Published in final edited form as:

Cell Signal. 2007 May ; 19(5): 958–967. doi:10.1016/j.cellsig.2006.11.007.

ZO-1 is required for Protein Kinase C Gamma-driven Disassembly of Connexin 43

Vladimir Akoyev^a and Dolores J. Takemoto^a

^aDepartment of Biochemistry, Kansas State University, Manhattan, Kansas 66506, United States, Email: akoev@ksu.edu, dtak@ksu.edu

Abstract

We have previously reported that protein kinase C gamma (PKC γ) is activated by phorbol-12-myristate-13-acetate (TPA) and that this causes PKC γ translocation to membranes and phosphorylation of the gap junction protein, connexin 43 (Cx43). This phosphorylation, on S368 of Cx43, causes disassembly of Cx43 out of cell junctional plaques resulting in the inhibition of dye transfer. The purpose of this study is to identify the specific role of zonula occludens protein-1 (ZO-1), a tight junction protein with recently established effects on gap junctions, in this PKC γ -driven Cx43 disassembly. For this purpose, ZO-1 levels in lens epithelial cells in culture were decreased by up to 70% using specific siRNA. The down-regulation of ZO-1 caused a stable interaction of PKC γ with Cx43 even without normal enzyme activation by TPA. However, after TPA activation of the PKC γ , the Cx43 did not disassemble out of plaques even though the PKC γ enzyme was activated and the Cx43 was phosphorylated on S368. Confocal microscopy demonstrated that the siRNA treatment caused a loss of ZO-1 from borders of large junctional Cx43 cell-to-cell plaques and resulted in the accumulation of Cx43 aggregates inside of cells. Loss of the specific “plaquesome” arrangement of large Cx43 plaques surrounded by ZO-1 was accompanied by a complete loss of functional dye transfer. These results suggest that ZO-1 is required for Cx43 control, both for dye transfer, and, for the PKC γ -driven disassembly response.

Keywords

connexin 43; Protein kinase C gamma; Zonula occludins protein-1; lens epithelial cells

1. Introduction

Connexin 43 (Cx43) plays a pivotal role in cell-to-cell communications and is controlled by numerous kinases at phosphorylation sites on the C-terminal tail [1,2]. PKC- γ is a well studied kinase, because of the phenomenon of TPA-induced phosphorylation of Cx43 on Ser368 [3-5]. Phosphorylation of Cx43 on Ser368 regulates gating, selectivity and permeability of the Cx43 channels [3-6]. At the cell level, the phosphorylation of Cx43 on Ser368 is responsible for disassembly of Cx43 junctional plaques [7] and disruption of cell-to-cell communications [4,5]. A second type of regulatory region on Cx43 is the PDZ-binding domain located on the very C-terminus [8]. PDZ-based interactions of Cx43 with the tight junction protein, ZO1,

Correspondence should be addressed to: Dolores J. Takemoto, Ph. D, Department of Biochemistry, 141 Chalmers Hall, Kansas State University, Manhattan, Kansas 66506, United States, Phone: 785-532-7009, Fax: 785-532-7278, E-mail: E-mail: dtak@ksu.edu.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

control further interactions with numerous cytoskeletal, tight, and adherent junction proteins through ZO1 [1,9]. Data reviewed by Giepmans demonstrates a close relationship between gap junction communication and tight junctions: many cell lines demonstrate inhibited gap junction communication after disruption of tight junctions [10]. Recent publications have been shown that Cx-43/ZO1 interactions control the size of Cx43 plaques [11-20]. Hunter has also demonstrated that ZO1 plays an important role in the process of accretion of cell surface, functional Cx43 plaques: ZO1 is associated at the periphery of Cx43 gap junction plaques [12], and the disruption of Cx-43/ZO1 interactions creates excessively large gap junction plaques [11,12]. The disruption of Cx43-ZO1 interactions, by use of competitive peptides, correlated with the accumulation of phosphorylated isoforms of Cx43. In contrast, Jin [18] has reported that ZO1 and Cx43 co-localize, regardless of disrupted interactions or reduced P₁ and P₂ phosphoisoforms of Cx43. Taken together these data raise questions of how the interaction between ZO1 and Cx43 controls the phosphorylation state of Cx43. The close proximity of the Ser368/PKC γ phosphorylation site to the PDZ/ZO-1-binding domain on the Cx43 C-terminus suggests that ZO-1/Cx43 effects and PKC γ effects may be connected. Here we present, for the first time, data that ZO1 can modulate the interaction of PKC- γ with Cx43 and, thus, can modulate the PKC- γ -driven gap junction disassembly of Cx43. Furthermore, we demonstrate that a specific cell surface “plaquetosome” arrangement of Cx43 and ZO-1 is required for both gap junction control by PKC γ and functional dye transfer activity.

2. Materials and Methods

2.1 Materials

Dulbecco's modified Eagle's medium (low glucose), RPMI 1640 medium, trypsin-EDTA, gentamicin and penicillin/streptomycin, were purchased from Invitrogen Corporation (Carlsbad, CA). Dithiothreitol (DTT), sodium fluoride (NaF), bovine serum albumin (BSA) were purchased from Fisher Scientific (Hampton, NH). Fetal bovine serum was purchased from Atlanta Biologicals (Norcross, GA). Phenylmethanesulfonyl fluoride (PMSF), protease inhibitor cocktail and ATX Ponceau S red staining solution were from Sigma-Aldrich (St. Louis, MO). Protein A/G PLUS-agarose beads were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). SuperSignal West Femto Substrate kit with secondary anti-mouse or anti-rabbit IgG conjugated with horseradish peroxidase were purchased from Pierce (Rockford, IL). Membrane blocking solution was purchased from Zymed-Invitrogen (San Francisco, CA). All electrophoresis reagents and protein molecular weight markers for electrophoresis were purchased from Bio-Rad Laboratories (Hercules, CA). Protein Assay dye (Bio-Rad Protein Assay) and Coomassie Brilliant Blue R-250 stain were purchased from Bio-Rad Laboratories (Hercules, CA). Whatman Optitran BA-S-85 nitrocellulose 0.45 μ m membrane was purchased from MidSci (St. Louis, MO). PepTag[®] non-radioactive protein kinase C assay system was purchased from Promega (Madison, WI). Phorbol-12-myristate-13-acetate (TPA, cat no. 524400) was purchased from Calbiochem (La Jolla, CA).

2.2 Antisera

Mouse monoclonal anti-N-terminal-Cx43 IgG were from Fred Hutchinson Cancer Research Center (cat no. Cx43NT1) Seattle, WA; polyclonal rabbit anti-N-terminal-ZO1 (cat no. 40-2300), polyclonal rabbit anti-Mid region-ZO1 (cat no. 40-2200), mouse monoclonal anti-ZO1 IgG₁ (against 334-634 amino acids region, cat no. 33-9100), mouse anti- α -tubulin loading control (cat no. 32-2500) were purchased from Zymed-Invitrogen (San Francisco, CA). Rabbit polyclonal anti-phospho-Cx43 (Ser-368) (cat no. AB3841), mouse monoclonal anti-Cx43 (against cytoplasmic C-terminus, cat no. MAB3068), and rabbit polyclonal anti-mouse Cx43 (against cytoplasmic C-terminus, cat no. AB1727), were purchased from Chemicon (Temecula, CA). Mouse anti-PKC- γ IgG (against C-terminal amino acids 449-697 region, cat no. P20420) was purchased from Transduction Lab, (Lexington, KY). Rabbit polyclonal anti-

phospho-PKC- γ (T514) IgG, (cat no. ab5778) and mouse anti- β -actin loading control IgG (cat no. ab8226), were purchased from Abcam (Cambridge, MA).

Alexa Fluor 466 and 568 goat anti-mouse or anti-rabbit IgG (H+L) conjugated secondary antibodies, lucifer yellow CH lithium salt (cat no. L-453), tetramethylrhodamine dextran 10,000 lysine fixable (cat no. D1817), 4',6-diamidino-2-phenylindole dihydrochloride (DAPI, cat no. D1306) and ProLong Gold antifade reagent (cat no. P36934) were purchased from Molecular Probes (Invitrogen, Eugene, OR).

2.3 Cell Culture

N/N1003A rabbit lens epithelial cells were cultured in 75-cm² flasks or in 6-well plates in Dulbecco's modified Eagle's medium (DMEM, low glucose; Invitrogen, San Diego, CA) supplemented with 10% fetal bovine serum and 50 μ g/mL gentamicin, 0.05 U/mL penicillin, and 50 μ g/mL streptomycin (pH 7.4) at 37°C in an atmosphere of 90% air and 10% CO₂. The cells were used for transfection when they reached 90% confluency. Prior to treatment with TPA (phorbol-12-myristate-13-acetate) cells were starved at least for 2 hours in DMEM without serum.

2.4 Anti-ZO1 siRNA

All siRNA transfection reagents: RNAiFect siRNA transfection reagent (cat no. 301605), negative non-silencing control siRNA (cat no. 102076), negative non-silencing control siRNA conjugated with Alexa Fluor 488 (cat no. 1022563) were purchased from Qiagen (Valencia, CA). Custom designed anti-ZO1 siRNAs, HPP grade, were purchased from Qiagen. To design siRNA, the mRNA sequence (NCBI accession no. **D14340**) of *Mus musculus* tight junction protein 1 (Tjp1, Zonula occludens protein, ZO1, NCBI accession no. **P39447**) was used. siRNAs were designed as 4 different siRNA duplex sequences. Sequence #1 was (Target: ACG AGG CAT CAT CCC AAA TAA. Offset: 2113. Sense: r(GAG GCA UCA UCC CAA AUA A)dTdT. Antisense: r(UUA UUU GGG AUG AUG CCU C)dGdT. Sequence #2 was (Target: CAG ATA AGC CTA AAT ATC AAA. Offset: 5184. Sense: r(GAU AAG CCU AAA UAU CAA A)dTdT. Antisense: r(UUU GAU AUU UAG GCU UAU C)dTdG). Sequence #3 was (Target: CAC GAT TTC TGT TTA GAT AAT. Offset: 6918. Sense: r(CGA UUU CUG UUU AGA UAA U)dTdT. Antisense: r(AUU AUC UAA ACA GAA AUC G)dTdG). Sequence #4 was (Target: CCG AAG ATA TTG TTC GAT CAA. Offset: 4410. Sense: r(GAA GAU AUU GUU CGA UCA A)dTdT. Antisense: r(UUG AUC GAA CAA UAU CUU C)dGdT.

2.5 Transfection with siRNA

Control non-silencing siRNA and anti-ZO1-siRNA were prepared according to Qiagen technical protocol in an RNase-free environment. Transfection of adherent cells with siRNA was carried out according to the protocol in Qiagen RNAiFect™ Transfection Handbook in 6-well plates using 5 μ g siRNA per well (2.5 μ g/mL per well). N/N1003A cells were seeded and grown into 6-well plates and when nearly 90% confluent, cells were transfected with siRNA with negative, non-silencing control siRNA or anti-ZO1 siRNA. After transfection, cells were grown for 24-96 hours. All four duplexes were tested and only two of them were efficient enough to suppress ZO1 protein synthesis after 24 hours. The efficiency of transfection was tested with negative, non-silencing control siRNA conjugated with Alexa Fluor 488 followed by confocal microscopy. The efficiency of suppression of ZO1 protein synthesis was tested by western blot analyses at 24, 48, 72 and 96 hours after transfection.

2.6 Whole Cell Homogenate Preparations (WCH)

Cells were collected by scraping from plates, sedimentation and washing 2 times in cold phosphate-buffered saline (PBS). The cell pellets were lysed on ice with cell lysis buffer

containing 20 mM Tris-HCl, pH 7.5, 0.5 mM EDTA, 0.5 mM EGTA, 0.5% Triton X-100, 0.1% protease inhibitor mixture cocktail, 5 mM NaF, and 2 mM PMSF. Lysates were sonicated for 20 seconds on ice. Protein concentration of each sample was measured using Bio-Rad Protein Assay and samples were diluted to make concentrations equal in all samples for future analyses.

2.7 Western Blot

Western blot analyses were carried out as previously described [21]. To block blots for anti-phosphorylated-Cx43 (S368) antibodies or anti-phosphorylated-PKC- γ (T514) special blocking solution, containing 3% bovine serum albumin and goat IgG (Zymed-Invitrogen, cat no. 00-0105) was used to block membranes overnight. Mouse anti- α -tubulin and mouse anti- β -actin were used as loading control primary antibodies for all samples. Western blots were visualized with SuperSignal West Femto Substrate kit supplied with secondary anti-mouse or anti-rabbit IgG, conjugated with horseradish peroxidase (Pierce, Rockford, IL, cat no. 34095) followed by exposure to X-ray film (Midwest Scientific, St. Louis, MO). For quantitative analyses, X-ray films were scanned and western blot bands were digitized using UN-SCAN-IT software (Silk Scientific, Inc, Orem, Utah). Digitizing procedure consisted of counting of total pixel numbers in every designated band. These total pixel numbers were used as a measure of amount of proteins in every band.

2.8 Coimmunoprecipitation

Immunoprecipitation analyses of WCH followed by western blot analyses were carried out as previously described [7,21]. All WCH had the same total protein concentration (2 mg/mL) before immunoprecipitation. Mouse monoclonal antibodies against the N-terminus (first 20 amino acids) of Cx43 or mouse monoclonal antibodies against C-terminus of Cx43 were used for immunoprecipitation. Five μ g/mL of antibodies were used for immunoprecipitation. Western blot was used to identify proteins in immunoprecipitates.

2.9 Immunofluorescence Confocal Microscopy

In order to reveal the location of Cx43 and ZO1 in whole cells confocal microscopy of immunolabeled Cx43 and ZO1 was used. N/N1003A lens epithelial cells were grown on glass coverslips in six-well plates until 90% confluency, transfected, starved in serum-free media, and treated with TPA at 100 - 400 nM for 20 min, at 37°C. The cells were fixed with 2.5% paraformaldehyde, quenched with 50 mM Glycine, washed with PBS, permeabilized with 0.05% Triton-X100 in PBS for 30 minutes, washed, and blocked with 3% BSA for at least 1 hour at room temperature. To identify target proteins, cells were simultaneously treated with mouse or rabbit primary antibodies directed against different target proteins in 3% BSA, overnight, at 4 °C with constant gentle shaking. After incubation with primary antibodies coverslips with cells were washed with blocking solution and stained with secondary Alexa Fluor-488 (green color) and/or Alexa Fluor-568 (red color) anti-mouse and anti-rabbit antibodies (Molecular Probes, Eugene, OR) for 2 hours at room temperature. The coverslips were then washed in cold PBS, 2 times, and mounted onto slides using ProLong Gold antifade reagent. Confocal microscopy was done using a Nikon C1 scanning confocal microscope with a 2-laser units system: air-cooled argon laser (454 nm) and He-Ne laser (543). Optical sections (images) of immunolabeled cells were taken using Nikon Plan Fluor 100X/1.3/WD 0.20/oil objective, standardized pinhole (30 μ m) and gain settings. For qualitative analyses, 20 images per slide were saved at resolution 2048 $\times$ 2048 pixels (127.3 $\times$ 127.3 μ m). Images were processed using Nikon EZ-C1 Viewer software. The total number of cell surface plaques, the number of plaques on the outer edge of cells and on the border between cells, the number of plaques inside cells and the number of plaques larger than 1 μ m² per single cells in each image were counted manually by marking every spot of interest with EZ-C1 Viewer software tools. To find and count plaques with area more than 1 μ m² a circle was drawn (1 μ m²) using EZ-C1 software

tools which measures the area for every plaque. Twenty cells in every saved image were processed. The number of Cx43 plaques was expressed as Mean $\pm$ S.D. At least three separate experiments were done in each case.

2.10 Dye Transfer - Gap Junction Activity Assay

To study the gap junction activities in cells the scrape-loading/dye-transfer (SL/DT) assay with lucifer yellow and rhodamine dextran was carried out as described before [21-23]. Gap junction activity was expressed as the number of cells transferring lucifer yellow minus cells with rhodamine dextran per total number of DAPI stained cells. Three different areas along each scrape were analyzed with at least 1000 cells counted. The number of cells transferring lucifer yellow were expressed as Mean $\pm$ S.D with $p < 0.05$ considered significant.

2.11 PKC- γ Activity Assay

To analyze the specific activity of PKC- γ immunoprecipitated from WCH the PepTag assay kit was used as described before [23].

2.12 Statistical Analyses

Origin software (Microcal Software Inc., Northampton, MA) was used for statistical analyses. Values of $p < 0.05$ were considered to be statistically significant.

3. Results

3.1 Anti ZO1 siRNA decreased ZO1 protein levels but not levels of Cx43 protein

In order to show that ZO1 siRNA transfection decreased expression of ZO1 protein, whole cell homogenates from transfected cells were subjected to western blot analyses with 3 different anti-ZO1 antibodies: polyclonal rabbit anti-N-terminal-ZO1, polyclonal rabbit anti-middle domain-ZO1 and monoclonal mouse anti-ZO1 IgG (against 334-634 amino acids region). These were used to rule out protein truncation, a common posttranslational process in the lens [1,24]. Western blot using ZO1 antiserum revealed the typical ZO1 α^+ and α^- isoforms at ~220 kDa as previously described (see band in Fig. 1, lanes in C) [25-28]. Treatment with anti-ZO1 siRNA decreased the level of expression of both isoforms of ZO1 after 24-96 hours of transfection by approximately 60% (Fig.1-A, lanes 6-9), compared to amount of ZO1 in control, non transfected N/N1003A cells (Fig.1-A, lane 1 and Fig.1-B, scanned gels) and to cells transfected with negative control, non silencing siRNA (Fig.1-A, lanes 2-5). Western blot analyses of total amount of Cx43 using Cx43 antiserum did not reveal changes after siRNA transfection (Fig.1-D). In control experiments, Cx43 always displayed three typical bands of Cx43 phosphoisoforms (Fig.1-D, lane 1): NP or P₀-Cx43 - nonphosphorylated form of Cx43, and 2 phosphorylated forms: P₁-Cx43 and P₂-Cx43 as previously described [18,29-31]. Treatment with TPA (at 100-400 nM), a well-known activator of PKC- γ increased the intensity of the P₁- and P₂- forms of Cx43 (Fig.1-D, lane 2) as previously described [2,4,31-33]. Treatment of N/N1003A cells with ZO1 siRNA did not decrease the intensities of P₁ and P₂ when compared to the level in control samples (Fig.1-D, lane 3 and 5) before or after TPA treatment (Fig.1-D, lane 4 and 6). We conclude that there are no changes in the total amount of Cx43 or in the composition of Cx43 phosphoisoforms after ZO1 siRNA treatment, and that the TPA treatment always caused increased appearance of the phospho-isoforms of Cx43. α -Tubulin (Fig.1-A and E) was used as a loading control. Eight separate experiments were done to collect statistical data for western blot analyses as shown in scans (Fig 1B).

3.2 ZO1 siRNA Transfection Does Not Change Levels or Activation of PKC- γ

In order to determine if ZO1 siRNA transfection would change the enzymatic activity of PKC- γ we used a non-radioactive PKC assay and immunoprecipitated PKC- γ as an enzyme source.

Our data demonstrated no changes in PKC- γ activity after ZO1 siRNA treatment. PKC- γ was active in all samples and was responsive to TPA activation (Fig.2-A and B). PKC- γ protein levels were also not altered when compared to negative control siRNA treatment (Fig.2-C). α -Tubulin was included as a loading control (Fig.2-D). These data demonstrate that ZO1 downregulation did not alter the amount, total activity, or the ability of PKC- γ to be activated after TPA stimulation.

3.3 Cx43 Forms ZO1-surrounded Plaques That Disassemble after TPA-activation of PKC- γ : ZO1 Downregulation Abolishes This Response and Structure

In order to study the ability of Cx43 to form plaques after siRNA transfection, scanning confocal microscopy was used. Typical confocal images of intact N/N1003A cells showed that immunolabeled Cx43 formed the well-known pattern of junctional “plaques” with their typical localization on the junctional interface between two adjacent cells and in the place of contacts between protrusions of one cell body with another cell (Fig.3-a, see arrow). Typical large plaques with area more than $1\mu\text{m}^2$ have elongated discontinuous staining (Fig.3-a, see arrow). Immunolabeled Cx43 was also found inside cells throughout the cytoplasm both evenly distributed as a diffuse green signal and as sparse, discernible single non-junctional aggregates (Fig.3-a). Confocal analyses determined that immunolabeled ZO1 was also assembled into cell surface aggregates (Fig.3-b, see arrow). ZO1 usually colocalized together with immunolabeled Cx43 on the junctional interface between two adjacent cells and on the edge of cells (Fig.3-c, see arrow) under our experimental conditions. For our statistical analyses we established a threshold for the size of countable plaques. We took into account data, presented by Bukauskas [17] that there are a critical number of channels and plaque size of more than 400 channels at which channels/plaques become capable of opening and of being functional. According to those estimations, plaques with a diameter of about $0.5\mu\text{m}^2$ contain about 2000 channels but only 20% among them are active. We set our threshold at $1\mu\text{m}^2$. Another reason to set a size limitation was a low signal/noise ratio for small particles what makes counting subjective. Plaques with the size of at least $1\mu\text{m}^2$ are clear and discernible for statistical analyses. Analyses of counted junctional and nonjunctional plaques in nontransfected, control N/N1003A cells, showed that, under our experimental conditions, 40% of all plaques per cell were junctional plaques with areas of more than $1\mu\text{m}^2$ (Fig.3-q, control, -TPA), and less than 10% of all plaques per cell were large nonjunctional plaques localized inside cells (Fig.3-r, control, -TPA). The rest of the junctional and nonjunctional plaques were “small” plaques with area less than $1\mu\text{m}^2$. Careful analyses of merged images of junctional plaques (Fig.3-c) revealed that a merged yellow signal represented $66\pm 22\%$ (Mean $\pm$ SD) of the whole area of plaques which demonstrates a high extent of colocalization of immunolabeled ZO1 with Cx43. Figure 3-d shows single scans for both green and red channels through the long axis of a plaque. Coincidence in the intensities changes demonstrates colocalization of two signals. However, the most typical feature of control junctional large plaques was that the rest ($44\pm 12\%$) of the labeled area for ZO1 were located on the edge of Cx43 plaques, and along the perimeter of plaque edges forming a ZO1 “ring” around the large yellow co-merged plaques (Fig.3-c, right insertion, red color). This pattern of colocalization of ZO1 and Cx43 was apparent for approximately 65% of all junctional plaques in control cells. Treatment with TPA, which is reported to both dissociate Cx43 plaques and inhibit dye transfer [4,5,34,35] changed the image of immunolabeled Cx43 drastically. TPA (100-400 nM, 20 min) induced a disassembly response of plaques by decreasing the number of junctional large plaques down to 10% of all plaques (Fig.3-e and q, control, +TPA) and induced the accumulation of nonjunctional plaques inside cells by 2.5 times (Fig.3-e and r, control, +TPA). The junctional localization of ZO1 in the cells was not changed after TPA treatment (Fig.3-f, g and right insertion) but we always found that the intensity of the red ZO1 signal in the plaques after TPA treatment was less than in control without TPA (Fig.3-d and h). Analyses of merged images (Fig.3-g and right insertion and Fig.3-h) showed that the merged yellow signal represented only $22\pm 13\%$ (Mean $\pm$ SD) of

the area of merged large plaques, which clearly demonstrated that TPA treatment decreased the amount of Cx43 in the plaques and, therefore, the extent of colocalization of Cx43 with ZO1 in the plaques on the outer edge of cells. However, in remaining surface plaques the “ring” of ZO1 around a Cx43 plaque remained. Previously, we have reported that disassembly of Cx43 plaques occurs after TPA-induced PKC- γ activation, and phosphorylation of Cx43 on Ser368 [7,36]. The absence of changes in ZO1 localization in the control culture cells suggested that TPA-induced PKC- γ activation has no effect on the normal location of ZO1 in the cell and on the location around the plaques but does cause a loss of Cx43 from the cell surface. Transfection with ZO1 siRNA changed the localization of immunolabeled Cx43 in N/N 1003A cells. Statistical analyses of counted Cx43 plaques showed that, in contrast to control N/N1003A cells, after ZO1 downregulation by siRNA, the number of junctional Cx43 plaques was decreased two times down to 20% (Fig.3-q, -TPA, ZO1 siRNA) and the amount of nonjunctional Cx43 plaques inside cells was increased more than two times up to 25% (Fig.3-r, -TPA, ZO1 siRNA). Nonjunctional plaques of immunolabeled Cx43 were located inside cells mostly in the perinuclear area (Fig.3-g, white arrow). These data demonstrated that ZO1 downregulation resulted in redistribution of Cx43. The greatest difference after siRNA treatment was a total loss of response of remaining junctional plaques to TPA. Cx43, which remained in the junctional plaques, no longer responded to TPA (Fig.3-m and q, right bars). Statistical analyses showed that, in contrast to the disassembly response of Cx43 plaques in control N/N cells, ZO1 downregulation abolished the disassembly response of Cx43 plaques to TPA-induced phosphorylation by PKC- γ (Fig.3-i and j, +TPA, ZO1 siRNA).

Further analyses of merged images of large plaques after ZO1 siRNA treatment showed that after ZO1 downregulation the Cx43 staining appeared more punctate than in control cells (Fig. 3-i and k, right insertion). Merged yellow color, representing colocalized ZO1 and Cx43, occupied only 35% of plaque areas. The rest of the space was occupied by immunolabeled Cx43 without red ZO1 signal on the border of plaques (Fig.3-k, insertion, right and Fig.3-l). Treatment with ZO1 siRNA induced drastic changes in the shape of plaques: plaques lost their continuous elongated shape (Fig.3-c, insertion) and became punctate (Fig.3-k, insertion). Treatment with TPA (100-400 nM, 20 min) had no effect on the image of plaques after ZO1 downregulation (Fig.3-m and n, insertion). These data demonstrate that ZO1 downregulation removed the ZO1 “ring” around the remaining cell surface Cx43 plaques, decreased the extent of colocalization with Cx43, and prevented the disassembly response of Cx43 plaques normally seen after PKC- γ activation. We next wished to determine if PKC- γ levels or activity were altered by ZO1 downregulation.

3.4 ZO1 Downregulation Caused Abnormal Interactions of PKC- γ With Cx43

In control immunoprecipitation experiments PKC- γ was able to interact and co-immunoprecipitate with Cx43 only after TPA activation (Fig.4-A, lanes 1 and 2 and D, left bar set, scans of blots). That type of enzyme activation and substrate binding is typical for conventional PKC- γ [33,36]. ZO1 downregulation by siRNA induced abnormal co-immunoprecipitation of PKC- γ with Cx43 regardless of TPA-induced PKC- γ activation (Fig. 4-A, lane 5 and 6, and Fig.4-D, right bar set). The results with Negative Control siRNA were identical to results with control cells (Fig.4-A, lanes 3, 4 and -D, middle bar set).

To further study the effect of ZO1 siRNA on the induction of abnormal interactions between PKC- γ and Cx43, without TPA activation, we probed both whole cell homogenates (Fig 4F and E, scans of blots) and immunoprecipitates (Fig 4 B and E, scans of blots) with anti-phosphorylated-S368-Cx43 antibody since this is the PKC- γ target. Whole cell homogenates of control cells always showed very little total amount of phosphorylated S368-Cx43 (Fig.4-F, lane 1), that was always increased by several times after treatment with TPA at concentrations of 300 nM (Fig.4-F, lane 2). The amount of phosphorylation was also greater

after TPA in both Negative Control and ZO1 siRNA treated cells (Fig.4 F and H). Although it appears that the amount of S368 reactivity is greater after siRNA, we could not find a statistically significant difference between the ZO1 siRNA and the negative control siRNA. Pull down experiments revealed a similar picture. In our control experiments, coimmunoprecipitation with anti-Cx43 antibodies followed by blotting with anti-phosphorylated-S368-Cx43 antibodies, showed very little amount of phosphorylated-S386-Cx43 (Fig.4-B, lane 1) in controls, and increased amount of phosphorylated-S386-Cx43 after TPA treatment (Fig.4-B, lane 2, Fig.5-E) as previously published [2,35]. However, of note, although PKC- γ co immunoprecipitated with Cx43 (Fig.4-A lane 5, -D, right two bars) after ZO1 downregulation, PKC- γ did not phosphorylate Cx43 (Fig.4-B, lane 5, Fig.4-E, right bar set). After TPA treatment, the increased amount of phosphorylated-S386-Cx43 was also found compared to control and negative control siRNA (Fig.4-B, lanes 2, 4, and 6, Fig.4-E). These results demonstrate that ZO1 downregulation induces an abnormal interaction of PKC- γ with Cx43 without consequent phosphorylation on Ser368 of Cx43. However, although normal S368 phosphorylation is observed after ZO1 siRNA treatment, plaque disassembly does not occur (see Figure 3).

3.5 ZO1 Downregulation Resulted in a Loss of Functional Dye Transfer - Gap Junction Activity

ZO1 downregulation prevented the PKC- γ -driven Cx43 disassembly and induced an abnormal interaction of PKC- γ with Cx43. After ZO1 downregulation, the “ring” of ZO1 around large Cx43 plaques was also absent. We wished to determine if ZO1 downregulation would also interfere with the function of gap junction dye transfer. Our experiments demonstrate that ZO1 downregulation inhibited inter-cell dye transfer by 5 times from 20% in intact cells down to 4% in cells transfected with ZO1 siRNA cells (Fig.5-A, B and C). This was similar to the decrease of dye transfer after PKC- γ activation by TPA in control cells (Fig.5). Treatment of transfected cells with TPA had no further inhibiting effect on dye transfer (Fig.5A, C and E).

4. Discussion

The purpose of this study was to identify a specific function for ZO1 in signal transduction between phosphorylation of Cx43 by PKC- γ , disassembly of Cx43 gap junction plaques and function of dye transfer in lens epithelial cells. For this purpose, we used transient transfection with ZO1 siRNA, which suppressed the expression of ZO1 protein by 60% and allowed us to analyze functional and structural aspects of gap junction activity related to ZO1. We believe that our data sheds new light on ZO1 functions and reveals a newly discovered regulatory role for ZO1 in the integrity of Cx43 gap junctions.

One functional role of ZO1 is quite well established. ZO1 mediates a broad range of interactions between tight junctions and the cytoskeleton [9]. Despite having numerous binding domains, it is still not clear whether ZO1 has functions that are more specific than participation in tight junction-cytoskeletal interactions. Recent data demonstrate that knockout of ZO1 in mouse epithelial cells was not essential for tight junction assembly and only caused a delay in formation of tight junctions [37].

It has also been demonstrated that ZO1 colocalizes with Cx43 [12-14,18-20,38-41]. Abolishing ZO1-Cx43 interactions inhibited gap junction communication in cardiac myocytes [20] and in osteoblastic cells [13], but not in Cx43-deficient MDCK cells [18]. Overexpression of ZO1 in osteoblastic cells even enhanced gap junction dye transfer [13]. However, the disruption of PDZ-mediated interactions between ZO1 and Cx43 using mutations of PDZ-2 domain or using peptides, based on PDZ-binding domain of Cx43 or N-terminal sequences of ZO1 resulted in an increased size of Cx43 plaques [11,12,19], disappearance of ZO1 from the plaque periphery [12], redistribution of Cx43 from membranes to cytoplasmic pools [20], redistribution of Cx43

between raft fractions [13] and redistribution of Cx43 from nonjunctional pools to plaques, where phosphorylated forms of Cx43 were found [12]. The disruption of ZO1-Cx43 interactions had little effect on colocalization and led to the accumulation of immunolabeled Cx43 inside cells [12-14,20,42].

Despite numerous data demonstrating the importance of Cx43-ZO1 interactions for gap junction assembly there have been no reports linking these interactions to functional control by phosphorylation. In order to analyze size and distribution of Cx43 plaques between outer edge and inner space of cells we employed the TPA-induced PKC- γ phosphorylation on Ser 368 of Cx43 as a powerful regulatory signal to modulate the amount, size and assembly-disassembly state of Cx43 plaques [2-4,35,43,44]. It has been shown that phosphorylation of Cx43 by PKC- γ induces the inhibition of intercellular communication and results in gap junction plaque disassembly [2,7,21]. Here we present for the first time data that ZO1 could modulate the interaction of PKC- γ with Cx43 and thus modulate Cx43 gap junction disassembly responses. First, we downregulated ZO1 by 40% in N/N1003A lens epithelial cells and this resulted in disruption of the interaction and structural organization of Cx43 and ZO1 merged plaques, the characteristic “plaquetosome” structure. This resulted in loss of dye transfer activity. We have demonstrated that ZO1 downregulation did not alter the total amount of Cx43 protein in the cells during our experimental time frame and did not affect the PKC- γ pathway: the amount of PKC- γ in the cells, the ability to respond to TPA activation and enzyme activity after TPA activation were not affected by ZO1 downregulation. This suggests that in our experiments the consequences of ZO1 downregulation are not caused by the downregulation of Cx43 or PKC- γ . We found that ZO1 colocalizes with Cx43 in large cell surface plaques in control cells. ZO1 always surrounds Cx43 plaques. This typical “ZO1 ring” morphology of the plaques represents a functional plaque, a “plaquetosome” in control cells. ZO1 downregulation inhibits dye transfer and this coincides with significant changes in the morphology and number of large plaques. ZO1 downregulation decreased the number of large (>one μm^2) Cx43 plaques on the interface between cells. However, the most striking finding is the morphological change in the rest of the large Cx43 plaques after ZO1 downregulation. ZO1 downregulation induced the disappearance of the ZO1 “ring”, the plaquetosome, from the plaque periphery and reduced the colocalization with Cx43. This was not the result of decreased size of Cx43 plaques because we analyzed the morphology of large plaques with the size of more than one μm^2 before and after ZO1 downregulation. The reduction of colocalization with Cx43 was also not a result of decreased amount of total Cx43 in the plaque and in the cells because there were no changes in the amount of Cx43 after ZO1 downregulation. The disassembly effect, reduced extent of colocalization and disappearance of ZO1 from the plaque periphery as well as inhibited dye transfer all resulted from ZO1 downregulation. That suggests that after ZO1 downregulation the rest of the large Cx43 plaques comprise the same amount of Cx43 as normal plaques but, in contrast to normal plaques, they are not able to transfer dye without ZO1 located in the specific “ring” arrangement around the plaque. We suggest that functional Cx43 gap junctions are built upon the “plaquetosome”: the composite membrane structure based not only on the interactions between Cx43 and ZO1 protein but also on a specific location of ZO1 around each plaque. The typical image of such a plaquetosome shown in Fig. 3-C, right insertion. We have also shown that after ZO1 downregulation, the rest of the large junctional Cx43 plaques, as well as inside-located large Cx43 aggregates, were unable to respond to TPA-induced phosphorylation by PKC. We suggest that ZO1 downregulation abolishes the response of Cx43 to TPA-induced phosphorylation. Considering the fact that the PKC- γ pathway is intact after ZO1 downregulation, the absence of response to TPA stimulation suggests that the plaquetosome structure is also required for an effective disassembly response.

One explanation of how ZO1 may modulate the effect of PKC- γ phosphorylation was found in the experiments with immunoprecipitation. We found, that ZO1 downregulation induced abnormal interaction of PKC- γ with Cx43 without activation by TPA and that abnormal amount

of bound PKC- γ had very low phosphorylation effects on Cx43 S368. That suggests that abnormal interaction of PKC- γ and Cx43 was not induced by some possible changes in the PKC- γ pathway but was because of conformational changes in the Cx43 induced by ZO1 depletion. These data suggest that interaction of PKC- γ with Cx43 followed by phosphorylation on Ser368 is not enough to induce this functionally significant event - disassembly of gap junction plaques.

5. Conclusion

These data demonstrate for the first time that: 1) ZO1 is required for PKC- γ to convey the phosphorylation signal to Cx43 for disassembly. 2) Cx43 needs to interact with the “right” protein (ZO1) and to be in the right conformation, the plaquetosome “ring” before phosphorylation can proceed to disassembly. 3) This plaquetosome arrangement is also needed for functional dye transfer.

Acknowledgments

We thank Dr. Guido Zampighi (UCLA) for helpful comments about our data. This work was supported by a grant from the National Institutes of Health, EY 13421 (to DJT).

References

1. Saez JC, Berthoud VM, Branes MC, Martinez AD, Beyer EC. *Physiol Rev* 2003;83:1359–1400. [PubMed: 14506308]
2. Solan JL, Lampe PD. *Biochim Biophys Acta* 2005;1711:154–163. [PubMed: 15955300]
3. Moreno AP, Saez JC, Fishman GI, Spray DC. *Circ Res* 1994;74:1050–1057. [PubMed: 7514508]
4. Lampe PD, TenBroek EM, Burt JM, Kurata WE, Johnson RG, Lau AF. *J Cell Biol* 2000;149:1503–1512. [PubMed: 10871288]
5. Bao X, Reuss L, Altenberg GA. *J Biol Chem* 2004;279:20058–20066. [PubMed: 14973142]
6. Ek-Vitorin JF, King TJ, Heyman NS, Lampe PD, Burt JM. *Circ Res* 2006;98:1498–1505. [PubMed: 16709897]
7. Wagner LM, Saleh SM, Boyle DJ, Takemoto DJ. *Mol Vis* 2002;8:59–66. [PubMed: 11951087]
8. Giepmans BN. *Cardiovasc Res* 2004;62:233–245. [PubMed: 15094344]
9. Sierralta J, Mendoza C. *Brain Res Brain Res Rev* 2004;47:105–115. [PubMed: 15572166]
10. Giepmans BN. *Adv Cardiol* 2006;42:41–56. [PubMed: 16646583]
11. Hunter AW, Jourdan J, Gourdie RG. *Cell Commun Adhes* 2003;10:211–214. [PubMed: 14681018]
12. Hunter AW, Barker RJ, Zhu C, Gourdie RG. *Mol Biol Cell* 2005;16:5686–5698. [PubMed: 16195341]
13. Laing JG, Chou BC, Steinberg TH. *J Cell Sci* 2005;118:2167–2176. [PubMed: 15855237]
14. Giepmans BN, Moolenaar WH. *Curr Biol* 1998;8:931–934. [PubMed: 9707407]
15. Falk MM, Lauf U. *Microsc Res Tech* 2001;52:251–262. [PubMed: 11180618]
16. Jordan K, Solan JL, Dominguez M, Sia M, Hand A, Lampe PD, Laird DW. *Mol Biol Cell* 1999;10:2033–2050. [PubMed: 10359613]
17. Bukauskas FF, Jordan K, Bukauskiene A, Bennett MV, Lampe PD, Laird DW, Verselis VK. *Proc Natl Acad Sci U S A* 2000;97:2556–2561. [PubMed: 10706639]
18. Jin C, Martyn KD, Kurata WE, Warn-Cramer BJ, Lau AF. *Cell Commun Adhes* 2004;11:67–87. [PubMed: 16247852]
19. Zhu C, Barker RJ, Hunter AW, Zhang Y, Jourdan J, Gourdie RG. *Microsc Microanal* 2005;11:244–248. [PubMed: 16060977]
20. Toyofuku T, Yabuki M, Otsu K, Kuzuya T, Hori M, Tada M. *J Biol Chem* 1998;273:12725–12731. [PubMed: 9582296]
21. Lin D, Boyle DL, Takemoto DJ. *Invest Ophthalmol Vis Sci* 2003;44:1160–1168. [PubMed: 12601045]

22. Nguyen TA, Boyle DL, Wagner LM, Shinohara T, Takemoto DJ. *Exp Eye Res* 2003;76:565–572. [PubMed: 12697420]
23. Lin D, Takemoto DJ. *J Biol Chem* 2005;280:13682–13693. [PubMed: 15642736]
24. Hoehenwarter W, Klose J, Jungblut PR. *Amino Acids* 2006;30(4):369–389. [PubMed: 16583312]
25. Balda MS, Anderson JM. *Am J Physiol* 1993;264:C918–C924. [PubMed: 7682777]
26. Gonzalez-Mariscal L, Islas S, Contreras RG, Garcia-Villegas MR, Betanzos A, Vega J, Diaz-Quinonez A, Martin-Orozco N, Ortiz-Navarrete V, Cereijido M, Valdes J. *Exp Cell Res* 1999;248:97–109. [PubMed: 10094817]
27. Willott E, Balda MS, Heintzelman M, Jameson B, Anderson JM. *Am J Physiol* 1992;262:C1119–C1124. [PubMed: 1590354]
28. Sheth B, Fesenko I, Collins JE, Moran B, Wild AE, Anderson JM, Fleming TP. *Development* 1997;124:2027–2037. [PubMed: 9169849]
29. Musil LS, Goodenough DA. *J Cell Biol* 1991;115:1357–1374. [PubMed: 1659577]
30. Musil LS, Cunningham BA, Edelman GM, Goodenough DA. *J Cell Biol* 1990;111:2077–2088. [PubMed: 2172261]
31. Kuroki T, Inoguchi T, Umeda F, Ueda F, Nawata H. *Diabetes* 1998;47:931–936. [PubMed: 9604871]
32. Dempsey EC, Newton AC, Mochly-Rosen D, Fields AP, Reyland ME, Insel PA, Messing RO. *Am J Physiol Lung Cell Mol Physiol* 2000;279:L429–L438. [PubMed: 10956616]
33. Newton AC. *Biochem J* 2003;370:361–371. [PubMed: 12495431]
34. Li H, Liu TF, Lazrak A, Peracchia C, Goldberg GS, Lampe PD, Johnson RG. *J Cell Biol* 1996;134:1019–1030. [PubMed: 8769424]
35. Solan JL, Fry MD, TenBroek EM, Lampe PD. *J Cell Sci* 2003;116:2203–2211. [PubMed: 12697837]
36. Lin D, Zhou J, Zelenka PS, Takemoto DJ. *Invest Ophthalmol Vis Sci* 2003;44:5259–5268. [PubMed: 14638725]
37. Umeda K, Matsui T, Nakayama M, Furuse K, Sasaki H, Furuse M, Tsukita S. *J Biol Chem* 2004;279:44785–44794. [PubMed: 15292177]
38. Singh D, Solan JL, Taffet SM, Javier R, Lampe PD. *J Biol Chem* 2005;280:30416–30421. [PubMed: 15980428]
39. Barker RJ, Price RL, Gourdie RG. *Circ Res* 2002;90:317–324. [PubMed: 11861421]
40. Nielsen PA, Baruch A, Shestopalov VI, Giepmans BN, Dunia I, Benedetti EL, Kumar NM. *Mol Biol Cell* 2003;14:2470–2481. [PubMed: 12808044]
41. Duffy HS, Ashton AW, O'Donnell P, Coombs W, Taffet SM, Delmar M, Spray DC. *Circ Res* 2004;94:215–222. [PubMed: 14699011]
42. Nielsen PA, Baruch A, Giepmans BN, Kumar NM. *Cell Commun Adhes* 2001;8:213–217. [PubMed: 12064591]
43. Laird DW, Castillo M, Kasprzak L. *J Cell Biol* 1995;131:1193–1203. [PubMed: 8522583]
44. Lampe PD, Lau AF. *Arch Biochem Biophys* 2000;384:205–215. [PubMed: 11368307]
45. Richards TS, Dunn CA, Carter WG, Usui ML, Olerud JE, Lampe PD. *J Cell Biol* 2004;167:555–562. [PubMed: 15534005]

Abbreviations List

Cx	Connexin
TPA	Phorbol-12-myristate-13-acetate
PDZ	Postsynaptic density protein/Drosophila tumor suppressor DLGA/ tight junction protein ZO1
PKC-γ	

Protein Kinase C- γ

ZO1

Zonula Occludens Protein-1

Figure 1.
(A) -Western blot analyses of whole cell homogenates (WCH): Lane#1 - control N/N1003A cells (CNT). Lanes # 2-5 - cells after 24-96 hours of Negative Control siRNA transfection (Neg.CNT.siRNA). Lanes #6-9 - after 24-96 hours of ZO1 siRNA transfection (ZO1 siRNA). (B) - ZO1 protein levels in WCH in percentages of control. (CNT, shaded bar), after transfection with ZO1 siRNA (ZO1 siRNA, gray bars), after transfection with negative control siRNA (Neg.CNT.siRNA, empty bars). Both 220 kDa bands were digitized by UN-SCAN-It gel software. Average pixel value for ZO1 bands from control cells was used as a control (CNT) 100% level of ZO1 content. The average pixel value for ZO1 bands in the every other sample was normalized to controls and expressed in percents of control, Mean±SD. Asterisk (*) means that there is a statistical difference (p<0.05) between control and ZO1 siRNA and (**) between negative control siRNA and ZO1 siRNA. (C), (D) and (E) - Western Blot analyses of WCH in the absence (-) and after (+) TPA treatment (300 nM, 20 min, 37°C). Lanes #1-2 - control cells (CNT). Lanes #3-4 - cells after 48h of Negative control siRNA treatment (Neg.CNT.siRNA). Lanes #5-6 - cells after 48h of ZO1 siRNA treatment (ZO1 siRNA). Multiple bands (45 and 47 kDa) for Cx43 are phosphorylated forms of Cx43 detected by anti-N-terminal-Cx43 antisera (Fred Hutchinson Cancer Research Center Seattle, WA) as previously reported [4,30,35,45].

Figure 2.

The effect of TPA on PKC- γ activity in whole cell homogenates (WCH) without (-) and after (+) TPA treatment (300 nM, 20 min and 37°C): **(A)** - An example of photo of the agarose gel demonstrating PKC- γ activity. **(CNT.)** - control. **(Neg.CNT.siRNA)** - negative siRNA control. **(ZO1 siRNA)** - ZO1 siRNA samples. **(B)** - PKC- γ activity plotted as % of control (Mean $\pm$ SD). The activity of the immunoprecipitated PKC- γ in the WCH before TPA treatment was considered as a 100%. **(CNT)** - Control cells, **(Neg.CNT.siRNA)** - after 48h of transfection with negative control siRNA, **(ZO1 siRNA)** - after 48h of transfection with ZO1 siRNA. PKC- γ was immunoprecipitated from WCH and was tested using a non-radioactive PKC-assay as described before [23]. Five separate experiments were carried out to gather statistical data for PKC- γ activity. Asterisk (*) means a statistical difference ($p < 0.05$) between control and TPA-treated samples. **(C)** - Western blot analyses of WCH: Lanes #1-2 - control cells (**CNT**). Lanes # 3-4 - cells after Negative control siRNA treatment (**Neg.CNT.siRNA**). Lanes # 5-6 - cells after ZO1 siRNA treatment (**ZO1 siRNA**). **(D)** - α -tubulin is included as a loading control.

Figure 3.

Confocal images of localized Cx43 and ZO1. Cells were double immunolabeled with both primary monoclonal mouse anti-C-terminal Cx43 IgG and rabbit polyclonal anti ZO1 (middle domain) IgG. Secondary anti-mouse Alexa Fluor-488 (green color) antibodies were used for Cx43 and anti-rabbit Alexa Fluor-568 (red color) antibodies were used for ZO1 (see Material and Methods for details). (a) - (h) - control cells before (a)-(d) and after (e) - (h) TPA treatment (300 nM, 20 min, 37 °C). (a) and (e)- immunolabeled Cx43, (b) and (f)- immunolabeled ZO1, (c) and (g)- merged images. Insertions at right of (c) and (g) demonstrate plaques under high magnification. White square box at lower right corners on the insertions represents area equal to $1 \mu^2$. d,h,l, and p are confocal intensity scans. (i-o) - images of cells after ZO1 siRNA

transfection (48h after transfection) without (**i-k**) and after (**m-o**) TPA treatment (300 nM, 20 min, 37 °C). Insertion to right of (**k and o**) demonstrates plaques under high magnification. White arrows indicate junctional plaques between cells or as described in text. Large white bar in the right bottom corner of every picture represents scale bars of 20 microns. (**q**) - (**r**) - The total number (Mean±SD) of large plaques ($>1\mu\text{m}^2$) on outer edge of cells (**q**) and inside cells (**r**) per cell without (-) and after (+) TPA treatment (300 nM, 20 min, 37 °C). (**Control**) - control cells; (**ZO1 siRNA**) - cells transfected with ZO1 siRNA (48 hours after transfection). Asterisk (*) means a statistical difference ($p<0.05$) between indicated data and control (-TPA).

PKC- γ in Immunoprecipitates

Phospho-S368-Cx-43 in Immunoprecipitates

Phospho-S368Cx-43 in WCH

Figure 4.

(A), (B) and (C), - Western blot analyses of proteins coimmunoprecipitated with anti-N-terminal-Cx43 antibodies in the absence (-) or after (+) TPA treatment (300 nM, 20 min, 37 °C). (F) and (G) - Western blot analyses of whole cell homogenates (WCH) in the absence (-) or after (+) TPA treatment (300 nM, 20 min, 37 °C). Lanes #1-2 - intact N/N1003A cells (CNT). Lanes #3-4 - cells after Negative control siRNA treatment (Neg.CNT.siRNA). Lanes #5-6 - cells after 48h of ZO1 siRNA transfection (ZO1 siRNA). Protein levels in immunoprecipitates (D, E) and in the WCH (H) as % of control - (CNT). (Neg.CNT.siRNA) - cells after transfection with negative control siRNA; (ZO1 siRNA) - cells

after transfection with ZO1 siRNA. Data plotted as percentage of control (Mean $\pm$ SD). Asterisk (*) means a statistical difference ($p<0.05$) between indicated data and controls.

Figure 5.

Dye transfer - Gap Junction Activity. (A) - Percent of cells with lucifer yellow without (-) and after (+) TPA treatment (300 nM, 20 min, 37 °C). (B – E) – Fluorescent microscopy images of cells along cut/scrape with lucifer yellow minus and plus TPA treatment (300 nM, 20 min, 37 °C). (Control) - Intact N/N1003A cells, (Negative siRNA) - cells treated with negative control siRNA, (ZO1 siRNA) - cells after 48h of transfection with ZO1 siRNA. White arrows show places of the cut. Data plotted as percentage of control (Mean±SD). Asterisk (*) means a statistical difference ($p < 0.05$) between indicated data and control.