Biochemical and Biophysical Research Communications 409 (2011) 603–609

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

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

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ARTICLE INFO

Article history:
Received 3 May 2011
Available online 12 May 2011

Keywords:
Connexons
Maximal transport capacity
Hill coefficient
Affinity constants

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. Thus, competitive inhibition of permeation through hemichannels might contribute to cellular retention of essential molecules and/or uptake inhibition of toxic compounds.

1. 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 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^+ [4–7], 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 [10–12] and quantitative kinetic properties of permeation of Cx HCs remain largely unknown. The aim of the present study was to determine the

Abbreviations: Cx, connexin; DCFS, divalent cation-free solution; Etd, ethidium; HCs, hemichannels; Panx, pannexin; Prd, propidium.

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doi:10.1016/j.bbrc.2011.05.031

diffusion kinetics of three cations of different size, shape and charge through mouse Cx43-EGFP and Cx43-WT HCs.

2. Materials and methods

2.1. 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 (VCYDKSFPISHVR, first extracellular loop domain of Cx43) and ^{10}pnx1 (WRQAAFVDSY, first extracellular loop domain of Panx1) peptides were obtained from NeoMPS, SA. (Strasbourg, France).

2.2. 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.

2.3. 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 (1000 iterations) (Supplementary data Fig. 1).

2.4. 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 63X 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).

2.5. 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), $MgCl_2$ (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.

2.6. 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.

3. Results

3.1. 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 data 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 μM Etd (Fig. 2D), ~100 μM Prd (Fig. 2E) and ~150 μM DAPI (Fig. 2F). The ranking of maximal uptake capacity (V_{max}) of these HCs was: Etd > DAPI > Prd (V_{max} = 17.8 ± 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