Chapter 34
Regulation of Intercellular Calcium Signaling Through Calcium Interactions with Connexin-Based Channels

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Abstract The synchronization of numerous cellular events requires complex electric and metabolic cell-cell interactions. Connexins are a family of membrane proteins that constitute the molecular basis of two kinds of channels: gap junction channels (GJCs), which allow direct cytoplasm-cytoplasm communication, and hemichannels (HCs) that provide a pathway for exchanges between the intra and extra-cellular milieu. Both kind of connexin-based channels support intercellular communication via intercellular propagation of calcium waves. Here, we review evidence supporting the role of Ca^{2+} in the regulation of GJCs and HCs formed by connexins. Also it is speculated how these connexin-based channels could contribute to the propagation of intercellular Ca^{2+} signals.

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M.S. Islam (ed.), *Calcium Signaling*, Advances in Experimental Medicine and Biology 740, DOI 10.1007/978-94-007-2888-2_34,

Keyword Hemichannels • Gap junctions • Connexons • Calcium waves • Dye coupling • Calmodulin

Introduction

The synchronization of numerous cellular events requires complex electric and metabolic cell-cell interactions. In vertebrates, these cellular interactions are in part mediated by low-resistance intercellular channels that most frequently are located at gap junctions. The latter are plasma membrane specializations formed by the aggregation of tens to thousands intercellular channels (gap junction channels, GJCs), which are believed to provide direct but selective cytoplasmic continuity between communicating cells. Each GJC span the appositional plasma membranes of contacting cells and is formed by the serial docking of two hemichannels (HCs) (Fig. 34.1). Each HC is composed of six protein subunits termed connexins, a family of highly conserved proteins encoded by at least 21 different genes in humans [1]. Connexins are named after their predicted molecular mass expressed in kDa, so that Cx43 has a molecular mass of ~43 kDa. GJCs allow the intercellular exchange of metabolites, such as ATP, ADP, glucose, glutamate and glutathione, and second messengers including cAMP and inositol 1,4,5-trisphosphate [2–7]. In addition, these channels permit the intercellular spread of electrotonic potentials in excitable and non-excitable tissues [8–10].

In the last decade, a new pathway for exchange of ions and molecules between the intra and extracellular milieu constituted by connexin HCs has received progressive attention [11]. For a long time, the HC docking in apposed membranes to form intercellular GJCs was the only function assigned to HCs (Fig. 34.1). However, diverse evidence obtained in mammalian cells during the last decade indicate that nonjunctional HCs can open at the unapposed cell surface, forming aqueous conduits permeable to ions and small molecules (e.g., ATP, glutamate, NAD^+ and PGE_2) that allow diffusional exchange between the intra and extracellular compartments constituting a route for autocrine/paracrine cellular communication [11]. Pioneering findings by Paul et al. [12] identified the first nonjunctional currents mediated by connexin HCs in an exogenous expression system and supported the rational that opening of these channels was incompatible with cellular life. Nevertheless, recent evidence indicate that HCs are involved in several physiological cell and tissue functions and/or responses, including cellular proliferation [13–16], regulation of aqueous humor outflow [17], ischemic tolerance [18, 19], and adhesive cell-cell interactions [20]. More recently, another gene family encoding a set of three membrane proteins, named pannexins, has been identified [21] (Fig. 34.1). So far, the absence of ultrastructural evidences for gap junction formation in mammalian cells suggests that the main function of pannexin-based channels is paracrine/autocrine communication acting predominantly in the form of HCs [22].

Changes in GJC and HC open probability can result from changes in covalent modifications of connexin subunits (e.g., phosphorylation, nitrosylation and S-glutathionylation) or variations in transmembrane physicochemical conditions (e.g.,

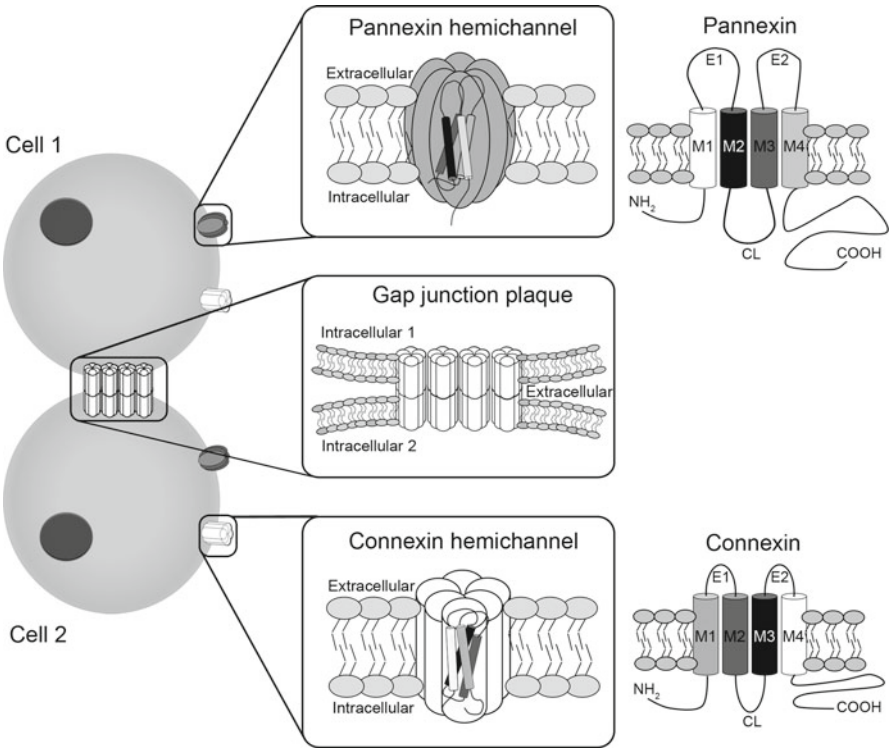

Fig. 34.1 Scheme showing the membrane topology of pannexins, connexins, hemichannels and gap junction channels. *Top and bottom right* correspond to pannexin and connexin proteins in the cell membrane, respectively. Both protein types have four transmembrane domains (*M1-4*) with amino (−*NH2*) and carboxy (−*COOH*) termini in the cytoplasmic side, two extracellular loops (*E1* and *E2*) and one cytoplasmic loop (*CL*). *Top and bottom centers* show hemichannels formed by six pannexin or connexin subunits each. The *middle center* shows an aggregate of connexin GJCs, namely a section through a gap junction "plaque", at a close contact between cells 1 and 2 as shown in the *left*. Each gap junction channel is formed by two hemichannels docked in the same plane (and rotated 30º with respect to one another). Each adjoining cell contributes with one of the hemichannels

transmembrane voltage, pH and concentration of cations) that affect the pore forming proteins. Here, we review the evidence supporting the role of Ca^{2+} in the regulation of GJCs and HCs formed by connexins. Also, it is speculated how these connexin-based channels could contribute to the propagation of intercellular Ca^{2+} signals.

Gating of Connexin-Based Channels by Ca^{2+}

Gap Junction Channels

Closure of GJCs presumably via a "gating mechanism" mediated by Ca^{2+} is a conserved mechanism in several connexin-based GJCs. Usually, an increase in

intracellular free Ca^{2+} concentration ($[Ca^{2+}]_i$) induces closure or drastic reduction gap junctional communication. The $[Ca^{2+}]_i$ that triggers this response in cell-cell communication via gap junction vary from nanomolar to micromolar range [23], and could occur by direct binding to connexins or indirectly, activating modulatory proteins.

Possible Direct Ca^{2+} Gating

Under physiological conditions, the $[Ca^{2+}]_i$ is tightly regulated by several mechanism. Thus, if Ca^{2+} directly affects connexin GJCs, a localized increase in $[Ca^{2+}]_i$ is likely to occur [23]. Once the increase in $[Ca^{2+}]_i$ is confined to a specific area, Ca^{2+} could bind directly to connexins that form GJCs. However, up to now there is no evidence of "calcium sparks" close to gap junctions. Moreover, Ca^{2+} binding sites usually consist of acidic residues contributed by carboxylate oxygen [24]. However, the variability of different connexins makes hard to indentify a potential conserved binding site. Therefore, due to the reasons mentioned above and the absence of crystal structures of different connexin-based GJCs the identification of a direct Ca^{2+} binding site at the cytoplasmic side of GJCs has been difficult [23].

A proposed mechanism for direct Ca^{2+}-dependent gating of GJCs involves the action of calmodulin (reviewed by [23, 25]). Calmodulin contains specialized domains in the N- and C-lobes that follow the $-NH_2$ terminus and Ca^{2+} binds to these domains, inducing conformational changes in the protein, enabling its interaction with several receptors. Such interaction has been observed with Cxs 38 [26], 32 [27], 37 [28], 43 [28], 44 [29], and 50 [30]. Moreover, a calmodulin mutant with high Ca^{2+}-sensitivity drastically increases the Ca^{2+} gating sensitivity of Cx32 GJCs and decreases their Vj sensitivity [28]. This was observed only when calmodulin is expressed before Cx32, suggesting that it interacts with Cx32 before gap junction formation [28]. Moreover, constructs of calmodulin and Cx32 tagged with fluorescent proteins show co-localization of both proteins when expressed in HeLa cells [28]. Similar results obtained in HeLa cells transfected with Cx50 were reported by Zhang and Qi [31]. They proposed the involvement of a Ca^{2+}-independent interaction between C-terminal and cytoplasmatic loop regions as mediator of a Ca^{2+}-dependent binding of calmodulin to Cx50 that induces closure of GJCs. In addition, Cx32 has two calmodulin interaction sites, one in the N-terminus and the other close to the C-terminal domain [32], while Cx43 has only one site in its N-terminus [23]. Moreover, the Cx32 HC activity induced by EGF is inhibited by W7, a calmodulin antagonist [33], suggesting that calmodulin could bind to Cx32 HCs, and potentially to GJCs.

Possible Indirect Ca^{2+} Gating

Indirect gating of connexin GJCs by Ca^{2+} might occur through activation of kinases, phosphatases or generation of metabolites/second messengers that modulate these

channels. Several connexins contain phosphorylation sites for more than one protein kinase, many of them with Ca^{2+}-dependent activity. These consensus sites vary between connexins and have been preferentially identified in their C-terminal domain [34]. The functional consequence could be an increase or decrease of junctional communication, response that depends on the connexin type and cell condition.

Calcineurin, a Ca^{2+} activated phosphatase highly localized in the central nervous system, is vulnerable to ischemic and traumatic insults particularly in neurons, and it is believed to play important roles in neuron-specific functions [35]. Rat astrocytes, normally expressing phosphorylated forms of Cx43, present a reduced a reduced gap junctional communication and increased Cx43 dephosphorylation when exposed to chemical hypoxia [36]. This condition rapidly increases the $[Ca^{2+}]_i$ [37], and while calcineurin inhibition with cyclosporin A or FK506 reduces the dephosphorylation of Cx43, okadaic acid or calyculin A, inhibitors of protein phosphatases 1 and 2A, had little effect. These results suggest that phosphorylated Cx43 GJCs might be substrates of calcineurin under conditions of cell stress, but perhaps not in normal astrocyte cultures, where calcineurin inhibitors had no effect on the phosphorylation state of Cx43 [13]. This would be consistent with the calmodulin-dependent activation of calcineurin [38, 39], suggesting that a calmodulin-dependent signaling systems play a relevant role in regulating GJCs.

Elevated $[Ca^{2+}]_i$ can lead to activation of calmodulin-dependent protein kinase II (CAM kinase II) that can phosphorylate Cx36 [40] and activation of CAM kinase II is associated to increase in electrical synapsis mediated by the Cx35 (ortholog of mammalian Cx36) in Mauthner neurons of the goldfish subjected to repetitive electrical stimulation that induces long term potentiation [41]. However, it remains unknown whether the increase in electrical coupling is due to a gating mechanism that activates more GJCs already present at the cell-cell interface or increase in number of GJCs due to reduced degradation and/or increased recruitment.

Ca^{2+} can induce the generation of arachidonic acid (AA), which, in turn, promotes closure of GJCs. AA is generated by activation of Ca^{2+}-dependent lipases during hypoxia-reoxygenation events and blocks intercellular coupling via GJCs in astrocytes [42] and in mouse hepatocytes expressing connexins 26 and 32 [43]. Blocking metabolization of AA with cyclooxygenase and lipoxygenase inhibitors preserve the GJC activity in astrocytes treated with AA, suggesting that downstream metabolic products of AA induce rapid channel closure or removal of Cx43 GJCs [42].

Hemichannels

A distinctive functional feature of connexin HCs is their sensitivity to variations in both extra and intracellular concentrations of divalent cations. In particular, homomeric HCs formed by all studied connexins are rapidly activated by reduction or removal of extracellular Ca^{2+} and Mg^{2+}, and their open probability is markedly reduced upon exposure to extracellular divalent cations [44]. However, the blocking effect of extracellular Mg^{2+} is less pronounced and possibly less functionally relevant

than that of Ca^{2+} [45–49]. Conversely, HCs formed by some connexins (e.g., Cxs 43 and 45) but not others (e.g. Cx26) increase the cell membrane permeability to dyes upon rises in $[Ca^{2+}]_i$ and this has been associated to increased level of HCs present at the cell surface [15]. The molecular mechanisms and functional implications of the complex/dual effect of Ca^{2+} on connexin HCs are not completely understood, but they are believed to be involved in both physiological and pathological regulation of HCs [50–53].

The first observation on connexin HC activation by reduction of extracellular Ca^{2+} concentration ($[Ca^{2+}]_e$) came from studies by DeVries and Schwartz [54]. In their seminal article using electrophysiological recordings and Lucifer yellow uptake by solitary horizontal cells of the catfish retina, they recognized undocked HCs working as single units by showing their increasing activation upon progressive reduction in $[Ca^{2+}]_e$ from 2 mM to <10 μM [54]. Afterwards and using diverse experimental models including mammalian cells with endogenous connexin expression and exogenous expression systems, HCs formed by diverse connexins were shown to be functionally modulated by variations in the $[Ca^{2+}]_e$, including HCs formed by Cxs 23 [55] [56], 26 [15, 37, 57–60], 30 [61], 30.2 [62], 31.9 [62], 32 [33, 37, 47], 35 [63], 37 [48], 38 [64–66], 43 [15, 67–75], 45 [15, 76, 77], 45.6 [78], 46 [45, 46, 79–82], 50 [83]; 52.6 [84] and 56 [85].

Homomeric HCs formed by Cxs 26 or 43 rapidly and reversibly increase their internal pore diameter upon extracellular Ca^{2+} removal [variation from 0.5 to 1.3 nm [86] and 1.8 to 2.5 nm [87], respectively] as measured by high-resolution atomic force microscopy. The latter findings support a prominent structural change of connexin-based hexamers induced by $[Ca^{2+}]_e$ variations.

Although the mechanism of HC blockade induced by extracellular Ca^{2+} is not completely understood, it is unlikely to occur solely as a consequence of conformational changes but rather involves direct polyvalent cation binding to the mouth of the HC pore. Indeed, extracellular Ca^{2+} and voltage sensitivities of Cx32 HCs in *Xenopus* oocytes are modified by substitution of two aspartate residues (D169 or D178) located at the second extracellular loop by uncharged asparagines [47]. In homomeric Cx32 HCs, these residues are believed to form a 12 aspartate ring located at the external vestibule of the pore. Binding of Ca^{2+} to these negatively charged aspartate residues could explain the blocking effect of Ca^{2+} through reduction of the effective pore diameter or stabilization of the close configuration of HCs as it has been reported for other ion channels [88, 89]. Interestingly, one naturally occurring mutation due to substitution of the aspartate residue located in position 178 by a neutral tyrosine residue (D178Y) of human Cx32 is associated to a complex human genetic disease termed X-linked Charcot-Marie-Tooth [90]. However and despite the high sequence homology of extracellular E1 and E2 loops among connexins subunits, only HCs formed by few connexins [e.g. human Cxs 30, 32, 43; rat Cxs 32, 43 and 46; mouse Cx30 [47]] share this 12 aspartate extracellular ring but those connexins lacking it are still Ca^{2+} sensitive. In addition, while mutations of the positioned E2 aspartates (D169 and D178) alter the Ca^{2+}-sensitivity of Cx32 HCs, they do not abolished the Ca^{2+} block entirely, indicating the involvement of additional molecular mechanisms. Alternatively, the presence of other negatively

charged amino acid residue such as glutamate or variations in the Ca^{2+} binding sites around or close the pore edge could account for the extracellular Ca^{2+} blockade of HCs formed by connexins lacking the anionic gate. In this regard, homomeric HCs formed by human Cx37 [lacking the anionic pore ring [47]] expressed in *Xenopus* oocytes are blocked by extracellular Ca^{2+} going through the pore (open by membrane depolarization) and reaching binding sites located at the cytoplasmic side, presumably acidic residues located in the N- and C-termini of the subunits [48]. In addition, single recordings of homomeric rat Cx46 HCs [possessing the 12 aspartate anionic pore ring [47]] expressed in Xenopus oocyte showed that divalent cations do not block directly, but rather modify the intrinsic electric gating though conformational stabilization of fully closed HCs [49]. Using excised patches, divalent cations are only effective from the extracellular side, implying that the binding sites are extracellular. Unfortunately, all available studies aimed to address the mechanism of extracellular Ca^{2+} blockade have been performed using electrophysiological measurements of HCs exogenously expressed in *Xenopus* oocytes and using membrane depolarization as activating stimulus. Several intra/extracellular molecules (e.g. ions, protons, nucleotides and cytokines), intracellular proteins (e.g. protein kinases and phosphatases) and binding partners (e.g., tight junction associated proteins and cytoskeleton proteins) have been shown to affect connexin HC structure and function [11] and may additionally be involved in the extracellular Ca^{2+} sensitivity and blocking effect. In addition, multiple gating mechanisms (e.g. chemical gating) have been identified for GJCs [91] and HCs might show similar functional complexity. In this regard, it was shown that replacement of extracellular Na^+ by K^+ (and other monovalent cations) modulates the extracellular Ca^{2+} sensitivity of homomeric Cx46 and Cx50 HCs, an effect residing in the cytoplasmic N-terminal domain of connexins [92]. In addition, diverse stimuli have been recently shown to overcome the extracellular Ca^{2+} blockade of connexin HCs even at resting negative transmembrane potentials (e.g. chemicals, mechanical stimulation, ischemia-like conditions, cytokines and growth factors) [15, 36, 93–97].

Augments in $[Ca^{2+}]_i$ increase connexin HC activation in the presence of physiological concentrations of extracellular divalent cations, possibly acting in an indirect fashion. In particular, cells expressing homomeric HCs formed by Cxs 32 or 43 increase their membrane permeability to cationic dyes and the HC-mediated ATP release in response to higher $[Ca^{2+}]_i$ and buffering the intracellular Ca^{2+} with chelating agents prevents HC activation in diverse experimental models [15, 33, 37, 74, 98]. In cells expressing Cx32 HCs, the intracellular Ca^{2+} activating concentration lies at ~500 nM and the HCs response is abolished by a selective calmodulin inhibitor [33]. Moreover, Cx32 HC activation induced my metabolic inhibition is partially prevented by a p38 MAP kinase inhibitor, totally prevented by intracellular Ca^{2+} chelators and mimicked by a Ca^{2+} ionophore in HeLa cells [37]. In HeLa cells expressing Cx43, increase HC activation induced by rising the $[Ca^{2+}]_i$ with FGF-1 or a Ca^{2+} ionophore is associated with higher surface HC levels, increased HC open probability and requires p38 MAP kinase activation [15]. Moreover, Cx43 HC activation is also triggered by intracellular concentration of ~500 nM Ca^{2+} and involves activation of complex intracellular signaling cascades including calmodulin,

calmodulin-dependent kinase II, p38 MAP kinase, phospholipase A_2, arachidonic acid, lipoxygenases, cyclooxygenases, reactive oxygen species, nitric oxide and membrane depolarization [97]. Finally, release of ATP and proliferation of the retinal pigment epithelial cells also requires Cx43 HC activation induced by increased $[Ca^{2+}]_i$ [74].

Interestingly, preventing the intracellular Ca^{2+} mobilization with BAPTA-AM or thapsigargin in cultured astrocytes does not inhibit glutamate release after exposure to a divalent cation free solution [73], indicating interdependence of the HC responses induced by extra and intracellular Ca^{2+}. However, the fact that at least some HCs are Ca^{2+} permeable [37, 99, 100], suggest possible activating loops and complex regulation of the HC responses by this cation.

Permeation of Calcium Ions Through Connexin-Based Channels

Gap Junction Channels

Up to now, only a couple of studies indicate that GJCs are permeable to Ca^{2+} [4, 101], meanly because it is difficult to rule out the involvement of cytoplasmic Ca^{2+}-mobilizing second messengers such as inositol (1,4,5)-trisphosphate (IP_3) and cyclic-ADP-ribose (cADPR) [102]. Moreover, another puzzle piece is that high $[Ca^{2+}]_i$ has been shown to reduce GJC activity in several cell types [103–105]. Nevertheless, the $[Ca^{2+}]_i$ required to block GJCs appears to be well above 1 μM [106], far higher than normal resting $[Ca^{2+}]_i$ that is between 50 and 100 nM. Therefore, it is expected that low Ca^{2+} concentrations could permeate GJCs. In fact, it has been reported that Ca^{2+} microinjected into individual hepatocytes or smooth muscle cells immediately increase the $[Ca^{2+}]_i$ in the injected cell [4, 101]. Importantly, the $[Ca^{2+}]_i$ increased within seconds in the contacting cells, while the initial rise in the $[Ca^{2+}]_i$ induced by IP_3 microinjection occur in discrete regions of the cytoplasm, which is inconsistent with simple diffusion of Ca^{2+}. The abovementioned studies indicate that IP_3 diffuses between cells to cause localized Ca^{2+} release from intracellular stores. Whereas changes in $[Ca^{2+}]_i$ seen in adjacent cells after Ca^{2+} microinjection in cells bathed in Ca^{2+} free solution are due to transjunctional Ca^{2+} diffusion from the injected cell and not to uptake from the extracellular solution [4, 101]. Accordingly, computational modeling of intercellular Ca^{2+} wave propagation support that both IP_3 and Ca^{2+} diffusion occur through GJCs [107–110].

Hemichannels

Most connexin HCs are highly sensitive to fluctuations in $[Ca^{2+}]_e$ and their open probability decreases in the presence of increasing concentrations of this cation [11]. Thus, HCs have been considered as Ca^{2+} sensors implicated in the mechanism by

which the $[Ca^{2+}]_e$ modulates the intracellular signaling [111]. Diverse studies have shown that ATP released via HCs increases the $[Ca^{2+}]_i$ via P2 receptor activation [14, 60, 74, 112–114] and thus, excluding the HC contribution as route for Ca^{2+} influx to the cytoplasm. The first experimental evidence suggesting Ca^{2+} influx through HCs was observed in cardiomyocytes subjected to metabolic inhibition. Under this treatment, cardiomyocytes exhibit an increase in intracellular free Ca^{2+} and Na^+ concentration, which are partially reduced by halothane or 1-heptanol, two HC and GJC blockers [73, 115]. However, voltage-operated Ca^{2+} and Na^+ channel blockers also reduce the intracellular increase of both cations, suggesting that HCs, as well as Ca^{2+} and Na^+ channels contribute to this phenomenon [115]. In support to the relevance of HCs in this response, the increase in $[Ca^{2+}]_i$ of cardiomyocytes subjected to ischemia is inhibited by Gap26 [116], a connexin mimetic peptide that blocks Cx43 HCs. Most experiments designed to elucidate the role of connexin HCs in Ca^{2+} influx induced by pathological or physiological conditions have been conducted in HeLa cells transfected with connexins [117, 118], since they possess all the cellular machinery required for Ca^{2+} signaling [119]. In fact, HeLa cells transfected with connexins 26, 32 or 43 exhibit Ca^{2+} waves in response to mechanical stimulation or extracellular ATP application that differ from those observed in parental HeLa cells [120]. Recent studies in HeLa cells transfected with mouse Cx43 and loaded with Fluo 4 used as calcium indicator, show that extracellular alkalinization increases the $[Ca^{2+}]_i$ in an $[Ca^{2+}]_e$-dependent way [100]. More relevant to this point, the alkalinization-induced rise in $[Ca^{2+}]_i$, was closely related to the level of Cx43 HCs present at the cell surface, suggesting that Ca^{2+} mobilization from the extracellular milieu is mediated by Cx43 HCs [100]. To demonstrate directly that Cx43 HCs are permeable to Ca^{2+}, purified Cx43 HCs were reconstituted into unilamelar liposomes loaded with Green-2 as Ca^{2+} indicator. When the $[Ca^{2+}]_e$ was increased from 5 to 20 μM the fluorescence of Green-2 increased almost twofolds. In contrast, liposomes without Cx43 HCs did not exhibit changes in $[Ca^{2+}]$ in their interior, indicating that Cx43 HCs are permeable to Ca^{2+} [100].

Cx43 HCs are not the only HCs permeable to Ca^{2+}. Cx32 HeLa transfectants subjected to metabolic inhibition show elevated $[Ca^{2+}]_i$, which does not occur within the same time course in parental cells, suggesting that Ca^{2+} influx could occur through Cx32 HCs [37]. Recently, Sánchez et al. [37] showed that activation of an endogenous Ca^{2+}-activated chloride channel in *Xenopus* oocytes occurs when Cx26 HCs are activated upon depolarization, suggesting that they allow the influx of Ca^{2+}, which further activates the chloride currents. Interestingly, a mutation positioned near TM1/E1 domain of Cx26 (G45E) associated with the Keratitis Ichthyosis Deafness syndrome (KID) leads to formation of HCs with increased Ca^{2+} permeability reflected by a robust chloride channel activation compared HC formed by with wild Cx26 [99]. Mutations in genes of Cxs 26, 32 and 43 have been associated with several human diseases, and some of them form HCs with aberrant conductances in exogenous expression systems. In light of the abovementioned findings, it is possible that altered permeability to Ca^{2+} occurs as a common mechanism for the development of these diseases [47, 99, 121]. Relevant to the lack of demonstration of this possibility are the experimental limitations of each approach used to demonstrate

Ca^{2+} influx via HCs. Reconstitution in liposomes might be a good approach since it discard other possible routes of Ca^{2+} influx that might be present in endogenous and exogenous expression systems and also eliminate all intracellular elements that regulate the functional state of HCs (e.g., kinases, phosphatase and scaffolding proteins) [44, 122]. An experimental strategy used to sense the direct passage of Ca^{2+} through GJCs has been the use of connexin-aequorin chimeras. However, in these studies it was found that some connexin-aequorin chimeras do not form functional GJCs, and remains to be demonstrate if they form functional HCs [123]. Therefore, this experimental strategy must be reviewed and its usefulness in studying the HC permeability to Ca^{2+} needs to be reevaluated.

Do Gap Junction Channels and Hemichannels Play a Role in the Propagation of Intercellular Calcium Waves?

Up to now, two mechanisms for intercellular calcium wave propagation have received more attention. One of them occurs between contacting cells and involves the diffusion through GJCs of cytoplasmic Ca^{2+}-mobilizing second messengers such as inositol (1,4,5)-trisphosphate (IP_3) [4, 124], cDAPR [125] and Ca^{2+} [4, 101] (Fig. 34.2). The another one is related to the activation of P2 purinergic receptors in neighbor cells by extracellular ATP released through vesicles [126] and/or HCs [112] (Fig. 34.2). Evidence supporting the first mechanism includes that the waves are: (i) gap junction dependent; (ii) not blocked upon ATP hydrolysis; (iii) not blocked by purine-receptor antagonists and (iv) do not jump a gap between cells [127, 128]. Whereas facts sustaining the second mechanism comprise: (i) hemichannel blockers prevent the waves; (ii) ATP is released by the initiator cell, and the calcium waves extend as far as the ATP diffuses; (iii) the waves are blocked by extracellular apyrase and by inhibitors of P2 receptors; (iv) the waves jump cell-free gaps and are deflected by flow of medium [129–132]. It is possible that these mechanisms coexist under physiological conditions. In support to this possibility, an organotypic model of mice cochlea show propagation of intercellular calcium waves at long distances through ATP released via Cx30 and Cx26 HCs, whereas GJCs composed by the same Cxs allow the simultaneous diffusion of IP_3 across coupled cells [133]. Importantly, both mechanisms occur in response to activation of P2Y/PLC/IP_3/Ca^{2+} signaling cascade and are propagated normally in cultures lacking either $P2X_7$ receptors or Panx1, indicating that the phenomena is not related to Panx1 based channels [133]. Moreover, intercellular Ca^{2+} waves induced by mechanical stimulation depend of GJCs and paracrine ATP-mediated signaling in human urothelial cells [134]. Similar calcium wave propagation has been proposed to occur in osteocytes [135]; ciliated tracheal epithelial cells [136] and astrocytes [137]. An alternative mechanism proposed for propagation of intercellular calcium waves is through the release of NAD^+ via HCs [138]. Most of cells, express CD38, an ectoenzyme that cycles NAD^+ to form cADPR, then cADPR cross the cell membrane to reach ryanodine receptors in the endoplasmic reticulum (ER), triggering release of

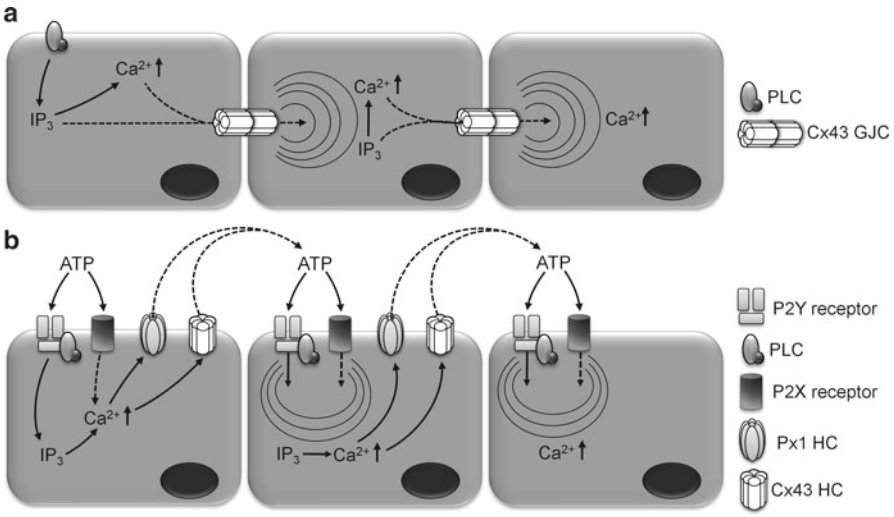

Fig. 34.2 Two models for conduction of Ca^{2+} waves in astrocytes. (**a**) Upstream receptor stimulation leads to activation of phospholipase C (*PLC*) and formation of cytoplasmic inositol (1,4,5)-trisphosphate (*IP*$_3$), which promote the release of Ca^{2+} stored in the endoplasmic reticulum. Both IP_3 and Ca^{2+} diffuse to neighboring cells through gap junction channels generating waves of rises in intracellular Ca^{2+} concentration $[Ca^{2+}]_i$. (**b**) ATP released from vesicles and/or ion channels diffuses through the extracellular space and activates membrane purinergic (P2) receptors. Stimulation of metabotropic P2Y receptors leads to activation of phospholipase C (*PLC*) and formation of IP_3. Whereas, activation of ionotropic P2X receptors leads to Ca^{2+} influx. The increase in free $[Ca^{2+}]_i$ induced by IP_3 and P2X receptor opening could promote ATP release through Cx43 and Panx1 hemichannels, extending the Ca^{2+} wave to neighboring cells

Ca^{2+} into the cytoplasm. Afterwards, subsequent release of NAD^+ via HCs and/or cADPR diffusion through GJCs [125] would propagate a calcium wave.

It is interesting that $[Ca^{2+}]_i$ at ~500 μM induces opening of HCs and closing of GJCs. This finding could have pathophysiological implications. For example, astrocytes subjected to ischemic-like conditions exhibit an increase in Cx43 HCs activity and decrease in intercellular gap junctional communication [36]. During these conditions a fast increase in $[Ca^{2+}]_i$ has been documented [37], while exposure to Ca^{2+} ionophore induces rapid closure of Cx43 GJCs [130]. A recent work from the group of Li and co-workers [139, 140] has elegantly demonstrated that only capacitative Ca^{2+} entry via store-operated channels is effective in blocking gap junctional communication, while Ca^{2+} ionophores were without effect. How $[Ca^{2+}]_i$ changes are linked to HC opening and GJC closure is currently unknown, but could depend on differential distribution of intracellular regulatory proteins in different microdomains, which may also vary in different cell types and physiological or pathophysiological states. Opening of HCs could be necessary to release toxic metabolites to the extracellular medium, and accelerate the intake of energetic metabolites. At the same time, closing GJCs might help to avoid the spreading of death signals from damaged to healthy cells, as occurs during propagation of spreading depression in nerve tissue.

Acknowledgements This work was partially supported by the CONICYT 79090028 (to JAO); FONDECYT 1111033 (to JCS); FONDEF DO7I1086 (to JCS); and ANILLO ACT-71 (to JCS) grants.

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