FEBS Letters 589 (2015) 1340–1345

journal homepage: www.FEBSLetters.org

Differential regulation of Connexin50 and Connexin46 by PI3K signaling

Jennifer M. Martinez, Hong-Zhan Wang, Richard Z. Lin, Peter R. Brink, Thomas W. White *

The Department of Physiology & Biophysics, Stony Brook University, Stony Brook, NY 11794-8661, USA

ARTICLE INFO

Article history:
Received 6 March 2015
Revised 6 April 2015
Accepted 16 April 2015
Available online 29 April 2015

Edited by Lukas Huber

Keywords:
Connexin
Lens
Gap junction
PI3K
Akt

ABSTRACT

Gap junction channels can modify their activity in response to cell signaling pathways. Here, we demonstrate that Connexin50 (Cx50) coupling, but not Connexin46 (Cx46), increased when co-expressed with a constitutively active p110α subunit of PI3K in *Xenopus* oocytes. In addition, inhibition of PI3K signaling by blocking p110α, or Akt, significantly decreased gap junctional conductance in Cx50 transfected HeLa cells, with no effect on Cx46. Alterations in coupling levels were not a result of reduced Cx50 unitary conductance, suggesting that changes in the number of active channels were responsible. These data indicate that Cx50 is specifically regulated by the PI3K signaling pathway.

1. Introduction

There are two main mechanisms of intercellular communication between adjacent cells: small molecules can be directly shared through gap junction channels linking the cytoplasm of neighboring cells, or, extracellular ligands binding to receptors can activate intracellular signaling pathways. Gap junction channels and cell signaling have been separately shown to be important elements for the maintenance of homeostasis and proper organ development in many organisms [1,2], however, less is known about the interplay between them [3]. One organ that has been well studied because of its dependence on intercellular communication is the ocular lens. Due to its absence of a vascular system, the lens relies heavily on gap junction channels for cell-to-cell communication during growth and differentiation [4–6].

Transport of ions, metabolites, and small signaling molecules to all cells of the lens requires direct communication between neighboring cells by gap junction channels [7,8]. Gap junctions are comprised of hexameric oligomers of connexin subunits that are inserted into the plasma membrane [9]. When two of these complexes from neighboring cells dock, they form a channel that connects the two cytoplasms [10,11]. Out of the ~20 connexin family members, three are present in the lens with distinct expression patterns: Connexin43 (Cx43) is expressed in the lens epithelium [12], Connexin46 (Cx46) is present in the differentiating and mature fiber cell types [13], and Connexin50 (Cx50) is expressed in all three cell types [14–16].

Genetic knockouts of lens connexins have identified the roles each have in development and homeostasis. Cx46 knockout mice developed severe nuclear cataracts [17]. Targeted deletion of Cx50 resulted in mild nuclear cataracts and an ocular growth defect [18]. Functional replacement of Cx50 with Cx46 restored ocular transparency but did not rescue the growth defect [19]. These results suggested that Cx46 and Cx50 lack redundancy and demonstrated the possibility that Cx50, specifically, was involved in proliferation and growth regulation of the lens [20,21].

It was previously shown that manipulation of the MAPK signaling pathway differentially regulated Cx50 and Cx46 [22], increasing Cx50 mediated coupling with no effect on Cx46. This was consistent with the hypothesis that Cx50, but not Cx46, was interacting with growth signaling pathways. Here, we investigated the effects on gap junctional conductance produced by Cx50 or Cx46 when the PI3K signaling pathway was either activated or inhibited. In cells incubated with PIK-75 and Akt Inhibitor-VIII (Akti) to specifically block p110α and Akt, respectively, there was a significant decrease in Cx50-mediated gap junctional conductance that was not seen in Cx46 transfected cells. We demonstrated that

☆ *Author contributions:* TWW conceived and supervised the study; PRB and TWW designed experiments; JMM and H-ZW performed experiments; RZL provided new tools and reagents; PRB and TWW analyzed data; JMM and TWW wrote the manuscript; TWW made manuscript revisions.

* Corresponding author at: Department of Physiology & Biophysics, Stony Brook University, T5-147, Basic Science Tower, Stony Brook, NY 11794-8661, USA. Fax: +1 (631) 444 3432.

E-mail address: thomas.white@stonybrook.edu (T.W. White).

http://dx.doi.org/10.1016/j.febslet.2015.04.029

Cx50 junctional coupling, but not Cx46, increased when co-expressed with a constitutively active PI3K subunit. Analysis of single channel conductance showed that alteration in Cx50 coupling mediated by PI3K did not result from changes in unitary conductance of the channel. These results suggest a mechanism of differential regulation of these two lens connexins by the PI3K signaling pathway.

2. Materials and methods

2.1. In vitro transcription, oocyte microinjection, and pairing

Cx50, Cx46, and constitutively active p110α-H1047R (caPI3K) [23] coding sequences were subcloned into pCS2+, linearized with NotI, and transcribed using the SP6 mMessage mMachine (Ambion, Austin, TX). *Xenopus laevis* oocytes were removed from adult females (Nasco, Fort Atkinson, WI), defolliculated by collagenase B and hyaluronidase digestion, and stage V-VI cells were selected and cultured in modified Barth's (MB) medium. Endogenous connexins were suppressed by injection of an antisense oligonucleotide to *Xenopus* Cx38 (10 ng/cell) [24] using a Nanoject II injector (Drummond, Broomall, PA). Oligo injected cells were subsequently injected with either Cx50, Cx46 cRNA (5 ng/cell), or H_2O as a negative control 24 h before pairing. Vitelline envelopes were removed, and oocytes were manually paired with vegetal poles apposed in MB medium. Paired cells were injected with caPI3K cRNA 4 h before electrophysiology recordings. Gap junctional conductance measurements were taken 16–24 h after pairing.

2.2. Dual whole-cell voltage clamp

Gap junctional conductance was measured by dual voltage clamp [25]. Current and voltage electrodes (1.2 mm diameter, omega dot; Glass Company of America, Millville, NJ) were pulled to a resistance of 1–2 MΩ with a vertical puller (Narishige, Tokyo, Japan) and filled with 3 M KCl, 10 mM EGTA, and 10 mM HEPES, pH 7.4. Voltage clamp recordings were performed using two GeneClamp 500 amplifiers controlled by pClamp software using a Digidata 1320A interface. (Axon Instruments, Foster City, CA). Both cells of a pair were clamped at −40 mV to eliminate any transjunctional potential. One cell was then subjected to alternating pulses of ±20 mV, while the current produced by the voltage change was measured in the second cell. The current delivered to the second cell was equal in magnitude to the junctional current (I_j), and gap junctional conductance (G_j) was calculated by dividing the measured current by the voltage difference between first cell (V1) and the second cell (V2), $G_j = I_j/(V1–V2)$.

2.3. Preparation of oocyte samples and Western blot

Oocytes were collected in 1 ml of ice-cold lysis buffer containing 5 mM Tris, pH 8.0, 5 mM EDTA, and protease inhibitors and homogenized using a series of mechanical passages through syringe needles of diminishing diameter (20, 22, and 26 Ga). Extracts were first centrifuged at 2000×*g* at 4 °C for 5 min, then the supernatants were centrifuged at 45 000×*g* at 4 °C for 30 min. Membrane pellets were resuspended in SDS sample buffer (2 μl/oocyte), separated on 10% SDS–PAGE gels, and transferred to nitrocellulose membranes. Protein blots were probed with antibodies specific for the carboxy tail of Cx46 (rabbit) [13,14], or the cytoplasmic loop of Cx50 (goat; Santa Cruz Biotechnology, Santa Cruz, CA) at a 1:1000 dilution followed by incubation with horseradish peroxidase-conjugated rabbit anti-goat, or goat anti-rabbit secondary antibodies (Jackson ImmunoResearch Laboratories, West Grove, PA). In addition, blots were probed with mouse monoclonal phospho (p)-Akt (Ser 473) or rabbit total (t)-Akt antibodies (Cell Signaling Technology, Danvers, MA.) at 1:1000 dilution, followed by incubation with ECL-mouse secondary antibody or horseradish peroxidase (HRP)-conjugated goat anti-rabbit secondary antibody (Santa Cruz Biotechnology, Santa Cruz, CA). Densitometry quantifications were performed using ImageJ (NIH). Band densities of three independent experiments were analyzed and the normalized mean values plotted.

2.4. Transient transfection and dual whole-cell patch clamp

HeLa cells were plated on glass coverslips, grown to 50% confluence and transiently transfected with 4–5 μg of Connexin46 DNA subcloned into a pIRES2-eGFP vector (Clontech) using Lipofectamine 2000 (Invitrogen, Carlsbad, CA). Gap junctional conductance of Cx46 expressing cells was measured after overnight incubation. Cx50 stably transfected HeLa cells [26], were also used. Junctional conductance of cell pairs was measured using dual whole-cell patch clamp with Axopatch 1D patch-clamp amplifiers (Axon Instruments) at room temperature. Cells were bathed in a solution containing 137.7 mM NaCl, 5.4 mM KCl, 2.3 mM NaOH, 1 mM MgCl2, 5 mM HEPES, 10 mM glucose, pH 7.4. Patch electrodes with resistances of 3–5 MΩ were filled with internal solution containing 120 mM aspartic acid, 120 mM KOH, 10 mM EGTA, 3 mM NaATP, and 5 mM HEPES, pH 7.2. Macroscopic and single-channel recordings were acquired using pClamp, sampled at 1–2 kHz and filtered at 0.2–0.5 kHz. Analysis of recordings was performed with pClamp and Origin software (MicroCal Software, Northampton, MA). Each cell of a pair was initially held at a common holding potential of 0 mV. To evaluate junctional coupling, 200-ms hyperpolarizing pulses from the holding potential of 0 to ±20 mV were applied to one cell to establish a transjunctional voltage gradient (V_j), and junctional current was measured in the second cell (held at 0 mV). To selectively block PI3K signaling, cells were incubated in either 50 nM PIK-75 or 10 uM Akt inhibitor VIII (Akti, which blocks phosphorylation of Akt by maintaining it in an inactive conformer) for 12–48 h prior to recordings. For unitary conductance measurements, alternating bipolar V_j pulses ranging from ±10 mV to ±110 mV were applied to low conductance cell pairs that had only one or two active channels. Single channel currents for the three treatment conditions were plotted against voltage and fit by linear regression to determine the slope which was equal to the unitary conductance. The correlation coefficients of all three linear fits were $r^2 \geqslant 0.99$.

3. Results

3.1. Treatment with PIK-75 or Akti differentially regulated Cx50 and Cx46-mediated conductance

Previous studies have shown that manipulation of signaling pathways can affect gap junctional conductance [22,27,28]. To investigate whether inhibiting PI3K signaling affected gap junctional conductance, we treated HeLa cells stably expressing Cx50 [26], with PIK-75 to inhibit the p110α catalytic subunit of PI3K. As shown in Fig. 1A, pairs expressing Cx50 without inhibitor had high coupling with a mean G_j of 22.2 nS. Treatment with PIK-75 for 24 h significantly decreased conductance by 3-fold to a mean G_j of 6.8 nS ($P < 0.05$, Student's t test). To test if the decrease in conductance was caused by inhibiting PI3K directly, or inhibition of the signaling cascade, we treated Cx50 HeLa cells with Akt Inhibitor-VIII (Akti) to specifically inhibit Akt, a downstream effector of PI3K signaling. Inhibition of Akt also showed a 3-fold decrease in Cx50-mediated conductance with a mean G_j of 8 nS. DMSO was used as a vehicle for both inhibitors, and incubation of Cx50 expressing cells with DMSO alone had no effect on coupling. To evaluate the effects of inhibiting PI3K signaling on the