairs by double whole-cell clamp electrophysiology. The cytosol was buffered for Ca^{2+} through the pipette solution with either BAPTA or EGTA. The uncoupling effect of AA (20 µM, 20 s) depends on $[Ca^{2+}]_i$ buffering. EGTA at concentrations as high as 2 mM was totally ineffective in inhibiting uncoupling by AA (A). In contrast, BAPTA caused a 20% inhibition at concentrations as low as 0.1 mM and completely eliminated the uncoupling effects of AA at 1-2 mM concentrations (B). Indeed, BAPTA is known to be a faster and more efficient intracellular Ca^{2+} buffer than EGTA. EGTA inhibited uncoupling by ~40% and ~80% at 5 and 10 mM concentrations, respectively. From Ref. 25, with permission.

versed by coexpression of deleted Cx43 and the deleted CT end (70,71). However, the deleted Cx43 was not insensitive to acidification, but just less sensitive, a finding hard to explain if the postulated gating "ball" (CT end) is missing. Nonetheless, this model may only be relevant for Cx43, because a Cx32 mutant in which 84% of the CT had been deleted (D219) was as sensitive to CO_2 as wild-type Cx32 (72,73).

For defining connexin domain(s) of Cx32 participating in CO_2-induced gating, we have studied the gating sensitivities to CO_2 of Cx32, Cx38, and various chimeras and mutants of the above, expressed in *Xenopus* oocytes (74). Cx32 is much less sensitive to CO_2 than Cx38. Our data show that two chimeras, Cx32/38I (Cx32 with an inner loop, IL, of Cx38) and Cx32/38I_2 (Cx32 with the second half of the inner loop, IL_2, of Cx38), are as sensitive to CO_2 as Cx38 (74,75) (Figure 5). This indicates that the second half of the inner loop plays an important role in pH gating sensitivity.

The mechanism by which IL_2 plays a role in CO_2 gating sensitivity is still unclear. Spray and Burt (76) have proposed that low-pH induced uncoupling follows protonation of H residues. An important role in determining the CO_2 sensitivity of Cx43 has been attributed to H95 (77), a residue located at the N-terminus of IL in most connexins. Both Cx32 and Cx38 have an H residue at that location, but their neighboring residues are different and this could account for their difference in pH sensitivity. However, in view of our data on the relevance of IL_2 (74), this residue may not play a key role in determining the CO_2 sensitivity of Cx32 and Cx38. More relevant to channel gating could be some of the H residues of IL_2. Recently, Hermans et al. (78) have provided preliminary evidence indicating that two H residues of Cx43 (H126 and H142) modulate in opposite ways the uncoupling effect of CO_2. In Cx32, IL_2 contains two H residues (H123 and H126). Although in preliminary experiments the replacement of H126 with R did not affect the CO_2 sensitivity of Cx32 (75), a more detailed evaluation of the potential role of these two residues is presently underway in our laboratory.

Since CT chimeras did not express functional channels, the potential role of CT could not be tested with chimeras, but interesting data were obtained with mutations of basic residues at its initial 18-residue segment (C_1) and with CT deletions. Although much of the C-terminus of Cx32 seems not to play a significant role in CO_2-gating sensitivity, as 84% deletion of it at residue 219 (Cx32-D219) does not affect CO_2 sensitivity (72,73) (Figure 6), the C_1 domain (Figure 7) appears to have an inhibitory role. This is suggested by our recent data with mutants in which some or all of the positively charged residues (R) of C_1 were replaced with neutral (polar) residues (N or T) (73). Progressive replacement of R with N residues resulted in

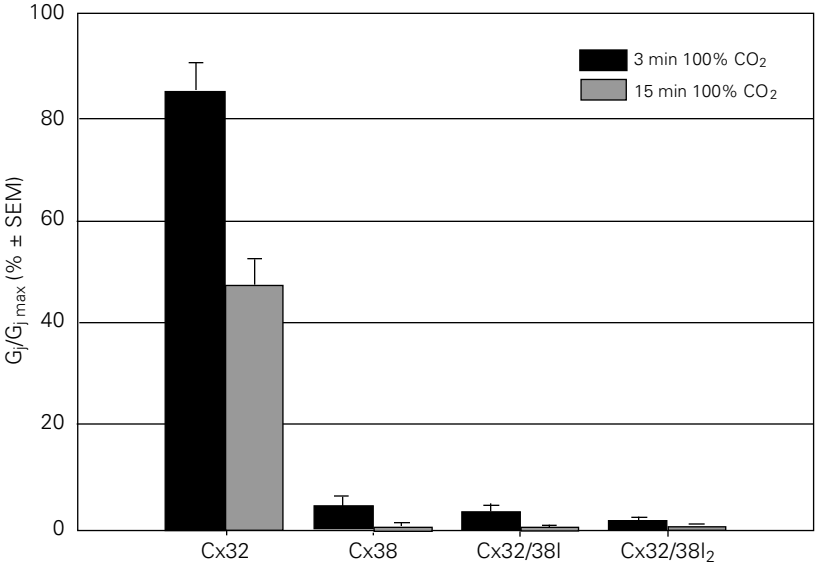


Figure 5 - Junctional sensitivity to CO_2, expressed as normalized junctional conductance ($G_j/G_{j\,max}$; 100% = control, pretreatment value), in oocyte pairs expressing Cx32, Cx38 or Cx32/38 chimeras (74,75). With Cx38, a 3-min exposure to CO_2 decreased G_j to nearly 0%, whereas with Cx32, even a 15-min CO_2 treatment decreased G_j by only ~55%. Two chimeras, Cx32/38I (inner loop of Cx32 replaced by that of Cx38) and Cx32/38I_2 (second half of inner loop, IL_2, of Cx32 replaced by that of Cx38), reproduced the uncoupling efficiency of Cx38. This indicates that IL_2 plays an important role in pH gating sensitivity. The N-terminal domain does not appear to be relevant because the chimera Cx32/38N (Cx32 with NT of Cx38) behaved similarly to Cx32 (see Ref. 74).

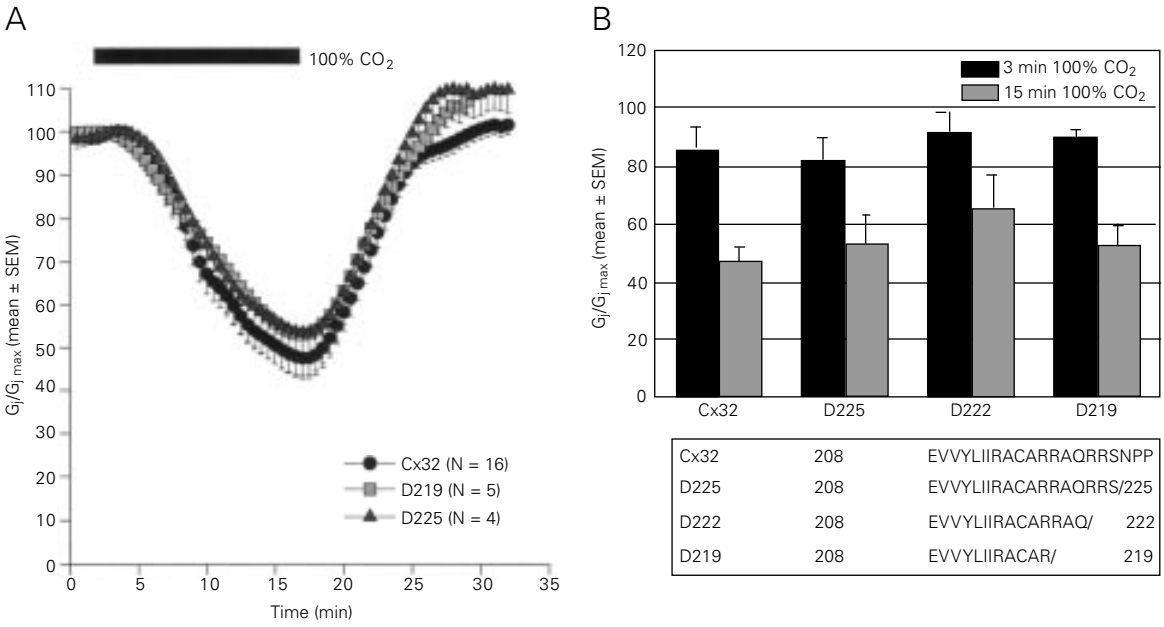
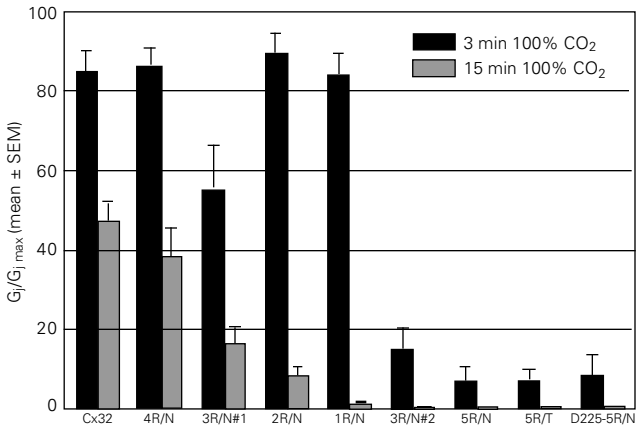


Figure 6 - Decrease in junctional conductance (G_j) in *Xenopus* oocyte pairs, expressing wild-type Cx32 or Cx32 deleted of most of the C-terminus, with exposure to 100% CO_2 for either 15 min (A and B) or 3 min (B). Note that deletion of the C-terminus by over 80% (D225, D222, D219) did not affect CO_2 sensitivity. With 3 min CO_2, G_j dropped to 82 ± 8%, 91 ± 7% and 90 ± 3% (mean ± SEM) with D225, D222 and D219, respectively, and with 15 min CO_2, to 53.5 ± 10%, 65 ± 11% and 53 ± 7% with D225, D222 and D219, respectively.



Cx32	215	RACARRAQRRSNPP
4R/N	215	R---NN--NN----
3R/N#1	215	N---RR--NN----
2R/N	215	R---NN--RR----
1R/N	215	N---RR--RR----
3R/N#2	215	N---NN--RR----
5R/N	215	N---NN--NN----
5R/T	215	T---TT--TT----
D225-5R/N	215	N---NN--NN-/225

Figure 7 - Summary of the effects of partial or total replacement of arginine (R) residues with asparagine (N) or threonine (T) residues in the initial domain (C_1) of the C-terminus chain, on normalized G_j ($G_j/G_{j\,max}$; 100% = control, pretreatment value), following 3-min or 15-min exposure to CO_2. Note that replacement of all of the 5 R with N or T residues greatly increased the CO_2 sensitivity of Cx32, whereas partial R/N replacement resulted in intermediate CO_2 sensitivities. This indicates that the R residues differ in their ability to inhibit the CO_2 sensitivity of Cx32. R215 appears to have greater inhibitory power than R219-220. In contrast, R223-224 seems to partly counteract the inhibitory activity of both R215 and R219-220, because 2R/N and 1R/N were more sensitive to 15-min exposure to CO_2 than 4R/N and 3R/N#1, respectively.

a progressive increase in Cx32 sensitivity to CO_2 (Figure 7). Interestingly, the 5 R residues were not all equally effective in inhibiting the Cx32 sensitivity to CO_2. R215 has greater inhibitory power than R219-220, whereas R223-224 seems to partly counteract the inhibitory activity of both R215 and R219-220. This is suggested by the fact that 2R/N and 1R/N were more sensitive to 15-min exposure to CO_2 than 4R/N and 3R/N#1, respectively (Figure 7).

A possible interpretation of these data is that the gating mechanism involves electrostatic interactions among intracellular domains of Cx32. IL_2 and C_1 are positively charged domains in all connexins, whereas IL_1 is the only cytoplasmic domain that is rich in negative charges. Although IL_1 contains positive charges as well, being the most heavily charged domain of connexins, in α-helical conformation it would have positive and negative charges partitioned on opposite sides of the helix (Figure 8); indeed, IL_1, IL_2 and C_1 of Cx32 are likely to be α-helical and IL may have a helix-loop-helix structure, in view of the presence of G (residues 110 and 112) and P (residue 114) residues at its mid-region.

As a working model, we are considering the possibility that in Cx32 under normal coupling conditions the negative charges of IL_1 are masked or somehow unavailable for electrostatic interaction with other domains. With CO_2, conformational changes, brought about by changes in the connexin phosphorylation state, protonation of H residues, CaM binding, a combination of the above, or other as yet unknown factors, may expose them, allowing IL_2 and C_1 to competitively interact with IL_1. IL_1-IL_2 interaction would result in a closed channel state, whereas IL_1-C_1 interaction would maintain the channel in an open state (Figure 8). Based on this hypothesis, uncoupling efficiency would de-

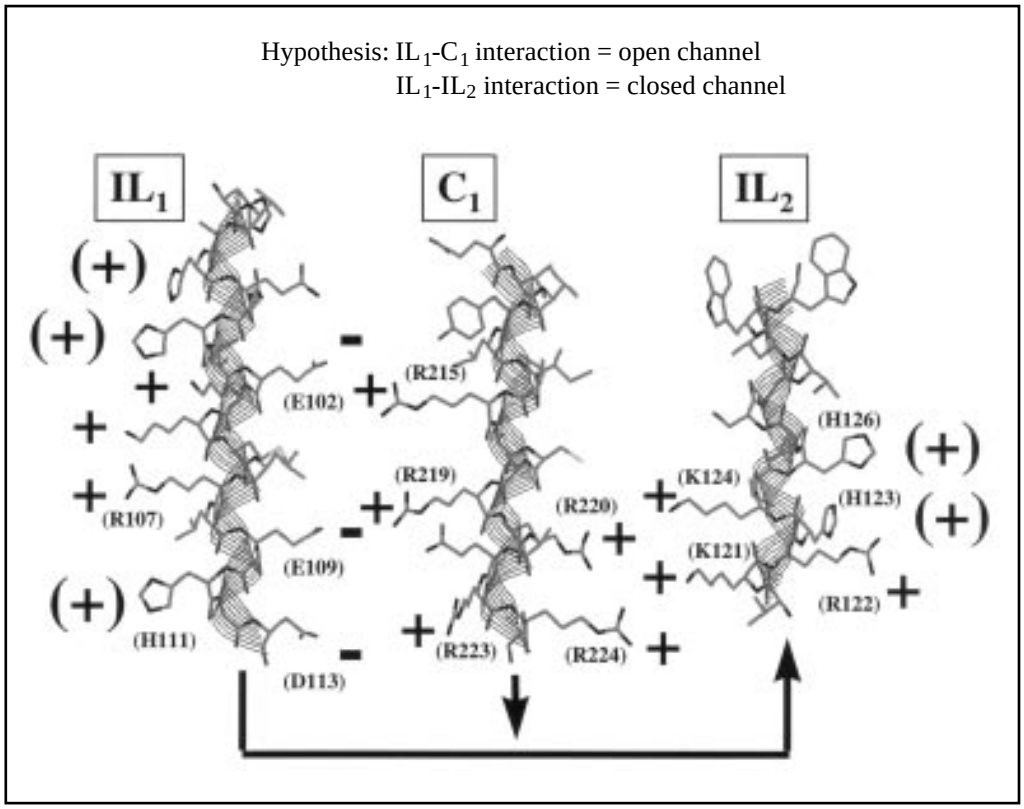


Figure 8 - Model of potential electrostatic interactions among three cytoplasmic domains (IL_1, IL_2 and C_1) of Cx32, displayed in alpha-helical conformation. In view of the fact that 1) IL_2 and C_1 are positively charged, 2) the inhibitory action of C_1 depends on its positive charges, and 3) the only cytoplasmic domain with negative charges is IL_1 (not considering some acidic residues of the C-terminal domain that can be deleted without gating consequences), we propose that open and closed channel states depend on charge interactions among IL_1, IL_2 and C_1. In coupled conditions the negative charges of IL_1 would be unavailable for interaction, whereas with CO_2 conformational changes would expose them, enabling IL_2 and C_1 to competitively interact with IL_1. IL_1-IL_2 interaction would result in closed channel, whereas IL_1-C_1 interaction would maintain the channel open.

pend on differences between IL_2 and C_1 in binding affinity to IL_1. In Cx32, one would predict C_1 to be a strong competitor of IL_2, whereas the opposite would be true for connexins more sensitive to CO_2, such as Cx38, Cx50, Cx45, etc. The gradual increase in Cx32 sensitivity to CO_2 that follows the progressive removal of positive charges in C_1 would be the consequence of a gradual decrease in the capacity of C_1 and IL_1 to interact with each other. The increased CO_2 sensitivity of the Cx32/38I_2 chimera, compared to wild-type Cx32, would indicate that IL_2 of Cx38 has greater affinity for IL_1 of Cx32 than IL_2 of Cx32. Therefore, IL_2 of Cx38 would compete more efficiently against the inhibitory domain (C_1) for binding to IL_1. Although one should be well aware that based on the very limited amount of data this and any other potential model should not be valued more than working hypotheses, we feel that the potential participation of electrostatic interactions among connexin domains in the gating mechanism is worth careful study.

A puzzling question is the apparent contradiction between our data on Cx32 and those of Delmar's group (40,70,71) on Cx43 regarding the relevance of CT in CO_2 gating. Whereas we found most of the CT of Cx32 to be irrelevant and its initial regions (C_1) to act as a gating inhibitor, Delmar's group found middle (residues 261-300) and end (residues 374-382) regions of the CT of Cx43 to be gating mediators (or activators); indeed, they have named the end of CT "the gating particle" of the ball-and-chain model (71). Of course, it is quite possible that connexins are gated by different molecular mechanisms. On the other hand, we think that there might be a common denominator for Cx32 and Cx43 data. A possibility is that in Cx43 the negatively charged region of the "gating particle" interacts with C_1 (a basic-amphiphilic domain, as in Cx32). By doing so, the "gating particle" would eliminate the inhibitory function of C_1, as it would prevent it from interacting with IL_1; its deletion would reduce CO_2 sensitivity because C_1 would then be free to bind to IL_1 and to act as inhibitor domain by competing against IL_2. Indeed, acidic residues of the "gating particle" were found to be crucial for its function (71). The proline-glycine-rich mid-region of CT may provide the hinge that enables the "gating particle" to bend backward and bind to C_1. Based on this interpretation, the reason why coexpressing Cx32 with the "gating particle" of Cx43 increases the CO_2 sensitivity of Cx32 (70,71) would be that the "gating particle" of Cx43 interacts electrostatically with C_1 of Cx32 and blocks its inhibitory function. Note that in Cx32 the end of the CT chain does not contain sequences even remotely similar to that of the "gating particle".

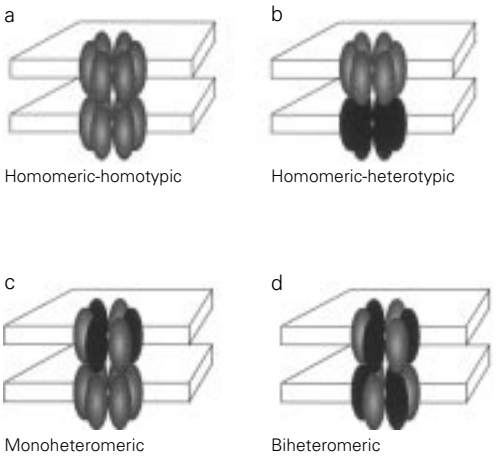


Figure 9 - Gap junction channels can be homotypic (made of two connexons expressing the same connexin) (a) or heterotypic (made of two connexons each expressing a different connexin) (b). Similarly, connexons can be homomeric (made of the same connexin) (a and b) or heteromeric (composed of different connexins) (c and d). Therefore, cell-cell channels can be homomeric-homotypic (a), homomeric-heterotypic (b), monoheteromeric (one connexon heteromeric and the other homomeric) (c), or biheteromeric (both connexons heteromeric) (d).

Does chemical gating require connexin cooperativity?

Coupling between cells expressing different connexins has been demonstrated in many systems (1), indicating that a cell-cell channel can be homotypic (made of two connexons expressing the same connexin, Figure 9a) or heterotypic (made of two connexons each expressing a different connexin, Figure 9b) (1). In turn, connexons can be homomeric (made of the same connexin,

Figure 9a and b) or heteromeric (composed of different connexins, Figure 9c and d) (79). Therefore, cell-cell channels can be homomeric-homotypic (Figure 9a), homomeric-heterotypic (Figure 9b), monoheteromeric (one connexon heteromeric and the other homomeric, Figure 9c), biheteromeric (both connexons heteromeric, Figure 9d), etc.

In view of this complexity, we have recently begun addressing questions on CO_2 gating in heteromeric connexons and heterotypic channels. We think that heteromeric connexons are an excellent tool for learning whether CO_2 gating requires cooperativity among the connexins of a connexon. Similarly, heterotypic channels can help determining whether the two connexons of a cell-cell channel influence each other's gating behavior. Our preliminary data indicate that connexin cooperativity within a connexon (hemichannel) may be necessary (80). If this were true, one would expect physiologically occurring heteromeric hemichannels to gate poorly, resulting in important functional consequences for tissue behavior.

We have tested oocyte pairs in which one oocyte expresses a 50/50 mixture of wild-type Cx32 and 5R/N mutant (*mixed* oocyte) and the other either wild-type Cx32 (*32* oocyte) or 5R/N mutant (*R/N* oocyte), creating *mixed-32* and *mixed-R/N* pairs (both with monoheteromeric channels, Figure 9c). Interestingly, these pairs were much less sensitive than *32-32* and *R/N-R/N* pairs, respectively (Figure 10). Since the 5R/N mutant is much more sensitive to CO_2 than the wild-type Cx32 (Figure 7), if connexins were gating independently from each other one would have expected the *mixed-32* to be more sensitive than *32-32* and the *mixed-R/N* to be only slightly less sensitive than *R/N-R/N* pairs. The presence of one or more 5R/N in most hemichannels should have increased the gating sensitivity of the heteromeric hemichannels.

Whereas connexin cooperativity within a connexon may be needed for efficient gating, cooperativity between two connexons forming a cell-cell channel may not be necessary. In our preliminary data, *32-R/N* pairs (homomeric heterotypic, Figure 9b) were less sensitive than *R/N-R/N* pairs and more sensitive than *32-32* pairs (Figure 10) to a level predicted for independent hemichannel gating.

Conclusion

The chemical gating of gap junction channels appears to be a complex phenomenon that involves more than one connexin domain. Both an increase in cytosolic calcium concentration and a decrease in pH_i appear to initiate the cell-cell uncoupling process, but based on our data the effect of low pH_i on gap junction channels appears to be primarily mediated by an increase in cytosolic free calcium concentration. The chain of events that link the increase in calcium and/or hydrogen ion concentration to the channel gating mechanism is unclear, although indirect evidence suggests a role of calmodulin in the uncoupling process. At the molecular level, two connexin domains have been considered important: the inner (cytoplasmic) loop and the carboxy-terminus chain. However, different functions have been attributed to the latter in Cx32 and in Cx43.

The absence of high resolution structural

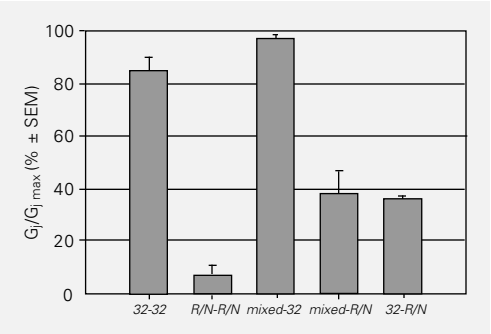


Figure 10 - Sensitivity to CO_2 presented as normalized junctional conductance ($G_j/G_{j\ max}$; 100% = control, pretreatment value) in oocyte pairs expressing heteromeric or heterotypic channels. Pairs in which one oocyte expressed a 50/50 mixture of Cx32 and 5R/N mutant (*mixed*) and the other either Cx32 (*32*) or 5R/N (*R/N*) were less sensitive to CO_2 than *32-32* and *R/N-R/N* pairs, respectively. Their sensitivity is consistent with the idea that in heteromeric hemichannels (*mixed*) gating is impaired and suggests that gating may require connexin cooperativity. In contrast, the sensitivity of heterotypic channels (*32-R/N*) was close to that theoretically predicted, indicating that the two hemichannels of a cell-cell channel are likely to gate independently from each other.

information is the major handicap for understanding channel gating mechanisms at the molecular level. Nonetheless, important information on the gating process will continue to accumulate through connexin chimeras and mutants. A thorough study of relevant connexin domains based on molecular genetics and biophysics, coupled to careful comparisons of connexin sequences and gating behaviors, should provide a good understanding of the molecular mechanism of channel gating even in the absence of a detailed portrait of the three-dimensional architecture of connexin.

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