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Review

Hunting for connexin hemichannels

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ABSTRACT

Connexin hemichannels (connexons) are building blocks of gap junctions but also function as free unapposed channels, which has become an active field of research. Defining functions of hemichannels and their involvement in any biological event requires ruling out possible participation of other channels that share biophysical and regulatory properties, for example pannexins, CALHM1 and P2X receptors. The lack of specific inhibitors for these channels has become an obstacle in elucidating the role of connexin hemichannels. Several experimental approaches are now available to identify hemichannels at the cell surface and to characterize their electrophysiological, permeability and regulatory properties. The use of connexin knockout/knockdown, and the development of peptides that target intracellular connexin domains and specific antibodies directed to extracellular domains have helped to dissect the role of hemichannels in endogenously expressing systems. Moreover, studies of connexin mutants in exogenous expression systems have provided convincing evidence on hemichannels in the pathogenesis of several human genetic diseases. We here present a brief overview of connexin hemichannels as functional channels and itemize a list of aspects to consider when concluding on their involvement.

1. The early beginnings and maturation of the concept of functional connexin hemichannels

Functional connexin hemichannels were first demonstrated as macroscopic voltage-gated currents associated to swelling and death of *Xenopus* oocytes used as exogenous expression system injected with the RNA of a mammalian lens Cx46 [1]. Interestingly, oocytes expressing the endogenous Cx38 do not die. A possible interpretation of this observation could be that oocytes express regulatory mechanism(s) that keep endogenous Cx38 hemichannels preferentially in the closed state while exogenously expressed Cx46 hemichannels have increased open probability or are permanently open because ancillary proteins partners or essential intracellular signals are either lacking or present at too low a concentration to interact with connexin proteins that are over expressed. Thereafter, cultured solitary horizontal cells of the catfish retina were shown to express an endogenous current that was activated upon exposure to low extracellular calcium ion (Ca^{2+}) concentration. This current was reduced by extracellular 1 mM Ca^{2+} or higher, treatment with dopamine, or a weak acid, and the same conditions also blocked Lucifer yellow uptake [2], a broadly used gap junction channel permeability probe and thus, was supposed to permeate connexin hemichannels. All together, the latter findings were interpreted as opening of hemichannels present in the cell surface. However, the molecular composition of the membrane pathway responsible for the different findings remained unidentified. Then, several articles described the electrophysiological characterization of macroscopic and single hemichannel currents for various connexins in exogenously expression systems including *Xenopus* oocytes and mammalian cell lines [3–15]. Consequently, characteristic unitary conductances for homomeric connexin hemichannels were identified and found to have approximately twice the conductance of the corresponding gap junction channels [14,16]. Single hemichannel conductance data combined with kinetic information on activation/deactivation and the lack of ion-selectivity (reversal potential equal to zero) emerged as an electrophysiological identity card of the channels and this significantly contributed to a better understanding of modulatory influences affecting hemichannels [13,14,17]. In some studies a direct correlation between the expression of Cx43, the membrane current

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and dye uptake mediated by hemichannels in mammalian cells was demonstrated [7,13,14]. Two direct demonstrations that connexins form functional membrane units have been the reconstitution of hemichannels in lipid bilayers and identification of the unitary conductance in voltage clamp recordings. In liposomes or planar lipid bilayers, the sensitivity to inhibitors like heptanol, carbenoxolone and lanthanum ions (La^{3+}), and permeability properties to small molecules and Ca^{2+} has been demonstrated [18–25], indicating that connexin hemichannels could operate without the necessity of accessory proteins and without involvement of other protein forming channels. A possible bias of the electrophysiological characterization of the biophysical hemichannel properties is that accessory proteins, phosphorylation state, ionic concentrations and nature of the permeating charge carriers may all influence the recorded parameters. Patch clamp in whole cell configuration is the standard mode for recording unitary hemichannel currents, because of the low open probability of hemichannels, but it has the intrinsic drawback of dialyzing crucial intracellular regulatory molecules.

The demonstration of the presence of hemichannels in the plasma membrane required the introduction of more refined approaches. Among them, hemichannels located at the cell surface were detected with antibodies directed to extracellular domains of connexins [26–28]. These observations were consistent with results of Western blot analysis of biotinylated and pull down cell surface proteins [28–31]. Another approach was the expression of connexins with a fluorescent tag and their localization in the cell surface using confocal microscopy [14,32]. A different approach was the demonstration of functional hemichannels at the cell membrane by measuring the release of fluorescent permeability probes. For example, in neurons and spinal cord astrocytes, the release of fluorescent probes from cells previously loaded with membrane permeable dyes such as calcein-AM or microinjected Lucifer yellow was demonstrated [33–35]. Additionally, cellular uptake of non-fluorescent permeability tracers that upon binding to intracellular nucleic acids become highly fluorescent (DAPI, ethidium bromide and propidium iodide) allowed real time measurements of membrane permeability properties via hemichannels [14,17,29–32,36–39]. Importantly, these dyes are standard tools for assessing cell death by plasma membrane rupture. However, it is not always established what exactly mediates the plasma membrane permeability for these dyes [40], and a contribution of hemichannels – if present- is definitely a possibility to consider.

The connexin hemichannel-dependent release of small molecules including ATP, amino acids, reduced glutathione, NAD^+, IP_3, prostaglandin E_2 and cyclic nucleotides has also been demonstrated [10,41–49]. Similarly, cellular uptake of nutrient (fluorescent glucose derivative) or signaling molecules (cADPR and IP_3) has equally been demonstrated to occur via hemichannels [50–53].

Simultaneous with the methods that became available to study hemichannel function, several groups reported the regulation of these channels by diverse conditions including the effect of growth factors, pro-inflammatory cytokines, intracellular free Ca^{2+} levels, concentration of physiological extracellular divalent cations (Zn^{2+}, Ca^{2+} and Mg^{2+}), Co^{2+}, Cd^{2+}, monovalent cations, membrane potential, redox potential, protein phosphorylation, membrane stretch, alkalinization, acidification, hypoxia-reoxygenation, metabolic inhibition, and cellular nutrients including extracellular concentration of glucose and polyunsaturated fatty acids [9,17–20,29,31,35,50,53–70]. Thus, hemichannels are recognized as membrane pathways for a new autocrine/paracrine signaling mechanism and also serve for diffusional transport of molecules across the cell membrane in addition to the well-known transporters of small molecules, for example amino acid and glucose transporter proteins [71,72].

2. How to hunt for a connexin hemichannel

Often, the first evidence for considering hemichannels in a particular biological response comes from experiments with classical gap junction/hemichannel blockers such as glycyrrhetinic acid and its derivative carbenoxolone, long-chain alcohols like heptanol and octanol or halogenated general volatile anesthetics like halothane (reviewed in [73]). However, all these substances display extensive off-target effects. The same applies for La^{3+} that blocks hemichannels but also Ca^{2+} channels [74,75]. Thus, the hunt for hemichannels only starts after these initial (often exciting) findings. Table 1 gives an overview of criteria to consider when acquiring evidence that supports a role for hemichannels in the biological response of interest. This table extends the criteria proposed previously in Ref. [76].

Criterion 1: In most studies conducted in either endogenous or exogenous expression systems, the interpretation of results obtained with non-selective hemichannel/gap junction channel blockers was supported by evidence for connexin expression adjoined by complementary approaches using a cell type deficient in connexin expression. Additionally, the presence of connexin hemichannels at the cell surface (or in the membrane of the organelle of interest) should be demonstrated using antibodies or biotinylation as mentioned in the previous section.

Criterion 2: Work with hemichannel-permeable molecular markers is essential to identify the permeability characteristics and demonstrate the bidirectional transport function of the channel. Like discussed before, Lucifer yellow, DAPI, ethidium bromide and propidium iodide have been extensively used to that purpose and hemichannel permeability to these probes has been analyzed in detail [36]. A recent account on using various fluorescent markers used to probe the pore size of calcium homeostasis modulator 1 (CALHM1) channels is presented in Ref. [77]. Importantly, negative control experiments with high molecular weight dyes that do not permeate hemichannels are essential. Unfortunately, molecular markers based on size or molecular weight cannot be used to distinguish between channels composed of connexins, pannexins or CALHM1. Connexins have a pore diameter in the order of 14–15 Å [78,79] while the pore diameter of CALHM1 is in the same order [77]. No pore diameter data are available for pannexin

Table 1
Criteria for involvement of connexin hemichannels.[a]

(1) The specific connexin under consideration is expressed in the cell type of interest and is present in non-junctional membrane areas
(2) The cells of interest release or take up molecules with a molecular weight below 1.5 kDa in response to triggers acting intracellulary or extracellulary; larger-sized markers are not taken up or released
(3) The cells of interest display single channel currents with unitary conductance and other biophysical properties that are compatible with reported values or with information derived from gap junction channel data
(4) The response of interest is significantly reduced in cells, tissues or animals deficient in the connexin of interest. Consider alterations of other putative membrane pathways as a consequence of connexin knock-down
(5) The response of interest is significantly reduced in cells, tissues or animals expressing mutant connexin versions characterized by loss-of-function (or increased by gain-of-function) of hemichannels that have no or opposite effects on gap junctions
(6) The response of interest is significantly reduced in cells, tissues or animals by specific connexin hemichannel blockers (or increased by specific activators) which do not influence or have an opposite effect on gap junctions
(7) Consider alternative membrane pathways that may be recruited by hemichannel opening and dominantly contribute to the response of interest

[a] The more criteria fulfilled, the stronger the evidence.

channels but these channels allow the permeation of molecules below 900 Da [80], which also permeate through connexin channels. Not only size and molecular weight play a role in permeation but also charge: the preference for connexin channels for positively or negatively charged molecular markers depends on the connexin type (reviewed in Ref. [81]). Panx1 channels are anion-selective [82] while CALHM1 channels appear to make little distinction between anions and cations [77].

Criterion 3: The most direct demonstration of functional hemichannels in the cell surface so far has been provided by their biophysical characterization in terms of unitary current events and the lack of selectivity for permeating ions. This was mainly based on previously characterized properties of gap junction channels and the fact that each hemichannel is, as already referred to above, expected to have a unitary conductance value twice that of a gap junction channel made of the same connexin type [14,16]. If an exogenous expression system of the connexin under study is used, it should be verified whether the hemichannels function as they do in cells natively expressing the concerned connexin. For example, exogenous expression of Cx43 in *Xenopus* oocytes gives hemichannels that cannot be activated unless extra C-terminal peptide is added [69], suggesting that essential binding partners are lacking or insufficiently present in some expression systems. It is therefore crucial to complement the evidence with recordings in the native cell of interest that endogenously expresses the specific connexin. However, cells expressing a single connexin type are rare and frequently they express two or more compatible connexins that can form heteromeric hemichannels with different stoichiometry increasing the difficulty for identifying the hemichannel based on its unitary conductance value. In addition, most cells co-express connexins with several other membrane channels that share permeability and/or gating properties including pannexin channels, $P2X_7$ but also $P2X_2$, $P2X_4$ and $P2X_5$ receptor channels [83], transient receptor potentials (TRP) channels and CALHM1 channels [84–87].

Criterion 4: The use of cells, tissues or animals deficient or overexpressing the connexin of interest is a crucial and necessary experiment to support involvement of the particular connexin under scrutiny. In many cases the putative hemichannel-mediated response was strongly and sometimes completely suppressed in the corresponding connexin-deficient cells. However, because this approach affects both gap junctions and hemichannels, it is by no means conclusive and necessitates complementation with other experiments (see criteria 5 and 6 discussed below). Additionally, this does not necessarily rule out the participation of other membrane pathways as possible protagonists in the phenomenon of interest (see criterion 7). It is furthermore necessary to verify whether suppression of connexin expression influences the expression of other membrane channels with similar permeability and pharmacological features as connexin hemichannels.

Criterion 5: Additional evidence can be obtained from experiments (overexpression, knock-in or endogenously expressed) with mutant versions of the connexin of interest that differentially influence hemichannels and gap junctions. For example, the Cx43 G138R mutant which has a gain of hemichannel function and loss of gap junction channel function [88,89]. Work with this mutant has provided supportive evidence on the role of hemichannels in changes in membrane permeability related to decreased extracellular Ca^{2+} in the brain [90] and on the etiopathogenesis of several human genetic diseases [71]. Of note, evidence at the unitary current level supporting distinctive effects of this mutant connexin on channels and hemichannels is still lacking.

Criterion 6: An alternative to mutants with differential effects on hemichannels and gap junctions is the use of antibodies or peptides. Antibodies directed to extracellular domains of the connexin protein offer an interesting approach because the extracellular loops of unapposed hemichannels are accessible while this is not the case in gap junctions where the loops are engaged in docking interactions. As a consequence, they are expected to specifically affect free hemichannels but not those in gap junctions. Specific antibodies directed to extracellular domains of Cx43 have been generated by several groups [26,91,92]. Such approach has the advantage that it allows visualization of the hemichannel pool in the plasma membrane supporting criterion 1. Importantly, Cx43 targeting antibodies have been documented to inhibit hemichannels at the single channel level [39]. A possible drawback relates to the fact that the extracellular loops are rather well conserved between different connexins, making it possible that the antibodies affect hemichannels composed of other connexin types.

Like antibodies, peptides have been introduced in the field to provide more specificity towards connexins. Peptides identical to defined domains on the extracellular loops of the connexin protein have provided useful insights, in part because they have been investigated more extensively than antibodies. Two prototypic examples of such peptides are Gap26 and Gap27 which were first described to inhibit gap junctions but later found to also inhibit hemichannels [53,93–96]. The kinetics of Gap26/Gap27 inhibition of gap junctions and hemichannels is different, with hemichannel inhibition occurring more rapidly (minutes) than inhibition of gap junctions (tens of minutes to hours) [97,98]. Peptide 5, which is similar to Gap27 but contains an extra 5 amino acids in N-terminal direction at the expense of 4 amino acids less at the C-terminal side has been demonstrated to block hemichannels at low (10 μM) concentration while inhibiting hemichannels and gap junctions at higher concentration [99]. At present, neither the interaction site of these peptides with the connexin protein nor the exact mechanism by which they inhibit gap junction channels and hemichannels is known. What is known is that they shift the voltage activation threshold for hemichannel opening to more positive values but that does not explain why they block hemichannel opening triggered by chemical rather than electrical stimuli [53,96]. Apparently, interaction of Gap26 or Gap27 with the closed hemichannel drives these channels into a stabilized closed state (deep closed state) that is not available for opening upon exposure to electrical or chemical triggers (Fig. 1). The notion of a stabilized/deep closed state derives from insights obtained on the block of gap junction channels or hemichannels by Mg^{2+} and Cd^{2+} [100,101]. While some evidence is available to support this notion [96], crucial evidence demonstrating altered interaction of Gap26/27 peptides with the connexin protein when the hemichannel is open is still lacking. Previous suggestions that Gap26 and Gap27 inhibit hemichannels because of size and not because of specific interaction with the connexin protein have been refuted [102]: Gap26/27 display maximal hemichannel inhibition at ~250 μM while non-specific (size-dependent) steric pore block is only observed at concentrations 4 times higher (1 mM) [53,96].

A drawback of Gap26/27 peptides is their low affinity for binding to connexins and their limited ability to distinguish between different connexins. The latter is related to the conserved nature of the extracellular loop domains they mimic. For example, Gap27 inhibits channels composed of Cx43 as well as Cx37 (and probably many more as this has not been systematically verified). As a result, efforts have been directed to develop peptides that are more selective towards specific connexins, based on mimicry of sequences located on intracellular parts of the connexin protein. In contrast to the extracellular loops, intracellular domains are very different between different connexins. A crucial insight came from studies with L2 peptide that is identical to a 26 amino acid sequence on the intracellular (cytoplasmic) loop of Cx43. L2 peptide is known to slightly promote gap junctional coupling (by less dwelling in a subconductance state) and to prevent gap junction closure upon exposure to acidic conditions [103]. In sharp contrast,

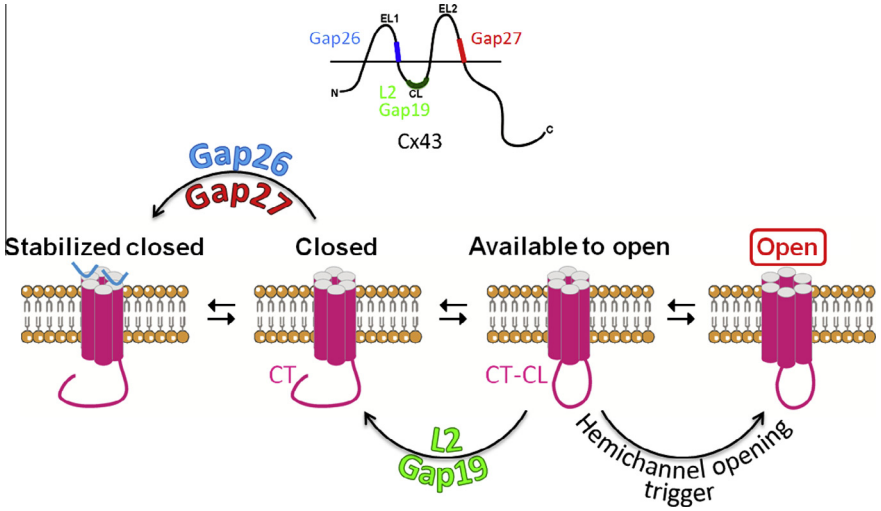

Fig. 1. Peptide inhibitors of hemichannels and their effect on the channel state. Four states are proposed, three of them are closed and only the rightmost is an open hemichannel. Gap26 and Gap27 are identical to a conserved sequence on respectively extracellular loop 1 (EL1) and extracellular loop 2 (EL2) of the connexin protein. They inhibit hemichannels by driving them in a stabilized (deep) closed state from which they cannot directly open [53,96]. L2 (26 amino acids) and Gap19 (9 amino acids) are identical to a sequence located on the cytoplasmic loop (CL) of Cx43. They bind to the C-terminal tail (CT) of Cx43, thereby preventing the CT–CL interaction. CT–CL interaction (depicted as 'CT–CL' in the available to open state) is necessary to bring hemichannels in a state where they are able to open when an electrical or chemical trigger is present. By preventing this CT–CL interaction, L2/Gap19 drives the hemichannels to the closed state, thereby not being available anymore for opening [105]. Note that L2/Gap19 specifically relate to Cx43 and not to other connexins. In addition to the effects illustrated, Gap26/27 and L2/Gap19 also shift the voltage activation threshold for Cx43 hemichannel opening to more positive values [96].

we found that L2 peptide inhibits hemichannels [69]. The exact reason why the responses of hemichannels and gap junction channels to L2 peptide are so different is currently unclear. However, in depth analysis has indicated that these distinctive responses are crucially linked to intramolecular interaction between the C-terminal tail (CT) and the cytoplasmic loop (CL) of Cx43 [69,104,105]. Currently, we do not know whether CT–CL interaction occurs within the same connexin subunit or between subunits. In gap junctions, CT–CL interaction closes the channel while the very same interaction is necessary to keep hemichannels in a state where they are available for opening in response to electrical or chemical triggers (Fig. 1). Thus, L2 peptide drives the hemichannels from the available-to-open state to the closed state. In a next step, an active small peptide sequence was identified within the L2 sequence consisting of the nonapeptide Gap19. This peptide inhibits Cx43 hemichannels like L2, but with higher affinity (half-maximal effect at 7 μM when applied intracellulary) and was demonstrated to have no effect on gap junctional conductance the first 30 min and to even slightly stimulate gap junctions upon 24 h exposure. Gap19 has a good membrane permeability (due to the high lysine content), but linking it to a TAT cell-internalization sequence significantly improves its cell permeation and gives half-maximal inhibition at 7 μM when applied extracellulary.

Criterion 7: Marked reduction of the response in connexin-deficient cells (criterion 4) does not necessarily mean that hemichannels are the exclusive membrane pathway contributing to the response of interest. It is well conceivable that membrane potential changes, Ca^{2+} entry or escape of ATP, all induced by hemichannel opening, recruits other channels activated by these alterations, for example Panx1 channels, maxi-anion channels, CAHLM1 channels and P2X receptor channels. Recent work has indicated that with the current tools available, maxi-anion channels can be distinguished from Cx43 and Panx1 channels [106]. However, the problem is that when other channels are functionally recruited, inhibiting the supposed primary hemichannel candidate may result in strong, almost complete, suppression of the response measured, leading to the false conclusion that the connexin hemichannel (or whatever other channel proposed as the primary candidate) is the dominant one. In this case, comparing the degree of response-inhibition of knocking out or blocking each candidate channel separately may shed some light on primary and secondary responses. However, the complex effects of electrical, Ca^{2+} and ATP signaling on multiple targets combined with extensive cross talk between these signals will make it difficult to disentangle this skein.

3. Concluding remarks

The concept of functional connexin hemichannels has nowadays achieved a status of acceptance in the scientific field. Many physiologically relevant substances have been demonstrated to be released or taken up via the hemichannel pathway and an even more extensive list of factors opening and closing hemichannels is available. Yet, new candidate channels with properties that overlap with connexin hemichannels have appeared, challenging once again the role and contribution of connexin hemichannels to the studied phenomenon. A typical example is the field of ATP release in taste cells where connexins, pannexins and CALHM1 channels have been sequentially proposed to play a role (reviewed in [77,107]). In the absence of specific inhibitors for the newer (younger) candidate channels, some 'criteria-hygiene' presented in this review is necessary. Obviously, the strength of evidence for each channel case should be weighted and luckily enough, not all cells display the competing channel candidates as much as the ubiquitous connexin channel family. An upcoming question for the future is how these channels mutually influence each other to produce a concerted response supported by multiple non-selective, large molecule-permeable channels. Interestingly, the strong interest and resulting information stream on connexin hemichannels has brought up perhaps the most enigmatic question of all: why do free, unapposed hemichannels behave so differently from their counterparts incorporated in gap junctions? Luckily enough, their sometimes distinct behavior (for example relating to the effect of CT–CL interaction) is key to develop strategies for selectively inhibiting hemichannels without inhibiting gap junctions. A handicap of the connexin field is that the protein forms two types of channels, a

problem that is less pronounced for pannexins and absent for CALHM1 channels. At least, the connexin field will definitely not suffer from lack of ideas for the years to come. We are looking at a bright future for gap junctions as well as functional connexin hemichannels.

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