

Review

Chemical gating of gap junction channels Roles of calcium, pH and calmodulin

Camillo Peracchia*

Department of Pharmacology and Physiology, University of Rochester, School of Medicine and Dentistry, 601 Elmwood Avenue,
Rochester, NY 14642-8711, USA

Received 16 June 2003; received in revised form 13 October 2003; accepted 13 October 2003

Abstract

Both Ca^{2+} and H^+ play a role in chemical gating of gap junction channels, but, with the possible exception of Cx46 hemichannels, neither of them is likely to induce gating by a direct interaction with connexins. Some evidence suggests that low pH_i affects gating via an increase in $[\text{Ca}^{2+}]_i$; in turn, Ca^{2+} is likely to induce gating by activation of CaM, which may act directly as a gating particle. The effective concentrations of both Ca^{2+} and H^+ vary depending on cell type, type of connexin expressed and procedure employed to increase their cytosolic concentrations; however, pH_i as high as 7.2 and $[\text{Ca}^{2+}]_i$ as low as 150 nM or lower have been reported to be effective in some cells. Some data suggest that Ca^{2+} and H^+ affect gating by acting synergistically, but other data do not support synergism. Chemical gating follows the activation of a slow gate distinct from the fast V_j -sensitive gate, and there is evidence that the chemical/slow gate is V_j -sensitive. At the single channel level, the chemical/slow gate closes the channels slowly and completely, whereas the fast V_j gate closes the channels rapidly and incompletely. At least three molecular models of channel gating have been proposed, but all of them are mostly based on circumstantial evidence.

© 2004 Elsevier B.V. All rights reserved.

Keywords: Gap junction; Connexin; Chemical gating; Calcium; pH; Calmodulin; Membrane channel

1. Introduction

The permeability of gap junction channels is regulated by specific changes in cytosolic ionic composition, posttranslational modifications (phosphorylation) and transjunctional voltage (V_j). As the channels close, cells uncouple from each other electrically and metabolically. Uncoupling is mainly a protective device for isolating healthy cells from damaged neighbors, but in some cells there is evidence for channel gating sensitivity to nearly physiological $[\text{Ca}^{2+}]_i$ and $[\text{H}^+]_i$, suggesting that modulation of channel permeability by these ions may be relevant to normal cell functions as well (reviewed in Ref. [1]).

The major goal of research focused on the regulation of gap junction channels is to understand how cell communication is physiologically controlled and how cell coupling modulation is linked to other cellular activities. For achieving this goal, one needs first to understand the molecular basis of channel gating by defining the role of gating agents,

identifying connexin domains relevant to gating, evaluating the likely participation of accessory molecules, and probing gating by pharmacological and genetic tools.

2. Role of Ca^{2+} in channel gating

In 1877, Engelmann [2] reported that cardiac cells in direct contact with each other during life became independent as they died. This phenomenon, named “healing-over”, was believed to result from the formation of ionic barriers between injured and uninjured cells. Almost a century later, Déléze [3,4] reported that cut heart fibers do not heal in the absence of external Ca^{2+} , but do so rapidly when Ca^{2+} is supplied. This provocative observation, published soon after the serendipitous discovery that most cells communicate directly with each other electrically and metabolically [5,6], suggested for the first time the role of Ca^{2+} in the regulation of gap junctional communication. Subsequently, the role of Ca^{2+} in cell uncoupling was confirmed through data, mostly indirect, correlating loss of electrical and dye coupling among cells exposed to treatments known

* Tel.: +1-585-275-2201; fax: +1-585-273-2652.

E-mail address: camillo_peracchia@urmc.rochester.edu (C. Peracchia).

to increase $[Ca^{2+}]_i$ (reviewed in Refs. [7–9]). Most convincing then was the evidence that in insect gland cells, electrical uncoupling coincides with an increase in $[Ca^{2+}]_i$, monitored by the Ca^{2+} indicator aequorin [10,11].

The role of Ca^{2+}_i was later confirmed in a number of cell types, including cardiac cells [12–19], amphibian embryonic cells [20], rat lacrimal cells [21], crayfish giant axons [22,23], Novikoff hepatoma cells [24,25], astrocytes [26–28], lens cultured cells [29], pancreatic β -cells and acinar cells [30–32], osteoblasts [33], and cochlear supporting cells [34,35], among others (reviewed in Refs. [1,36]).

Significantly, there is evidence that Ca^{2+} may self-limit its cell–cell diffusion. This was first reported in pancreatic acinar cells [32] and recently confirmed in SkHep1 cultured cells [37]. The latter study [37] reported that Ca^{2+} , uncaged by flash photolysis in one SkHep1 cell expressing either connexin32 (Cx32) or Cx43, never spread to its neighbors. This suggests that an increase in $[Ca^{2+}]_i$ rapidly closes gap junction channels to complete cell–cell uncoupling. However, the possibility that Ca^{2+} was buffered so rapidly that it was unable to reach the junctional area cannot be discarded.

2.1. Which $[Ca^{2+}]_i$ affects gating?

While it is clear that Ca^{2+} affects channel gating, the $[Ca^{2+}]_i$ that activates the gating mechanism is still uncertain, and may vary among connexins and cell types. Some studies reported channel gating sensitivity to $[Ca^{2+}]_i$ as high as 40–400 μ M in ruptured insect gland cells [38], internally perfused amphibian embryonic cells [20], and rat cardiac cells dialyzed with different patch pipette solutions [19]. Other studies reported that $[Ca^{2+}]_i$ ranging from high nanomolar to low micromolar were effective in insect salivary gland cells [11], mammalian and amphibian cardiac cells [13,16,17], rat lacrimal cells [21], crayfish giant axons [22,23] and Novikoff hepatoma cells [24,25].

Novikoff cells were studied in detail by dual whole-cell patch clamp using pipette solutions buffered for Ca^{2+} with BAPTA and for H^+ with HEPES (pH 7.2) or MES (pH 6.1). In these cells, junctional conductance (G_j) was affected by $[Ca^{2+}]_i = 500$ nM or greater, whereas $[Ca^{2+}]_i = 100$ nM was ineffective either at pH=7.2 or at pH=6.1 [24] (Fig. 1A). Similar results were obtained in these cells following brief application of arachidonic acid [25]. A 20-s exposure to 20 μ M arachidonic acid increased $[Ca^{2+}]_i$ to 0.7–1.5 μ M and simultaneously brought G_j to zero in 10–15 s. Significantly, the uncoupling effect of arachidonic acid was exquisitely sensitive to Ca^{2+} buffering, intracellular BAPTA being 10 times more effective in preventing channel closure than EGTA, a slower and less efficient Ca^{2+}_i buffer [25] (Fig. 1B). The increase in $[Ca^{2+}]_i$ resulted primarily from arachidonic acid-induced Ca^{2+} entry, because $[Ca^{2+}]_i$ increased only minimally in cells bathed in Ca^{2+} -free external solutions without affecting G_j [25]. In contrast, prolonged exposure to arachidonic acid (20 μ M, 1 min) slowly uncoupled the cells even in Ca^{2+} -free media,

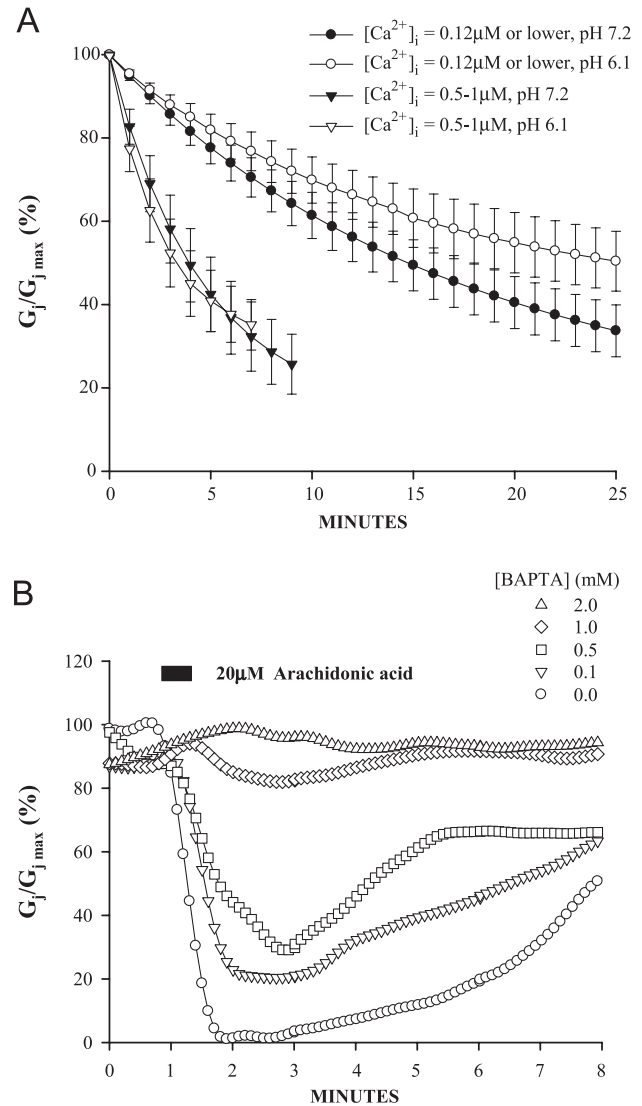


Fig. 1. Calcium affects gating at nanomolar concentrations independently of pH_i . (A) Single exponential decay of normalized G_j monitored by dual whole-cell patch clamp in Novikoff hepatoma cell pairs internally buffered for Ca^{2+} with BAPTA and for H^+ with either HEPES (pH 7.2) or MES (pH 6.1). With $[Ca^{2+}]_i = 0.12 \mu M$ or lower, G_j decays with mean τ 's of 35.2 and 22.3 min, at $pH_i = 6.1$ and 7.2, respectively. With $[Ca^{2+}]_i = 0.5-1.0 \mu M$, G_j decays with mean τ 's of 5.9 and 6.2 min, at $pH_i = 6.1$ and 7.2, respectively. These data indicate that the Cx43 channels of Novikoff cells are sensitive to $[Ca^{2+}]_i$ in the physiological range (≥ 500 nM) and that low pH_i neither decreases G_j nor increases channel sensitivity to Ca^{2+} . From Ref. [24]. (B) In Novikoff hepatoma cells the effect on G_j of brief applications of arachidonic acid (20 μM , 20 s) is strongly inhibited by buffering cytosolic Ca^{2+} with BAPTA through the pipette solution. BAPTA causes a 20% inhibition at concentrations as low as 0.1 mM and completely eliminates the uncoupling effects of arachidonic acid at 1–2 mM concentrations. From Ref. [25].

indicating that arachidonic acid has both a rapid Ca^{2+} -dependent effect and a slow Ca^{2+} -independent effect on channel gating [25].

Evidence for gap junction channel sensitivity to nanomolar $[Ca^{2+}]_i$ has been recently confirmed in astrocytes co-injected with lucifer yellow and Ca^{2+} . In these cells,

the blockage of cell–cell dye diffusion was linearly related to $[Ca^{2+}]_i$ ranging from 150 to 600 nM [26]. Consistent with these data is evidence that addition of 20 mM BAPTA to patch pipette solutions substantially increases coupling between astrocytes [28], which suggests that channel gating is even sensitive to resting $[Ca^{2+}]_i$, known to be in the low nanomolar range. Similarly, dye coupling was blocked in ionomycin-treated astrocytes by an increase in $[Ca^{2+}]_i$ to 500 nM [27], and comparable data were reported in lens cultured cells [29, 39], HeLa cells transfected with Cx43 [39], and pancreatic β -cells [30]. In contrast, higher $[Ca^{2+}]_i$ appear to be needed to uncouple cardiac myocytes [40–42].

The reason for the wide range of threshold $[Ca^{2+}]_i$ reported to be effective on channel gating is unclear. Perhaps, in some cells the increase in $[Ca^{2+}]_i$ is too brief to activate the relatively slow gating mechanism. Alternatively, the efficient internal buffering mechanisms of the Ca^{2+} stores and the cytosol may create large $[Ca^{2+}]_i$ gradients within the cell, such that the cytosolic medium bathing the junctional surface may be affected only minimally by even large changes in $[Ca^{2+}]_i$ occurring in other cellular regions. This is supported by evidence for very slow Ca^{2+} diffusion in the cytosol, as demonstrated by Allbritton et al. [43] who reported Ca^{2+} diffusion coefficients ranging from 13 to 65 $\mu m^2/s$ for $[Ca^{2+}]$ ranging from 90 nM to 1 μM in cytosolic extracts of *Xenopus* oocytes; for comparison, inositol triphosphate diffused with a diffusion coefficient of 283 $\mu m^2/s$ [43]. Indeed, in some cases, an increase in $[Ca^{2+}]_i$ resulting from Ca^{2+} entry appears to be much more effective than that caused by Ca^{2+} release from internal stores. In Novikoff cells, for example, an increase in $[Ca^{2+}]_i$ to values as high as 1.2 μM , which followed Ca^{2+} release from stores induced by external application of 100 μM ATP, only decreased coupling by 30–40% [44], whereas even a smaller increase in $[Ca^{2+}]_i$ resulting from arachidonic acid-induced Ca^{2+} entry rapidly reduced G_j to zero [25]. Consistent with the idea that Ca^{2+} entry may be more effective than Ca^{2+} release from stores is an interesting study demonstrating the close relationship between capacitative Ca^{2+} entry and gap junction channel gating in pancreatic acinar cells exposed to acetylcholine (ACh) [31]. In this study, application of low concentrations of ACh, enough to cause only partial Ca^{2+} release from stores, did not affect coupling, whereas higher ACh concentrations (1 μM , 5 min), sufficient to empty the Ca^{2+} stores and consequently induce capacitative Ca^{2+} entry, uncoupled the cells. Ca^{2+} entry was confirmed as the cause of uncoupling because the same ACh application was ineffective in Ca^{2+} -free media [31].

2.2. Does Ca^{2+} act directly on the channel gates?

A direct Ca^{2+} effect on connexins would be expected to involve clusters of negative charges facing the cytosol. Among connexins, the only conserved acidic residue facing the cytosol is a glutamate that marks the transition between

fourth transmembrane (TM4) and COOH-terminus (CT) domains (residues 208 in Cx32). However, an isolated acidic residue per connexin, even though there would be six of them per connexon, would be incapable of binding Ca_i^{2+} with sufficiently high affinity to account for gating-effective $[Ca^{2+}]_i$ in the nanomolar range (see Section 2.1), and would not distinguish between Ca_i^{2+} and Mg_i^{2+} ; furthermore, this CT region is not believed to be near the pore. Since the channels are not sensitive to Mg^{2+}_i , in spite of its intracellular concentration in the millimolar range, a Ca^{2+} effect at nanomolar concentrations would require Ca^{2+} specific sites like those of Ca^{2+} modulated proteins (EF-hands) [45], none of which are present in connexins. The ineffectiveness of millimolar $[Mg^{2+}]_i$ also eliminates a possible involvement of membrane phospholipids, as they would not distinguish between Ca^{2+} and Mg^{2+} .

Thus, it is reasonable to believe that the Ca^{2+} effect on gating is mediated by an intermediate component. Indeed, in the last two decades a number of studies have supported the role of calmodulin (CaM) as the most likely intermediate of the Ca^{2+} effect on gating, possibly via direct interaction with connexins (see Section 4).

3. Role of H^+ in channel gating

In 1977, the undisputed role of Ca^{2+} as the only regulator of gap junction channel permeability was challenged by evidence that CO_2 -induced cytosolic acidification causes uncoupling [46]. In this and a subsequent study, cells of early *Xenopus* embryos were reversibly uncoupled by lowering pH_i to 6.4–6.2 with exposure to external solutions gassed with 100% CO_2 [46,47]. The uncoupling effect of lowered pH_i was soon confirmed in insect gland cells [48], pancreatic acinar cells [49], crayfish axons [50,51], *Ambystoma* and *Fundulus* embryonic cells [52], embryonic chick lens cells [53], and cardiac cells [54], among others.

3.1. Which $[H^+]_i$ affects gating?

In the past two decades, pH_i sensitivity has been tested in various cell types and it is generally accepted that in virtually all cells cytosolic acidification decreases G_j . However, the sensitivity to pH_i varies among cell types and is at least in part related to the type of connexin expressed. We have reported that the rat liver connexin (Cx32) expressed in *Xenopus* oocytes is much less sensitive than the native oocyte connexin, Cx38 [55]. Indeed, Cx32 and Cx38 are at the opposite end of the spectrum in terms of pH_i sensitivity. For example, with brief (3 min) superfusions of solutions gassed with 100% CO_2 , sufficient to lower pH_i from 7.6 to 6.5 [56], G_j only decreases to $\sim 75\%$ of control values with Cx32 channels, but it drops to 0% with Cx38 channels [55].

Delmar and coworkers have systematically tested the pH_i sensitivity of G_j in oocyte pairs expressing different con-

nexins, by a method that monitors G_j and pH_i while pH_i is lowered in steps by application of different CO_2 fractional concentrations [57,58]. A comparison of pH_i sensitivities among eight connexin channels, based on pK_a (the pH at which the normalized G_j is 50% of maximum), confirmed 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 falling in between in the following order of decreasing sensitivity: Cx50>Cx46>Cx45>Cx26>Cx37>Cx43>Cx40>Cx32 [59]. The type of connexin expressed, however, is not the only factor in determining pH_i sensitivity, as similar cytosolic acidifications achieved by different procedures have different effects on gating, and different cells expressing the same connexin often have different pH_i sensitivities (see Section 3.2).

3.2. Does H^+ act directly on the channel gates?

Early data on amphibian embryonic cells and other cell systems seemed to suggest a direct effect of H^+ on connexin channels, but some inconsistencies were apparent (reviewed in Ref. [60]). Turin and Warner [47] showed changes in both coupling ratio (α) and pH_i in the same cells, but plotted α versus pH_i only for the recoupling phase; thus, the possibility of curve hysteresis in the relationship between pH_i and α could not be evaluated. Spray et al. [52], using recessed-tip pH microelectrodes, reported a hysteresis in the relationship between pH_i and α , but interpreted it as an artifact of the effects of CO_2 on non-junctional membrane conductance and concluded that protons act directly on connexins ($\text{pK}_a = 7.3$). Subsequent work from the same group, using neutral carrier pH microelectrodes, reevaluated the original data, reporting the presence of more pronounced hysteresis and a sensitivity to $[\text{H}^+]_i$ almost one order of magnitude lower than previously stated ($\text{pK}_a = 6.5$) [61]. Campos de Carvalho et al. [62], using recessed-tip pH microelectrodes, reported that in crayfish axons, G_j measured when pH_i decreases indeed falls nearly along the same Hill relationship as G_j measured when pH_i is recovering, but only with brief exposures to acetate, whereas with longer exposures G_j recovers slowly and incompletely in spite of the fact that pH_i recovers at a normal rate. Significant hysteresis in the relation between $[\text{H}^+]_i$ and junctional resistance and several other inconsistencies were also reported in sheep cardiac fibers [63].

We have studied the relationship between $[\text{H}^+]_i$ and junctional resistance (R_j) in crayfish axons, uncoupled by acetate superfusion, by measuring $[\text{H}^+]_i$ with neutral carrier microelectrodes [22]. Plots of the time course of R_j and $[\text{H}^+]_i$ differed markedly in shape, and $[\text{H}^+]_i$ maxima preceded R_j maxima by 40–90 s, resulting in marked curve hysteresis in the relationship between R_j and $[\text{H}^+]_i$. In addition, R_j maxima obtained with slow acidification rates were three times greater than those resulting from faster acidifications, in spite of the fact that both protocols resulted in the same pH_i minima. Similar results were obtained in *Xenopus*

oocytes expressing the native connexin, Cx38, by monitoring G_j by dual voltage clamp and pH_i by BCECF, a fluorescein derivative pH-indicator [56]. The data showed a drastic difference in time course between G_j and pH_i , as pH_i minima preceded G_j minima by ~ 4 min and pH_i recovered much faster than G_j [56].

Cx43 channels displayed a pK_a of 6.7 in oocytes [59], whereas a pH_i of 6.6 had only a small effect on coupling in mammalian heart fibers, which express primarily Cx43 [54], and H^+ affected healing-over in the heart only at $\text{pH} = < 5$ [64]. Internally perfused oocyte pairs expressing Cx43 were completely insensitive to pH_i [65], and double whole-cell clamped Novikoff hepatoma cells that also express Cx43 were insensitive to internal solutions well buffered to pH 6.1 (Fig. 1A) [24]. A $\text{pH}_i = 6.4$, achieved by superfusion with acetate, a weak acid that shuttles H^+ by diffusing through the plasma membrane in its neutral H^+ -associated form, uncoupled almost completely crayfish giant axons [22], whereas $\text{pH}_i = 6.0$ did not change G_j in internally perfused axons [66,67]. Low-pH media did not alter the permeability of Cx32 hemichannels incorporated into liposomes [68], and several inconsistencies in the relation between pH_i and electrical coupling were reported in insect gland cells [48].

In several studies, similar pH_i achieved by different procedures seemed to have very different effects on coupling. Cytosolic acidification of amphibian blastomeres to $\text{pH}_i = 6$, caused by superfusion of solutions gassed with 100% CO_2 , brought G_j to zero, whereas a much larger degree of acidification was required to decrease G_j by the same amount when low pH_i was induced by injection of HCl, as pK_a shifted from ~ 6.4 to ~ 5.7 [61]. Furthermore, unilateral HCl injection caused a small degree of acidification in the uninjected cells [61], which indicates that H^+ does not self-limit its cell–cell diffusion. A similar discrepancy in uncoupling efficiency between cytosolic acidification induced by weak diffusible acids (CO_2 , acetate, etc.) and acidification caused by cytosolic dialysis with a strong acid (HCl) was reported by Dunina-Barkowska et al. [69]. Bevan and Harris [68] found that heteromeric Cx32/Cx26 hemichannels incorporated into liposomes were insensitive to low pH when H^+ was buffered with maleate, bicarbonate or Tris, but showed some pH sensitivity in the presence of aminosulfonate buffers, and concluded that H^+ affects gating indirectly via protonation of aminosulfonates (taurine, in intact cells; reviewed in Ref. [70]). Finally, the relative insensitivity of coupling to acidification alone has been confirmed in astrocytes subjected to ischemia [28].

Although much of these data seems to suggest an indirect effect of H^+ on channel gating, in one study it appears that direct protonation affects gating [71]. In this study, the activity of Cx46 hemichannels, expressed in *Xenopus* oocytes and monitored by patch clamp in excised patches, was found to be sensitive to cytoplasmic pH. Cx46 hemichannels rapidly and reversibly closed with short exposures to low pH, although channel closure was poor reversibility

with longer exposures. Half-maximal reduction of open channel probability occurred at pH 6.4. Further work is needed to determine whether the direct effect of protons is peculiar to these hemichannels only and whether other connexins behave like Cx46 when tested with this approach.

3.3. Does Ca^{2+} mediate the effect of H^+ on the channel gates?

In crayfish axons a marked curve hysteresis was observed in the R_j – $[\text{H}^+]_i$, but not in the R_j – $[\text{Ca}^{2+}]_i$ relationship, as R_j and $[\text{Ca}^{2+}]_i$ curves were similar in shape and their maxima coincided in time [22]. This led us to conclude that in these cells G_j is a function of $[\text{Ca}^{2+}]_i$, and that H^+ probably acts as a trigger to Ca^{2+} release [22,23]. Similarly, in *Xenopus* oocytes expressing Cx38, we observed a drastic difference in time course between G_j and pH_i , whereas pCa_i , measured with fura- C_{18} (a membrane associated Ca^{2+} -indicator), and G_j minima were closely matched [56]. Furthermore, in these cells injection of BAPTA to attain an intracellular concentration of ~ 2 mM strongly inhibited (halved) the effect of CO_2 on G_j [56].

In cardiac cells studied by dual whole-cell clamp, G_j decreased markedly with 100% CO_2 when the pipette solutions were buffered for Ca^{2+} with EGTA [72]. We obtained the same result in Novikoff hepatoma cells internally buffered with EGTA, but not with BAPTA [24]. Indeed, EGTA is not as reliable as BAPTA as intracellular Ca-buffer for several reasons. First, the Ca-EGTA affinity constant drops by two orders of magnitude with a decrease in pH from 7 to 6, whereas that of Ca-BAPTA decreases only slightly with the same pH drop [24]. Second, calculations of the gradient of $[\text{Ca}^{2+}]$ in the vicinity of a channel pore show that EGTA solutions are very ineffective in buffering $[\text{Ca}^{2+}]$ within macromolecular distances from the pore, while faster buffers such as BAPTA are quite effective [73,74]. This is consistent with our data showing strong dependence of CO_2 uncoupling efficiency on the type of Ca-buffer used (EGTA versus BAPTA), and further indicates that acidification uncouples via an increase in cytosolic Ca^{2+} that is not adequately buffered by EGTA [24]. Indeed, one would expect the opposite result if H^+ , rather than Ca^{2+} , were affecting the channels directly, because with CO_2 EGTA would bind H^+ and release Ca^{2+} such that $[\text{Ca}^{2+}]_i$ would increase more, and $[\text{H}^+]_i$ less, than with BAPTA. Thus, with lowered pH_i , EGTA becomes a Ca^{2+} releasing agent, rather than a Ca^{2+} chelator.

Two reports on cardiac cells suggested synergism between Ca^{2+} and H^+ in reducing G_j [14,18]. Burt [14] tested the effect of cytosolic acidification in the presence or absence of treatments expected to raise $[\text{Ca}^{2+}]_i$, as well as the effect of elevation of $[\text{Ca}^{2+}]_i$ alone, on dye diffusion between cultured neonatal myocardial cells. In this study, acidosis without increase in $[\text{Ca}^{2+}]_i$ only reduced dye coupling by 10%, whereas acidosis with elevation of $[\text{Ca}^{2+}]_i$ reduced dye coupling by 78%. In contrast, treatments expected just to

increase $[\text{Ca}^{2+}]_i$ had no effect on dye coupling. In this study, however, the extent of $[\text{Ca}^{2+}]_i$ and $[\text{H}^+]_i$ changes could not be determined as neither $[\text{Ca}^{2+}]_i$ nor $[\text{H}^+]_i$ was measured. White et al. [18] reported that cytosolic acidification by 100% CO_2 in the presence of normal $[\text{Ca}^{2+}]_o$ decreased pH_i to 6.0, increased $[\text{Ca}^{2+}]_i$ from ~ 150 to ~ 500 nM, and substantially reduced G_j . In contrast, the same CO_2 application in low $[\text{Ca}^{2+}]_o$ decreased pH_i to 5.9–6.0, only increased $[\text{Ca}^{2+}]_i$ from ~ 60 to ~ 240 nM, and had no effect on G_j . These results were interpreted to indicate that, although other factors may be involved, H^+ and Ca^{2+} affect G_j synergistically [18].

In contrast, our data on Novikoff cells, which also express Cx43, do not suggest synergism between Ca^{2+} and H^+ in reducing G_j , but rather further confirm evidence that Ca^{2+} mediates the effect of H^+ on channel gating [24]. Novikoff cell pairs were buffered to different pH_i and pCa_i values and monitored for G_j decay over time. With $\text{pCa}_i \geq 6.9$ (pH 7.2 or 6.1), G_j decreased following single exponential decays with a time constant (τ) of ~ 28 min; with $\text{pCa}_i = 6$ –6.3 (pH 7.2 or 6.1), G_j decreased with a τ of ~ 6 min; with $\text{pCa}_i 5.5$ (pH 7.2) the cells uncoupled in less than 1 min ($\tau = \sim 20$ s). Low pH_i affected neither time course nor shape of G_j decay at any of the pCa_i tested. Indeed, just glancing at the data, it seems that at $\text{pCa}_i \geq 6.9$ coupling is preserved better at $\text{pH}_i = 6.1$ than at $\text{pH}_i = 7.2$, as the τ 's are 35.2 and 22.3 min, respectively (Fig. 1A); however, this difference is not statistically significant ($P = 0.06$). These data indicate that the Cx43 channels of Novikoff cells are sensitive to $[\text{Ca}^{2+}]_i$ in the physiological range (≥ 500 nM) and that low pH_i neither decreases G_j nor increases channel sensitivity to Ca^{2+} [24].

Lack of synergism between Ca^{2+} and H^+ was further supported in astrocytes subjected to ischemia [28]. Cotrina et al. [28] reported that the reduction of gap junction permeability resulting from application of a calcium ionophore (lasalocid) was not potentiated by cytosolic acidification to $\text{pH}_i = 6.4$ or 6.0 induced by exposure to lactic acid (a weak, diffusible acid); furthermore, exposure to the calcium ionophore by itself uncoupled the astrocytes without changing pH_i .

4. Are the $\text{Ca}^{2+}/\text{H}^+$ effects on gating mediated by a soluble intermediate?

Johnston and Ramón [66] reported the inability of Ca^{2+} and H^+ to uncouple internally perfused crayfish axons. These data, later confirmed by Arellano et al. [67], prompted these investigators to propose that a soluble cytosolic intermediate mediates the $\text{Ca}^{2+}/\text{H}^+$ -induced cell–cell uncoupling [66]. In the same year, we proposed calmodulin (CaM) as soluble intermediate [75,76]. Recently, the likelihood that a soluble intermediate participates in the uncoupling mechanism has been further supported by evidence that internally perfused oocytes expressing Cx43 are insen-

sitive to acidification [65]. Consistent with this idea may also be the observation that neither low pH nor high Ca^{2+} media alter the permeability of Cx32 hemichannels incorporated into liposomes [68]. This study also reported that heteromeric Cx32/Cx26 hemichannels display some pH sensitivity in media buffered with aminosulfonate, while they are insensitive to pH with maleate, bicarbonate or Tris buffer. This led to the conclusion that H^+ may gate indirectly via protonated aminosulfonates (taurine, in intact cells; reviewed in Ref. [70]).

In the last two decades, a number of studies have supported the idea that chemical gating induced by changes in $[\text{Ca}^{2+}]_i$ or $[\text{H}^+]_i$ may be mediated by CaM, although the mechanism by which CaM activation may lead to channel gating is still unclear. Evidence for CaM participation in channel gating is based on data generated by various experimental approaches, including: application of CaM blockers, inhibition of CaM expression, overexpression of CaM mutants, colocalization of CaM and gap junctions by immunofluorescence microscopy, and in vitro testing of CaM binding to connexins and synthetic connexin peptides (see Sections 4.1–4.3).

4.1. Role of calmodulin in channel gating

CaM is an ubiquitous protein of 148 amino acids whose sequence is extremely well conserved from plants to mammals. It is a soluble acidic protein shaped like a dumbbell due to the presence of two roughly spherical convoluted lobes. A short NH_2 -terminus chain is followed by the N-lobe which is joined to the C-lobe by a flexible linker. Each lobe contains two specialized domains known as EF-hands [45] that bind Ca^{2+} with affinities in the nanomolar range. The Ca^{2+} affinity of the two EF-hand domains of the C-lobe is approximately one order of magnitude greater than that of the EF-hand domains of the N-lobe. Ca^{2+} binding to apo-CaM induces a conformational change that exposes two hydrophobic pockets, one in each lobe. Ca^{2+} -CaM binds to a receptor domain, usually structured as a basic amphiphilic α -helix, by interacting with it hydrophobically and electrostatically. In a number of cases, CaM has also been shown to bind to certain target domains in Ca^{2+} -independent manners.

During the past two decades, a significant amount of data in support of the CaM hypothesis for gap junction channel gating has been accumulated, but only recently some evidence for a direct CaM role in channel gating has surfaced. If proven, this would add gap junction channels to a family of CaM regulated membrane channels that include: Ca^{2+} -activated Na^+ and K^+ channels of *Paramecium*, Trp1 (Transient-receptor-potential-like) nonspecific Ca^{2+} channels of *Drosophila melanogaster*, ryanodine receptor channels, small-conductance Ca^{2+} -activated K^+ channels, intermediate conductance Ca^{2+} -dependent K^+ channels, L-type Ca^{2+} channels, P/Q-type Ca^{2+} channels, and Na^+ channels, among others (reviewed in Ref. [77]).

4.2. Effect of CaM inhibitors and inhibition of CaM expression

In 1981, a CaM role in chemical gating was suggested by the ability of a CaM antagonist (trifluoperazine) to prevent CO_2 -induced uncoupling of *Xenopus* embryonic cells [75,76]. Subsequently, more specific CaM blockers (calmidazolium and W7) were found to prevent uncoupling of *Xenopus* embryonic cells [78] and crayfish axons [79], respectively. Similar results were reported in cardiac [80,81] and lens [39,82] cells. In pairs of guinea pig ventricular myocytes, in which one cell was voltage-clamped and G_j was measured after perforation of the non-junctional membrane of the partner cell, the gap junction sensitivity to Ca^{2+} -induced gating increased from pCa 5.7 to pCa 7 upon perfusion with 10 μM CaM, and W7 (but not W5) protected the cells from Ca^{2+} -induced uncoupling [83]. In some cases, CaM inhibitors have also been shown to improve coupling [78,84]. In contrast, CaM inhibitors were found to uncouple insect epidermal cells [85] and Hansen cells of the guinea pig cochlea [86].

The CaM idea was also tested by monitoring the effect of CO_2 on G_j in *Xenopus* oocytes in which CaM expression was previously inhibited by injection of oligonucleotides antisense to CaM mRNA [56]. CaM mRNA was permanently degraded in 5 h and the effect of CO_2 on G_j was reduced by $\sim 60\%$ in 24 h, by $\sim 76\%$ in 48 h and by $\sim 93\%$ in 72 h (Fig. 2). Oocytes that had lost gating sensitivity to CO_2 partially recovered gating competency following calmodulin injection [56].

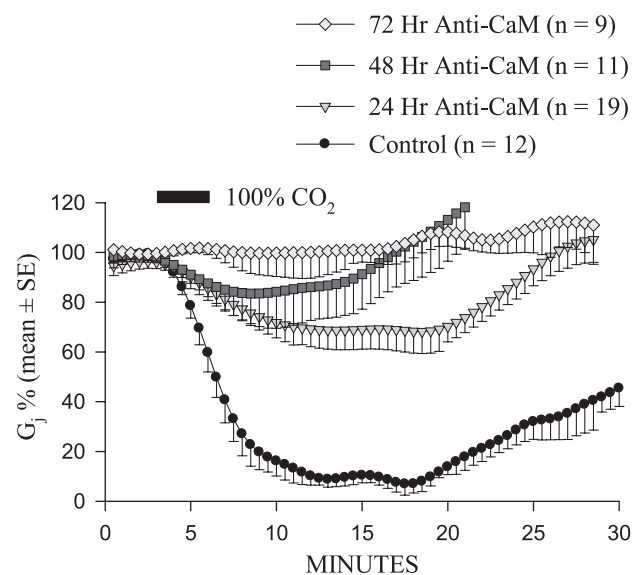


Fig. 2. Normal CaM expression is needed for uncoupling by CO_2 . In *Xenopus* oocyte pairs expressing the native connexin (Cx38), inhibition of CaM expression with oligonucleotides antisense to CaM mRNA prevents CO_2 -induced uncoupling. With 3-min CO_2 application, G_j decreased by 96% in control oocytes, and only by 35%, 18% and 1% at 24, 48 and 72 h, respectively, after CaM-antisense injection. From Ref. [56].

4.3. Testing the CaM hypothesis with CaM mutants

To further explore the CaM participation in gating, we have tested the effects of expressed CaM mutants on chemical and V_j gating of Cx32 channels [87]. Two mutants were tested: CaMCC and CaMNN [87]. In CaMCC, the N-terminal EF hand pair (residues 9–76) is replaced by a duplication of the C-terminal pair (residues 82–148), whereas in CaMNN the N-terminal pair replaces the C-terminal pair. Since the Ca^{2+} affinity constant of the C-terminal EF hand pair is almost one order of magnitude greater than that of the N-terminal pair [88], we felt that expression of CaMCC might result in increased chemical gating sensitivity. Indeed, in oocytes expressing CaMCC, the G_j of junctions made of Cx32 was very low, but dramatically increased when $[\text{Ca}^{2+}]_i$ was lowered with BAPTA superfusion, suggesting that CaMCC increases the Ca^{2+} sensitivity of gating to such an extent that even basal $[\text{Ca}^{2+}]_i$ affects gating (Fig. 3A). This was confirmed by testing the effect of CO_2 application. With 3-min superfusions of solutions gassed with 100% CO_2 , G_j rapidly dropped to zero, whereas in controls it decreased only by $\sim 15\%$. Significantly, CaMCC was effective only when it was expressed before Cx32 (Fig. 3B). The relevance of the CaMCC/Cx32 expression sequence suggests that CaMCC, and by extension native CaM, associates with Cx32 before connexon assembly; this indicates that CaM may be an integral, regulatory subunit of the connexon. The intimate relationship between CaMCC and Cx32 was confirmed by a large reduction in V_j sensitivity (Fig. 3C). Significantly, expression of CaMCC after Cx32 did not affect V_j sensitivity (Fig. 3C). These data suggest that CaMCC, and by extension CaM, is closely associated with Cx32 channels, because V_j gating is believed to be an inherent property of the Cx molecule. In interpreting the data obtained with

CaMCC, we have considered a potential source of artifact involving Cx38, the native oocyte connexin known to make channels highly sensitive to CO_2 . A possible scenario is that CaMCC blocks the expression of functional Cx32 channels and that the BAPTA treatment opens Cx38 channels. Although this scenario is possible, we feel that it is very unlikely: first, because the Cx32/CaMCC channels were studied 48–96 h after the oocytes were injected with oligonucleotides antisense to Cx38 mRNA, which elimi-

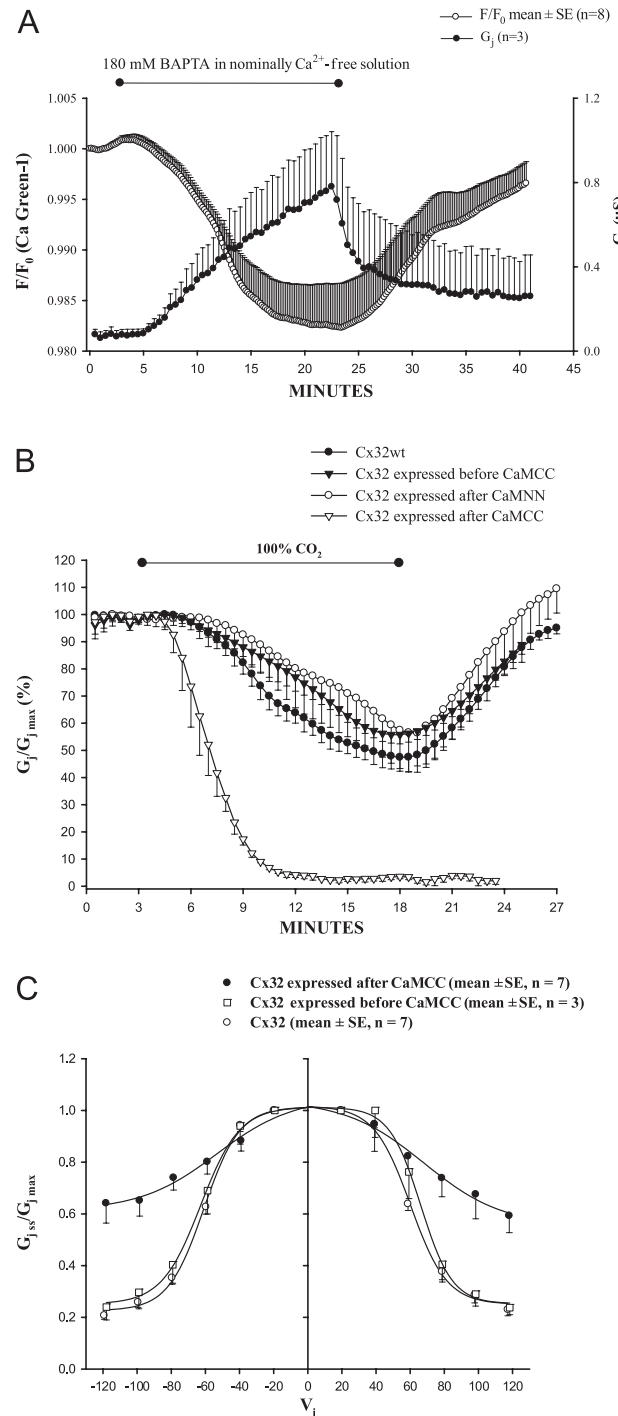


Fig. 3. Expression in *Xenopus* oocytes of CaMCC (a CaM mutant with higher overall Ca^{2+} affinity) alters both chemical (A and B) and V_j (C) gating sensitivities of Cx32 channels. (A) In oocytes expressing CaMCC before Cx32, G_j is very low, but increases reversibly with superfusion of 180 μM BAPTA in nominally Ca-free solutions, simultaneously with a drop in $[\text{Ca}^{2+}]_i$ (monitored with Calcium Green-1, A). (B) Cx32 channels expressed after CaMCC are much more sensitive to CO_2 than controls; in contrast, expression of CaMNN (a CaM mutant with lower Ca^{2+} affinity) or expression of CaMCC after Cx32 has no effect on chemical gating. The relevance of CaMCC–Cx32 expression sequence indicates that CaMCC must be present in the oocyte when Cx32 is being synthesized and/or assembled in the connexon. This suggests that CaM is an integral subunit of the connexon. (C) With CaMCC expressed before Cx32, the V_j sensitivity of Cx32 channels is drastically reduced, as shown here by the relationship between normalized G_j and V_j . With CaMCC expressed before Cx32, G_j drops with V_j : (?120 mV) by only $\sim 40\%$, whereas with native CaM or with CaMCC expressed after Cx32, it drops by $\sim 75\%$. In addition, with CaMCC expressed before Cx32, the number of equivalent gating charges (η) moving through the applied field is halved; note the sharply reduced steepness of the Boltzmann curve. The effect of CaMCC on V_j sensitivity further suggests that CaM is intimately associated with Cx32. From Ref. [87].

nates virtually all of the Cx38 channels; second, because Cx38 channels are very sensitive to V_j , their Boltzmann values being $V_0 = 35$ mV, $G_j \text{ min} = 0.3$ and $\eta = 3$ [55], whereas the Cx32/CaMCC channels were very insensitive to V_j , their Boltzmann values being $V_0 = 61$ mV, $G_j \text{ min} = 0.6$ and $\eta = 1$ (Fig. 3C) [87]. In the unlikely event that the channels studied were Cx38 channels, one would have to conclude that CaMCC blocks Cx32 channel formation and that Cx38 channels are sensitive to sub-basal $[\text{Ca}^{2+}]_i$ (in the low nanomolar range), a value that would only activate proteins endowed with high affinity EF-hand Ca^{2+} binding domains [45], such as CaM.

Recently, we have also tested CaM mutants lacking one or more of the four high affinity Ca^{2+} -binding sites. In these CaM mutants, glutamates (E) relevant for Ca^{2+} binding have been replaced with alanines (A) in CaM's EF-hand domains; these mutations were shown to dramatically reduce the Ca^{2+} affinity of the Ca^{2+} -binding EF-hand loops [89]. Expression in oocytes of CaM_{1,2,3,4} (E32A, E68A, E105A, E141A) or CaM_{1,2} (E32A, E68A), preceding expression of Cx32, effectively blocked the formation of functional Cx32 channels (Fig. 4A and B), whereas expression of CaM_{3,4} (E105A, E141A) had no effect (Fig. 4C). The effectiveness of CaM_{1,2,3,4} and CaM_{1,2} indicates that both of these CaM mutants are able to successfully compete with wild-type CaM in binding to CaM target sites in Cx32 or in other proteins relevant for Cx32 channel formation. The fact that CaM_{1,2,3,4} is effective suggests that CaM interacts with the relevant target(s) in Ca^{2+} -independent way. Conversely, the observation that CaM_{1,2}, but not CaM_{3,4}, prevents channel formation indicates that a normal Ca^{2+} -affinity of the EF hand domains of CaM's N-lobe is needed for channel formation. As a hypothesis, we propose that the C-lobe of CaM binds to a Cx32 site in a Ca^{2+} -independent way and that Ca^{2+} -binding to the N-lobe enables CaM to bind to another site of Cx32 or to a neighboring Cx32 monomer initiating the oligomerization of connexin monomers into hexameric connexons.

4.4. Colocalization of CaM and connexins

The CaM–Cx32 association was tested by immunofluorescence microscopy. In HeLa cells stably transfected with Cx32, CaM and Cx32 colocalized in punctated or linear areas of cell–cell contact as well as in a few punctated areas of the cytoplasm, likely to correspond to gap junctions retrieved from the plasma membrane and/or to Cx32 in the Golgi apparatus (Fig. 5) [87]. Similar results were obtained with Cx43 and Cx37 [90]. These results confirm earlier data by immuno-electron microscopy reporting CaM binding to gap junctions of myocardial cells stained in frozen thin sections with colloidal gold-labeled CaM [91].

Direct CaM–Cx32 interaction was also confirmed by confocal immunofluorescence microscopy in HeLa cells expressing Cx32 linked to green fluorescent protein (Cx32-CFP) and CaM linked to red fluorescent protein (CaM-RFP).

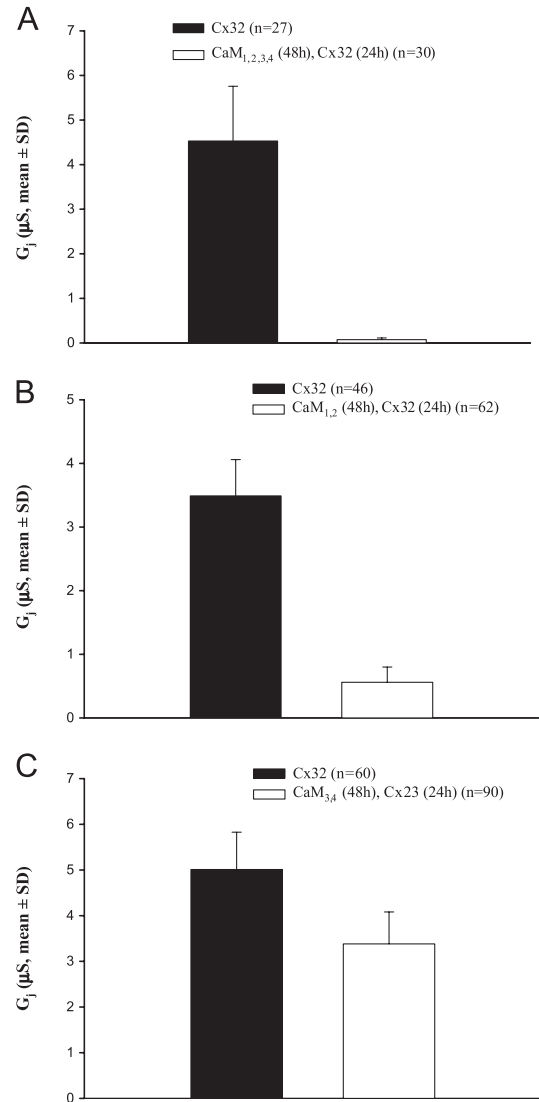


Fig. 4. Effect of expressing Ca^{2+} -insensitive CaMs on the formation of functional Cx32 channels in *Xenopus* oocytes. Expression of CaM_{1,2,3,4} (E32A, E68A, E105A, E141A) or CaM_{1,2} (E32A, E68A) before Cx32 strongly inhibits Cx32 channel formation, as demonstrated in A and B, respectively, by the drastic difference in G_j after 2–6 h of pairing. In contrast, expression of CaM_{3,4} (E105A, E141A) had no significant effect (C). These data indicate that normal Ca^{2+} binding to EF-hand sites of CaM's N-lobe is needed for channel formation. Since CaM_{1,2,3,4} appears to successfully compete against native CaM, it is likely that CaM binds to Cx32 or an accessory molecule in a Ca^{2+} -independent way.

However, in these samples CaM and Cx32 only colocalized at cytoplasmic spots, as these cells did not form junctional plaques (Fig. 6) [90]. The absence of junctional plaques in cells co-expressing Cx32-GFP and CaM-RFP may be due to steric hindrance, as the large size of the two fusion proteins may be excessive to permit the oligomerization of connexins into connexons. Similar results were obtained by expressing Cx32 linked to cyan fluorescent protein (Cx32-CFP) and CaM linked to yellow fluorescent protein (CaM-YFP) [90].

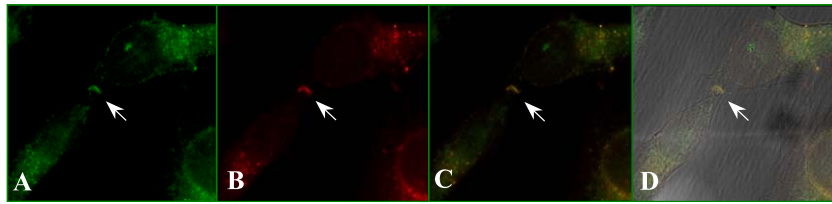


Fig. 5. Immunofluorescence microscopy of HeLa cells stably transfected with Cx32 and sequentially labeled for Cx32 and CaM. A and B show labeling for CaM and Cx32, respectively. C shows the overlay of A and B, and D adds to the overlay the bright field image. Note the colocalization of CaM and Cx32 at the junctional site (arrow) and at most, but not all, of the cytoplasmic spots.

4.5. Evidence of CaM binding to connexins and connexin peptides

Hertzberg and Gilula [92] demonstrated the ability of CaM to bind to Cx32 in gel overlays. Similar data of CaM binding to Cx32 and Cx32 fragments were later reported by various groups [93–95]. CaM binding to Cx32 was also indirectly demonstrated by evidence that CaM prevents both Cx32 proteolysis by *m*-calpain [96] and Cx32 phosphorylation by EGF receptor tyrosine kinase [97]. CaM interaction with connexins is also indirectly suggested by evidence for its participation in Cx32 oligomerization into connexons in vitro [98].

There are three potential CaM binding sites in Cx32, two at the NH₂-terminus (NT): residues ~ 1–22 (MJWTGL-YTLLSGVNRHSTAIGR; site-N1) and residues ~ 18–33 (TAIGRVWLSVIFIFRI; site-N2), and one at CT: residues ~ 208–226 (EVVYLIIRACARRAQRSSN; site-C1). Sites N1 and C1 are most likely in the cytoplasm, whereas site-N2 may be at the transition between NT and the first transmembrane domain (TM1). Site-N2 and site-C1 had been identified early on [99] through a computer program that selects basic amphiphilic α -helical domains. The CaM binding capacity of synthetic peptides matching site-C1 of Cx32, Cx38 and Cx43 was demonstrated by spectrofluorometry and circular dichroism spectroscopy [100,101]. More recently, Török et al. [95] have used a fluorescent derivative of CaM (TA-CaM) to identify CaM-binding

domains of Cx32. Several Cx32 peptides were tested, corresponding to NT, the cytoplasmic loop (CL), the initial segment of CT, and the two extracellular loops. Only two of these peptides bound CaM in Ca²⁺-dependent way: a peptide matching site-N1 and one matching most of site-C1 (residues 216–230). The dissociation constants (K_d) of TA-CaM binding to these peptides were 27 nM and 1.2 μ M, respectively [95]. In collaboration with Katalin Török, we are presently further testing the CaM binding to sites N1, N2 and C1 using FRET-based DA-CaM [102]. Preliminary data indicate that site-N1 binds DA-CaM with ~ 1 μ M K_d and is displaced by the Trp peptide of the Myosin Light Chain Kinase (MLCK), whereas site-C1 shows a complex binding behavior, it is not displaced by the Trp peptide of MLCK even at >10 μ M concentrations, and appears to bind with stoichiometry >1:1.

Whether corresponding domains of other connexins also bind CaM needs to be determined. Significantly, a triptophan (W) residue believed to be most relevant for CaM binding at site N-1 [95] is present within the first four residues of NT in all connexins except for Cx29, a connexin that does not form functional channels (Peracchia, unpublished).

4.6. Participation of cam activated enzymes

Although there is evidence for a direct role of CaM in gating, one should keep in mind that there are several

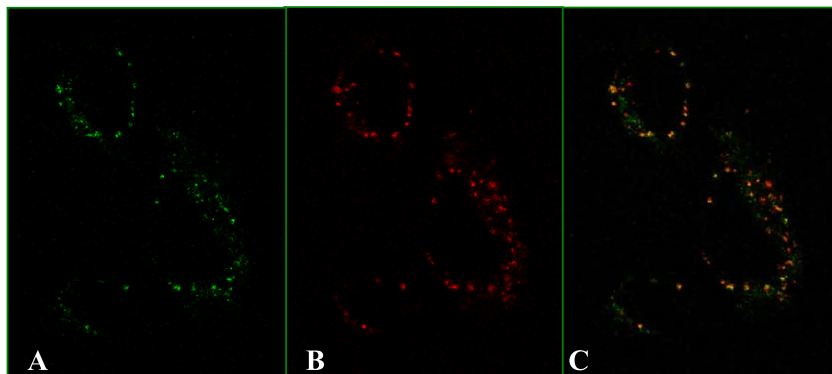


Fig. 6. Fluorescence microscopy of two HeLa cells co-expressing Cx32 linked GFP (Cx32-GFP) and CaM linked to RFP (CaM-RFP). CX32-GFP (A) and CaM-RFP (B) colocalize at most, but not all, of the cytoplasmic spots, as seen in the overlay (C) of A and B. Significantly, these cells did not form junctional plaques, as no fluorescence was seen at cell–cell contacts. This may be due to steric hindrance, as the larger size of the two fusion proteins may impair the oligomerization of Cx32 into connexons.

indirect ways in which Ca^{2+} -CaM could affect channel function; for example, via Ca^{2+} /CaM-dependent protein kinases and phosphatases. Indeed, phosphorylation of Cx32 by Ca^{2+} /CaM kinase II has been shown to occur, but only in isolated junctions, as intact hepatocytes exposed to the Ca^{2+} -ionophore ionomycin did not become phosphorylated [103]. Recently, activation of Ca^{2+} /CaM kinase II has been shown to increase junctional conductance of mouse astrocytes [104] and goldfish Mauthner cells [105], but whether mechanisms other than connexin phosphorylation are involved is still unclear.

Over the years, we have frequently questioned the possibility that CaM participates in channel gating via enzyme activation, by testing the effect of inhibitors and/or activators of various enzymes (unpublished data). For calcineurin (Ca–CaM-dependent phosphatase 2B), we tested FK506 and calcineurin injection; for nitric oxide (NO) synthase: nitroprusside; for Ca^{2+} /CaM kinase II: KN-62; for MAP kinase kinase: PD098,059; for phospholipase A2: aristolochic acid; for cytochrome 480: FKS 525A; for most kinases: staurosporin; for phosphatase 1 and 2A: okadaic acid. None of them significantly affected G_j and/or CO_2 -induced gating. A role of Ca^{2+} -activated proteases is also unlikely because connexin proteolysis would be irreversible, and the recovery rate of Ca^{2+} -induced uncoupling is considerably faster than the turnover time of connexins (half life = ~ 3 h) [106]. Therefore, although an indirect CaM participation cannot be entirely ruled out yet, based on present evidence, a direct CaM role in gating seems more likely.

5. Connexin domains potentially relevant to chemical gating

In an attempt to identify Cx32 domains that may participate in the mechanism of chemical gating, we have studied in *Xenopus* oocytes the CO_2 sensitivity of channels made of chimeras and mutants of Cx32 and Cx38, two connexins whose channels are at the opposite end of the spectrum in CO_2 sensitivity. The replacement of Cx32's CL with Cx38's CL conferred to Cx32 channels the high CO_2 sensitivity of Cx38 channels, suggesting that CL plays a key role in CO_2 sensitivity [55]. In contrast, NT swap between Cx32 and Cx38 did not alter significantly CO_2 gating sensitivity [55].

CT chimeras did not express functional channels, but basic residue mutations at its initial 18 residue segment (site-C1), and CT deletions yielded interesting results [106,107]. Although much of Cx32's CT is irrelevant, as 84% deletion of it (at residues 219) does not affect CO_2 sensitivity [107,109], the presence of basic residues in site-C1 appears to be one of the reasons for the low CO_2 sensitivity of Cx32 channels. This is suggested by the behavior of mutants in which the five positively charged arginines (R215, R219, R220, R223 and R224) of site-C1

are replaced either with neutral polar residues, asparagine (N) or threonine (T), or with other basic residues, lysine (K) or histidine (H). 5R/N and 5R/T mutations greatly increased the sensitivity of Cx32 channels to CO_2 (Fig. 7). The increase in CO_2 sensitivity resulting from 5R/N mutation was seen both with full-length Cx32 (5R/N, Fig. 7) and with Cx32 in which CT was deleted at residue 225 (D225-5R/N, Fig. 7). In contrast, 5R/K and 5R/H mutant channels were as sensitive as Cx32 wild-type (Cx32wt) [107,108]. The five R residues were not equally effective, as CO_2 sensitivity appears to be strongly inhibited by R215 and mildly by R219, whereas R220, R223 and R224, rather than inhibit, may slightly increase CO_2 sensitivity [107,108]. Mutation of the five R residues to E (5R/E) increases the CO_2 gating sensitivity even more [110]. Thus, the mutants rank is as follows in CO_2 sensitivity: 5R/E > 5R/N > Cx32wt. Interestingly, site-C1 has been identified as a CaM binding domain [95,99] and theoretically its basic residues would be expected to be relevant for CaM interaction. Therefore, the increased gating sensitivity of 5R/N and 5R/E mutants is somewhat paradoxical, because it suggests that the greater the theoretical reduction in CaM binding affinity the greater the CO_2 gating sensitivity. This may mean that CaM or one of the two CaM lobes needs to be released from this site for gating to occur.

In Cx43, CT regions more distal than the corresponding site-C1 of Cx32 are believed to play a major role in chemical gating, because deletion of CT at residue 257

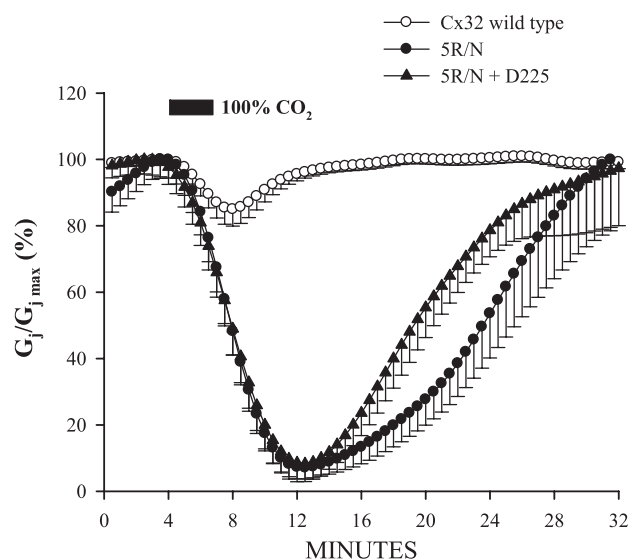


Fig. 7. CO_2 gating sensitivity of Cx32 mutants in which five arginines (R215, R219, R220, R223 and R224) of the initial CT domain (site-C1; potential CaM binding domain) were replaced with asparagines (N) in either Cx32wt (5R/N) or Cx32 deleted at residue 225 (D225-5R/N). Note that the R/N replacement strongly enhances the CO_2 sensitivity of both 5R/N and D225-5R/N with respect to control Cx32wt (the CO_2 gating sensitivity of the Cx32 mutant D225 is the same as that of Cx32wt, data not shown). This suggests that the presence of basic residues in site-C1 is at least one of the reasons for the low CO_2 sensitivity of Cx32 channels. From Ref. [107].

strongly reduces the sensitivity to CO₂ [57,111]. Data showing that co-expression of CT-deleted Cx43 and CT as separate molecules results in full recovery of CO₂ sensitivity prompted Delmar and co-workers to propose a “particle-receptor” gating model, in which the CT would close the channel by binding to a receptor domain located somewhere else in Cx43 [58,112] (see Section 7).

6. Slow gate, chemical gate and calmodulin

Gap junction channels are believed to possess more than one gate. V_j gradients activate two types of gates: a fast and a slow gate. Chemical uncouplers activate a chemical gate that behaves in terms of kinetics and efficiency identically to the slow V_j gate [113,114]. Changes in membrane potential (V_m) activate an additional gate (V_m gate), which is only present in few connexin channels. Although chemical gate and slow V_j gate are usually referred to as separate gates, evidence is accumulating in favor of the idea that they may in fact be the same gate.

6.1. Chemical uncouplers close single channels by activating a slow gate

The electrical behavior of individual gap junction channels can be monitored by double whole-cell clamp (DWCC) when cell coupling is minimal, such as in poorly coupled cell pairs, during channel formation, or at the final stages of chemical uncoupling. Interesting data on single channel gating were obtained in rat fibroblasts and HeLa cells stably transfected with Cx43 during exposure to solutions gassed with 100% CO₂ [113]. In this study, junctional current (I_j),

single channel conductance and I_j kinetics were studied during uncoupling and recoupling at different V_j gradients, which enabled us to distinguish the behavior of the chemical (CO₂-sensitive) gate from that of the fast V_j -sensitive gate. Since in Cx43 channels V_j gating only occurs at V_j gradients >40–50 mV, by monitoring CO₂-induced chemical gating at three V_j gradients: V_j =30 mV (fast V_j gates open), V_j =55 mV (fast V_j gates flickering) and V_j =70 mV (fast V_j gates mostly closed), the individual behavior of the two gates as well as their potential interplay could be studied in detail. In the absence of V_j -gating (V_j =30 mV), CO₂ caused exclusively slow I_j transitions from open to closed channel states (mean transition time: ~10 ms), corresponding to a single channel conductance of ~120 pS. At V_j =55 mV, the V_j -gating induced fast I_j flickering between open, γ_j (main state), and residual, γ_j (residual), states (transition time: ~2 ms), with a γ_j (residual)/ γ_j (main state) ratio of 20–25% (Fig. 8). At V_j =70 mV, in addition to slow I_j transitions between open and closed states, CO₂ induced slow transitions between residual and closed states. This indicates that chemical and fast V_j gates operate independently. During recoupling (Fig. 8), each channel reopened by a slow transition (mean transition time: ~10 ms) from closed to open state (rarely from closed to residual state). Fast I_j flickering between open and residual states followed (Fig. 8).

The data are consistent with the idea that gap junction channels possess two major gating mechanisms [115] and indicate that CO₂ induces channel gating exclusively by the slow gating mechanism. Similar results were obtained in cells uncoupled by long-chain *n*-alkanols or arachidonic acid [116], indicating that slow-gating-mediated single-channel closure is not restricted to CO₂, but is common to

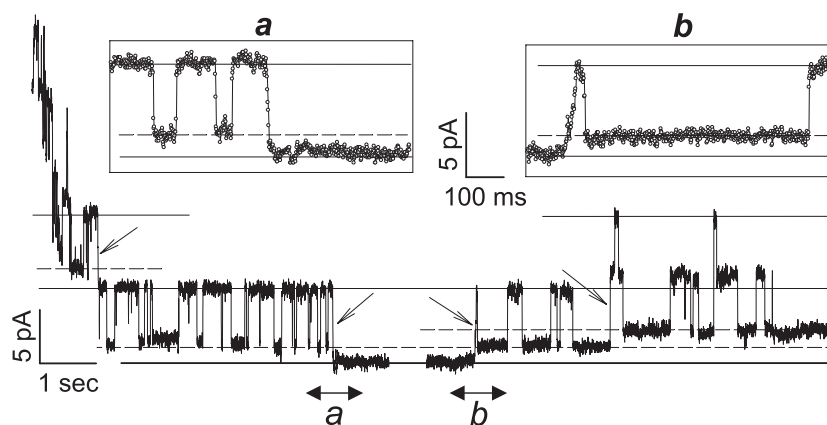


Fig. 8. Single channel evidence for two distinct gating mechanisms. Effect of CO₂ on single channel gating in gap junctions of fibroblast pairs studied by dual whole-cell patch clamp and subjected to V_j =55 mV. Exposure to 100% CO₂ reversibly reduces G_j to zero. With only a few channels operational, the gating behavior of individual channels is monitored just before complete uncoupling (left trace and inset a) and at the beginning of recoupling (right trace and inset b). Each channel closes by a single 125-pS transition of slow kinetics (~10 ms; slow gating) from open state, γ_j (main state), to closed state (left trace, arrows). This follows the conventional channel flickering behavior characterized by frequent 90–95-pS transitions of fast kinetics (<1 ms; fast V_j gating) between γ_j (main state) and γ_j (residual state; dashed line). The channels reopen, during recoupling, by first undergoing a conductance transition of slow kinetics from closed state to open state (right trace, arrows), followed by the usual flickering behavior between open and residual states (fast V_j gating). The difference in kinetics between fast V_j gating and slow gating is clearly visible in insets a and b, which display segments of the main trace (a and b, respectively) at an expanded scale (sampling rate=1 ms). From Ref. [113].

other chemical gating agents as well. A subsequent study provided evidence for V_j sensitivity of the chemical gate, suggesting that the chemical gate is a slow V_j -sensitive gate [110] (see Section 6.3).

6.2. Connexin mutations unmask a slow V_j -sensitive gate

In most types of connexin channels, with the possible exception of Cx45 channels [117,118], the activity of the slow V_j gate is rarely observed in the absence of chemical uncouplers. However, this gate appears to spontaneously manifest itself in a variety of channels made of connexin mutants. Recently, we have studied several Cx32 mutants that generate channels with pronounced slow V_j gating characteristics when paired heterotypically with Cx32wt [110,119]. The mutants are: tandem, 5R/E, 5R/N, ML/NN, ML/CC, 3R/N and ML/NN+3R/N. In a tandem, two Cx32 monomers are linked N- to C-terminus. In 5R/E and 5R/N, five CT arginines (R215, R219, R220, R223 and R224) are replaced with glutamates (E) and asparagines (N), respectively. In ML/NN and ML/CC, two CL residues, methionine (M105) and leucine (L106), are replaced with N and cysteines (C), respectively. In 3R/N, the residues R215, R219 and R220 are replaced with N. In ML/NN+3R/N, two mutations listed above are combined. The slow V_j gating behavior of these mutant channels is qualitatively the same; therefore, for simplicity it will be exemplified here by that of heterotypic channels generated by pairing tandem expressing oocytes with Cx32wt oocytes (tandem-32).

In contrast to homotypic Cx32 junctions (32–32), which show a characteristic sensitivity to V_j , with I_j decaying exponentially with time for $V_j > \pm 40$ mV, tandem-32 channels display a unique I_j – V_j behavior (Fig. 9A). With mutant side negative, as V_j is increased in 20-mV steps from 20 to 120 mV, the initial and final I_j progressively decrease to very low values, and V_j sensitivity is apparent even at the lowest V_j . With mutant side positive, I_j progressively increases to high values, so that I_j at the end of the pulse is greater than the initial I_j . Only at the largest V_j gradients (100–120 mV), a more conventional I_j behavior starts appearing (Fig. 9A). This results in a large asymmetry in the relation between V_j and normalized G_j ($G_{j\text{ ss}}/G_{j\text{ max}}$; Fig. 9B).

This I_j/V_j behavior suggested that V_j negative or positive at mutant side progressively closes or opens, respectively, an increasing number of channels. This was tested with trains of long 60-mV V_j pulses positive at tandem side. Three distinct I_j behaviors were observed during the train of 60-mV pulses: a monophasic I_j increase (pulses #1–3); a biphasic I_j time-course (pulses #4–9), characterized by initial progressive I_j increase followed by exponential I_j decay; and a conventional I_j behavior (pulses #10–18), depicted by initial I_j peak followed by exponential I_j decay to a steady-state level (Fig. 9C). These data confirmed the hypothesis that repeated application of pulses positive at mutant side eventually opens (renders operational) all of the

available channels (from pulse #10 onwards), which then display the normal behavior of the fast V_j gate of the adjoined Cx32wt hemichannels.

To determine the time course of G_j changes in response to positive and negative V_j at mutant side, mutant-32 channels were subjected to prolonged, steady-state, V_j gradients. Steady-state V_j of 40 mV, positive at tandem side, slowly increased G_j by as much as 400% (Fig. 10A), whereas V_j negative at tandem side decreased it by over 85% (Fig. 10B). Upon return to $V_j = 0$ from $V_j = 40$ mV (tandem side positive), G_j increased abruptly before dropping, due to the reopening of the conventional V_j -sensitive gates of Cx32wt hemichannels (Fig. 10A). As expected, this was not observed when the V_j polarity was reversed from positive to negative (Fig. 10B).

The slow change in G_j was interpreted as a gating phenomenon based on the activity of a slow V_j gate distinct from the fast V_j gate. There are several reasons for making this distinction [110], one being that in all of the connexins tested the slow V_j gate always closes at the negative side of V_j , whereas the fast V_j gate closes at negative or positive side of V_j depending on the type of connexin tested. For example, heterotypic channels between Cx26 and a Cx26 mutant (4pos/E), in which the basic residues of CT were mutated to E (R215E, K220E, K222E and R223E), behaved qualitatively as tandem-32 channels when exposed to V_j gradients [1], in spite of the fact that the fast V_j gates of Cx26 and Cx32 are sensitive to opposite voltage polarities [120].

An intriguing question is why most disparate connexin mutations unmask the same slow gating mechanism. In Cx32, a variety of point mutations in NT, TM1, CL or CT have been shown to generate the slow gating behavior when mutant hemichannels are paired heterotypically to Cx32wt hemichannels. In NT: S11D, R15W, R15Q [121] and R15N [122]; in TM1: M34T, V35M and V38M [123]; in CL: deletion of residues 111–116 [124] and a double mutation: M105N and L106N [119]; in CT: the mutation of five arginines (R215, R219, R220, R223 and R224) to N or E, individually or in groups [110,119], or combined with M105N and L106N [119].

In the absence of uncouplers, Cx32 channels may be in balance between two states: open and closed state. In Cx32wt the balance may be in favor of the open state, whereas disparate mutations unbalance the mutant hemichannel favoring to different degrees the closed state. Closed and open state could be interconverted by V_j , with V_j positive and negative at mutant side opening and closing, respectively, the mutant hemichannel. In any event, we feel that slow gating is a very intriguing phenomenon that may very well hold the key for unlocking the mystery of the chemical gating mechanism.

6.3. Is the chemical gate V_j -sensitive?

Mutant-32 channels were much more sensitive to CO_2 than 32–32 channels, and G_j , reduced to low values by

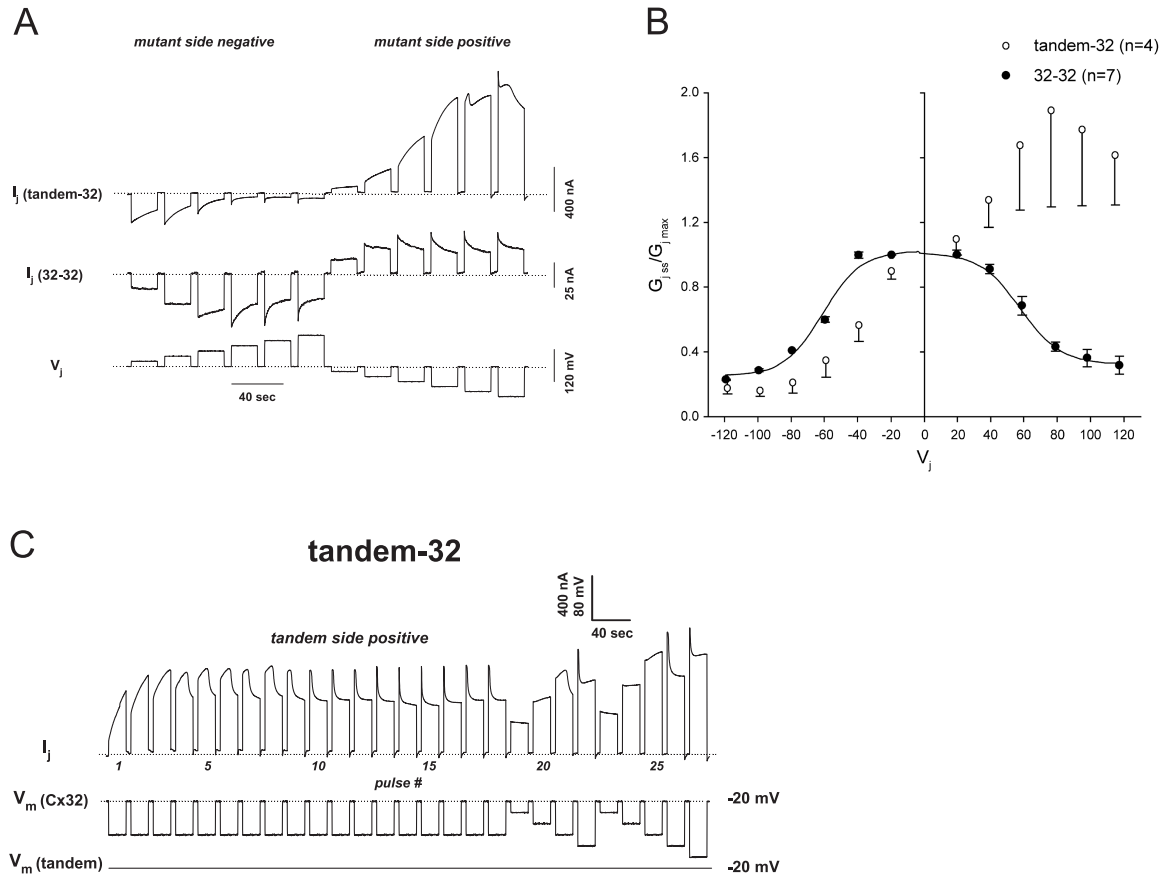


Fig. 9. Slow gating of heterotypic mutant-Cx32 channels. (A) Response of I_j to V_j pulses in *Xenopus* oocyte pairs expressing homotypic Cx32wt channels (32–32) or heterotypic channels between tandem and Cx32wt (tandem-32). 32–32 channels display a characteristic sensitivity to V_j , as I_j decays exponentially with time for V_j values ≥ 40 mV. In contrast, tandem-32 channels displays a unique I_j/V_j behavior. With tandem side negative (left trace), as V_j is increased from 20 to 120 mV, initial and final I_j progressively decrease to very low values, and V_j sensitivity seems present even at $V_j = 20$ mV. With tandem side positive (right trace), I_j progressively increases to high values, and I_j increases rather than decreases from the initial I_j ; only with V_j values of 100–120 mV two more conventional I_j levels are seen. (B) The asymmetrical I_j/V_j behavior of tandem-32 channels is clearly demonstrated by plots of normalized G_j versus V_j . (C) Response of I_j to repeated V_j pulses in *Xenopus* oocyte pairs expressing tandem-32 channels. With trains of 60-mV V_j pulses (tandem side positive), three distinct I_j behaviors are observed: a monophasic increase in I_j (pulses #1–3); a biphasic I_j time-course (pulses #4–9), with initial I_j increase followed by exponential I_j decay; and finally, a conventional I_j behavior (pulses #10–18). Subsequent applications of conventional V_j protocols (tandem side positive) result in a fairly conventional I_j behavior (pulses 19–27). This intriguing voltage behavior suggest that V_j gradients positive at tandem side progressively open an increasing number of channels; thus, the normal V_j behavior can only be observed when all of the channels have opened (have become operational). From Ref. [110].

CO₂, increased dramatically and reversibly upon application of V_j positive at mutant side, indicating that V_j opened channels that were closed by the CO₂ treatment (Fig. 11A) [110,119]. Conversely, V_j negative at mutant side further reduced G_j , indicating that negative V_j complements CO₂-induced gating (Fig. 11A).

This observation raised the possibility that the chemical gate is voltage-sensitive and further suggested that chemical gate and slow V_j gate are the same [110]. Evidence for it is also provided by the observation that the degree of slow gating sensitivity to V_j of the mutants tested corresponds reasonably well to their CO₂ sensitivity [119]. An effect of voltage on chemical gating has also been reported in insect cells [116], but in this case the chemical gate is sensitive to membrane potential (V_m) rather than to V_j . Additional evidence favoring the idea that chemical gate and slow V_j gate are the same comes from data on mutant channels

expressed in oocytes in which CaM expression was inhibited with oligonucleotides antisense to CaM mRNA [119]. Within 24–48 h after the injection of CaM antisense oligonucleotides, the “slow gating” behavior of mutant-32 channels was greatly reduced or eliminated (Fig. 11B) and tandem-32 channels assumed a symmetrical G_j/V_j relationship very similar to that of 32–32 channels (Fig. 11C). This observation, in conjunction with previous CaM data [125], suggests that CaM may be involved in both chemical gating and “slow gating” mechanisms.

In apparent contradiction with the idea that chemical gate and slow gate are the same are recent single channel data indicating that CT-truncation of Cx40 at residue 249 [126] or Cx43 at residue 257 [127] does not eliminate slow V_j -sensitive gating, while it eliminates completely fast V_j -sensitive gating. This was demonstrated by evidence for loss of residual conductance state and presence of slow single

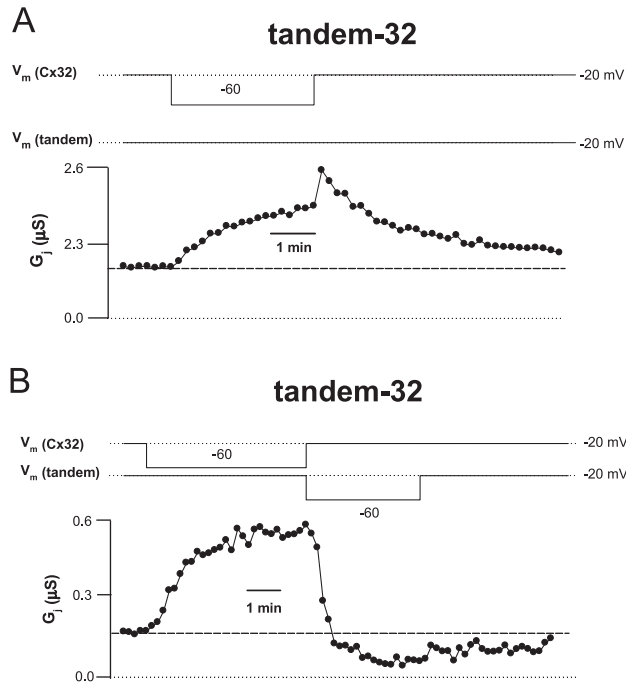


Fig. 10. Slow gating of heterotypic tandem-Cx32 channels. G_j response to steady state V_j gradients in *Xenopus* oocyte pairs expressing tandem-32 channels. (A) In oocytes initially clamped at $V_m = -20$ mV ($V_j = 0$), a 40-mV V_j step (tandem side positive) exponentially increases G_j with a $\tau \sim 1$ min. Upon return to $V_j = 0$ mV, G_j slowly returns to control values. (B) With V_j reversal to from 40 mV (tandem side positive) to 40 mV (tandem side negative), G_j decreases exponentially to nearly 0 μ S, indicating that the relative negativity at the tandem side actively closes channels. Interestingly, upon return to $V_j = 0$ from $V_j = 40$ mV, tandem side positive, G_j increases abruptly at first, before dropping (A). This is due to the reopening of the fast V_j -sensitive gates. Indeed, the abrupt increase in G_j is not observed when V_j is reversed from positive to negative at tandem side (B), because in this case, as the fast V_j gates open at the Cx32wt side they close at the tandem side. From Ref. [110].

channel current transitions from open state to fully closed state, and vice versa, typical of the behavior of both slow gating and chemical gating. The preservation of the slow gating mechanism in these CT-truncated connexins seemed at odd with previous claims for loss of chemical gating with CT-truncation [59,111,112], because, based on the ball-and-chain model [112], one would have expected the opposite result, namely: loss of slow V_j -gating and preservation of fast V_j -gating. A possibility is that slow gating and chemical gating involve different molecular mechanisms, in spite of their virtually identical efficiency and kinetics at the single channel level [113,114]. However, one should note that CT-truncation of Cx43 and Cx40 does not eliminate chemical gating sensitivity, but rather reduces it [57,59,]. Indeed, early data showed that CT-truncation of Cx43 reduced the pK_a of the G_j -pH_i relationship from 6.6 to 6.1, and resulted in a much steeper Hill coefficient (6.0 versus 4.2) such that at pH_i values just below 6 all of the channels made of CT-truncated Cx43 were closed [57]. This clearly indicates that CT-truncation does not eliminate chemical gating by cytosolic acidification,

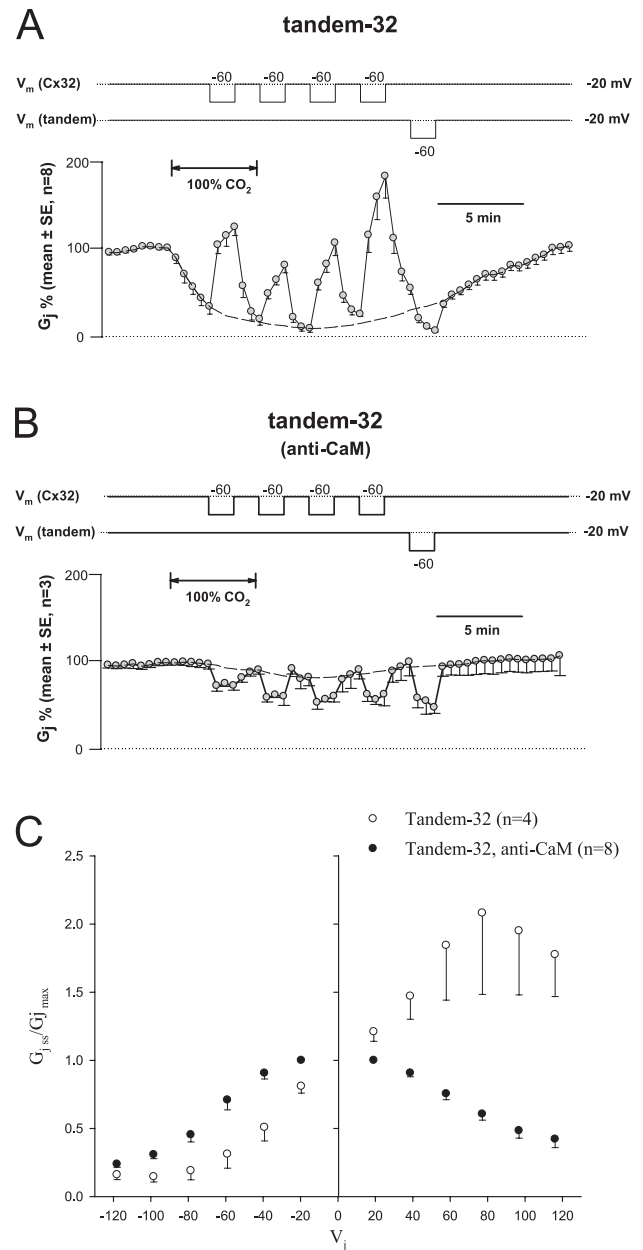


Fig. 11. Chemical gating, V_j gating and calmodulin. (A) Effect of V_j on G_j during exposure to 100% CO_2 in oocyte pairs expressing tandem-32 channels. G_j , reduced to low values by CO_2 at $V_j = 0$, increases significantly and reversibly with the application of V_j gradients positive at tandem side, whereas V_j negative at tandem side reversibly reduces G_j to very low values. (B) With inhibition of CaM expression, CO_2 has minimal effect on G_j , and, as in homotypic Cx32wt channels [110], both positive and negative V_j at tandem side decrease G_j . These data indicate that both chemical and slow V_j gating phenomena depend on CaM. The dashed lines in A and B indicate the predicted time course of G_j in the absence of V_j gradients. From Refs. [110,119]. (C) The asymmetrical V_j behavior of tandem-32 channels is virtually abolished with inhibition of CaM expression, such that tandem-32 channels behave virtually identically to 32–32 channels (Fig. 9B). This further indicates that the slow V_j gating phenomenon manifested in tandem-32 channels depends on CaM. From Ref. [119].

but just reduces gating sensitivity by shifting the G_j – pH_i relationship. Furthermore, it is very possible that with CT-truncation, the chemical/slow gate of Cx43 and Cx40 maintains a virtually normal V_j -sensitivity in spite of decreasing significantly in chemical sensitivity. Thus, it is not unreasonable to believe that in C-terminus truncated Cx43 and Cx40 the chemical/slow gate is still present.

6.4. Is the V_j -gate chemically sensitive?

Over a decade ago, CO_2 applications were shown to increase the V_j -sensitivity of Cx32 channels expressed in oocytes [109], suggesting that V_j -gating is sensitive to cytosolic changes induced by CO_2 . Recently, we have tested this hypothesis by studying the CO_2 sensitivity of V_j -gating in a number of connexin channels expressed in oocytes. In Cx45 channels, with CO_2 -induced drop in G_j , the speed of V_j -dependent inactivation of junctional current (I_j) and V_j sensitivity reversibly increased [118]. With 40 mV V_j , the τ of single exponential I_j decay reversibly decreased by $\sim 40\%$ with CO_2 , and $G_{j \text{ steady state}}/G_{j \text{ peak}}$ decreased multiphasically, suggesting that both speed and sensitivity of V_j -dependent inactivation of Cx45 channels are significantly increased by changes in $[H^+]_i$ and/or $[Ca^{2+}]_i$ induced by CO_2 [118]. Similar results were obtained with Cx32 channels [122]. In contrast, sensitivity and speed of V_j gating of Cx40 channels decreased, rather than increased, with CO_2 application, suggesting that there might be two populations of connexin channels that manifest opposite V_j gating behaviors following cytosolic acidification [128]. These observations indicate that further probing of the effect of chemicals on V_j gates may provide important clues on gating mechanisms.

7. Models of chemical gating

In spite of four decades of work, the molecular mechanism of chemical gating is still unclear. Among possible mechanisms one could conceive global conformational changes in connexins, physical obstruction of the pore by flexible connexin domains, participation of cytosolic molecules either as pore plugging elements or activators of conformational changes in connexins, or other mechanisms altogether. Over the years, at least three models have been proposed. However, none of them should be considered more than working hypotheses, as they are based mainly on circumstantial evidence.

An early model proposed that channel gating results from a rotation of the six hemichannel's connexins [129,130], with displacement toward the lumen of large hydrophobic residues (phenylalanines, F) [131]. Indeed, the third transmembrane segment (TM3), a connexin domain believed to participate in lining the pore, contains F residues, ranging from one to four among connexins, that would line up more or less on the same side of an α -helical domain. Of the four

F residues of Cx32: F141, F145, F149 and F152, the most conserved among connexins is F149. This model is certainly attractive, but tests of it through F mutations have given disappointing results. The combined Cx32 mutation of F145 and F149 to valine (V), a smaller hydrophobic residue unlikely to be bulky enough to obstruct the pore, generated channels indistinguishable from Cx32wt channels in CO_2 gating sensitivity (Wang and Peracchia, unpublished data).

More recently, Delmar co-workers have proposed a gating model similar to the “ball-and-chain” type of voltage-dependent sodium and potassium channels [132,133]. This model [58,111,112], later referred to as “particle-receptor” model, envisions gating as the result of interaction between a distal CT domain and a receptor domain likely to be part of CL. Consistent with this model, at least for Cx43, may be recent in vitro evidence from experiments combining resonant mirror technology, enzyme-linked sorbent assay and nuclear magnetic resonance (NMR), that demonstrate an interaction between CT (residues 255–382) and a peptide corresponding to the second half of CL (residues 119–144), that is enhanced by low pH [134]. The particle-receptor model, however, may only be applicable to few connexins [59], because aside from Cx43, CT deletion was reported to be partially effective in Cx40 channels, minimally in Cx37, and ineffective in Cx45 and Cx32 [59,107,109], and obviously in Cx26, whose CT is very short (18 residues). In addition, recent evidence of normal gating sensitivity to CO_2 or heptanol in HeLa cells expressing channels made of Cx43 linked at its CT to Green Fluorescent Protein (GFP) [114] is difficult to reconcile with the particle-receptor model, as it is hard to believe that a protein (GFP), similar in size to connexins, linked to CT, has no effect on the proposed interaction between the distal CT domain and its CL receptor needed for CO_2 -induced gating. Indeed, this study further supported the role of CT in fast V_j gating, as Cx43-GFP channels lost the residual conductance state [114], behaving like in CT-truncated Cx43 [127] and Cx40 [126] channels.

While Cx43 channels may be gated by a particle-receptor type mechanism, different models must be considered for other connexin channels such as those made of Cx32, in which CT deletion does not alter chemical gating sensitivity [107,109]. For Cx32 channels, we have recently proposed a “cork-type” gating model in which gating would involve physical obstruction of the cytoplasmic mouth of the pore by a CaM lobe [1]. What induced us to propose a cork-type gating mechanism was the evidence that the chemical/slow gate is likely to be a sizable, negatively charged particle [110,119]. This interpretation was based on the behavior of heterotypic channels between a number of Cx32 mutants and Cx32wt. As described above, in these channels, G_j slowly increased by severalfold or decreased to nearly zero with V_j positive or negative, respectively, at mutant side, and V_j positive at mutant side partially reversed CO_2 -induced uncoupling. Channel closing with V_j negative at mutant side would result from the attractive force exerted on the

negatively charged gate by positive V_j at the Cx32wt side, sensed through the channel lumen. Conversely, channel opening with V_j positive at mutant side would result from the repulsive force exerted on the negatively charged gate by the negative V_j at Cx32wt side. CaM seemed a likely gating candidate because: (1) it is a negatively charged protein known to bind to Cx32; (2) each of its lobes are ~ 25 Å in diameter, which is the approximate size of the cytoplasmic mouth of the channel [135]; (3) both chemical and slow gating efficiencies are eliminated by inhibition of CaM expression [56,118,119]; and (4) a CaM mutant (CaMCC) with higher overall Ca^{2+} affinity alters chemical and V_j gating sensitivities [87]. The cork model envisions two CaM-mediated gating mechanisms: CaM-driven and Cx-driven. In the CaM-driven mechanism, gating would be initiated by a change in CaM (Ca^{2+} -activation); in the Cx-driven mechanism, gating would require a change in Cx caused by Cx mutation or posttranslational modification.

The CaM-driven mechanism is exemplified by the CO_2 -induced gating of homotypic Cx32wt channels. In these channels, CO_2 -induced changes in $[\text{Ca}^{2+}]_i$ would activate CaM and enable a CaM lobe to physically obstruct the pore by binding to a receptor site near the pore, perhaps both electrostatically and hydrophobically. The tight interaction between the CaM lobe and its Cx receptor would render this gating mechanism insensitive to V_j (not reversible by positive V_j at the gated hemichannel side).

The Cx-driven gating mechanism is exemplified by the behavior of mutant-32 channels. At the mutant hemichannel side, a CaM lobe would be free to interact with the channel's mouth in the absence of uncouplers. The CaM lobe would interact loosely (only electrostatically?) with the positively charged channel's mouth and could be displaced by the electric field generated by V_j negative at the Cx32wt hemichannel side. The protein regions forming the cytoplasmic mouth of the channel are likely to be positively charged, as the cytoplasmic connexin domains have a high ratio of basic versus acidic residues; for example, in Cx32 if we neglect most of CT, which can be deleted by over 80% without affecting chemical gating sensitivity [107,109], and assign a charge value of 1 for R, K, D and E residues and 1/2 for H residues, we count 18 basic and 6 acidic residues per connexin. Obviously, based on the short range of electric-field's effectiveness, the V_j -sensitive slow gating phenomena observed with Cx32 mutants could only occur if the relevant CaM lobe were already very close to the channel's mouth.

Due to the high Ca^{2+} affinity constant of the C-lobe's EF-hand pair, which is almost one order of magnitude greater than that of the N-lobe's pair [88], it is likely that the N-lobe becomes activated only when $[\text{Ca}^{2+}]_i$ increases above basal level, whereas Ca^{2+} may be bound to the C-lobe at all times; thus, in the cork model one may envision the N-lobe as being the gating element, activated by an increase in $[\text{Ca}^{2+}]_i$ above basal levels, and the C-lobe as being the anchoring element, linking CaM to Cx32 at basal $[\text{Ca}^{2+}]_i$. The C-lobe may be anchored to Cx32's NT

(perhaps site-N1), and Ca^{2+} -activation of CaM may cause the N-lobe to interact with another receptor site (perhaps site-N2) electrostatically and hydrophobically (CaM-driven type). At basal $[\text{Ca}^{2+}]_i$, the N-lobe may be latched to site-C1 in Ca^{2+} -independent way and become unlatched with an increase in $[\text{Ca}^{2+}]_i$. Indeed, some CaM modulated proteins paradoxically bind CaM with higher affinity in the absence than in the presence of Ca^{2+} , a typical example being neuromodulin [136].

In mutant-32 channels, a weakening of the interaction between the N-lobe and its latching site (site-C1?) at the mutant hemichannel may free the N-lobe allowing it to interact electrostatically with the channel's mouth (Cx-driven type). This would explain why mutations of the five R residues of site-C1 to N (5R/N), and even more so to E (5R/E), which are expected to reduce significantly or eliminate entirely the CaM affinity to this site, paradoxically enhanced both slow V_j gating sensitivity and CO_2 sensitivity of heterotypic mutant-32 channels [110,119]. Ca^{2+} -activation of CaM would consolidate the interaction of the N-lobe with the channel's mouth by causing it to bind to its receptor site (site-N2?) electrostatically and hydrophobically, as in Cx32wt channels (CaM-driven type).

Cx-driven and CaM-driven gating mechanisms may imply that there are at least two closed-states. Indeed, some evidence for both V_j -sensitive and V_j -insensitive closed-states was provided by the behavior of heterotypic mutant-32 channels subjected to V_j gradients during CO_2 uncoupling [110,119]. In these channels, V_j positive at mutant side reopened channels less and less effectively as uncoupling progressed, to such an extent that at full uncoupling the effect on G_j of positive V_j at mutant side was barely

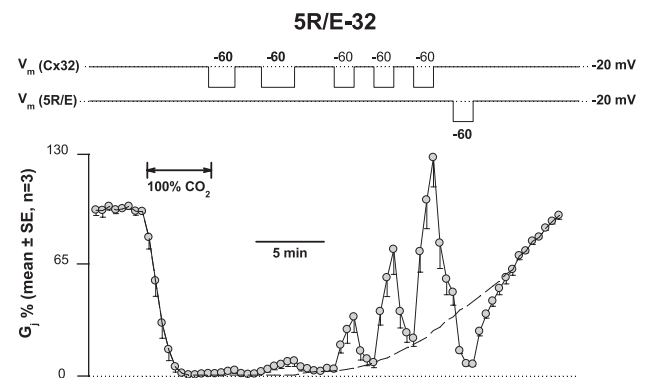


Fig. 12. Evidence for two closed channel states: V_j -sensitive and V_j -insensitive. As seen with tandem-32 channels (Fig. 11A), with 5R/E-32 channels, G_j reduced to low values by CO_2 at $V_j=0$ increases significantly and reversibly upon application of V_j gradients positive at the 5R/E side; however, in these channels, G_j increases much less at maximal uncoupling. This phenomenon, which is particularly obvious with mutant-32 channels very sensitive to CO_2 such as 5R/E-32, suggests that there might be two closed states: V_j -sensitive and V_j -insensitive. The former may involve only electrostatic interactions between the gate and the channel mouth; the latter, both electrostatic and hydrophobic interactions. The dashed line indicates the predicted time course of G_j in the absence of V_j gradients. From Ref. [110].

detectable (Fig. 12). This seemed to imply that, as uncoupling progressed, more and more channels underwent a transition from a closed-state reversible by V_j to one not reversible by V_j . We thought that the former might involve only weak (electrostatic?) interactions between CaM's N-lobe and Cx32 (Cx-driven type) and the latter, more stable (electrostatic and hydrophobic?) interactions (CaM-driven type).

Present gating models are believed to be relevant to specific connexin channels only. However, although it is possible that channels made of different connexins close by different gating mechanisms, such as the particle-receptor mechanism for Cx43 and the cork mechanism for Cx32 channels, it seems unlikely that evolution has generated a variety of connexin-specific mechanisms of chemical and voltage gating. Therefore, it would not be surprising if, in the future, findings that presently appear contradictory and seem to point to different mechanisms, in fact, turn out to have a common ground and, at the molecular level, reflect the same basic gating mechanism.

References

- [1] C. Peracchia, X.G. Wang, L.L. Peracchia, Behavior of chemical- and slow voltage-sensitive gating of connexin channels: the "cork" gating hypothesis, in: C. Peracchia (Ed.), *Gap Junctions. Molecular Basis of Cell Communication in Health and Disease*, Academic Press, San Diego, CA, 2000, pp. 271–295.
- [2] T.W. Engelmann, Vergleichende Untersuchungen zur Lehre von der Muskel und Nerven elektricität, *Arch. Ges. Physiol.* 15 (1877) 116–148.
- [3] J. Déléze, Calcium ions and the healing-over in heart fibers, in: B. Taccardi, C. Marchetti (Eds.), *Electrophysiology of the Heart*, Pergamon, Elmsford, New York, 1965, pp. 147–148.
- [4] J. Déléze, The recovery of resting potential and input resistance in sheep heart injured by knife or laser, *J. Physiol.* 208 (1970) 547–562.
- [5] W.R. Loewenstein, Y. Kanno, Studies on an epithelial(gland) cell junction: I. Modifications of surface membrane permeability, *J. Cell Biol.* 22 (1964) 565–586.
- [6] S.W. Kuffler, D.D. Potter, Glia in the central nervous system: physiological properties and neuron–glia relationship, *J. Neurophysiol.* 27 (1964) 290–320.
- [7] W.R. Loewenstein, Permeability of membrane junctions, *Ann. N. Y. Acad. Sci.* 137 (1966) 441–472.
- [8] W.R. Loewenstein, Junctional intercellular communication: the cell-to-cell membrane channel, *Physiol. Rev.* 61 (1981) 829–913.
- [9] C. Peracchia, Structural correlates of gap junction permeation, *Int. Rev. Cytol.* 66 (1980) 81–146.
- [10] B. Rose, W.R. Loewenstein, Permeability of cell junction depends on local cytoplasmic calcium activity, *Nature* 254 (1975) 250–252.
- [11] B. Rose, W.R. Loewenstein, Permeability of a cell junction and the local cytoplasmic free ionized calcium concentration: a study with aequorin, *J. Membr. Biol.* 28 (1976) 87–119.
- [12] W.C. De Mello, Effect of intracellular injection of calcium and strontium on cell communication in heart, *J. Physiol.* 250 (1975) 231–245.
- [13] G. Dahl, G. Isenberg, Decoupling of heart muscle cells: correlation with increased cytoplasmic calcium activity and with changes of nexus ultrastructure, *J. Membr. Biol.* 53 (1980) 63–75.
- [14] J.M. Burt, Block of intercellular communication: interaction of intracellular H^+ and Ca^{2+} , *Am. J. Physiol.* 253 (1987) C607–C612.
- [15] P. Maurer, R. Weingart, Cell pairs isolated from adult guinea pig and rat hearts: effects of $[Ca^{2+}]_i$ on nexal membrane resistance, *Pflügers Arch.* 409 (1987) 394–402.
- [16] A. Noma, N. Tsuboi, Dependence of junctional conductance on proton, calcium and magnesium ions in cardiac paired cells of guinea-pig, *J. Physiol.* 382 (1987) 193–211.
- [17] R.D. Veenstra, R.L. DeHaan, Cardiac gap junction channel activity in embryonic chick ventricle cells, *Am. J. Physiol.* 254 (1988) H170–H180.
- [18] R.L. White, J.E. Doeller, V.K. Verselis, B.A. Wittenberg, Gap junctional conductance between pairs of ventricular myocytes is modulated synergistically by H^+ and Ca^{2+} , *J. Gen. Physiol.* 95 (1990) 1061–1075.
- [19] L. Firek, R. Weingart, Modification of gap junction conductance by divalent cations and protons in neonatal rat heart cells, *J. Mol. Cell. Cardiol.* 27 (1995) 1633–1643.
- [20] D.C. Spray, J.H. Stern, A.L. Harris, M.V. Bennett, Gap junctional conductance: comparison of sensitivities to H and Ca ions, *Proc. Natl. Acad. Sci. U. S. A.* 79 (1982) 441–445.
- [21] J. Neyton, A. Trautmann, Single-channel currents of an intercellular junction, *Nature* 317 (1985) 331–335.
- [22] C. Peracchia, 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.* 113 (1990) 75–92.
- [23] C. Peracchia, 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.* 117 (1990) 79–89.
- [24] A. Lazrak, C. Peracchia, Gap junction gating sensitivity to physiological internal calcium regardless of pH in Novikoff hepatoma cells, *Biophys. J.* 65 (1993) 2002–2012.
- [25] A. Lazrak, A. Peres, S. Giovannardi, C. Peracchia, Ca-mediated and independent effects of arachidonic acid on gap junctions and Ca-independent effects of oleic acid and halothane, *Biophys. J.* 67 (1994) 1052–1059.
- [26] M.O.K. Enkvist, K.D. McCarthy, Astroglial gap junction communication is increased by treatment with either glutamate or high K^+ concentration, *J. Neurochem.* 62 (1994) 489–495.
- [27] C. Giaume, L. Venance, Characterization and regulation of gap junction channels in cultured astrocytes, in: D.C. Spray, R. Dermietzel (Eds.), *Gap Junctions in the Nervous System*, RG Landes Medical Pub, Austin, TX, 1996, pp. 135–157.
- [28] M.L. Cotrina, J. Kang, J.H. Lin, E. Bueno, T.W. Hansen, L.L. He, Y.L. Liu, M. Nedergaard, Astrocytic gap junctions remain open during ischemic conditions, *J. Neurosci.* 18 (1998) 2520–2537.
- [29] J.M. Crow, M.M. Atkinson, R.G. Johnson, Micromolar levels of intracellular calcium reduce gap junctional permeability in lens cultures, *Investig. Ophthalmol. Vis. Sci.* 35 (1994) 3332–3341.
- [30] D. Mears, N.F. Sheppard Jr., I. Atwater, E. Rojas, Magnitude and modulation of pancreatic β -cell gap junction electrical conductance in situ, *J. Membr. Biol.* 146 (1995) 163–176.
- [31] M. Chanson, P. Mollard, P. Meda, S. Suter, H.J. Jongsma, Modulation of pancreatic acinar cell to cell coupling during ACh-evoked changes in cytosolic Ca^{2+} , *J. Biol. Chem.* 274 (1999) 282–287.
- [32] D.I. Yule, E. Stuenkel, J.A. Williams, Intercellular calcium waves in rat-pancreatic acini: mechanism of transmission, *Am. J. Physiol.* 271 (1996) C1285–C1294.
- [33] K. Schirmacher, D. Nonhoff, M. Wiemann, E. Peterson-Grine, P.R. Brink, D. Bingmann, Effects of calcium on gap junctions between osteoblast-like cells in culture, *Calcif. Tissue Int.* 59 (1996) 259–264.
- [34] L. Lagostena, J.F. Ashmore, B. Kachar, F. Mammano, Purinergic control of intercellular communication between Hensen's cells of the guinea-pig cochlea, *J. Physiol.* 531 (2001) 693–706.
- [35] L. Lagostena, F. Mammano, Intracellular calcium dynamics and membrane conductance changes evoked by Deiters' cell purinoreceptor activation in the organ of Corti, *Cell Calcium* 29 (2001) 191–198.

- [36] C. Peracchia, A. Lazrak, L.L. Peracchia, Molecular models of channel interaction and gating in gap junctions, in: C. Peracchia (Ed.), *Handbook of Membrane Channels. Molecular and Cellular Physiology*, Academic Press, San Diego, 1994, pp. 361–377.
- [37] M.F. Leite, K. Hirata, T. Pusch, A.D. Burgstahler, K. Okazaki, J.M. Ortega, A.M. Goes, M.A. Prado, D.C. Spray, M.H. Nathanson, Molecular basis for pacemaker cells in epithelia, *J. Biol. Chem.* 277 (2002) 16313–16323.
- [38] G.M. Oliveira-Castro, W.R. Loewenstein, Junctional membrane permeability: effects of divalent cations, *J. Membr. Biol.* 5 (1971) 51–77.
- [39] M.M. Lurtz, C.F. Louis, Effect of a sustained increase in intracellular calcium concentration on connexin43 gap junction permeability, *Biophys. J.* 84 (2003) 525.
- [40] F. Verrecchia, J.C. Hervé, Reversible blockade of gap junctional communication by 2,3-butanedione monoxime in rat cardiac myocytes, *Am. J. Physiol.* 272 (1997) C875–C885.
- [41] B. Déléage, J. Déléage, Increase in gap junction conductance of adult mammalian heart myocytes by intracellular calcium ions, in: R. Werner (Ed.), *Gap Junctions*, IOS Press, Amsterdam, 1998, pp. 72–75.
- [42] F. Duthe, I. Plaisance, D. Sarrouilhe, J.C. Hervé, Endogenous protein phosphatase 1 runs down gap junctional communication of rat ventricular myocytes, *Am. J. Physiol.* 281 (2001) C1648–C1656.
- [43] N.L. Allbritton, T. Meyer, L. Stryer, Range of messenger action of calcium ion and inositol 1,4,5-trisphosphate, *Science* 258 (1992) 1812–1815.
- [44] A. Lazrak, A. Peres, S. Giovannardi, C. Peracchia, Calcium participation in ATP and arachidonic acid but not in oleic acid and halothane induced gating of gap junctions in Novikoff cells, in: Y. Kanno, K. Kataoka, Y. Shiba, Y. Shibata, T. Shimazu (Eds.), *Inter-cellular Communication Through Gap Junctions*, Elsevier, Amsterdam, 1995, pp. 451–454.
- [45] R.H. Kretsinger, Hypothesis: calcium modulated proteins contain EF-hands, in: E. Carafoli, F. Clementi, W. Drabnowski, A. Margreth (Eds.), *Calcium Transport in Contraction and Secretion*, Elsevier, Amsterdam, 1975, pp. 469–478.
- [46] L. Turin, A.E. Warner, Carbon dioxide reversibly abolishes ionic communication between cells of early amphibian embryo, *Nature* 270 (1977) 56–57.
- [47] L. Turin, A.E. Warner, Intracellular pH in early *Xenopus* embryos: its effect on current flow between blastomeres, *J. Physiol.* 300 (1980) 489–504.
- [48] B. Rose, R. Rick, Intracellular pH, intracellular Ca, and junctional cell–cell coupling, *J. Membr. Biol.* 44 (1978) 377–415.
- [49] N. Iwatsuki, O.H. Peterson, Pancreatic acinar cells: the effect of carbon dioxide, ammonium chloride and acetylcholine on intercellular communication, *J. Physiol.* 291 (2003) 317–326.
- [50] C. Giaume, M.E. Spira, H. Korn, Uncoupling of invertebrate electrotonic synapse by carbon dioxide, *Neurosci. Lett.* 17 (1980) 197–202.
- [51] C. Giaume, H. Korn, Ammonium sulfate induced uncoupling of septate axons with and without increased junctional resistance, *Neuroscience* 7 (1982) 1723–1730.
- [52] D.C. Spray, A.L. Harris, M.V. Bennett, Gap junctional conductance is a simple and sensitive function of intracellular pH, *Science* 211 (1981) 712–715.
- [53] S.M. Schuetze, D.A. Goodenough, Dye transfer between cells of the embryonic chick lens becomes less sensitive to CO₂ treatment with development, *J. Cell Biol.* 92 (1982) 694–705.
- [54] W.R. Reber, R. Weingart, Ungulate cardiac Purkinje fibres: the influence of intracellular pH on the electrical cell-to-cell coupling, *J. Physiol.* 328 (1982) 87–104.
- [55] X.G. Wang, L.Q. Li, L.L. Peracchia, C. Peracchia, Chimeric evidence for a role of the connexin cytoplasmic loop in gap junction channel gating, *Pflügers Arch.* 431 (1996) 844–852.
- [56] C. Peracchia, X.G. Wang, L.Q. Li, L.L. Peracchia, Inhibition of calmodulin expression prevents low-pH-induced gap junction uncoupling in *Xenopus oocytes*, *Pflügers Arch.* 431 (1996) 379–387.
- [57] S. Liu, S. Taffet, L. Stoner, M. Delmar, M.L. Vallano, J. Jalife, A structural basis for the unequal sensitivity of the major cardiac and liver gap junctions to intracellular acidification: the carboxyl tail length, *Biophys. J.* 64 (1993) 1422–1433.
- [58] G.E. Morley, S.M. Taffet, M. Delmar, Intramolecular interactions mediate pH regulation of connexin43 channels, *Biophys. J.* 70 (1996) 1294–1302.
- [59] K. Stergiopoulos, J.L. Alvarado, M. Mastroianni, J.F. Ek-Vitorin, S.M. Taffet, M. Delmar, Hetero-domain interactions as a mechanism for the regulation of connexin channels, *Circ. Res.* 84 (1999) 1144–1155.
- [60] C. Peracchia, Possible involvement of caffeine- and ryanodine-sensitive calcium stores in low pH-induced regulation of gap junction channels, in: C. Peracchia (Ed.), *Biophysics of Gap Junction Channels*, CRC Press, Boca Raton, FL, 1991, pp. 13–28.
- [61] M.V.L. Bennett, R.L. Verselis, R.L. White, D.C. Spray, Gap junctional conductance: gating, in: E.L. Hertzberg, R.G. Johnson (Eds.), *Gap Junctions*, Alan R. Liss, New York, 1988, pp. 207–304.
- [62] A. Campos de Carvalho, D.C. Spray, M.V. Bennett, pH dependence of transmission at electrotonic synapses of the crayfish septate axon, *Brain Res.* 321 (1984) 279–286.
- [63] M.L. Pressler, Intracellular pH and cell-to-cell transmission in sheep Purkinje fibers, *Biophys. J.* 55 (1989) 53–65.
- [64] W.C. De Mello, The influence of pH on the healing-over of mammalian cardiac muscle, *J. Physiol.* 339 (1983) 299–307.
- [65] B.J. Nicholson, L. Zhou, F. Cao, H. Zhu, Y. Chen, Diverse molecular mechanisms of gap junction channel gating, in: R. Werner (Ed.), *Gap Junctions*, IOS Press, Amsterdam, 1998, pp. 3–8.
- [66] M.F. Johnston, F. Ramón, Electrotonic coupling in internally perfused crayfish segmented axons, *J. Physiol.* 317 (1981) 509–518.
- [67] R.O. Arellano, F. Ramón, A. Rivera, G.A. Zampighi, Lowering of pH does not directly affect the junctional resistance of crayfish lateral axons, *J. Membr. Biol.* 94 (1986) 293–299.
- [68] C.G. Bevans, A.L. Harris, Regulation of connexin channels by pH-direct action of the protonated form of taurine and other aminosulfonates, *J. Biol. Chem.* 274 (1999) 3711–3719.
- [69] A.Y. Dunina-Barkowska, I.M. Bujurina, V.S. Pivovarov, V.A. Frolov, Effects of transmembrane gradients of bicarbonate and ammonium on junctional and non-junctional membrane conductances in bhk cells, *Biol. Membr.* 17 (2000) 637–652.
- [70] A.L. Harris, Emerging issues of connexin channels: biophysics fills the gap, *Q. Rev. Biophys.* 34 (2001) 325–472.
- [71] E.B. Trexler, F.F. Bukauskas, M.V.L. Bennett, T.A. Bargiello, V.K. Verselis, Rapid and direct effects of pH on connexins revealed by the connexin46 hemichannel preparation, *J. Gen. Physiol.* 113 (1999) 721–742.
- [72] J.M. Burt, D.C. Spray, Single-channel events and gating behavior of the cardiac gap junction channel, *Proc. Natl. Acad. Sci. U. S. A.* 85 (1988) 3431–3434.
- [73] A. Marty, E. Neher, Potassium channels in cultured bovine adrenal chromaffin cells, *J. Physiol.* 367 (1985) 117–141.
- [74] M.D. Stern, Buffering of calcium in the vicinity of a channel pore, *Cell Calcium* 13 (1992) 183–192.
- [75] C. Peracchia, G. Bernardini, L.L. Peracchia, A calmodulin inhibitor prevents gap junction crystallization and electrical uncoupling, *J. Cell Biol.* 91 (1981) 124a.
- [76] C. Peracchia, G. Bernardini, L.L. Peracchia, Is calmodulin involved in the regulation of gap junction permeability? *Pflügers Arch.* 399 (1983) 152–154.
- [77] Y. Saimi, C. Kung, Calmodulin as an ion channel subunit, *Annu. Rev. Physiol.* 64 (2002) 289–311.
- [78] C. Peracchia, Communicating junctions and calmodulin. Inhibition of electrical uncoupling in *Xenopus* embryo by calmidazolium, *J. Membr. Biol.* 81 (1984) 49–58.

- [79] C. Peracchia, Calmodulin-like proteins and communicating junctions. Electrical uncoupling of crayfish axons is inhibited by the calmodulin inhibitor W7 and is not affected by cyclic nucleotides, *Pflügers Arch.* 408 (1987) 379–385.
- [80] J.A. Wojtczak, Electrical uncoupling induced by general anesthetics: a calcium-independent process? in: M.V.L. Bennett, D.C. Spray (Eds.), *Gap Junctions*, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York, 1985, pp. 167–175.
- [81] W. Tuganowski, I. Korczynska, K. Wasik, G. Piatek, Effects of calmidazolium and dibutyl cyclic AMP on the longitudinal internal resistance in sinus node strips, *Pflügers Arch.* 414 (1989) 351–353.
- [82] S.A. Gandolfi, G. Duncan, J. Tomlinson, G. Maraini, Mammalian lens inter-fiber resistance is modulated by calcium and calmodulin, *Curr. Eye Res.* 9 (1990) 533–541.
- [83] J. Toyama, H. Sugiura, K. Kamiya, I. Kodama, M. Terasawa, H. Hidaka, Ca^{2+} -calmodulin mediated modulation of the electrical coupling of ventricular myocytes isolated from guinea pig heart, *J. Mol. Cell. Cardiol.* 26 (1994) 1007–1015.
- [84] V. Verselis, A. Campos de Carvalho, R.L. White, M.V.L. Bennett, D.C. Spray, Calmodulin and gap junction regulation, *J. Cell Biol.* 91 (1986) 73.
- [85] J.P. Lees-Miller, S. Caveney, Drugs that block calmodulin activity inhibit cell-to-cell coupling in the epidermis of *Tenebrio molitor*, *J. Membr. Biol.* 69 (1982) 233–245.
- [86] A. Blodow, A. Ngezahayo, A. Ernst, H.A. Kolb, Calmodulin antagonists suppress gap junction coupling in isolated Hensen cells of the guinea pig cochlea, *Pflügers Arch.* 446 (2003) 36–41.
- [87] C. Peracchia, A. Sotkis, X.G. Wang, L.L. Peracchia, A. Persechini, Calmodulin directly gates gap junction channels, *J. Biol. Chem.* 275 (2000) 26220–26224.
- [88] A. Persechini, K.J. Gansz, R.J. Paresi, Activation of myosin light chain kinase and nitric oxide synthase activities by engineered calmodulins with duplicated or exchanged EF hand pairs, *Biochemistry* 35 (1996) 224–228.
- [89] J.R. Geiser, D. van Tuinen, S.E. Brockerhoff, M.M. Neff, T.N. Davis, Can calmodulin function without binding calcium? *Cell* 65 (1991) 949–959.
- [90] A. Sotkis, X.G. Wang, T. Yasumura, L.L. Peracchia, A. Persechini, J.E. Rash, C. Peracchia, Calmodulin colocalizes with connexins and plays a direct role in gap junction channel gating, *Cell Adhes. Commun.* 8 (2001) 277–281.
- [91] K. Fujimoto, N. Araki, K.-S. Ogawa, S. Kondo, T. Kitaoka, K. Ogawa, Ultracytochemistry of calmodulin binding sites in myocardial cells by staining of frozen thin sections with colloidal gold-labeled calmodulin, *J. Histochem. Cytochem.* 37 (1989) 249–256.
- [92] E.L. Hertzberg, N.B. Gilula, Liver gap junctions and lens fiber junctions: comparative analysis and calmodulin interaction, *Cold Spring Harbor Symp. Quant. Biol.* 46 (1981) 639–645.
- [93] L.J. Van Eldik, E.L. Hertzberg, R.C. Berdan, N.B. Gilula, Interaction of calmodulin and other calcium-modulated proteins with mammalian and arthropod junctional membrane proteins, *Biochem. Biophys. Res. Commun.* 126 (1985) 825–832.
- [94] D.B. Zimmer, C.R. Green, W.H. Evans, N.B. Gilula, Topological analysis of the major protein in isolated intact rat liver gap junctions and gap junction-derived single membrane structures, *J. Biol. Chem.* 262 (1987) 7751–7763.
- [95] K. Török, K. Stauffer, W.H. Evans, Connexin 32 of gap junctions contains two cytoplasmic calmodulin-binding domains, *Biochem. J.* 326 (1997) 479–483.
- [96] M. Elvira, A. Villalobo, Calmodulin prevents the proteolysis of connexin32 by calpain, *Bioelectrochem. Bioenerg.* 42 (1997) 207–211.
- [97] J.A. Diez, M. Elvira, A. Villalobo, The epidermal growth factor receptor tyrosine kinase phosphorylates connexin32, *Mol. Cell. Biochem.* 187 (1998) 201–210.
- [98] S. Ahmad, P.E.M. Martin, W.H. Evans, Assembly of gap junction channels: mechanism, effects of calmodulin antagonists and identification of connexin oligomerization determinants, *Eur. J. Biochem.* 268 (2001) 4544–4552.
- [99] C. Peracchia, The calmodulin hypothesis for gap junction regulation six years later, in: E.L. Hertzberg, R.G. Johnson (Eds.), *Gap Junctions*, Alan R. Liss, New York, 1988, pp. 267–282.
- [100] S.J. Girsch, C. Peracchia, Gap junction proteins and calmodulin. Interaction between calmodulin and C-terminus peptides of the rat heart, rat liver and early *Xenopus* embryo connexins, *Biophys. J.* 61 (1992) A506.
- [101] C. Peracchia, L. Shen, Gap junction channel reconstitution in artificial bilayers and evidence for calmodulin binding sites in MIP26 and connexins from rat heart, liver and *Xenopus* embryo, in: J.E. Hall, G.A. Zampighi, (eds.), *Gap Junctions*, Prog. Cell Res., vol. 3, pp. 163–170.
- [102] K. Török, A. Tzortzopoulos, Z. Grabarek, S.L. Best, R. Thorogate, Dual effect of ATP in the activation mechanism of brain Ca^{2+} /calmodulin-dependent protein kinase II by Ca^{2+} /calmodulin, *Biochemistry* 40 (2001) 14878–14890.
- [103] J.C. Saez, A.C. Nairn, A.J. Czernik, D.C. Spray, E.L. Hertzberg, P. Greengard, M.V. Bennett, Phosphorylation of connexin 32, a hepatocyte gap-junction protein, by cAMP-dependent protein kinase, protein kinase C and Ca^{2+} /calmodulin-dependent protein kinase II, *Eur. J. Biochem.* 192 (1990) 263–273.
- [104] M.H. Pina-Benabou, M. Srinivas, D.C. Spray, E. Scemes, Calmodulin kinase pathway mediates the K^{+} -induced increase in gap junctional communication between mouse spinal cord astrocytes, *J. Neurosci.* 21 (2001) 6635–6643.
- [105] A.E. Pereda, T.D. Bell, B.H. Chang, A.J. Czernik, A.C. Nairn, T.R. Soderling, D.S. Faber, Ca^{2+} /calmodulin-dependent kinase II mediates simultaneous enhancement of gap-junctional conductance and glutamatergic transmission, *Proc. Natl. Acad. Sci. U. S. A.* 95 (1998) 13272–13277.
- [106] D.W. Laird, K.L. Puranam, J.-P. Revel, Turnover and phosphorylation dynamics of connexin43 gap junction protein in cultured cardiac myocytes, *Biochem. J.* 273 (1991) 67–72.
- [107] X.G. Wang, C. Peracchia, Positive charges of the initial c-terminus domain of Cx32 inhibit gap junction gating sensitivity to CO_2 , *Biophys. J.* 73 (1997) 798–806.
- [108] X.G. Wang, C. Peracchia, Molecular dissection of a basic COOH-terminal domain of Cx32 that inhibits gap junction gating sensitivity, *Am. J. Physiol.* 275 (1998) C1384–C1390.
- [109] R. Werner, E. Levine, C. Rabadan-Diehl, G. Dahl, Gating properties of connexin32 cell–cell channels and their mutants expressed in *Xenopus* oocytes, *Proc. R. Soc. Lond.* 243 (1991) 5–11.
- [110] C. Peracchia, X.G. Wang, L.L. Peracchia, Is the chemical gate of connexins voltage sensitive? Behavior of Cx32 wild-type and mutant channels, *Am. J. Physiol.* 276 (1999) C1361–C1373.
- [111] J.F. Ek-Vitorin, G. Calero, G.E. Morley, W. Coombs, S.M. Taffet, M. Delmar, pH regulation of connexin43: molecular analysis of the gating particle, *Biophys. J.* 71 (1996) 1273–1284.
- [112] M. Delmar, K. Stergiopoulos, N. Homma, G. Calero, G. Morley, J.F. Ek-Vitorin, S.M. Taffet, A molecular model for the chemical regulation of connexin43 channels: the “ball-and-chain” hypothesis, in: C. Peracchia (Ed.), *Gap Junctions. Molecular Basis of Cell Communication in Health and Disease*, Academic Press, San Diego, CA, 2000, pp. 223–248.
- [113] F.F. Bukauskas, C. Peracchia, Two distinct gating mechanisms in gap junction channels: CO_2 -sensitive and voltage-sensitive, *Biophys. J.* 72 (1997) 2137–2142.
- [114] F.F. Bukauskas, A. Bukauskiene, M.V.L. Bennett, V.K. Verselis, Gating properties of gap junction channels assembled from connexin43 and connexin43 fused with green fluorescent protein, *Biophys. J.* 81 (2001) 137–152.
- [115] F.F. Bukauskas, R. Weingart, Voltage-dependent gating of single gap junction channels in an insect cell line, *Biophys. J.* 67 (1994) 613–625.

- [116] R. Weingart, F.F. Bukauskas, Long-chain n-alkanols and arachidonic acid interfere with the V_m -sensitive gating mechanism of gap junction channels, *Pflügers Arch.* 435 (1998) 310–319.
- [117] F.F. Bukauskas, A.B. Angele, V.K. Verselis, M.V.L. Bennett, Coupling asymmetry of heterotypic connexin 45/connexin 43-EGFP gap junctions: properties of fast and slow gating mechanisms, *Proc. Natl. Acad. Sci. U. S. A.* 99 (2002) 7113–7118.
- [118] C. Peracchia, K.C. Young, X.G. Wang, L.L. Peracchia, Is the voltage gate of connexins CO_2 -sensitive? Cx45 channels and inhibition of calmodulin expression, *J. Membr. Biol.* 195 (2003) 53–62.
- [119] C. Peracchia, X.G. Wang, L.L. Peracchia, Slow gating of gap junction channels and calmodulin, *J. Membr. Biol.* 78 (2000) 55–70.
- [120] V.K. Verselis, C.S. Ginter, T.A. Bargiello, Opposite voltage gating polarities of two closely related connexins, *Nature* 368 (1994) 348–351.
- [121] P.E.M. Purnick, S.H. Oh, C.K. Abrams, V.K. Verselis, T.A. Bargiello, Reversal of the gating polarity of gap junctions by negative charge substitutions in the N-terminus of connexin 32, *Biophys. J.* 79 (2000) 2403–2415.
- [122] K.C. Young, C. Peracchia, Carbon dioxide sensitive voltage gating of connexin 32 and connexin 32/45 chimeric channels, *Mol. Biol. Cell* 13 (2002) 351.
- [123] S. Oh, Y. Ri, M.V. Bennett, E.B. Trexler, V.K. Verselis, T.A. Bargiello, Changes in permeability caused by connexin 32 mutations underlie X-linked Charcot–Marie–Tooth disease, *Neuron* 19 (1997) 927–938.
- [124] C. Ressot, D. Gomès, A. Dautigny, D. Pham-Dinh, R. Bruzzone, Connexin32 mutations associated with X-linked Charcot–Marie–Tooth disease show two distinct behaviors: loss of function and altered gating properties, *J. Neurosci.* 18 (1998) 4063–4075.
- [125] C. Peracchia, X. Wang, L. Li, L.L. Peracchia, Inhibition of calmodulin expression prevents low-pH-induced gap junction uncoupling in *Xenopus oocytes*, *Pflügers Arch.* 431 (1996) 379–387.
- [126] J.M.B. Anumonwo, S.M. Taffet, H. Gu, M. Chanson, A.P. Moreno, M. Delmar, The carboxyl terminal domain regulates the unitary conductance and voltage dependence of connexin40 gap junction channels, *Circ. Res.* 88 (2001) 666–673.
- [127] A.P. Moreno, M. Chanson, J. Anumonwo, I. Scerri, H. Gu, S.M. Taffet, M. Delmar, Role of the carboxyl terminal of connexin43 in transjunctional fast voltage gating, *Circ. Res.* 90 (2002) 450–457.
- [128] C. Peracchia, J.T. Chen, L.L. Peracchia, CO_2 reduces the sensitivity of Cx40 channels to transjunctional voltage, *Biophys. J.* 86 (2004) 583a.
- [129] P.N.T. Unwin, G.A. Zampighi, Structure of the junction between communicating cells, *Nature* 283 (1980) 545–549.
- [130] P.N.T. Unwin, P.D. Ennis, Two configurations of a channel-forming membrane protein, *Nature* 307 (1984) 609–613.
- [131] P.N. Unwin, Gap junction structure and the control of cell-to-cell communication, *Ciba Found. Symp.* 125 (1987) 78–91.
- [132] C.M. Armstrong, F. Bezanilla, Inactivation of the sodium channel: II. Gating current experiments, *J. Gen. Physiol.* 70 (1977) 567–590.
- [133] W.N. Zagotta, T. Hoshi, R.W. Aldrich, Restoration of inactivation in mutants of *Shaker* potassium channels by a peptide derived from ShB, *Science* 250 (1990) 568–571.
- [134] H.S. Duffy, P.L. Sorgen, M.E. Girvin, P. O'Donnell, W. Coombs, S.M. Taffet, M. Delmar, D.C. Spray, pH-dependent intramolecular binding and structure involving Cx43 cytoplasmic domains, *J. Biol. Chem.* 277 (2002) 36706–36714.
- [135] G. Perkins, D. Goodenough, G. Sosinsky, Three-dimensional structure of the gap junction connexon, *Biophys. J.* 72 (1997) 533–544.
- [136] K.A. Alexander, B.T. Wakim, G.S. Doyle, K.A. Walsh, D.R. Storm, Identification and characterization of the calmodulin-binding domain of neuromodulin, a neurospecific calmodulin-binding protein, *J. Biol. Chem.* 263 (1988) 7544–7549.