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Review

Cell membrane permeabilization via connexin hemichannels in living and dying cells

Juan C. Sáez[a,b,*], *Kurt A. Schalper*[a], *Mauricio A. Retamal*[c], *Juan A. Orellana*[a,b], *Kenji F. Shoji*[a,b], *Michael V.L Bennett*[d]

[a]*Departamento de Ciencias Fisiológicas, Pontificia Universidad Católica de Chile, Santiago, Chile*
[b]*Núcleo Milenio de Inmunología e Inmunoterapia, Santiago, Chile*
[c]*Laboratorio de Fisiología-Electrofisiología, Clínica Alemana, Universidad del Desarrollo, Santiago, Chile*
[d]*Department of Neuroscience, Albert Einstein College of Medicine, Bronx, NY, USA*

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ABSTRACT

Vertebrate cells that express connexins likely express connexin hemichannels (Cx HCs) at their surface. In diverse cell types, surface Cx HCs can open to serve as a diffusional exchange pathway for ions and small molecules across the cell membrane. Most cells, if not all, also express pannexins that form hemichannels and increase the cell membrane permeability but are not addressed in this review. To date, most characterizations of Cx HCs have utilized cultured cells under resting conditions have and revealed low open probability and unitary conductance close to double that of the corresponding gap junction channels. In addition, the cell membrane permeability through Cx HCs can be markedly affected within seconds to minutes by various changes in the intra and/or extracellular microenvironment (i.e., pH, pCa, redox state, transmembrane voltage and intracellular regulatory proteins) that affect levels, open probability and/or (single channel) permeability of Cx HC. Net increase or decrease in membrane permeability could result from the simultaneous interaction of different mechanisms that affect hemichannels. The permeability of Cx HCs is controlled by complex signaling cascades showing connexin, cell and cell stage dependency. Changes in membrane permeability via hemichannels can have positive consequences in some cells (mainly in healthy cells), whereas in others (mainly in cells affected by acquired and/or genetic diseases) hemichannel activation can be detrimental.

Contents

* *Corresponding author.* Departamento de Ciencias Fisiológicas, Pontificia Universidad Católica de Chile, Santiago, Chile.
E-mail address: jsaez@bio.puc.cl (J.C. Sáez).

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Introduction

Connexin hemichannels (Cx HCs)

Connexin hemichannels are hexameric oligomers of transmembrane proteins termed connexins (Fig. 1), and docking of two hemichannels in apposed membranes forms intercellular gap junction channels. When open at the unapposed cell surface, non-junctional hemichannels are aqueous pores (Fig. 1) permeable to ions and small molecules that allow diffusional exchange between the intra and extracellular compartments and also constitute a route for autocrine/paracrine cellular communication [1,2]. Cx HCs and gap junction channels frequently are oppositely influenced by various experimental and physiological conditions [3–8].

Although synthesis and trafficking pathways of Cx HCs might differ from those of intercellular channels, hemichannels formed by most studied connexins (all but Cx26 [9]) oligomerize in the Golgi/trans-Golgi network [10–13]. Upon assembly, hemichannels (except Cx26 HCs [9]) are transported to the non-junctional plasma membrane via a cytoskeleton-mediated route [14–16]. Once inserted in the plasma membrane, they diffuse laterally to join the external aspect of gap junctional plaques [17,18], where they dock with hemichannels from a neighboring cell to form intercellular channels. However, Cx43 HCs may also be directly targeted to the region where gap junctional plaques are found via a microtubule/dynein/β-catenin/N-cadherin-dependent pathway [19]. It might be possible that hemichannels in non-junctional membrane and those in gap junctions are derived from different precursor pools. In support of this notion, fluorescent-tagged Cx43 can be delivered to cell protrusions or to cell surface domains that lack a contacting cell [20].

In cells expressing wild-type connexins, changes in surface hemichannel levels and/or intrinsic properties of hemichannels

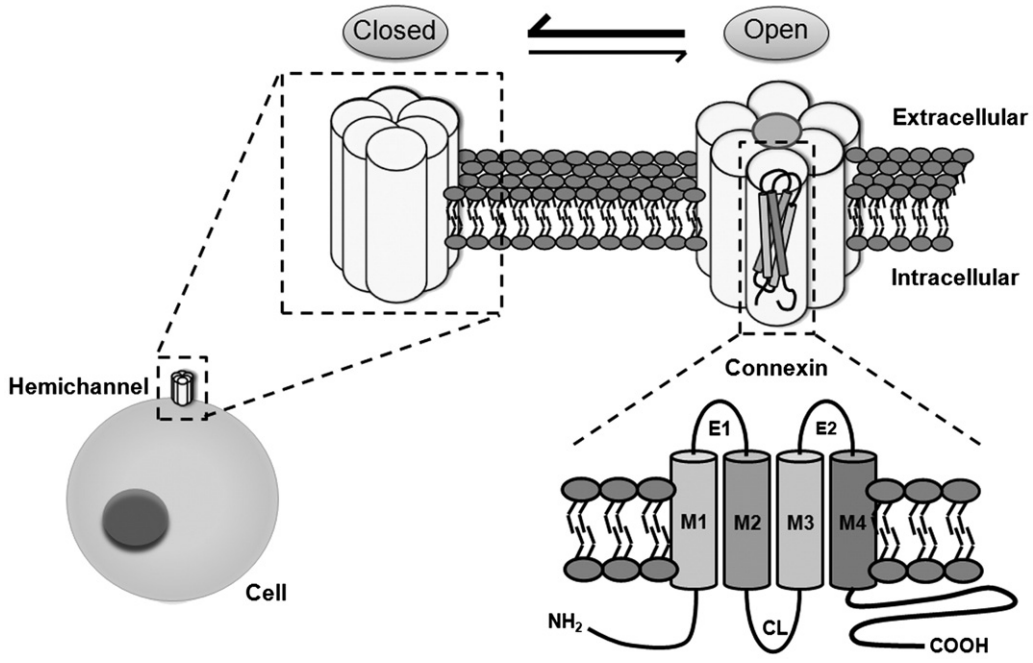

Fig. 1 – Diagram illustrating basic structures of connexins and undocked hemichannels present at the cell surface. The membrane topology of a connexin consists of 4 membrane-spanning domains (M1-M4), 2 extracellular loops (E1 and E2) and 1 cytoplasmic loop (CL). The amino (−NH_2) and carboxy (−COOH) terminal tail are intracellular. A hemichannel is formed by six connexins that oligomerize laterally leaving a central pore. In cultured cells under resting conditions hemichannels remain preferentially closed, but they can be activated by diverse physiological and pathological conditions, offering a diffusional transmembrane route between the intra and extracellular milieus.

found at the cell surface (e.g., open probability and permeability) can significantly affect the cell membrane permeability. Increases in hemichannel levels have been clearly associated with rises in intracellular-free Ca^{2+} concentration ($[Ca^{2+}]_i$) [6,21]. Variations in hemichannel open probability can result from changes in covalent modifications of connexin subunits (e.g., redox and phosphorylation state) or variations in transmembrane physicochemical conditions (e.g., transmembrane voltage, pH and concentration of monovalent cations) that affect the pore forming proteins. Although changes in hemichannels unitary conductance upon application of specific stimuli have not yet been reported, they are likely to occur in Cx43 HCs after changes in phosphorylation state that reduce their size cutoff [22]. Additionally, several connexin mutations affect the open probability and/or permeability of hemichannels [23–31] and in most cases possible permeability changes have not been yet evaluated.

Cultured cells (primary cultures or cell lines) under "resting" conditions show low hemichannel activity defined as low frequency of unitary current events and/or low membrane permeability to small molecules (e.g., ATP and small fluorescent dyes). However, cells within organisms are exposed to diverse stimuli, which may transiently increase the hemichannel activity. Moreover, there may also be endogenous hemichannel inhibitors that contribute to control transient increases in hemichannel activity. With regard to positive regulators, increased hemichannel activity has been recorded in cells exposed to endogenous ligands associated with cell proliferation or differentiation, such as lipophilic molecules and growth factors [4,6,32]. Thus, the activity of hemichannels in cultured cells under "resting" conditions might not reflect the importance of these transmembrane pathways in cells *in vivo*.

Activation of hemichannels has also been associated with cell death under diverse pathological conditions (for review see [33]). In acquired diseases, the primary cause of cell death is directly related to the pleiotropic effects of the injury, and a high level of hemichannel activity makes the cells more susceptible to the injury (e.g., generation of reactive oxygen species, ATP depletion and increase in $[Ca^{2+}]_i$ as occurs after ischemia-reperfusion). In contrast, sustained and uncompensated high hemichannel activity found in human genetic diseases associated with point connexin mutations and gain of hemichannel function could be the primary cause of death, as observed in cells transfected with mutant connexins [23–25,27–29,31]. Under these conditions, the deleterious effect of hemichannel activation is possibly related to the long lasting and unbalanced Ca^{2+} influx leading to activation of intracellular Ca^{2+}-dependent hydrolases (e.g., proteases, phospholipases and endonucleases) and cellular depletion of vital small solutes and metabolites such as ATP and glutathione.

Do cells in tridimensional tissues express functional hemichannels in their surface? To our knowledge, demonstrations of hemichannel activity *in vivo* or in tissue slices bathed in extracellular solution containing divalent cations have not been reported. However, ATP is released through hemichannels in whole-mount retinal pigment epithelium [34], and high Cx43 HC activity occurs in colonocytes during infectious diarrhea where it may mediate water release [35].

Structure–function relationship

Connexin proteins are encoded by at least 21 different genes in humans and are named according to their predicted molecular mass in kDa from Cx23 to Cx58 [36]. All studied connexins share a common membrane topology with four transmembrane domains, two extracellular loops, one intracellular loop, and both N- and C-terminus in the cytosolic side of the cell membrane (for a review, see [37]). The main divergences in length and amino acid sequence among connexins are located in the C-terminal tail and cytoplasmic loop. Several recent studies have focused on how each connexin protein domain affects the functional state of hemichannels (see below).

Post-translational modifications of connexins affect preferentially amino acid residues located in the C-terminus and include palmitoylation, myristoylation, nitrosylation, protease cleavage and phosphorylations [38–42]. But the possible role of most of these covalent modifications on hemichannels remains unknown. Phosphorylation is the most extensively studied covalent modification of connexins (for reviews, see [43,44]). Electrophysiological studies of Cx43 HCs using PKC inhibitors suggested that activation of a novel PKC isoform completely inhibits hemichannels, whereas activation of a conventional isoform (βII) only partially reduces the hemichannel-mediated currents [45]. Moreover, MAP kinase or PKC-mediated phosphorylation of Cx43 HCs reconstituted in liposomes reduces their permeability to molecules [22,46], and PKC-mediated phosphorylation of Cx43 at serine 368 is required to decrease the hemichannel-mediated permeabilization in this model [47]. In addition, opening of Cx46 HCs expressed in *Xenopus* oocytes is also decreased by PKC-mediated protein phosphorylation [48].

In astrocytes and exogenous expression systems, *S*-nitrosylation of Cx43 and Cx46, i.e., covalent binding of NO to Cys, affects the activity of hemichannels [39,49]. In cortical rat astrocytes, treatment with the NO donors, GSNO or Nor-1 or metabolic inhibition increases hemichannel-mediated dye uptake [39]. NO is also involved in Cx43 HC-mediated cell permeabilization induced by proinflammatory cytokines in astrocytes [5] or Cx43 transfected C6 glioma cells [4]. Although NO does not affect the open probably of Cx46 HCs under control condition, it changes the opening and closing kinetics at positive voltages as well as the permeability to large molecules [49]; the possible relevance of NO 46 HCs expressed by lens fibers remains to be demonstrated. Proteolysis of Cx46 is another putative post-translational modification that might affect Cx46 HCs. In support to this possibility, it has been shown that proteolysis shifts the pK_a of gap junction channels formed by rat Cx46 from 6.8 to 6.6 [50], but its functional consequences on Cx46 HCs remains to be studied.

In gap junction channels formed by Cxs 43 or 45, the C-terminal tail may participate in channel gating induced by intracellular acidification or membrane potential through a "ball and chain mechanism" [51,52]. Moreover, the Cx43 C-terminus binds to the second half of the cytoplasmic loop in a pH-dependent action [54]. It remains unknown whether similar conformational changes account for the hemichannel sensitivity to intracellular pH variations. In support of this possibility, intracellular acidification rapidly reduces Cx46 HC current in *Xenopus* oocytes [53]. In addition, nuclear resonance magnetic experiments reveal that acidification induces α-helical structure and protonation of histidine residues in a Cx43 peptide corresponding to the second half of the intracellular loop, without mayor structural changes in the C-terminus.

In ^{15}N-HSQC NMR analyses, the carboxy terminal (CT) domain of Cx43 was shown to be highly disorganized with two α-helical

regions one at Ala^{311}–Ser^{325} and the other at Asp^{339}–Lys^{345} [55]. The CT can interact with several intracellular and membrane proteins, such as ZO-1 [56], ZO-2 and ZO-3 [57], ubiquitin protein ligase, Nedd4 [58], several protein kinases (for reviews, see [43,44]), aquaporin-0 [59] and NOV, a member of the CCN gene family [60]. The interaction between connexins and other proteins has been supported by co-localization and pull-down experiments [61,62]. However, it has not been determined whether connexins forming surface hemichannels interact with any proteins other than protein kinases. Truncation of Cx43 CT changes the subcellular localization, number and size of Cx43 gap junctions but does not prevent their assembly into hemichannels [6,63]. Accordingly, mutant Cxs 32, 43, 46 and 50 lacking most of their CT form functional hemichannels and gap junction channels [6,39,64,65], showing that the CT is not essential for channel function. Hemichannels and gap junction channels formed by most CT mutants studied show relevant differences in their electrophysiological and permeability properties [6,39,64,65]. Truncation of Cx43 C-terminus affects the location, decreases the number and increases the size of gap junction plaques in cardiomyocytes, suggesting its involvement in the regulation of critical steps in hemichannels trafficking [63]. In addition, the Cx43 CT alone seems to play a regulatory function in glioma cell invasion [66], inhibition of cell growth [67], determination of the infarct size and susceptibility to ventricular tachyarrhythmias after acute myocardial infarction [68] and neuroprotection during cerebral ischemia [69]. To our knowledge, similar studies addressing the possible role of surface hemichannels have not been reported and it remains to be determined the extent to which many biological responses are mediated by gap junction channels or hemichannels or both.

Much less is known about the functional role of the N-terminal tail of connexins. Mutation of the N-terminus of different connexins affects some hemichannel properties including gating polarity [70], charge selectivity [71] and perm-selectivity [72] as well as formation of functional hemichannels [73]. Cx43 tagged with enhanced green fluorescent protein (EGFP) on its N-terminus shows formation of gap junction plaques but fails to form functional gap junction channels or hemichannels in HeLa cells transfectants [74]. The latter might be due to a pore blocking action of the altered N-terminal tail, as has been proposed for channels composed of wild-type Cx26, in which the N-terminus forms a plug that physically closes the pore [75]. The N-terminus has α-helical region [76,77], which at least in Cx32 has a flexible turn around glycine 12 allowing the N-terminus to extend into the channel vestibule [78]. Together, these data indicate that the N-terminus is important in formation of Cx HCs as well as in control of their functional state.

The intracellular loop (IL) of connexins has been less studied. However, it plays a major role in pH sensitivity of gap junction channels composed of Cx38 [79]. Functionally, the IL has been shown to participate in the modulation of permeability, conductance and voltage gating of Cx26 and Cx30 based gap junction channels [80]. Probably, the IL affect functions (but not formation) of gap junction channels [81] because it interacts directly with the connexin C-terminus [82,83] and other proteins such as aquaporin-0, calmodulin, beta-tubulin and possibly cadherins [84–87]. This intramolecular interactions (IL–C-terminus) can be modulated by changes in intracellular pH [88]. Moreover, the IL of fish Cx35 and chicken Cx56 can be phosphorylated [89,90], suggesting that channel functions can be modulated through changes in post-translational covalent modifications of the IL. Since phosphate can be protonated, which changes the net charge of the phosphorylated amino acid residues, this covalent modification may affect other hemichannels properties such as pH and voltage sensitivity.

Each extracellular loop (EL) has three conserved cysteine residues (Cys), which can form disulfide bridges with Cys located in the same or other loops [91]. The ELs, are implicated in slow hemichannel gating (also call loop gating) [92], which is modulated by extracellular divalent cations [93]. Loop gating involves movement of the TM1-E1 region resulting in a reduction of diameter of the hemichannel lumen [94], mainly due to a rotation and inward tilt of all six M1 segments [95]. Interestingly, different peptides sharing the EL sequence block Cx HCs in diverse models [5,96–98] and prevent the formation of new gap junction channels [99,100]. However, a nonspecific allosteric effect was proposed as the mode of action of these peptides [101]. More selective peptides for pannexin hemichannels are now available [102], and future studies might provide similar reagents for Cx HCs together with more knowledge on their mechanism of action.

The transmembrane domains (TM) are believed to form the hemichannel aqueous pore, but little clarity exists regarding the specific amino acid residues lining it. Important advances have been made using SCAM (substituted Cys accessibility method) experiments. Prominent reduction in the hemichannel activity occurred after binding of the sulfhydryl-specific methanethiosulfonate MTS to the M1 domain of Cx46 [103] and M3 domain of Cx32 [104], suggesting that these TMs line the pore of hemichannels formed by those connexins. However, the recently reported 3.5 A° resolution map of wild-type human Cx26 [77], indicate that M3 and M4 are facing the hydrophobic membrane environment, while M1 and M2 line the pore interior.

Permeability

Most studies of hemichannel permeability have measured the uptake of synthetic molecules with different mass, shape and net charge such as Lucifer yellow [46,105], ethidium [3,5,6,74], calcein [106], 5(6)-carboxyfluorescein [47,107] and DAPI [108]. Release or uptake measurements of biologically relevant molecules such as ATP [109,110], glutamate [111], NAD^+ [112], glutathione [113], prostaglandin E_2 [114], 2-NBDG [5] and ascorbate [115] have also provided information on permeability of hemichannels. For paracrine/autocrine actions, transient opening of hemichannels may allow release of enough signaling molecule to reach effective concentrations in a confined discrete extracellular space. There is still the question of whether Cx HCs provide a diffusional pathway for passive uptake of metabolites, generally thought to be mediated by relatively specific membrane transporters. An answer may be found through calculation of apparent affinity constants and maximal transport velocity of hemichannels for particular permeant molecules, which could be compared to those of transporters for the same molecules (e.g., glucose).

Is permeability a fixed or a plastic feature of hemichannels? Recent evidence indicates that hemichannel permeability can be affected by covalent modification of the hemichannel subunits, which likely cause conformational changes in the hemichannel structure. For example, in *Xenopus* oocytes the permeability of Cx46 HCs to Lucifer yellow and ethidium is slightly lowered (~15%) after treatment with nitric oxide (NO) without changes in

5(6)-carboxyfluorescein uptake, suggesting that NO reduces the size cutoff of Cx46 HCs, possibly through *S*-nitrosylation of connexin subunits [49]. Moreover, the permeability of Cx43 HCs reconstituted in liposomes to charged organic dyes is reduced by PKC phosphorylation of Cx43, which presumably reduces the pore diameter [22]. Interestingly, when all six connexin subunits are phosphorylated, the sucrose permeability becomes close to zero, but hemichannels remain permeable to ethyleneglycol (*MW* 62) [22]. Thus, even when impermeable to larger molecules, phosphorylated Cx43 HCs remain permeable to small molecules and most likely to ions. Therefore, in addition to changes in number of surface hemichannels and/or open probability of hemichannels, changes in cell membrane permeability via hemichannels could result from changes in intrinsic hemichannel permeability features. To date, reduction in hemichannel permeability as consequence of putative covalent modifications have been described but increases in hemichannel permeability may also occur and further research will be required to validate or disapprove this possibility.

Regulation of the activity of hemichannels at the cell surface

Number of hemichannels in the cell membrane

Cell membrane permeability via hemichannels is affected by relatively rapid changes in the amount of surface hemichannels, and biotinylation of surface membrane proteins with subsequent "pull down" has proved a valuable tool to study the magnitude and velocity of these changes [21,33,39]. Cortical astrocytes or Cx32 HeLa transfectants exposed to metabolic inhibition for only 15 min show an abrupt increase of ~2-fold in their surface Cx43 and Cx32 levels, respectively [21,39]. Moreover, Cx43 transfected HeLa cells treated with a Ca^{2+} ionophore (4Br-23187) or FGF-1 require ~30 min and ~7 h, respectively, to reach a ~2-fold increase in surface hemichannel levels [6].

Rises in $[Ca^{2+}]_i$ appear to be a key regulator of surface hemichannel amount. Increased hemichannel levels in Cx43 HeLa transfectants after FGF-1 requires sustained rises in $[Ca^{2+}]_i$ and p38 MAPK activation [6]. Accordingly, blockade of intracellular Ca^{2+} rises with BAPTA (cells preloaded with BATA-AM) or application of SB202190, a p38 MAPK inhibitor, similarly prevent the increase in surface Cx43 HC levels, suggesting that $[Ca^{2+}]_i$ rises and p38 MAPK activation are in the same activation pathway. In Cx32 HeLa transfectants, the level of surface hemichannels increase after metabolic inhibition, which requires Cx HC-mediated Ca^{2+} influx [21]. Interestingly, the increase in surface hemichannel levels of cells treated with metabolic inhibitor, FGF-1 or a Ca^{2+} ionophore is not paralleled by proportional rises in total connexin levels, suggesting that the mechanism involves connexin redistribution and not synthesis *de novo* [6,21]. In addition, cytosolic stress induced by different stimuli reduces Cx43 HC internalization/degradation and increases the number of surface hemichannels in rat kidney fibroblasts [116]. Different forms of metabolic stress are associated with increased cytosolic Ca^{2+} levels. It is likely that amount of hemichannels at the cell membrane can be modulated by increases in $[Ca^{2+}]_i$ that affect insertion and/or removal of hemichannels from the cell surface. Thus, increase in $[Ca^{2+}]_i$ and consequently the number of surface hemichannels could possibly be mediated by numerous membrane receptors and ion channels; receptors linked to G proteins that lead to IP_3 generation and release of Ca^{2+} from intracellular stores or activation of several TRP channels, P2X receptors and pannexin hemichannels (Fig. 2).

In addition to the above mechanisms, which take place over a time course of several, the cell membrane permeability could change much more quickly via gating of Cx HCs.

Gating mechanisms

Transmembrane voltage

In cultured cells under resting conditions, hemichannels have a low open probability at negative membrane potentials and open probability is increased at positive potentials (for review see [117]). Two gating mechanisms have been described for rat Cx46 HCs, termed fast and slow gating (slow gating is also known as loop gating) [92]. Normally, positive membrane potentials activate fast gating which corresponds to fast transitions between the fully open state and a substate. At negative membrane potentials, the loop gating activates slow transitions, perhaps involving multiple substates, between the fully open state, substates and fully closed state [117]. Recently, Verselis and Srinivas [93] showed that slow but not fast gating of Cx46 HCs is modulated by extracellular divalent cations. Negative cell membrane voltages cause closure by loop gating, and divalent cations help to stabilize the closed conformation. This phenomenon may be due to an interaction between divalent cations and the extracellular loops, which induces a narrowing of the hemichannel pore [94]. In general, the biological importance of Cx HC activation by voltage remains to be demonstrated.

Extracellular cations

Increasing extracellular Ca^{2+} concentration ($[Ca^{2+}]_o$) reduces the open probability of all Cx HCs examined (for a review, see [118]). In Cx32 HCs, Ca^{2+} ions bind to a 12 Asp ring at the external vestibule of the hemichannel pore [119]. Alternatively, Cx37 HCs may be directly blocked by extracellular Ca^{2+} going through the pore and reaching binding sites located at the cytoplasmic side [120]. Using atomic force microscopy, a 1.8-nm external pore diameter was visualized in Cx43 HCs reconstituted in lipid bilayers under control conditions. Moreover, removal of extracellular Ca^{+2} increased the outer hemichannel pore diameter to 2.5 nm [121,122]. In addition, Ca^{+2}-induced inhibition of Cx50 HCs is modulated by extracellular concentration of monovalent cations [123] and Ca^{+2} probably acts through the stabilization of the closed configuration of the loop gate [93]. Mg^{2+}, the other main extracellular divalent cation, also affect the functional state of hemichannels [124]. Increasing extracellular Mg^{2+} shifts the voltage dependence of activation, i.e., opening, to more positive potentials, decreases the maximum conductance, accelerates deactivation and slows activation [124]. These effects of Mg^{2+} on Cx46 HCs may result from sequential activation with two independent divalent cation binding sites. In addition, a mutation in the first extracellular domain of hCx46 (hCx46*N63S) results in hemichannels with increased sensitivity to Mg^{2+}-induced blockade (124), which may provide further insight on the mechanism of action of Mg^{2+}. The reduction in hemichannel activation caused by extracellular divalent cations can be overcome by other agents. For example, nitric oxide donors and inflammatory cytokines increase the hemichannel activity in astrocytes, in the presence of divalent cations [5,39]. Under these conditions, the number of hemichannels in the cell surface is not elevated, and the increase in hemichannel activity is rapidly blocked by DTT, a cysteine reducing agent, suggesting that nitrosylation of Cx43 reduces the sensitivity of Cx43 HCs to extracellular divalent cations.

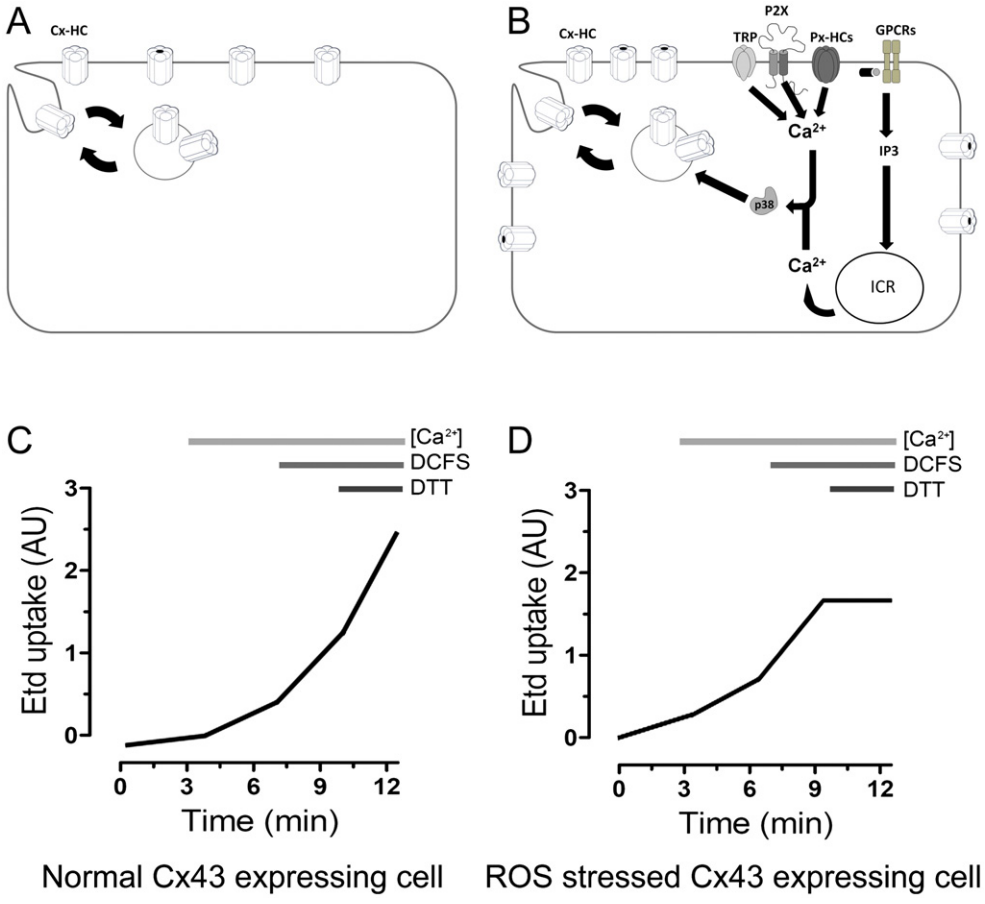

Fig. 2 – Scheme of several conditions that affect membrane permeability via Cx43 HCs. (A) Few Cx43 HCs at the cell membrane of a normal single cell under resting condition. Open hemichannels (black dot at the center) are few. Arrows indicate insertion and removal of Cx43 HCs contained in transport vesicles. (B) Single cell under metabolic stress, where diverse membrane channels [Ca^{2+} permeable TRP channels (TRPs), purinergic P2X receptors (P2Xs), pannexin hemichannels (Px HCs) known to be activated by reactive oxygen species lead to Ca^{2+} influx]. Also, G-protein-coupled receptors (GPCRs) can increase $[Ca^{2+}]_i$ by generation of IP_3, which acts on intracellular Ca^{2+} stores (ICS) to release Ca^{2+} into the cytoplasm. Under both conditions, the increase in $[Ca^{2+}]_i$ leads to activation of p38 MAP kinase (p38) that either reduces (−) removal from or enhances (+) insertion of Cx43 HCs into the cell membrane. Under both conditions, the net number of surface Cx43 HCs is augmented. (C and D) Graphs illustrate time lapse measurements of Etd uptake in Cx43 expressing cells under control conditions (left) or conditions that generate an oxidant stress due to excessive generation of reactive oxygen species (ROS) (right). In both cases, Etd uptake is first shown (~3 min) under control conditions and then treatment with agents that increase $[Ca^{2+}]_i$ (i.e., FGF-1 in normal cell and metabolic inhibitors in ROS stressed cell), followed by change of the extracellular medium to divalent cation-free solution (DCFS) followed at about 10 min of recording with 10 mM DTT. DTT induced opposite effects in normoxic and ROS stressed cells.

Hemichannels formed by Cx50, a main lens connexin, are sensitive to extracellular monovalent cations [125]. Substitution of extracellular Na^+ with K^+, Cs^+, Rb^+ or $NH4^+$, in the presence of physiological extracellular Ca^{2+} concentration, results in >10-fold potentiation of macroscopic Cx50 HC currents, an effect reversed upon returning to Na^+. Replacement of Na^+ with Li^+, choline or TEA does not potentiate the current. The magnitude of potentiation of Cx50 HC currents is progressively decreased as external Ca^{2+} is reduced. The potentiation is reduced in low Ca^{2+} solution, and the main effect of K^+ appears to be a reduction in the ability of divalent cations to close Cx50 HCs. Cx46 HCs exhibit only a modest increase in open probability upon replacing Na^+ with K^+. Studies using reciprocal chimeric hemichannels demonstrate that the difference in regulation by monovalent ions in Cx46 and Cx50 HCs resides in the region from the NH_2-terminal through the cytoplasmic loop [125]. A possible biological relevance of hemichannel regulation by extracellular monovalent ions is in ephaptic communication as in the retina [126].

Other divalent cations that affect hemichannel activity are Zn^{2+}, Co^{2+}, Cd^{2+}, Mg^{2+} and Ba^{2+} [127]. In bass retinal hemichannels, currents generated by depolarization in Ca^{2+}-free saline and in saline containing 1 mM Ca^{2+} medium are markedly inhibited by extracellular Zn^{2+}. The Zn^{2+} effect on hemichannel currents is concentration dependent (half-maximum inhibitory concentration = 37 μM) and is more potent as compared to other divalent cations ($Zn^{2+} > Cd^{2+}$, $Co^{2+} > Ca^{2+} > Ba^{2+} > Mg^{2+}$). Interestingly, Zn^{2+} and Ca^{2+} modulate hemichannels independently in additive experiments. Cell treatment with *N*-bromosuccinimide to modify histidine residues suppresses the inhibitory action of Zn^{2+}, and Zn^{2+} may act through histidine residues on the extracellular domain

of hemichannels independent of $[Ca^{2+}]_o$ [127]. The effect of Zn^{2+} on Cx38- or Cx35-HCs is biphasic; low $[Zn^{2+}]$ (10 μM) enhances the currents generated by depolarizing voltages 40 mV, and higher Zn^{2+} (1 mM) reduces the currents [128]. This differential effect of Zn^{2+} on hemichannel activity may be explained by differences in connexins as well as by differences in the cell system used to evaluate its effects.

pH

Hemichannels composed of different connexin types are sensitive to intracellular acidification [53,129,130]. A direct effect of H^+ on the connexin subunit was suggested in Cx46 HCs, since the response is very fast and is observed in the excised patch configuration [53]. Chemical gating of Cx46 HCs seems to affect a pH sensor located close to the channel pore [53] and pH reduction shifts the voltage activation to more positive voltages [131]. However, intracellular acidification might rapidly affect $[Ca^{2+}]_i$ in intact cells, which could affect activation of Cx32 and Cx43 HCs.

Redox potential

S-nitrosylation of Cx43 increases dye uptake in cortical astrocytes [39] and is probably associated with changes in the voltage sensitivity of Cx46 HCs [49]. The open probability of Cx43 HCs increases several fold after treatment of normal cells with dithiothreitol (DTT) [41], suggesting that both reduction and oxidation can modulate hemichannel gating, possibly depending on the cell type and its metabolic state. In agreement with this possibility, the increase in Cx43 HC activity observed in cells under oxidative stress, such as that induced by NO donors [39], metabolic inhibition [39], inflammatory cytokines [5] and hypoxia-reoxygenation [98], is completely inhibited by DTT.

The increase in Cx43 HC activity induced in normal cells by DTT (Fig. 2) or nitric oxide donors occurs in seconds and without changes in levels of surface Cx43 [39,41], suggesting that both result from increase in open probability of existing surface hemichannels. The effect of DTT is not abolished by buffering the $[Ca^{2+}]_i$ and the effect of both NO donors and DTT occurs in the presence of extracellular divalent cations. The increase in Cx43 HC activity induced by DTT is not reproduced by extracellular application of reduced glutathione, but application of glutathione in the recording pipette increases the open probability [41]. Moreover, the recovery in membrane permeability induced by DTT in cells during metabolic inhibition is reproduced by application of glutathione ethyl ester, which is membrane permeable, but not by extracellular reduced glutathione [39]. These data indicate that intracellular but not extracellular Cys residues mediate the changes in Cx43 HC activity induced by alterations in redox potential. Changes in redox potential may not affect all Cx HCs, because not all connexins have cysteine residues in intracellular domains, e.g., Cx45.

Mechanosensitivity

Mechanical stimulation induces ATP release through Cx43 HCs in osteoblastic cells and chondrocytes [132,133]. Moreover, mechanical stimulation enhances the activity of Cx46 HCs at negative transmembrane potentials but inhibits the increase in hemichannel activity induced by positive membrane potentials [134]. The mechanosensitivity of lens hemichannels may assist accommodation by providing a transient path for volume flow as the lens changes shape [134]. Cx30 HCs are also mechanosensitive and may be involved in regulation of pressure-induced natriuresis by releasing ATP into the tubular fluid [135]. Additionally, Cx43 HCs in osteocytes activate after shear stress, accelerating PGE_2 release [136]. The mechanism of action of mechanical stress on Cx HC activity remains unknown but clearly occurs in the presence of extracellular divalent cations and most likely without changes in intracellular redox potential.

Interaction between different hemichannel-mediated membrane permeabilization mechanisms

Cx46 HCs expressed in *Xenopus* oocytes show a progressive current facilitation after repetitive positive pulses to potentials greater than +20 mV, which depends on the time between pulses (<20 s) [137,138]. The mechanism of hemichannel current facilitation by repetitive depolarizing voltages remains unknown. However, it is unlikely to occur as a consequence of increased $[Ca^{2+}]_i$ because increases in $[Ca^{2+}]_i$ require >15 min to significantly increase the hemichannel-mediated cell permeabilization to dyes [3,6,21,39]. Facilitation of hemichannel currents is also observed in oocytes expressing Cx46 with a truncated C-terminus; nominal removal of divalent cations from the extracellular side causes maximal current activation of these truncated hemichannels and also prevents facilitation, indicating that Cx46 HCs show activation independent of their C-terminal domain [138]. Similarly, application of consecutive electrical pulses to Cx46 expressing oocytes in the absence of extracellular divalent cations does not affect the maximal current, which is ~3-fold higher than the maximal current generated by repetitive voltage steps at physiological $[Ca^{2+}]_o$ (Fig. 3). Moreover, repetitive application of depolarizing voltages from a holding potential of −60 mV to +80 mV in the presence of extracellular divalent cations induces current facilitation to a maximal value, which is lower than that recorded in the absence of divalent cations (Fig. 3). However, the current facilitation depends on the amplitude of the depolarization, reaching a maximum at approximately +60 mV [138], suggesting that repetitive voltages open a smaller fraction of Cx46 HCs than does divalent cation-free solution. These results also suggest that Cx46 HC currents would increase when repetitive voltage steps are applied to cells bathed with saline solution containing physiological concentrations of Ca^{2+} and Mg^{2+}.

Potentiation occurs after sequential stimulation of cells with different treatments known to enhance hemichannel activity. Incubation of Cx43 HeLa transfectants with 20 ng/ml FGF-1 induces a ~2-fold increase in the dye uptake rate [6]. The increase in hemichannel activity induced by FGF-1 requires elevation of intracellular $[Ca^{2+}]_i$ and is associated with increase in Cx levels at the cell surface [6]. Application of DTT to Cx43 HeLa transfectants treated with FGF-1 further increases Cx HC activity (Figs. 4A and B) and to a level higher than that induced by DTT alone (Fig. 4B), effects that are not observed in parental cells (Fig. 4B). The effect induced by FGF-1 + DTT is greater than the sum of the effects of the two stimuli by themselves and is completely blocked by La^{3+}, indicating the participation of Cx HCs and absence of pannexin hemichannels. As expected, a similar response occurs after adding DTT to cells treated with the Ca^{2+} ionophore 4-Br-A23187 (Figs. 4C and D). Again, the response is higher than with either compound alone and was also prominently inhibited by La^{3+}. Permeabilization by multiple stimuli may well be greater than that induced by any of them singly, particularly if there is synergistic action. Similarly, exposure of cortical astrocytes to a divalent cation-free

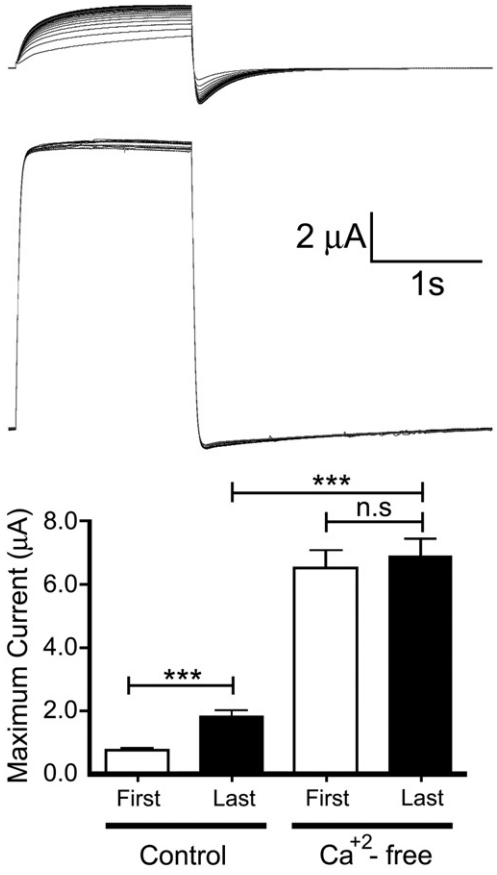

Fig. 3 – Facilitation of Cx46 HC current induced by successive depolarizations might not result in activation of all hemichannels available at the plasma membrane. *Xenopus laevis* oocytes were injected with cRNA of rat Cx46 and 24 h later electrophysiology recordings were performed. The pulse protocol was 2.5 s at −60 mV following by 1.5 s at +80 mV for 25 repetitions. Two current records are shown; the top record is superimposed traces showing progressive increase in outward and tail currents induced by the pulses under control conditions (in ND96 bath solution, plus 1.8 Ca^{+2} and 1.0 Mg^{+2}). The bottom record was obtained with the same protocol as in (A), but the cell was bathed in ND96 recording solution without Ca^{+2} and Mg^{+2} (Ca^{+2} free). Under this condition the initial current was ~4.0 times bigger than the maximal current in control medium ($n=3$, $^{***}p<0.05$) and there was little variation in Cx46 HC currents during successive pulses. The bar graph shows mean ± SEM of several experiments, pointing out the difference between the first and the last pulse under control conditions ($^{***}p<0.05$). However, no difference was found between the first and last current in oocytes in Ca^{+2}-free solution (n.s., $p>0.05$).

solution plus DTT induces a higher rate of ethidium uptake than the sum of each stimulus acting alone, and the effect is abrogated by β-glycyrrhetinic acid (β-GA), a known hemichannel blocker (Fig. 5). In summary, permeabilization responses *in vivo* may involve multiple hemichannel activating mechanisms, and may induce an unexpectedly great increase in cell permeability (Fig. 2). Increased insertion and increase in P_o of inserted channels would be multiplicative and could account for these observations.

Cell permeabilization through hemichannels induced by metabolic inhibition (iodoacetic acid and antimycin A) in HeLa-Cx43 and HeLa-Cx32 transfectants exposed to metabolic inhibitors show a higher (La^{3+} sensitive) ethidium uptake rate increase after exposure to a divalent cation-free solution than without metabolic inhibition [21]. However, under this condition, the total response is not higher than the sum of each stimulus by itself, indicating that potentiation was less than by summation. Moreover, application of metabolic inhibitors after divalent cation-free solution does not further affected the cell permeabilization, suggesting a requirement for Ca^{2+} influx in the metabolic inhibition induced hemichannel response or saturation of available Cx32 HCs. Therefore, not all activating stimuli induce potentiation of the Cx HC activity, possibly depending on the cell state, stimulus nature and molecular mechanism.

Concluding remarks

Historically, hemichannels were regarded simply as precursors of gap junction channels, since there was limited evidence that hemichannels were not open to the external milieu prior to gap junction channel formation. Moreover, their permeability properties predicted from that of gap junction channel was so high that their opening was thought to be deleterious. A possible error has been the extrapolation of gap junction channel permeability to that of the constituent hemichannels. The human genome encodes 21 connexins and most cell types express two or more, which frequently offers the formation of heteromeric hemichannels instead. Moreover, most permeability studies have been carried out in homomeric gap junction channels formed by no more than 40% of connexins and the most relevant information obtained in those studies is that gap junction channels could show charge selectivity. Nonetheless, the size cutoff of the few homo and heteromeric gap junction channels so far characterized remain unknown and numerous reports still quote that gap junctions are permeable to molecules of up to 1–1.2 kDa based on studies obtained in insect cells which do not express connexins. Now it is clear that hemichannels should be viewed as intercellular communication pathways independent of gap junctions and in parallel with their role as precursors of gap junction channels. Clarification of their role as a pathway between cytoplasm and extracellular space will require further work. For example, it is unknown if cellular sorting mechanisms for delivery of hemichannels that will be functional as hemichannels differ from those hemichannels that are destined to form gap junction channels. In agreement with this possibility, in epithelial and endothelial cells, gap junctions are often located in the baso-lateral membrane below the apicaltight juctions. Hence, in these tissues it is clear that if there are hemichannels in the apical membrane, they do not form gap junction channels.

There is little doubt that surface hemichannels not forming gap junctions will be found interacting with other membrane or cytoplasmic proteins in the same way as gap junction channels, but at this point identity of binding partners is uncertain. As discussed here, intercellular communication through Cx HCs is a highly regulated process, and cell signaling that upregulates Cx HC activity frequently down regulates gap junction based intercellular communication. Although these differences could be explained by spatial separation of regulatory pathways not yet described, they

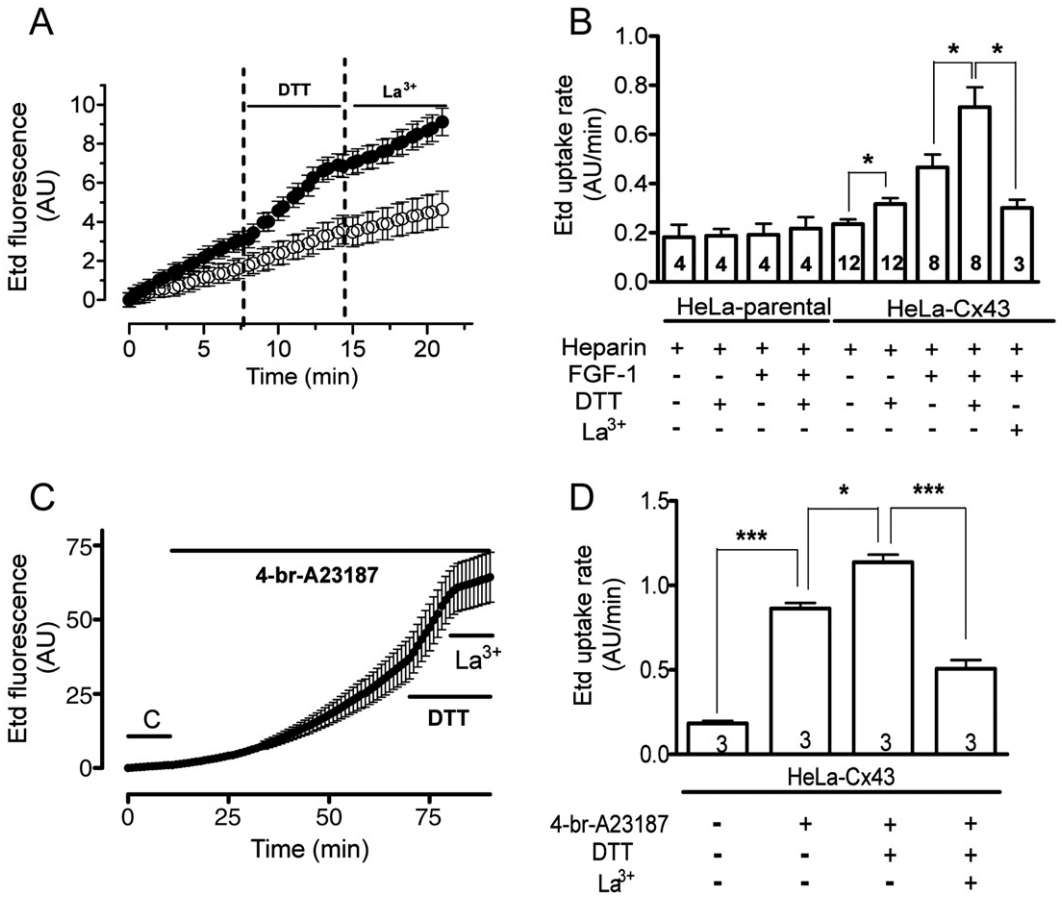

Fig. 4 – DTT induces additional increase in the Cx HC-mediated dye uptake of HeLa-Cx43 cells treated with FGF-1 or a Ca^{2+} ionophore. (A) Time lapse recording showing the Etd uptake of HeLa-Cx43 cells or parental HeLa cells incubated for 7 h with 20 ng/ml FGF-1 plus heparin (filled circles) or heparin alone (white circles), after addition of 10 mM DTT and after treatment with 200 μM La^{3+}. Representative experiment showing mean ± SEM of 20 cells. (B) Average Etd uptake rates of parental HeLa cells or HeLa-Cx43 cells in the control condition (heparin alone), after addition of 10 mM DTT or incubated for 7 h with 20 ng/ml FGF-1 plus heparin and treated with DTT; and after addition of La^{3+}. (C) Time lapse recording showing Etd uptake by HeLa-Cx43 cells in the control condition (c) and after addition of 2.5 μM 4-Br-A23187, 10 mM DTT and La^{3+} (150 μM). Representative experiment showing mean ± SEM of 20 cells. (D) Average Etd uptake rates of HeLa-Cx43 cells in the control condition, after addition of 4 Br-A23187, then DTT and then La^{3+}. The number of independent experiments is indicated within each bar. 20 cells were recorded in each experiment. $^{*}p<0.05$; $^{***}p<0.001$.

could also be explained by differences in post-translational hemichannel modifications before or during delivery to different membrane domains.

We know that the activity of Cx HCs is modulated through changes in their surface levels, permeability and electrophysiological properties. Stimuli activating Cx HCs through different mechanisms induce responses that can be connexin specific. In addition, healthy cells are likely capable of adapting to transient activation of Cx HCs through active compensatory mechanisms, whereas damaged cells (metabolically compromised, under proinflammatory environments and/or carrying hemichannel defective Cx mutations) may not be able to overcome the metabolic consequences of hemichannel activation. The increase in number of hemichannels in the cell surface membrane induced by rises in $[Ca^{2+}]_i$ is a response common to healthy and sick cells. In both cases, hemichannels are a pathway that contributes to increase in $[Ca^{2+}]_i$, but sick cells may be unable to restore their $[Ca^{2+}]_i$. Under the same thought, hemichannels might be part of responses generated by different drugs or biologically relevant compounds acting on molecular targets that lead to increases in $[Ca^{2+}]_i$ and/or changes in intra and/or extracellular compartments including redox state, pCa, pH, membrane potential and activity of protein phosphatases and kinases. Thus, a component of cell responses elicited by compounds that increase $[Ca^{2+}]_i$ may be due to changes in hemichannel activity rather than changes mediated exclusively by the primary target. An example of this possibility is the rise in membrane permeability induced by FGF-1, which also leads to a delayed increase in intracellular $[Ca^{2+}]_i$ in HeLa transfectants [6]. This Cx HC transactivation is connexin dependent since it occurs in Cx43 and Cx45 but not in Cx26 HeLa transfectants. Thus, conditions that potentiate hemichannel activity may differ in cells expressing different connexins.

To date, the demonstration that functional hemichannels exist at the cell surface and appear relevant to numerous cellular responses has utilized mainly *in vitro* studies. Now, it is important to acquire evidence from *in vivo* experimental models. Although *in*

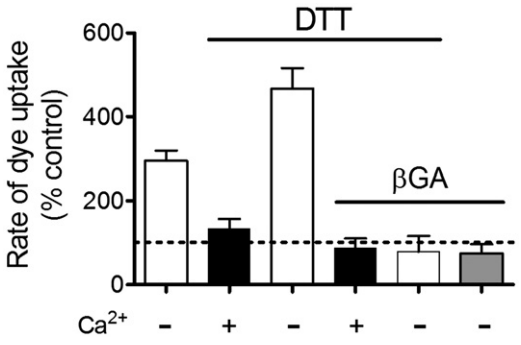

Fig. 5 – Treatment with divalent cation-free solution and DTT causes a synergistic potentiation of the dye uptake response mediated by Cx43 HCs in HeLa transfectants. Time lapse measurements of Etd uptake rate were performed in physiological concentrations of divalent cations (Ca^{2+} +, filled bars) or in Ca^{2+} and Mg^{2+}-free solution (Ca^{2+} , white bars), without (gray bar) or with addition of 100 mM DTT followed by application of 18β-glycyrrhetinic acid (βGA, 150 μM). Uptake rates were normalized to the control value of each experiment. The dotted line indicates control dye uptake rate expressed as 100%. Each plotted number corresponds to the mean ± SEM of 4 independent experiments including 20 cells in each of them.

vitro preparations are convenient for analysis of the effects of single variables, *in vivo* data where cells are under the influence of multiple regulatory mechanisms will be required to establish functional relevance of hemichannels in both physiological and pathophysiological conditions.

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