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EGF induces efficient Cx43 gap junction endocytosis in mouse embryonic stem cell colonies via phosphorylation of Ser262, Ser279/282, and Ser368

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ABSTRACT

Gap junctions (GJs) traverse apposing membranes of neighboring cells to mediate intercellular communication by passive diffusion of signaling molecules. We have shown previously that cells endocytose GJs utilizing the clathrin machinery. Endocytosis generates cytoplasmic double-membrane vesicles termed annular gap junctions or connexosomes. However, the signaling pathways and protein modifications that trigger GJ endocytosis are largely unknown. Treating mouse embryonic stem cell colonies – endogenously expressing the GJ protein connexin43 (Cx43) – with epidermal growth factor (EGF) inhibited intercellular communication by 64% and activated both, MAPK and PKC signaling cascades to phosphorylate Cx43 on serines 262, 279/282, and 368. Upon EGF treatment Cx43 phosphorylation transiently increased up to 4-fold and induced efficient (66.4%) GJ endocytosis as evidenced by a 5.9-fold increase in Cx43/clathrin co-precipitation.

1. Introduction

Direct intercellular communication by gap junction (GJ) channels is a hallmark of normal cell and tissue physiology. GJs are the only cell–cell junction type that allows direct cell–cell communication via the transfer of molecules between cells. Examples include small metabolites such as glucose, amino acids, and ATP; ions such as Na^+, Ca^{2+}, and Cl^-; cell signaling molecules such as IP3 and cAMP; and potentially functional RNAs, such as miRNAs in glioma cells [1] and siRNAs in NRK cells ([2], reviewed in [3]). Complete double-membrane spanning GJ channels are formed when two hexameric hemi-channels (connexons) dock in the extracellular space. In addition, based on their double-membrane configuration GJs likely contribute significantly to cell–cell adhesion. Clearly, these cellular GJ functions require precise modulation. Remarkably, docked GJ channels cannot be separated into individual hemi-channels under physiological conditions [4,5]. Yet, analyses in many different cell and tissue types revealed that cells endocytose their GJs constitutively, and efficiently after treatment with inflammatory mediators such as thrombin and endothelin, in response to treatment with the non-genomic carcinogen lindane, and under many physiological and pathological conditions that require cell–cell uncoupling and/or physical cell–cell separation such as cell migration in development and wound healing, tissue differentiation, mitosis, apoptosis, leukocyte extravasation, ischemia, hemorrhage, edema, and cancer cell metastasis [6–14]. Constitutive and acute GJ endocytosis correlates with the short half-life of 1–5 h reported for GJ proteins (connexins, Cxs) and GJs [7,15–17]. We have previously shown that GJs are endocytosed as a whole in a clathrin-mediated endocytic process [6,12,18,19]. However, the specific post-translational modifications such as phosphorylation, ubiquitination, etc. that may render Cx proteins in GJs endocytosis competent are still poorly understood.

Twenty Cx isoforms are found in mouse with Cx43 being the most prominent isoform. Cx43 is a well-known phospho-protein. Numerous serine residues in the Cx43 C-terminus are phosphorylated to up-regulate (Ser325, Ser328, Ser330, Ser364/365, and Ser373) or down-regulate (Ser255, Ser262, Ser279/282, and

Abbreviations: AGJ, annular gap junction; CME, clathrin mediated endocytosis; EGF, epidermal growth factor; GJ, gap junction; GJIC, gap junction intercellular communication; LY, lucifer yellow; MAPK, mitogen activated protein kinase; MEF, mouse embryonic fibroblast; mES cells, mouse embryonic stem cells; PKC, protein kinase C; PM, plasma membrane; RTK, receptor tyrosine kinase

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Ser368) GJ mediated intercellular communication (GJIC) (reviewed in [20]). Protein kinase C (PKC) is thought to phosphorylate Cx43 at Ser368 to down-regulate GJIC [21,22]. Cx43 has also been shown to be a substrate of mitogen activated protein kinase (MAPK) that upon mitogen stimulation phosphorylates Cx43 at Ser255, Ser262, and Ser279/Ser282 to down-regulate GJIC [23,24]. Epidermal growth factor (EGF), a well-studied mitogen, binds to the EGF receptor (a receptor tyrosine kinase [RTK] family member) to activate both MAPK and PKC signaling pathways, to for example promote cell proliferation. Mouse embryonic stem (mES) cells are known to express the GJ proteins Cx31, Cx43, and Cx45 and to form functional GJs [25,26]. ES cells are actively proliferating cells with a relatively short cell cycle (see Supplemental Movie 1). They can infinitely self-renew while maintaining their pluripotency that is mediated via GJIC among the cells in the colony [27–29]. Treatment of mES cells with EGF is known to down-regulate GJIC [30]. However, the mechanism/s that lead to GJIC inhibition are not known. Since EGF can stimulate cell proliferation, and mitotic cells are known to remove their GJs at onset of mitosis [13], we hypothesized that EGF-treatment may lead to GJ endocytosis to down-regulate GJIC. To test this hypothesis, and to characterize signals that may lead to GJ endocytosis, we treated mES cell colonies with EGF. We found that EGF-treatment induced inhibition of GJIC that correlated with clathrin recruitment and Cx43–GJ endocytosis, and that GJ endocytosis was initiated by phosphorylation of Cx43 at serines 262, 279/282, and 368.

2. Materials and methods

2.1. Cell culture

E14TG2a mouse embryonic stem (mES) cells (ATCC, Cat. No. CRL-1821) were seeded on mouse embryonic fibroblasts (MEFs) (Millipore, Cat. No. PMEF-NL). Under established culture conditions described below mES cells remain undifferentiated and grow into 3-dimensional colonies that are only loosely attached to the culture dishes. Colonies were passaged and maintained in 0.1% gelatin-coated dishes (MEF-free) in humidified atmosphere containing 5% CO_2 at 37 °C in KO DMEM (Gibco, Cat. No. 10829). Media were supplemented for a final concentration of 15% with KO serum replacement (Gibco, Cat. No. 10828), 3 mM L-glutamine (Gibco, Cat. No. 25030), 50 I.U/ml penicillin and 50 μg/ml streptomycin (Gibco, Cat. No. 15070), 1 mM sodium pyruvate (Gibco, Cat. No. 11360, stock 100 mM), 1× non-essential amino acids (Millipore, Cat. No. TMS-001-C), 1× β-mercaptoethanol (Millipore, Cat. No. ES-007-E), and 1000 U/ml ESGRO mLIF (Millipore, Cat. No. ESG1106) to prevent cells from differentiating. Prior to EGF treatment (100 ng/ml EGF, Sigma, Cat. No. E4127), media were replaced with serum free media (to starve cells of growth factors) supplemented either with 50 μM PD98059 (MEK, MAPK pathway inhibitor; Sigma, Cat. No. P215), 100–500 nM chelerythrine-Cl (PKC inhibitor; Cayman Chemical, Cat. No. 11314), or both for 1 h and cultured under standard conditions.

2.2. Dye transfer assays

mES cell colonies were cultured in 3.5 cm diameter dishes for 2 days, then pre-incubated with serum free media as described above. Appropriate culture dishes were then treated with PD98059, chelerythine-Cl, or both and cultured for 1 h before adding 100 ng/ml EGF and incubating for additional 30 min. Media were replaced with 0.1% Lucifer Yellow (LY; Invitrogen, Cat. No. L682) in OPTIMEM (Gibco, Cat. No. 31985). To wound cells and allow for LY dye uptake, mES cell colonies were carefully cut with a sharp scalpel in the presence of LY, incubated at room temperature (RT) for 5 min to allow dye to transfer to neighboring cells, then washed 3 times with OPTIMEM followed by 3.7% paraformaldehyde fixation for 10 min. Paraformaldehyde was removed by washing 3 times with 1 × PBS containing Ca^{2+} and Mg^{2+}. LY fluorescence and Phase Contrast images were acquired using a 20× objective. Dye spreading from the injured cells to the farthermost receiving cells was measured using MetaVue software version 6.1r5 (Molecular Devices, Sunnyvale, CA), averaged and plotted. In addition, fluorescence intensities along lines placed perpendicular to the cut at representative areas were measured and plotted as well.

2.3. Immunofluorescence microscopy and image analyses

mES cell colonies were grown on glass cover slips pre-treated with 0.1% gelatin (Fisher Scientific, Cat. No. G-7) to improve colony adhesion; fixed and permeabilized in pure ice-cold ethanol for 10 min; blocked with 10% FBS in PBS at RT for 1 h, and incubated with primary rabbit polyclonal anti-Cx43 antibodies (Cell Signaling Technology, Cat. No. 3512) at 1:500 dilution at 4 °C overnight. Secondary antibodies (Alexa488-conjugated goat anti-rabbit, Molecular Probes/Invitrogen, Cat. No. A11008) were used at 1:500 dilution at RT for 1 h. Plasma membranes were visualized using 1 μg/ml Alexa594-conjugated wheat germ agglutinin (WGA; Molecular Probes/Invitrogen, Cat. No. W11262), or a monoclonal antibody directed against the membrane-associated protein, ZO-1 (at 1:300 dilution, Zymed Laboratories, Cat. No. 33-9100) combined with an Alexa568-conjugated secondary antibody (goat anti-mouse, at 1:500 dilution, Molecular Probes/Invitrogen, Cat. No. A-11031) after fixation in 3.7% formaldehyde and permeabilization in 0.2% Triton X-100. Cell nuclei were stained with 1 μg/ml DAPI. Cells were mounted using Fluoromount-G™ (SouthernBiotech, Cat. No. 0100-01) and examined. Wide-field fluorescence microscopy was performed on a Nikon Eclipse TE 2000E inverted fluorescence microscope equipped with a 40×, NA 1.4, Plan Apochromat oil immersion objective. Images were acquired using MetaVue software. Confocal microscopy was performed on a Zeiss Axiovert 200 M inverted fluorescence microscope equipped with an LSM510 META scan head and a 63×, NA 1.4, Plan Apochromat oil immersion objective. Argon-ion and Helium–Neon lasers were used to generate 488 and 543 nm excitation lines, and pinholes were typically set to 1 airy unit. Images were acquired and analyzed using ZEN software. Quantitative analyses were performed using ImageJ (National Institutes of Health, USA).

2.4. Electron microscopic analyses

mES cell colonies were cultured under feeder-free conditions in 3.5 cm diameter dishes, then fixed with 2.5% glutaraldehyde (Sigma, Cat. No. G7651) in 0.1 M sodium cacodylate buffer at RT for 2 h. Cells were washed, treated with tannic acid, dehydrated, uranyl acetate-stained and flat-embedded in the dishes as described in Falk [31]. Embedded cells were mounted, trimmed, thin-sectioned and examined with a Phillips CM100 electron microscope.

2.5. Western blot analyses

Denatured protein samples derived from mES cell lysates were analyzed on 10% SDS–PAGE mini-gels (BioRad). Biotinylated protein ladder (Cell Signaling Technology, Cat. No. 7727S) was used as a molecular weight marker. Proteins were transferred onto nitrocellulose membranes (Whatman, Cat. No. 10439396) on ice at 120 V for 1 h before blocking with 5% non-fat dry milk, or 5% BSA in TBST at RT for 1 h. Membranes were then incubated with primary antibodies at 4 °C overnight. Antibodies used were: rabbit anti-Cx43 (Cell Signaling Technology, Cat. No. 3512) at 1:2500 dilution, phospho-specific rabbit anti-Cx43 Ser255 (Santa Cruz,