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Cation permeation through connexin 43 hemichannels is cooperative, competitive and saturable with parameters depending on the permeant species

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Abstract

Kinetics of permeation through connexin 43-EGFP hemichannels (Cx43-EGFP HCs) were evaluated in divalent cation-free solutions, which enhance HC open probability and thus, allow measurements during initial velocity. Three cations that become fluorescent upon binding to intracellular nucleic acids [ethidium (Etd), propidium (Prd) and 4',6-diamidino-2-phenylindole (DAPI)] and Cx43-EGFP or Cx43 wild type HeLa cell transfectants (Cx43-EGFP- and Cx43-WT-HeLa cells, respectively) were used. Levels of Cx43-EGFP at the cell periphery and rate of dye uptake were directly related. The rate of uptake of each dye reached saturation consistent with a facilitated transport mechanism. Before saturation, the relation between rate of uptake and concentration of each dye was sigmoidal with Hill coefficients >1, indicating positive cooperativity of transport at low concentrations. The maximal rate of Etd uptake was not affected by the presence of DAPI and vice versa, but under each condition the apparent affinity constant of the main permeant molecule increased significantly consistent with competitive inhibition or competition for binding sites within the channel. Moreover, Cx43-EGFP and Cx43-WT HCs had similar permeability properties, indicating that EGFP bound to the C-terminal of Cx43 does not significantly alter the permeability of Cx43 HCs to positively charged molecules.

Keywords

connexons; maximal transport capacity; Hill coefficient, affinity constants

Introduction

The coordination of numerous cellular processes requires complex electric and metabolic cell-cell interactions. In vertebrate cells, these interactions are in part mediated by low-resistance intercellular channels at gap junctions (GJs). In addition, cells release chemical signals that act in an autocrine or paracrine manner, and one mode of release is through

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connexin (Cx) “hemichannels” (HCs), the precursors of gap junction channels each of which is formed by two HCs, one from each apposed cell.

HCs are formed by the oligomerization of six connexin subunits, which are encoded by 21 different genes in humans [1]. HCs can be formed of a single Cx (homomeric) or multiple Cxs (heteromeric). Members of a recently described three-member protein (1-3) family unrelated to the Cxs, termed pannexins (Panxs), can also form HCs in the cell membrane of vertebrates. Frequently, these two HC types are co-expressed, and their roles in membrane permeability can be differentiated by several means including their differential sensitivity to shRNA, to pharmacological inhibitors, to mimetic peptides with sequences the same as the extracellular loops of the Cx or Panx1, to extracellular divalent cations (opening of Cx HCs but not Panx HCs is enhanced by divalent free extracellular saline solutions), and to trivalent cations (e.g., La^{3+}), which block Cx HCs but not Panx HCs [2].

Cx HC-mediated cell permeabilization can be enhanced by both physiological and pathological stimuli [3]. Supporting their role in paracrine and/or autocrine cell signaling, Cx HCs have been shown to be permeable to various metabolites, including glucose, glutathione, ascorbate, and NAD^+ [4; 5; 6; 7], which is in agreement with their proposed role as pathways for cellular nutrition, detoxification, and propagation of Ca^{2+} waves [3].

The functional expression of Cx proteins fused to enhanced green fluorescent protein (EGFP) has facilitated the electrophysiological characterization of Cx43 HCs [8; 9]; in only a few studies were qualitative permeability properties of homomeric HCs assessed [10; 11; 12] and quantitative kinetic properties of permeation of Cx HCs remain largely unknown. The aim of the present study was to determine the diffusion kinetics of three cations of different size, shape and charge through mouse Cx43-EGFP and Cx43-WT HCs.

Materials and methods

Reagents

HEPES, LaCl_3 and ethidium (Etd) bromide were from Sigma-Aldrich (St. Louis, MO, USA). Propidium (Prd) iodide and 4',6-diamidino-2-phenylindole (DAPI) dihydrochloride were from Molecular Probes, Inc. (Eugene, OR, USA). Gap26 (VCYDKSFPIHVSR, first extracellular loop domain of Cx43) and $^{10}\text{pnx1}$ (WRQAAFVDSY, first extracellular loop domain of Panx1) peptides were obtained from NeoMPS, SA. (Strasbourg, France).

Cell Culture

Experiments were performed on previously described parental HeLa cells or on these cells transfected with cDNA encoding for mouse Cx43 wild type (Cx43-WT) [8; 9; 13] or Cx43 with an enhanced green fluorescent protein (EGFP) appended to its C-terminus (Cx43-EGFP) [8; 9]. Cells expressing Cx43 or Cx43-EGFP were selected as described before [9] and kept at 37°C in a 5% CO_2 /95% air atmosphere at nearly 100% relative humidity. As described previously [2], transfection of parental HeLa cells with vector alone did not affect basal dye uptake in either control extracellular solution or divalent cation-free solution (DCFS; not shown), indicating that transfection did not induce expression of Cxs or other molecules that increased membrane permeability.

Modeling of tracer molecules

Space filling models of permeability tracers were generated using the Molecular Modeling Pro software (ChemSW, Inc. CA, USA). Molecules were constructed using individual aromatic rings and common functional groups. Energy was minimized using the simplex

minimization option with the minimize geometry item provided by the program (1,000 iterations) (**Supplementary Fig. 1**).

Confocal microscopy

Cells plated onto glass coverslips were fixed with 70% ethanol for 20 min at -20°C. Then, coverslips were mounted using Fluoromont G and confocal images were taken with a 63 X oil objective at 500 nm intervals and acquired sequentially with an argon (488 nm) laser using a Leica TBCS SP2 laser-scanning confocal microscope (Wetzlar, Germany). Z projections were reconstructed using Leica confocal software and analyzed with ImageJ (NIH).

Time-lapse microscopy

Time-lapse experiments were performed as described before [9]. Cells were bathed in a divalent cation-free solution (DCFS) [in (mM): NaCl (148), KCl (5), MgCl₂ (1), glucose (5), HEPES (5), EGTA (10), pH = 7.4] containing 5 μM Etd, Prd or DAPI; and fluorescence intensity was recorded in selected cells (ROIs, regions of interest). Images were captured every 15 s using a Q Imaging model Retiga 13001 fast-cooled monochromatic digital camera (12-bit) (Qimaging, Burnaby, BC, Canada). Metafluor software (version 6.2R5, Universal Imaging Co., Downingtown, PA, USA) was used for off-line image analysis and fluorescence quantification. Slopes of dye uptake were calculated using Microsoft Excel software and expressed as AU/min. Affinity constants for each dye were calculated using the following equation for allosteric sigmoidal binding kinetics: $Y = (V_{\max} [S]^h) / (K_d + [S]^h)$, where $V_{\max}$ represents the maximal transport velocity, $[S]$ the concentration of permeant, h the Hill coefficient, and K_d the permeant concentration needed to achieve half-maximal transport velocity.

Data analysis and statistics

Data are reported as mean ± SEM. Statistical analyses were performed using GraphPad Prism software. Dye uptake was analyzed by one-way ANOVA. In all cases, a p value < 0.05 was considered statistically significant.

Results

Uptake of cationic permeability tracers by Cx43-EGFP HeLa cells is cooperative at low tracer concentration

HeLa cells transfected with specific Cxs express HCs at the cell surface, through which they take up and release small molecules including fluorescent dyes commonly used to assay whether they are open [8]. However, in cultured cells under resting conditions the presence of physiological concentrations of extracellular divalent cations reduces the open probability of HCs [8; 14; 15], so that longer times are required to determine HC permeability. Since divalent cation-free solution (DCFS) increases open probability within seconds and does not affect the level of Cxs at the cell surface [2; 8; 9], we used this solution to examine HC permeability.

Previous studies have shown that mouse Cx43-EGFP forms functional HCs through which Etd uptake occurs and its uptake rate correlates directly with the total fluorescence levels of Cx43-EGFP both in the presence [8] or absence of extracellular divalent cations [8; 9] (**Fig. 1A**). Here, we extended this observation to two additional tracer cations, propidium (Prd) and DAPI (**Fig. 1B-C**). As compared to control extracellular solution, in DCFS the dye uptake increased ~ 8 times for propidium (Prd), ~3 times for Etd and ~ 2 times for DAPI (**Fig. 1A-C**). We also found a direct relation between levels of Cx43-EGFP located at the cell margin and Etd uptake with $r^2 = 0.98$ (**Fig. 1G**) and between total EGFP levels

measured with conventional fluorescent microscopy and levels of EGFP localized in the cell margin measured by confocal microscopy ($r^2 = 0.92$, **Fig. 1H**), indicating that fluorescence intensity present in the cell membrane is directly related to total levels of EGFP. Thus, in dye uptake experiments results can be normalized to either total EGFP levels or EGFP located at the cell margin. In addition, the EGFP fluorescence detected at the cell margin and total EGFP fluorescence were not affected by exposing the cells to DCFS for 30 min (not shown). Moreover, uptake induced by DCFS in Cx43-EGFP HeLa cells did not involve Panx1 HCs, since it was completely blocked by Gap26 and was not significantly affected by 10 panx1 mimetic peptide (**Supplementary Fig. 2**).

The observed maximal uptake rate ($V_{\max}$) will be affected by the number of surface HCs (and is also likely to be characteristic for each HC type). We evaluated $V_{\max}$ in Cx43-EGFP cells with similar size, shape and fluorescence intensity to minimize variations due to differences in the number of HCs per cell, and we normalized dye uptake to the EGFP fluorescence of the cell (**Fig. 2D-F**). Uptake of Etd, Prd and DAPI by Cx43-EGFP cells was concentration dependent (**Fig. 2D-F**) and reached a maximal rate at concentrations of ~ 50 μ M Etd (**Fig. 2D**), ~ 100 μ M Prd (**Fig. 2E**) and ~ 150 μ M DAPI (**Fig. 2F**). The ranking of maximal uptake capacity ($V_{\max}$) of these HCs was: Etd > DAPI > Prd ($V_{\max} = 17.8 \pm 0.3$, 6.9 ± 1.3 and 1.6 ± 0.1 AU/min, respectively, $n=4$) (**Fig. 2D-F**). The uptake rate curves versus different dye concentrations were sigmoid, suggesting cooperative interactions between permeant molecules and HCs at low tracer concentrations. In support of this, the Hill coefficients were all positive and >1: 4.7 ± 0.6 for Etd, 4.0 ± 0.7 for Prd and 1.8 ± 0.4 for DAPI. The apparent affinity constants (K_d) of Cx43-EGFP HCs were 33.3 ± 1.5 μ M for Etd, 67.6 ± 3.3 μ M for Prd and 79.2 ± 5.7 μ M for DAPI.

Since fluorescence of Etd, Prd and DAPI depends on binding to intracellular nucleic acids, we examined whether saturation of binding contributed to the plateau in dye uptake rate (although uptake should slow if saturation were approached) (**Figs. 2D-F**). We measured fluorescence intensity during the first 3.5 min (initial velocity) in DCFS [time and dye concentration at which $V_{\max}$ was reached at least for Prd (**Fig. 2B and E**)], and cells were permeabilized with 70% ethanol (in PBS), which resulted in a pronounced increase in rate of increase of fluorescence for all three dyes (uptake rate of Etd, Prd and DAPI were: 39.2 ± 5.1 A.U./min; 27.6 ± 8.2 A.U./min; and 31.3 ± 4.9 A.U./min, respectively) showing saturation at about 250 A.U. (**Supplementary Fig. 3**). The plateau value was attained at about 250 A.U. was due to saturation of the camera. Since the fluorescence intensity measured in cells with intact membrane was about 10 or less A.U., it is unlikely that the maximal transport determined for each permeability tracer were affected by the binding capacity of nucleic acids. Moreover, a linear relationship between dye uptake and concentration of Etd, Prd or DAPI in Cx43-EGFP HeLa cells permeabilized with ethanol was found (**Supplementary Fig. 4**), which is suggestive of passive diffusion rather than facilitated transport with a $V_{\max}$ as described here for HCs. The direct relationship between the fluorescence intensity measured during the first 45 seconds after the application of each dye (initial velocity) and dye concentration also indicates that the different dyes present a similar and single way of interaction with the nucleic acids. Alternatively, the similar steady-state A.U. for the different dyes might be consequence of measuring close to their maximal emission value and at initial velocity (first 45 s). Independently of the explanation, it is unlikely that saturation of the nucleic acid binding sites contributes to the cooperative (S shape) transport shown in Figure 2.

Cx43-EGFP HCs allow the simultaneous passage of more than one molecule through a competitive process

Single Cx43-EGFP cells exposed to increasing concentrations of Etd showed prominent dye uptake (**Fig. 3A-I, cell 2**) that was almost completely blocked by 200 μ M La^{3+} (**Fig. 3I, cell**

2), a Cx HC blocker [9]. However, HeLa parental cells showed a much smaller and linear uptake (**Fig. 3A-I, cell 1**). The relation between Etd uptake rate and Etd concentration was well fit by a sigmoid curve (**Fig. 3J**). To examine if different permeants competed for occupancy of the HC pathway, we used Etd and DAPI, which emit fluorescence at very different wave lengths and therefore, the presence of one does not interfere with the recording of the other molecule. Thus, transfected cells with Cx43-EGFP were exposed to different concentrations of Etd or DAPI in the presence of a increasing concentrations of DAPI or Etd, respectively (**Fig. 3K and L, respectively**). The experimental $V_{\max}$ at 125 μM Etd was 17.8 ± 0.4 AU/min and in the presence of 125 μM DAPI was 17.3 ± 0.6 AU/min (**Fig. 3K**).

Using the data presented in Fig. 3K-N, the equation described in Material and Method and Graph Pad both K_d and $V_{\max}$ were calculated in the absence or presence of a second permeant. The $V_{\max}$ was comparable in the presence or absence of DAPI (18.3 ± 1.6 and 20.3 ± 2.4 AU/min, respectively, $n=3$), which were very close to the experimental $V_{\max}$ described above. The Etd K_d was shifted from 43.5 ± 1.6 μM to 74.3 ± 2.4 μM (**Fig. 3K**). Similarly, the presence of increasing Etd concentrations shifted the DAPI K_d from 61.4 ± 3 μM to 93.4 ± 4.5 μM (**Fig. 3L**), and again, the $V_{\max}$ was similar in the presence or absence of the second permeant (6.79 ± 0.6 and 6.73 ± 0.5 AU/min, respectively, $n=3$). In separate experiments, Cx43-EGFP HeLa cells were exposed to a single concentration (125 μM) of DAPI or Etd, respectively (**Fig. 3M and N, respectively**). The Etd K_d was shifted from 43.5 ± 1.6 μM in absence to 92.3 ± 1.5 μM in the presence of 125 μM DAPI (**Fig. 3M**). The $V_{\max}$ was similar in the presence or absence of 125 μM DAPI (17.6 ± 0.5 and 20.3 ± 2.4 AU/min, respectively, $n=3$) (**Fig. 3M**). Similarly, the presence of 125 μM Etd shifted the DAPI K_d from 61.4 ± 3 μM to 104.1 ± 3.1 μM (**Fig. 3N**). The $V_{\max}$ was similar in the presence or absence of Etd (6.55 ± 0.4 and 6.73 ± 0.5 AU/min, respectively, $n=3$).

The permeability of Cx43-EGFP HCs is indistinguishable from that of Cx43-WT HCs—To determine whether permeability properties of Cx43 HCs are affected by an EGFP tag, we compared Cx43-EGFP and Cx43-WT HCs. In Cx43-WT HeLa cells, the relation between dye uptake rate and dye concentration was sigmoidal and very similar to that found for Cx43-EGFP HCs (**Supplementary Fig. 5**). The apparent affinity constant of Cx43-EGFP and Cx43-WT HCs were for Etd (33.3 ± 1.5 μM and 35.2 ± 1.7 μM , respectively), Prd (67.6 ± 3.3 μM and 64.8 ± 4.2 μM , respectively) and DAPI (79.2 ± 5.7 μM and 77.8 ± 5.7 μM , respectively) (**Supplementary Fig. 5**). Furthermore, the Hill coefficient of Cx43-EGFP and Cx43-WT HCs were for Etd (4.7 ± 0.6 and 4.8 ± 0.4), for Prd (4.0 ± 0.7 and 4.2 ± 0.8) and for DAPI (1.8 ± 0.4 and 1.7 ± 0.2). Moreover, the $V_{\max}$ of Cx43-EGFP and Cx43-WT HCs for each dye were also similar, suggesting strongly that the two HeLa transfectants express similar levels of HCs in their surface membranes (**Supplementary Fig. 5**). The slight difference between $V_{\max}$ of Cx43-EGFP HCs (6.9 ± 1.3 AU/min; $n=4$) and Cx43-WT HCs (5.4 ± 1.6 AU/min; $n=4$) for DAPI was not statistically significant ($p>0.295$).

Discussion

Here, we show that Cx43-EGFP HCs in cells bathed with DCFS allow positive cooperative transport across the cell membrane for low concentrations of Etd, Prd and DAPI and saturation at higher concentrations. Moreover, Cx43-EGFP HCs have different numbers of affinity sites for each permeant as inferred from the Hill coefficients, and at least Etd and DAPI compete for passage through the same channels.

This work provides the first kinetic properties of HC permeability to small cationic molecules in DCFS. Permeabilization of the cell membrane with 70% ethanol caused a pronounced increase in dye uptake rate to levels far above the maximal value observed with

unpermeabilized cells. Therefore, under the recording conditions used, the number of intracellular binding sites did not affect the kinetics or saturating uptake rate. Moreover, recording in cells of similar size and shape and the use of an EGFP-tagged Cx that allowed normalization of the dye uptake in relation to the protein expression levels, made the dye uptake measurement in different cells relatively independent of the number of HCs. Therefore, the difference in uptake rate corresponded to different permeabilities to Etd, Prd and DAPI of surface Cx43-EGFP HCs.

The close correlation ($r^2 = 0.91-0.98$) between Etd, Prd and DAPI uptake rates and Cx43-EGFP expression levels, either in the presence or absence of extracellular divalent cations, suggest the existence of a direct relationship between surface and total Cx43-EGFP HC levels. Moreover, we found no change in EGFP fluorescence intensity due to changes in extracellular divalent cation concentrations and previously, we demonstrated no changes in surface Cx43 levels in DCFS as measured by biotinylation of surface proteins [2]. Thus, the increase in total fluorescence intensity of each permeant likely reflects a change in net dye flow through HCs due to variations in open probability rather than in permeability of HC induced by the lack of Ca^{2+} and/or Mg^{2+} . In agreement with this interpretation, the unitary conductance of Cx43-EGFP HCs recorded in the absence or presence of $\text{Ca}^{2+}/\text{Mg}^{2+}$ has been reported previously to be the same [8].

The dye uptake rate versus dye concentration curves for all three tracers showed a sigmoid shape and Hill coefficients >1 , indicating positive cooperative transport of the same permeant, and suggesting that Cx43-EGFP HCs might have differing binding sites for Etd, Prd and DAPI, respectively. Moreover, the presence of DAPI reduced the K_d of Etd and vice versa, which occurred without changes in $V_{\max}$ and thus, it is consistent with a competitive process. These data demonstrate that Etd and DAPI, and most likely physiological permeants, such as cations including metabolites (e.g., Ca^{2+} and NAD^+), cross the cell membrane through a common diffusional transport pathway, corresponding to Cx43 HCs. We have recently demonstrated that Cx43 HCs are permeable to a fluorescent glucose analogue [4], which can also be transported to the cell interior via GLUTs (more selective transporters). If a glucose competitive permeant is present in the cell exterior it can be predicted that transport of this metabolite will preferentially occur via GLUTs. However, in the absence of the inhibitor and high HC open probability a significant uptake of glucose might take place via Cx HCs. If kinetic properties of uptake are essentially the same as that of release, competitive inhibition might contribute to that cell expressing HCs with high open probability do not lose relevant quantities of essential molecules (e.g., metabolite or signaling molecule); the essential molecule would stay into the cells if other less permeable competitive molecule are present in the cell interior.

Cx43 HCs have a fully open state of about 220 pS and a substate of about 75 pS, whereas Cx43-EGFP HCs have only fully open and closed states [8]. Presumably the fully open state is permeable to major cytoplasmic charge carriers in the cytoplasm and extracellular space. However, the subconductance state is permeable to the major charge carriers in cytoplasm and bathing medium [16; 17], but it remains to be demonstrated if it is permeable to small molecules. The kinetic properties evaluated in the present work for Cx43 and Cx43-EGFP HCs were similar, suggesting that tracers used diffused preferentially if not exclusively through the full open state of the HCs formed by either Cx43 or Cx43-EGFP proteins. The similarity of the permeability properties of Cx43-EGFP and Cx43-WT HCs also suggests that the tag with EGFP does not significantly modify the intrinsic permeability properties of Cx43 HCs or its level of expression.

Drastic reductions in extracellular Ca^{2+} and Mg^{2+} concentrations sufficient to increase the open probability of HCs are known to occur in brain during ischemia [18; 19] and thus, the

above findings might be relevant under such conditions. However, it remains to be demonstrated if the permeability properties of HCs opened by reduction in extracellular divalent cation concentration are similar to those of HCs opened by other means including pro-inflammatory conditions, positive voltages and protein phosphorylation/dephosphorylation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

Cx	connexin
DCFS	divalent cation-free solution
Etd	ethidium
HCs	hemichannels
Panx	pannexin
Prd	propidium

References

1. Sohl G, Willecke K. Gap junctions and the connexin protein family. *Cardiovasc Res.* 2004; 62:228–32. [PubMed: 15094343]
2. Schalper KA, Palacios-Prado N, Orellana JA, Saez JC. Currently used methods for identification and characterization of hemichannels. *Cell Commun Adhes.* 2008; 15:207–18. [PubMed: 18649191]
3. Orellana JA, Sáez PJ, Shoji KF, Schalper KA, Palacios-Prado N, Velarde V, Giaume C, Bennett MV, Sáez JC. Modulation of brain hemichannels and gap junction channels by pro-inflammatory agents and their possible role in neurodegeneration. *Antioxid Redox Signal.* 2009; 11:369–99. [PubMed: 18816186]
4. Retamal MA, Froger N, Palacios-Prado N, Ezan P, Sáez PJ, Sáez JC, Giaume C. Cx43 hemichannels and gap junction channels in astrocytes are regulated oppositely by proinflammatory cytokines released from activated microglia. *J Neurosci.* 2007; 27:13781–92. [PubMed: 18077690]
5. Rana S, Dringen R. Gap junction hemichannel-mediated release of glutathione from cultured rat astrocytes. *Neurosci Lett.* 2007; 415:45–8. [PubMed: 17222973]
6. Ahmad S, Evans WH. Post-translational integration and oligomerization of connexin 26 in plasma membranes and evidence of formation of membrane pores: implications for the assembly of gap junctions. *Biochem J.* 2002; 365:693–9. [PubMed: 11985493]
7. Bruzzone S, Guida L, Zocchi E, Franco L, De Flora A. Connexin 43 hemi channels mediate Ca²⁺-regulated transmembrane NAD⁺ fluxes in intact cells. *FASEB J.* 2001; 15:10–12. [PubMed: 11099492]
8. Contreras JE, Sáez JC, Bukauskas FF, Bennett MV. Gating and regulation of connexin 43 (Cx43) hemichannels. *Proc Natl Acad Sci U S A.* 2003; 100:11388–93. [PubMed: 13130072]
9. Schalper KA, Palacios-Prado N, Retamal MA, Shoji KF, Martinez AD, Sáez JC. Connexin hemichannel composition determines the FGF-1-induced membrane permeability and free [Ca²⁺]_i responses. *Mol Biol Cell.* 2008; 19:3501–13. [PubMed: 18495870]

10. Bukauskas FF, Kreuzberg MM, Rackauskas M, Bukauskiene A, Bennett MV, Verselis VK, Willecke K. Properties of mouse connexin 30.2 and human connexin 31.9 hemichannels: implications for atrioventricular conduction in the heart. *Proc Natl Acad Sci U S A*. 2006; 103:9726–31. [PubMed: 16772377]
11. Valiunas V. Biophysical properties of connexin-45 gap junction hemichannels studied in vertebrate cells. *J Gen Physiol*. 2002; 119:147–64. [PubMed: 11815665]
12. Oh S, Verselis VK, Bargiello TA. Charges dispersed over the permeation pathway determine the charge selectivity and conductance of a Cx32 chimeric hemichannel. *J Physiol*. 2008; 586:2445–61. [PubMed: 18372303]
13. Elfgang C, Eckert R, Lichtenberg-Frate H, Butterweck A, Traub O, Klein RA, Hülser DF, Willecke K. Specific permeability and selective formation of gap junction channels in connexin-transfected HeLa cells. *J Cell Biol*. 1995; 129:805–17. [PubMed: 7537274]
14. Ebihara L, Liu X, Pal JD. Effect of external magnesium and calcium on human connexin46 hemichannels. *Biophys J*. 2003; 84:277–86. [PubMed: 12524281]
15. Gomez-Hernandez JM, de Miguel M, Larrosa B, Gonzalez D, Barrio LC. Molecular basis of calcium regulation in connexin-32 hemichannels. *Proc Natl Acad Sci U S A*. 2003; 100:16030–5. [PubMed: 14663144]
16. Bukauskas FF, Bukauskiene A, Verselis VK. Conductance and permeability of the residual state of connexin43 gap junction channels. *J Gen Physiol*. 2002; 119:171–85. [PubMed: 11815667]
17. Qu Y, Dahl G. Function of the voltage gate of gap junction channels: selective exclusion of molecules. *Proc Natl Acad Sci U S A*. 2002; 99:697–702. [PubMed: 11805325]
18. Nicholson B, Dermietzel R, Teplow D, Traub O, Willecke K, Revel JP. Two homologous protein components of hepatic gap junctions. *Nature*. 1987; 329:732–4. [PubMed: 2823143]
19. Lin MC, Huang YL, Liu HW, Yang DY, Lee CP, Yang LL, Cheng FC. On-line microdialysis-graphite furnace atomic absorption spectrometry in the determination of brain magnesium levels in gerbils subjected to cerebral ischemia/reperfusion. *J Am Coll Nutr*. 2004; 23:561S–565S. [PubMed: 15466966]

Figure 1. Uptake of Etd in Cx43-EGFP HeLa cells is directly proportional to EGFP fluorescence at the cell margins and to total EGFP fluorescence

Dye uptake was measured in Cx43-EGFP HeLa cells exposed to 5 μ M Etd (**A**), Prd (**B**) or DAPI (**C**) for 10 min in DCFS and then Cx43-EGFP fluorescence and rate of uptake of each dye were plotted through a linear regression analysis in control saline ($\circ$) or in DCFS ($\bullet$). Each graph shows analysis of 13 cells from four independent experiments. (**D-F**) Representative confocal images of Cx43-EGFP HeLa cell cultures. (**D**) Cx43-EGFP fluorescence. (**E**), After 10 uptake from 5 μ M Etd in DCFS. (**F**) In merged phase and Cx43-EGFP confocal fluorescence views, Cx43EGFP at the cell margins was measured as the fluorescence between inner and outer green lines (shown for cells 1-4; the measured values are indicated on panels **G**, **H**). (**G**) Etd uptake rate in DCFS and total Cx43-EGFP fluorescence at the cell margins were linearly related ($r^2 = 0.98$). The graph is from 15 cells in one of four independent experiments. (**H**) Total Cx43-EGFP fluorescence and that at the cell margins were linearly related ($r^2 = 0.92$) in the same cells as in Fig. 4. Calibration in E: 4 μ m.

Figure 2. The uptake rate mediated by Cx43-EGFP HCs is cooperative and characteristic for each permeant cationic molecule

(A-B) Representative time lapse measurements showing Etd (A), Prd (B) and DAPI (C) uptake of single Cx43-EGFP HeLa cells in DCFS at different concentrations of the respective permeability tracer. . Also, representative photomicrographs are shown of HeLa cells expressing Cx43-EGFP (green) and incubated for 3 min in 25 μ M Etd (red, A), Prd (red, B) or DAPI (blue, C). (D-F) Etd (D), Prd (E) and DAPI (F) uptake rates of Cx43-EGFP HeLa cells incubated with several concentrations of the corresponding permeability tracer. Each value represents average $\pm$ S.E.M. of four experiments including 20 cells normalized to the respective EGFP fluorescence.

Figure 3. Transport of Etd and DAPI across the cell membrane via Cx43-EGFP HCs is competitive

(A-H) Representative photomicrographs showing Etd (in red, B-H) fluorescence after exposure to increasing Etd concentrations (1-125 μM) in a parental HeLa cell (cell 1) and a Cx43-EGFP HeLa cell (cell 2) exposed to DCFS. Panel A is a phase image of the fields depicted in panels B-H. (I) Time lapse measurements showing the Etd uptake of cell 1 and 2 in panels B-H. (J) Etd uptake rates of Cx43-EGFP HeLa cells incubated with increasing concentrations of Etd in DCFS. The discontinuous line corresponds to the best fit obtained with the equation described in Material and Methods. (K) Etd uptake rates of Cx43-EGFP HeLa cells incubated with several concentrations of Etd alone (white circles) or in combination with cumulative concentrations of DAPI (black circles) in DCFS. (L) DAPI uptake rates of Cx43-EGFP HeLa cells incubated with increasing concentrations of DAPI alone (white circles) or in combination with cumulative Etd concentrations (black circles) in DCFS. (M) Etd uptake rates of Cx43-EGFP HeLa cells incubated with cumulative concentrations of Etd alone (white circles) or in the presence of 125 μM DAPI (black circles) in DCFS. (N) DAPI uptake rates of Cx43-EGFP HeLa cells incubated with increasing concentrations of DAPI alone (white circles) or in combination with 125 μM Etd (black circles) in DCFS. Each value represents average $\pm$ S.E.M. of four experiments including 20 cells per experiment and normalized with the respective EGFP fluorescence. Bar = 8 μm .