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A Cross-Link Between PKA and Rho-family GTPases Signaling Mediates Cell-Cell Adhesion and Actin Cytoskeleton Organization in Epithelial Cancer Cells

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Running Title Page

Running title: PKA mediates AJC and actin cytoskeleton assembly.

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A list of non-standard abbreviations: PKA, protein kinase A; AJC, apical junctional complex; AJ, adherens junction; TJ, tight junction; ROCK, Rho-associated kinase; TEM, transmission electron microscopy; TER, transepithelial electrical resistance; EMT, epithelial-to-mesenchymal transition.

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Abstract

Disassembly of the apical junctional complex (AJC) together with actin cytoskeleton alterations are among the initial events for the development of epithelial cancer. The cell signaling pathways for these processes have been analyzed separately. However the existence of a link between these two events has not been defined. In this study, using the extracellular calcium depletion model, we analyzed the signaling pathways regulating AJC disassembly together with actin cytoskeleton organization in colon adenocarcinoma cells (Caco-2). Changes in the location of AJC proteins were examined by immunofluorescence and immunoblotting and tight junction (TJ) functionality was observed by measuring the transepithelial electrical resistance and permeation to ruthenium red. The actin cytoskeleton was stained with rhodamine-phalloidin and analyzed by confocal microscopy. Rho-GTPases activation was assessed by its translocation to the membrane (a hallmark of RhoA activation) and immunoblotting. Pharmacological inhibition of PKA with H-89 prevented AJC disassembly and actin disorganization at the apical and medial regions caused by calcium depletion. Rho inhibition using Toxin A induced AJC disassembly and actin cytoskeleton reorganization. Y-27632, a ROCK inhibitor reversed redistribution of E-cadherin, but not of TJ proteins and actin disorganization caused by calcium depletion. Calcium depletion and Forskolin treatment caused activation of Rho, as evidenced by their translocation to the membrane, an event concurrent to Rac and RhoGDI translocation, and this effect was also reverted by H-89. Thus, our findings demonstrate a central role of a regulatory cascade that integrates PKA and Rho-family GTPases in the AJC disassembly and actin organization in tumor epithelial cells.

Introduction

The apical junctional complex (AJC) is the structure responsible to maintain epithelial cell-cell adhesions and it has important functions such as, polarity, mechanical integrity and cell signaling (Troyanovsky et al., 1999). Tight junctions (TJs) and the subjacent adherens junctions (AJs) form the AJC. Both structures are deregulated in epithelial-to-mesenchymal transition (EMT). EMT is a process that occurs in normal development and at the beginning of epithelial cancer in which cells acquire a mobile phenotype, characterized by the loss of cell-cell adhesion structures and actin cytoskeleton rearrangement (Thiery and Sleeman, 2006). E-cadherin, which is considered the most important protein of AJs, binds to *Armadillo* family proteins that include β-catenin and placoglobin (γ-catenin), which in turn modulate the interaction to α-catenin in a dynamic fashion (Drees et al., 2005). Integral membrane proteins in TJs include claudin family proteins, occludin and the junctional adhesion molecule (JAM) (Schneeberger and Lynch, 2004). These proteins interact with adaptor proteins of the *zonula occludens* family ZO-1, ZO-2 and ZO-3, to link TJs with the actin cytoskeleton (Shin et al., 2006).

There is some evidence suggesting that actin cytoskeleton participates in AJC biogenesis and function. Ivanov and co-workers showed that actin polymerization directly mediates recruitment and maintenance of AJ/TJ proteins at intercellular contacts, whereas myosin II regulates cell polarization and correct positioning of the AJC within the plasma membrane (Ivanov et al., 2004, 2005). Also, the Rho family of small GTPases, made up of Rho, Rac, and Cdc42, has been shown to play an important role in the regulation of epithelial structure, function and assembly (Braga et al., 1997) and in the regulation of F-actin dynamics (Hall, 1998). A potential downstream effector candidate of RhoA to modulate cell-cell adhesion and actin cytoskeleton organization is the Rho-associated kinase (ROCK), which is involved in many

cellular processes and participates in actin cytoskeletal rearrangement, including stress fiber formation and tumor cell invasion (Riento and Ridley, 2003). Some studies have reported a role of ROCK in the regulation of AJC structure and function because the actin cytoskeleton is central in regulating epithelial TJs with the participation of the RhoA protein (Walsh et al., 2001; Sahai and Marshall, 2002). Recently, using intestinal epithelial cell lines and the classical extracellular Ca^{2+} depletion model, the Rho exchange factor, GEF-H1, was reported to act upstream of Rho/ROCK-II signaling resulting in actomyosin contraction and disassembly of AJC (Samarin et al., 2007).

Signaling of PKA has long been known to regulate both the assembly and opening of the paracellular route of epithelial and endothelial cells. Various studies have led to the conclusion that in brain endothelial cells, PKA activation promotes the barrier function of TJs, however in other endothelial cells and in epithelial cells a generalization cannot be made, as PKA can produce contrary actions in different cells models (Gonzáles-Mariscal et al., 2008). In addition, PKA seems to inhibit RhoA functions in many different cell types. For instance, it was reported that cAMP inhibits RhoA-induced cytoskeletal changes, smooth muscle contraction and endothelial and tumor cell migration (O'Connor et al., 2000; Dong et al., 1998). Chen et al., (2005), showed that cAMP /PKA inhibited RhoA activation and that serine^{188} phosphorylation on RhoA was necessary for PKA to exert its inhibitory effect on RhoA activation in the human gastric epithelial cell line and prostate cancer. Therefore, although the role of PKA on RhoA activity has not yet been defined, the existence of a link between PKA and RhoGTPases can be hypothesized to mediate the loss of cell-cell adhesion, a crucial event in epithelial cancer development.

The aim of this study was to investigate a signaling network guiding a cross-link between the actin cytoskeleton and AJC organization in epithelial cancer cells. We used Caco-2 cells, a

human colon adenocarcinoma cell line and the well-known Ca^{2+} depletion model and reported that PKA modulates the AJC disassembly and the actin cytoskeleton organization through a network involving RhoA/ROCK and Rac signaling.

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Methods

Antibodies and reagents

Mouse monoclonal anti-E-cadherin (36 clone) was purchased from BD Biosciences (San Diego, CA) and anti-claudin-1 (JAY.8), occludin, ZO-1 (Z-R1) and RhoGDI-α (NGA-25) rabbit polyclonal antibodies from Zymed Laboratories, Inc. (South San Francisco, CA). Mouse monoclonal anti-Rac (23A8) and rabbit monoclonal anti-Rho (A-B-C) and TRITC-conjugated phalloidin were purchased from Sigma Chemical Co. (St Louis, MO). Tetramethyl rhodamine iso-thiocyanate (TRITC)-conjugated goat anti-rabbit and peroxidase-conjugated goat anti-rabbit were obtained from Zymed Lab. H-89 (N-[2-(p-Bromocinnamylamino) ethyl]–5–isoquinolinesulfonamide), PD98059 (2´-Amino-3´-methoxyflavone), PD153035 (4-[(3-Bromophenyl)amino]-6,7-dimethoxyquinazoline and LY294002 (2-(4-Morpholinyl)-8-phenyl-4H-1-benzopyran-4-one) inhibitors were purchased from BIOMOL Res. Labs. Inc. (Plymouth Meeting, PA). Toxin A from *Clostridium difficile* and Y-27632 ((R)-(+)-trans-N-(4-Pyridyl)-4-(1-aminoethyl)-ciclohexanecarboxamide) were purchased from EMD Biosciences, Inc. (San Diego, CA), and forskolin from Sigma.

Cell culture

Caco-2 cells, a human colon adenocarcinoma cell line (ATCC, # HTB-37) were passaged weekly with 0.05% trypsin/0.02% EDTA in PBS solution. The cells were grown in Dulbecco Modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS), penicillin G (60 mg/l) and streptomycin (100 mg/l) at 37° C in humidified atmosphere of 5% CO_2/air. For experimental purposes, cells were plated at high density (0.5 x 10^6 cells/mL) and the culture medium was changed every other day to avoid nutrient depletion.

Depletion of extracellular Ca^{2+} and pharmacological treatments

Low calcium (LC) culture medium was prepared in DMEM without FBS using a stock solution of 200 mM EGTA, pH 8.0, to give a final concentration of 4 mM EGTA (Citi, 1992). Cell monolayers were washed three times and incubated in LC medium for 2.5 h at 37° C. Control monolayers were washed and incubated with normal calcium (NC) culture medium without FBS.

For pharmacological assays, the inhibitors were diluted in DMSO to give a final concentration of H-89 (20 μM), PD98059 (50 μM), PD153035 (100 nM), LY294002 (10 μm), Y-27632 (10 μM) and Toxin A from *Clostridium difficile* (10 μM). Cells were also treated with 10 μM forskolin, a PKA activator. Cell monolayers were pre-incubated in NC medium containing inhibitors and forskolin for 1 h, followed by incubation in LC medium for 2.5 h containing the pharmacological agents as described above.

Immunofluorescence and confocal microscopy analysis

Caco-2 cell monolayers were grown on sterile glass coverslips and after the treatments they were washed in PBS, fixed in 4% paraformaldehyde and incubated in NH_4Cl for 10 min at room temperature. After, the cells were permeabilized with 0.5% Triton X-100 for 5 min and blocked with 3% BSA in PBS for 2h. Subsequently, they were incubated overnight at 4º C with primary antibodies against E-cadherin (1:100), occludin (1:20), claudin-1 (1:25) and ZO-1 (1:25) followed by 1h with the respective secondary antibodies at 37º C. The coverslips were washed then mounted using n-propyl-gallate and the cell staining was detected using an Axiovert S 100 immunofluorescence microscope equipped with a CCD camera and KS 300 image analyzer program (Carl Zeiss Inc., Germany).

For visualization of F-actin distribution, cell monolayers were fixed and permeabilized as described above and incubated with 500 ng/mL TRITC- phalloidin for 30 min at room temperature. After washing, stained monolayers were analyzed using a Zeiss LSM510 Meta Laser Scanning Confocal Microscopy, with 543 nm excitation laser. Individual images through the cell volume of similar confluent regions were collected and optical sections near the apical, medium (5 μm) and basal (9 μm) planes from monolayers (X-Y plane) and perpendicular plane (X-Z plane) were obtained. Images shown are representative of at least three independent experiments.

Cell extraction in Triton X-100 and immunoblotting

Samples were rinsed three times in PBS and incubated for 20 min at 4° C in extraction buffer CSK (50 mM NaCl, 10 mM piperazine-1, 4-bis (2-ethanesulfonic acid) (Pipes), pH 6.8, 3 mM $MgCl_2$, 0.5% TritonX-100, 300 mM sucrose) containing 1 mM orthovanadate, 20 mM NaF, and protease inhibitor cocktail (1:100, Sigma Chemical Co.). Cells were scratched from plates, homogenized and centrifuged at 10,000 g for 10 min at 4° C. The supernatant corresponding to the TX-100 soluble fraction (cytosolic proteins) was removed and stored at –20° C. The pellet was resuspended in SDS buffer (20 mM Tris-HCl, pH 7.5, 5 mM EDTA, 2.5 mM EGTA, 1% SDS and boiled at 100º C for 10 min. After centrifugation for 10 min at 10,000 g the supernatant, corresponding to the TX-100 insoluble fraction (cytoskeleton-linked proteins), was gently removed and stored at –20 °C. Equal amounts of protein (30 μg per lane), of cell fractions were electrophoretically separated by SDS-PAGE in 7.5% or 12% gels and transferred to nitrocellulose sheets. Then, the membranes were blocked and incubated overnight with primary antibodies: anti-occludin, anti-E-cadherin, anti-claudin-1 and anti-ZO-1. After washing, membranes were incubated for 1 h with peroxidase-conjugated goat anti-rabbit or peroxidase-conjugated goat anti-

mouse secondary antibodies. Proteins were visualized using an enhanced chemiluminescence kit (Amersham Biosciences, Buckingham, UK). Band images were quantified by optical density using the LabWorks 4.6 software (Bio Rad Lab. Hercules, CA).

Western blot analysis of RhoGTPases activation

After the different treatments, plasma membrane and cytosolic fractions were obtained by a classic cell fractionation. Cells were scraped into 10 mM Tris-HCl buffer, pH 7.5, containing 250 mM sucrose, 1 mM $MgCl_2$, 1 mM orthovanadate, 20 mM NaF and protease inhibitor cocktail (1:100) and then homogenized in a Potter type homogenizer. The cell lysates were centrifuged at 3,000 g for 10 min at 4°. The supernatant was collected and centrifuged at 30,000 g for 1 h at 4° C. The resulting supernatant was collected as the cytosolic fraction and the pellet as the plasma membrane fraction. The membrane fraction was resuspended in 10 mM Hepes buffer, pH 7.3, containing 2 mM EDTA, 0.2% SDS, 0.5% sodium deoxycholate, 0.1% Triton X-100, 150 mM NaCl, 2 mM orthovanadate, 20 mM NaF and a cocktail of protease inhibitors. Equal amounts of membrane and cytosolic fractions (50 μg protein per lane), were separated by SDS-PAGE in 12% gels and transferred to nitrocellulose sheets. They were blocked and then incubated overnight with anti-Rho(A-B-C), anti-Rac and anti- RhoGDI-α antibodies. Membranes were washed and incubated for 1h with horseradish peroxidase-conjugated anti-rabbit or anti-mouse secondary antibodies for enhanced chemiluminescence detection using an ECL kit (Amersham Biosciences, Buckingham, UK). Total lysates were obtained homogenizing cells in a Potter type homogenizer with lysis buffer (1% Triton X-100, 0.5% deoxycholate, 0.2% SDS, 150 mM NaCl, 10 mM Hepes, pH 7.3, 2 mM EDTA, containing 20 mM sodium fluoride, 1 mM sodium orthovanadate and a cocktail of protease inhibitors. Western blotting for Rho, Rac and RhoGDI were measured

as an internal control. Band images were quantified by densitometry using the LabWorks 4.6 software (Bio Rad Lab. Hercules, CA).

Transmission Electron Microscopy

The TJ functionality was also assessed using the electron-dense dye complex, ruthenium red and electron microscopy analysis. Cells were cultured on Transwell polycarbonate filters and fixed for 60 min on the apical side of the monolayer with a solution containing 2.5% glutaraldehyde, 1% freshly prepared paraformaldehyde, 8% sucrose, 2 mM $CaCl_2$, and 6 mg/mL of ruthenium red in 0.1 M cacodylate buffer, pH 7.4. After washing with cacodylate buffer containing ruthenium red for 10 min, they were then post-fixed with 1% OsO4 and 6 mg/mL ruthenium red in cacodylate buffer for 45 min. Then, monolayers were washed with cacodylate buffer, dehydrated with acetone series and embedded in Epon resin. Ultrathin sections (70 nm) were stained with lead citrate and observed in a Zeiss CEM-900 transmission electron microscope (Carl Zeiss Inc., Germany).

Transepithelial Electrical Resistance (TER) measurements

Caco-2 cells were seeded at confluence onto Transwell polycarbonate filters, 0.4 μm pore size and 0.33 cm^2 surface area (Costar, Cambridge, MA). After the treatments the cultures were washed and the TER values were determined using a Millicel-ERS system (Millipore Co, Billerica, MA, USA). All TER values were normalized for the area of the filter and obtained after filter and bath solution subtraction of blank Transwell that had been cultured in parallel.

Statistical Analysis

Data obtained from three independent TER measurements were expressed as percentages in relation to the control group (100%). Statistical analysis was performed using one-way ANOVA with a post-hoc Bonferroni test. Densitometric analyses are presented as the means ±

S.D. Comparison between non-treated, (which was normalized to 1) and treated samples in three independent experiments was carried out using Student's *t*-test for membrane and cytosolic fractions, and ANOVA for TX-100 insoluble and soluble fractions with a post-hoc Dunnett test.

Results

PKA modulates AJC protein disassembly and TJ functionality

In order to determine signaling pathways involved in the AJC disassembly caused by calcium depletion, Caco-2 cells were grown in NC and LC medium and pretreated with specific kinase inhibitors prior to incubation in LC medium and the distribution of the junctional proteins was initially analyzed by immunofluorescence. In NC medium, E-cadherin, occludin, claudin-1 and ZO-1 were observed to be preferentially at the cell-cell contacts. However, in LC medium the cells had a rounded shape and a loss of contacts between neighboring cells with the internalization of AJC proteins was observed, but the attachment to the substratum was maintained. The AJC disassembly and protein internalization caused by LC medium were prevented by pretreatment with the PKA inhibitor, H-89 but not by EGFR, MAPK and PI3K inhibitors (Fig. 1A). Forskolin, an agent known to raise AMPc levels and activate PKA, caused a significant internalization of AJC proteins (Fig. 1B). It is well known that junctional proteins are functional when they are cytoskeleton –linked. Thus, we further analyzed by immunoblotting the subcellular distribution of E-cadherin and claudin-1, a TJ protein that plays a important role in the paracellular permeability, using TX-100 soluble and insoluble fractions after incubation in NC and LC medium, as well as prior treatment with H-89. The distribution pattern and densitometry analysis of E-cadherin and claudin-1 showed a significant translocation from the insoluble to the soluble fraction in cells that were incubated in LC medium, and this effect was prevented by H-89 (Fig. 1C). It is important to point out that, although the IC_{50} of this inhibitor for PKA is 48 or 135 nM (Davies et al., 2000), we used the concentration of 20 μM on the basis of previous studies showing that it is also able to inhibit PKA activity (Klingler et al., 2000; Blanco-Aparicio et al., 1999).

We verified the TJ functionality by monitoring the TER in cell monolayers and in individual cell junctions using the ruthenium red technique and electron microscopy analysis. As seen in Figure 2A, permeability to ions of confluent Caco-2 cells assessed by TER measurements, showed a value of about 300 $\Omega.cm^2$ (100 %) in cells cultured in NC medium. However in LC medium there was an accentuated drop of the TER after 1 h and after 150 min values near zero were observed. This effect was also significantly prevented by H-89, which restored about 80 % of TER values, whereas EGFR, MAPK and PI3K inhibitors did not prevent the TER drop, showing that these kinases are not involved in the cell-cell contact disruption caused by LC medium. This result was confirmed by the ruthenium red technique. The electron-dense dye added to the apical region did not permeate the TJs of cells in NC medium (Fig. 2B), but it permeated cells incubated in LC medium (Fig. 2C). Of all the kinase inhibitors used, only H-89 abrogated the ruthenium red permeation caused by LC medium (Fig. 2D). Other kinase inhibitors such as PD98059, LY294002 and PD15035, did not revert the effect of LC medium on TJs functionality as analyzed also by this technique (data not shown). Together these results indicate that PKA activation is required to regulate AJC protein disassembly caused by LC medium and it is also required to regulate TJ barrier function.

PKA modulates actin cytoskeleton organization in a differential fashion through the cell volume

To explore the relationship between actin reorganization and AJC disassembly, we stained the actin cytoskeleton and carried out a confocal microscopy analysis. Figure 3 shows the actin filament distribution in the apical, medial and basal regions (Fig. 3A) and in the X-Z plane (Fig. 3B) of cell monolayers grown in NC medium with or without forskolin and LC medium in the presence or absence of H-89. In the NC medium, cells presented a punctual labeling in the apical

region, characterizing the presence of actin at microvillus and in cell-cell contacts. At the medial level, actin was predominant at intercellular junctions and on the basal side, stress fibers were observed. Forskolin treatment of cells in NC medium disorganized the actin cytoskeleton mainly in the apical and medial regions, but did not interfere with the stress fibers. In LC medium, cells acquired a rounded morphology showing F-actin labeling as a thick layer or cortical microfilaments at the cell periphery on the apical and medial sides, as well as stress fiber disruption on the basal side. H-89 prevented this effect, but the stress fibers were not reestablished, on the contrary it was possible to observe lamellipodia formation in the basal region. This data indicates that PKA activity modulates AJC assembly in parallel to actin cytoskeleton organization at apical and medial levels, but not at stress fibers.

Pharmacological inhibition of ROCK does not prevent TJ disruption but abrogates E-cadherin internalization.

On the basis of previous studies showing that PKA is a modulator of RhoA function, altering its signal transduction function (Cardone et al., 2005) and since it may be mediated by the RhoA target protein, Rho-kinase (ROCK) (Sahai and Marshall, 2002), we decided to investigate whether Rho/ROCK signaling could be involved in AJC disassembly in Caco-2 cells, by using Toxin A, an inhibitor of the Rho-GTPases family and Y-27632, a highly potent selective inhibitor of ROCK-I, but that also inhibits ROCK-II with almost equal potency. Initially, the distribution of AJC proteins was analyzed by immunofluorescence. Figure 4 shows that in NC medium, Toxin A caused an expressive cell-rounding and diffuse labeling of E-cadherin, occludin and claudin-1 proteins, as compared to the normal distribution at cell-cell contacts of cells. Interestingly, Toxin A caused an increase of cell size and intense membrane invaginations as seen by ZO-1 labeling. The ROCK inhibitor did not prevent the internalization of occludin, ZO-1 and claudin-1, but abrogated E-cadherin internalization caused by LC medium. We confirm

this result by immunoblotting using TX-insoluble and soluble fractions and observed that Y-27632 prevented the E-cadherin translocation from the insoluble to soluble fraction, but not of claudin-1 (Fig. 4B). ROCK inhibitor had no effect on TX-100 solubility of occludin and ZO-1 protein (data not shown). Next, we analyzed the effect of the ROCK inhibitor on TJ functionality using TER measurements and the ruthenium red technique (Fig. 5). The ROCK inhibitor did not prevent the TER drop caused by the LC medium and in NC medium it caused a non-significant decrease of the TER (Fig. 5A). By electron microscopy, we observed that the presence of the inhibitor in NC medium did not cause TJ permeation of the ruthenium red marker (Fig. 5B) showing a similar pattern to control cells, as showed in Figure 1B, but interestingly a morphological alteration with elongated microvilli in some apical areas near the TJs was observed. Moreover, the ROCK inhibitor did not abrogate ruthenium red permeation caused by LC medium (Fig. 5C). These data strongly suggest that ROCKs proteins in calcium-depleted cells independently modulate AJs and TJs.

ROCK does not regulate actin cytoskeleton organization in calcium-depleted cells

Next, we investigated the effect of Y-27632 and Toxin A on F-actin organization by confocal microscopy. Optical sections of cells in NC medium pretreated with Toxin A showed an enlargement of the cell causing actin distribution alteration at the cell-cell contacts in the apical and medial regions and stress fiber reorientation, as compared to control cells. ROCK inhibitor did not prevent the LC medium effect on the actin cytoskeleton organization at the cell volume but caused an intense stress fiber disruption and lamellipodia formation on the basal side (Fig. 6). Furthermore, Toxin A was seen to cause stress fiber disorganization. Collectively, these data argue against the involvement of ROCK in the disorganization of actin cytoskeleton during calcium depletion in Caco-2 cells.

Calcium depletion-induced membrane translocation of Rho, Rac and RhoGDI in Caco-2 cells

We finally decided to evaluate the effects of calcium depletion, forskolin, a cAMP-elevating agent and H-89 on Rho membrane translocation, which has been reported to be a reflection of RhoA activation (Hirakawa et al., 2007) as well as of Rac and RhoGDI. Figure 7A shows that calcium depletion and Forskolin treatment induced a significant membrane translocation of Rho and this effect was abrogated by H-89. The increase of Rho membrane translocation was concomitant to a decrease at the cytosolic fraction (data no shown). This result strongly supports the notion that AJC disassembly induces Rho activation through a mechanism that requires PKA. Here, we demonstrated in Figure 6 that LC medium induced disruption of stress fiber and lamellipodia formation and since these effects are related to Rac activity (Hall, 1998), we decided to verify in parallel whether PKA would also modulate Rac activity. As see in Figure 7B, Rac was predominantly localized at the membrane fraction in cells grown in LC medium whereas it is reduced by forskolin treatment. Surprisingly, H-89 prevented this effect indicating that PKA could also modulate Rac activity. A minority band with a molecular weight slightly higher than Rac1 seen in membrane fractions probably corresponds to the Rac1b isoform, which has been described in colon cell lines (Matos et al., 2003). It is known that Toxin A causes mono-glucosylation of Rho and Rac preventing its interaction with RhoGDI. Since RhoGDI is able to sequestrate Rho-GTPases from biological membranes blocking the interaction with their effectors (Genth et al. 1999), it results in the accumulation of these GTPases at the membranes. Here we used this device as a control of the Rho and Rac translocation to the membrane and as shown in Figure 7A and 7B, both GTPases were predominantly localized at the membrane after Toxin A treatment.

Although the results are conflicting it was reported that *in vivo* phosphorylation increases the RhoA interaction with RhoGDI, prevents RhoA activation, uncouples RhoA from interactions with downstream effectors and dissociates RhoA from the plasma membrane (Dong et al., 1998; Forget et al., 2002; Dransart et al., 2005). Thus, in order to verify whether Rho activation induced by PKA is RhoGDI-mediated, we analyzed the location of this protein by immunoblotting in membrane fractions. As shown in Figure 7C, in LC medium as well as in cells treated with forskolin, RhoGDI was predominantly localized at the membrane fraction, however pretreatment with H-89 prevented this effect. Taken together these results show that PKA-induced RhoA and Rac activation is mediated through association of these GTPases with RhoGDI and translocates them to the membrane.

Discussion

The participation of GTPases and PKA has long been implicated in AJC disassembly, as well as in actin cytoskeleton organization, however both cellular mechanisms have been studied separately and the existence of a link between GTPases and PKA to mediate regulation of these events in tumor cells remains to be defined. In the present work, we identified a cascade of regulatory molecules that integrate PKA, Rho/ROCK and Rac to regulate the AJC disassembly and actin cytoskeleton organization. This conclusion is supported by the observations that a) PKA inhibition prevented AJC protein redistribution, TJ functionality loss and actin disorganization in the apical and medial regions, b) Rho inhibition caused AJC disruption and actin cytoskeleton reorganization at all levels of cell volume c) ROCK inhibition abrogated redistribution of E-cadherin, but not of TJ proteins and actin cytoskeleton disorganization and d) forskolin and LC medium caused translocation of RhoA, Rac and RhoGDI proteins from the cytosol to the plasma membrane and this effect was reverted by H-89.

The participation of PKA on AJC disassembly has been related to TJ proteins. Our results showing that PKA is involved in increased paracellular permeability and redistribution of AJC proteins are in agreement with previous observations (Klingler et al., 2000). Additionally, we reported that other cell signaling pathways such as MAPK, EGFR and PI3K are not involved in this event. Because AJC proteins are cytoskeleton-associated, we verified if PKA participates in cytoskeleton organization in parallel with AJC modulation. We showed that LC medium caused actin cytoskeleton reorganization at all levels of cell volume and PKA activation by forskolin reorganized the cytoskeleton at the apical and medial levels, however at the basal level caused reorientation, but not stress fiber disruption (Fig. 3). Since forskolin was able to induced stress fiber disruption in fibroblasts (Ridley and Hall, 1994), our results indicate that PKA could be

involved in the acquisition of a mobile phenotype altering substratum anchorage, as suggested by Thiery and Sleeman (2006).

We have shown in the present study that LC medium caused cell rounding and actin ring formation, both typical characteristics of motility acquisition that occur in a Rho-dependent manner (Cáceres et al., 2005). Here, we explored this possibility using Toxin A, a Rho inhibitor that perturbs the epithelial barrier function (Nusrat et al., 2001) and verified that Toxin A caused AJC protein redistribution (Fig. 4) concomitantly with actin disorganization at apical and medial regions and stress fiber formation (Fig. 6). Next, we hypothesized that the involvement of RhoA in calcium depletion-induced AJC disassembly could be ROCK-dependent since ROCK represents the classical effector for Rho-GTPase (Matsui et al., 1996). Our experiments showed that Y-27632 did not abrogate TJ protein translocation from the areas of cell-cell contacts; did not abrogate the loss of the paracellular barrier function and did not prevent actin reorganization. However it blocked E-cadherin internalization in calcium-depleted cells (Figs. 4, 5 and 6). On the other hand, the presence of Y-27632 in NC medium did not significantly affect the TER. Recently, it was reported that inhibition of ROCK with Y-27632 completely blocked the reorganization of F-actin and translocation of AJC proteins in calcium-depleted cells (Samarin et al., 2007). These authors using selective expressional down-regulation of ROCK-I and ROCK-II reported the involvement of only the latter isoform in the AJC disassembly. It is known that the ROCK family of protein kinases consists of two highly homologous members, ROCK-I and ROCK-II, which present 65% overall identity and 92% identity in their kinase domain (Riento and Ridley, 2003). With this consideration and as both ROCKs have been previously implied in the regulation of AJC and actin cytoskeleton (Walsh et al., 2001; Sahai and Marshall, 2002), it is possible to suggest that these kinases participate in the regulation of TJs and AJs in an independent fashion. In other words, ROCK-I would be responsible to regulate AJs whereas

ROCK-II would regulate TJs and actin cytoskeleton organization. We showed lamellipodia formation at the basal level in calcium-depleted cells (Fig. 3) indicating Rac activation. Additionally, pretreatment with Y-27632 did not abrogate this effect; conversely an apparent enhancement of these structures was observed (Fig. 6). Since RhoA and Rac usually act in an opposite fashion in epithelial cells (Caron, 2003), our results suggest that in LC medium at the basal level Rac could be activated whereas Rho is inhibited. Additionally, as LC medium caused AJ disruption our findings further indicate that it could also inhibit Rac (Nakagawa et al., 2001) at the apical level. The fact that H-89 prevented actin reorganization at apical and medial levels (Fig. 3) suggests that PKA induced a differential modulation of Rac activity. The existence of a spatial distribution of PKA during chemotactic cell migration in fibroblasts and Rac activation by PKA during pseudopodia formation (Howe et al., 2005) supports this suggestion.

Considering that membrane translocation of Rho is a hallmark of RhoA activation (Kranenburg et al., 1997), we analyzed this event after calcium depletion and pretreatment with forskolin and H-89. We observed that LC medium and forskolin induced a membrane translocation of Rho and this effect was reverted by H-89, indicating that AJC disassembly could also be a consequence of RhoA activation which is mediated by PKA. There are conflicting results of the mechanisms of PKA action on RhoA in calcium-depleted cells, i.e., recent studies showed that calcium depletion induced Rho/ROCK-mediated MLC phosphorylation (Fan et al., 2007) and that RhoA activation resulted in actomyosin contraction and AJC disassembly (Samarin et al., 2007). In this latter study Rho inhibition abrogated AJC disruption, however other studies showed that RhoA or ROCK inhibition cause disorganization of this complex (Riento and Ridley, 2003; Miyake et al., 2006). Our results showing that H-89 prevented RhoA membrane translocation and that forskolin induced a similar effect to LC medium indicate that PKA is involved in RhoA activation. We suggest that PKA activation causes AJC disassembly

and subsequently this event could activate Rho at the apical cell side. Apparently, there is a contradiction in these results since LC medium increases RhoA at the membrane whereas it disrupts stress fibers, which are formed by RhoA inhibition (Hall, 1998). However, local activation of Rho-GTPases during cell migration and membrane protrusion has been suggested (Wozniak et al., 2005; Pertz et al., 2006). Thus, from these results we hypothesize that PKA-activation subsequent to calcium-depletion is able to inhibit RhoA on the basal cell side and cause stress fiber disruption and activate it on the apical side to recruit ROCK-I or ROCK-II to modulate AJ or TJ disassembly, respectively. Three mechanisms may explain Rho activation at the apical region: 1) PKA activation causes AJC disassembly 2) translocation of Rho to membrane with subsequent recruitment of effector proteins after AJC protein internalization and 3) Rac inhibition after AJ disassembly since these two GTPases present opposite activities (Fig. 8A). Previous studies showed that active Rho-GTPases are membrane-linked, while in an inactive state are predominantly located at the cytoplasm due to association of GTPase-GDI-GDP (Forget et al., 2002). Here, we observed that LC medium induced Rac membrane translocation, an event that was also abrogated by H-89. Thus, from this observation it is possible to suggest that calcium depletion is also able to cause modulation of PKA-mediated Rac activity. This suggestion is supported by the observation that although Rac does not present a region where it could be directly phosphorylated by PKA, this kinase might cause Rac activation through GAP protein (O'Connor and Mercurio, 2001). It has been proposed that RhoGDI could serve as a translocater of active GTPases (DerMardirossian et al., 2005) and a model suggests that RhoGDI can bind to membranes by three mechanisms: a) electrostatic attraction, b) recruitment of Rho-RhoGDI complex by receptors and c) destabilization of Rho-RhoGDI complex by displacement factors (GDFs) or phosphorylation (Dransart et al., 2005). Here, we observed that LC medium and forskolin induced RhoGDI membrane translocation concomitantly

with RhoA and Rac indicating that PKA activation could modulate the association of Rho-GTPases with RhoGDI to translocate them to the membrane. Additionally, since the binding sites for RhoGDI and Rho-GEF overlap, it is possible to suggest that the Rho-RhoGDI complex at the membrane should dissociate before it can be activated by a Rho-GEF. One possible candidate to mediate RhoA activation after Rho-RhoGDI translocation to the membrane could be GEF-H1, as this GEF protein is activated in calcium-depleted cells (Samarin et al., 2007). However, further studies are necessary to confirm this possibility.

In conclusion, our results demonstrate a cross-link between PKA and Rho-family GTPases signaling that mediates AJC disassembly and actin cytoskeleton organization in calcium-depleted epithelial cells. We suggest a novel mechanism involving spatial activation of a regulatory cascade consisting of PKA, Rho/ROCKs, RhoGDI and Rac (Fig. 8) and propose that a similar molecular event may regulate initial steps of EMT and cell migration in epithelial cancer and may potentially lead to novel therapeutic approaches.

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Footnotes

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Legends for Figures

Figure 1: Pharmacological inhibition of PKA prevents AJC protein redistribution. (A) Caco-2 cells were incubated in normal (NC) and in low calcium (LC) medium for 2.5 h, or pretreated with different kinase inhibitors as indicated: H-89 (PKA), PD98059 (MAPK), PD153035 (EGFR) and LY294002 (PI3K) prior to incubation with LC medium. (B) Cells were also incubated in NC medium containing forskolin, a PKA activator. Cells were fixed and stained for E-cadherin, occludin, claudin-1 and ZO-1. In (A) note that AJC proteins are redistributed in LC medium and this effect is abrogated by H-89, only. Forskolin caused a significant internalization of the AJC proteins (B). Scale bar, 12 μm. (C) Representative immunoblots and densitometric analysis of E-cadherin and claudin-1 of insoluble (i) and soluble (s) fractions in Triton X-100 of cells that were incubated in NC and LC medium and pretreated with H-89. Observe that H-89 prevented the redistribution of the proteins from the insoluble to soluble fractions caused by the LC medium. In each case the score was calculated using the following equation: Arbitrary score = (amount of the protein in the soluble fraction)/(amount of the protein in the insoluble fraction). The score for cells in NC medium was normalized as 1 in each case. Average scores S.E.M of three independent experiments is shown. Significantly different: * ($P < 0.05$).

Figure 2: TJ functionality is regulated by PKA. Cells were cultured on Transwell polycarbonate filters and the TJ functionality was analyzed by measurement of the Transepithelial electrical resistance (TER) (A) and by the ruthenium red technique (B-D). A: TER was measured in different conditions as indicated. Observe that H-89 dramatically abrogated the drop in TER induced by LC medium (*, $P<0.01$ compared with LC medium). None of the other kinase inhibitors reverted the LC medium effect on the TER. B: Representative images of thin sections of control cells showing the ruthenium red in the apical region, only. C:

cells incubated in LC medium for 2.5 h revealed extensive spaces in the junctional complex area (*) and permeation of the marker between the intercellular spaces. D: Addition of H-89 to the LC medium blocked this effect. N: Nucleus; TJ: Tight Junction; AJ: Adherens Junction; Arrows: ruthenium red marker. Bar, 4 μm.

Figure 3: PKA modulates actin cytoskeleton organization in a differential fashion through the cell volume. Cell monolayers were incubated in NC medium, NC plus forskolin (FK), LC medium, or LC plus H89, and probed with TRITC-conjugated phalloidin. (A) Representative X-Y confocal images of optical sections close to the apical, medial (5 μm) or basal (9 μm) cell sides. (B) Confocal images in the X-Z plane. Note that the LC medium disorganized the actin cytoskeleton and this effect was abrogated on the apical and medium sides by H-89, but not on the basal side. *Arrows*: stress fibers; *arrowheads*: lamellipodia. Scale bar, 20 μm.

Figure 4: Pharmacological inhibition of ROCK does not prevent TJ disassembly but abrogates E-cadherin internalization. (A) Cell monolayers were treated with Y-27632 either in LC medium or with Toxin A in NC medium, fixed and stained for E-cadherin, occludin, claudin-1 and ZO-1. Observe the redistribution of the AJC protein and an increased size of the cells caused by Rho inhibition using Toxin A (TXA). The ROCK inhibitor in LC medium seems to preserve the E-cadherin localization, but does not prevent TJ protein internalization. Bar, 12 μm. (B) Representative immunoblots and densitometric analysis of E-cadherin and claudin-1 of insoluble (i) and soluble (s) fractions in Triton X-100 of cells that were incubated in NC and LC medium and pretreated with Y-27632. Note that ROCK inhibitor prevented the redistribution of E-cadherin from the insoluble to soluble fractions caused by the LC medium, but not of claudin-1. In each case the score was calculated as in Figure 1C. Significantly different: * ($P < 0.05$) in relation to control group; # ($P < 0.05$) in relation to LC medium.

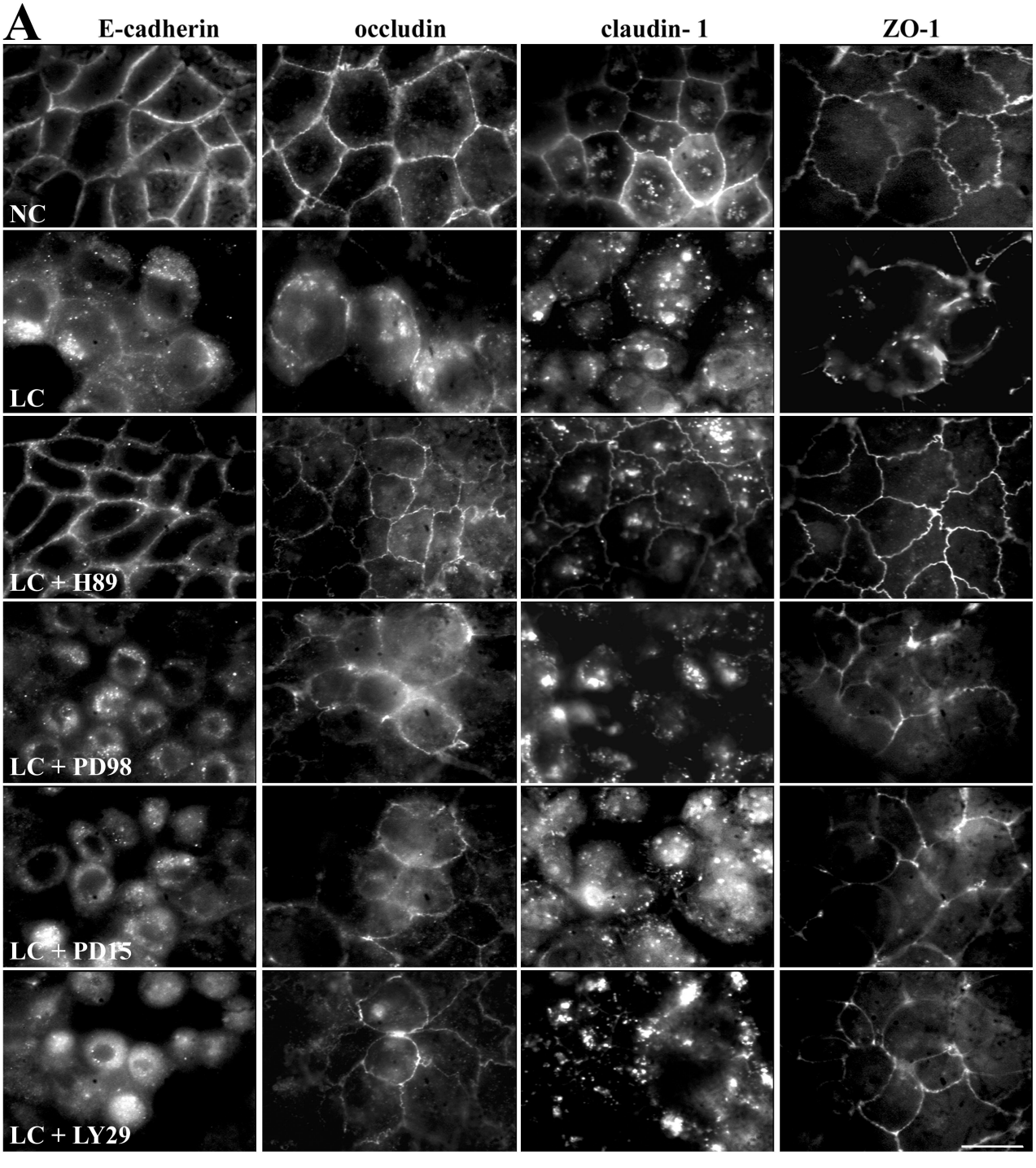
Figure 5: ROCK inhibition does not mediate AJC functionality in calcium-depleted cells. Cells were cultured on Transwell polycarbonate filters and the TJ functionality was analyzed by measurement of the Transepithelial electrical resistance (TER) (A) and by the ruthenium red technique (B-C). A: Showed a non significant (*, P> 0.05 compared with NC medium) decrease of the TER caused by ROCK inhibitor and the drop of TER in LC medium was not abrogated by Y-27632. The data are representative of three independent experiments. B-C: Cell monolayers were cultured on Transwell polycarbonate filters and after treatments were processed for electron microscopy using the ruthenium red technique. Caco-2 cells cultivated in NC medium plus Y-27632 (B), and LC medium plus Y-27632 (C). Wide spaces in the junctional complex area are indicated (*) in LC medium showing permeation of the trace. The ROCK inhibitor does not prevent the tracer permeation caused by calcium depletion. Incubation in NC medium restricted ruthenium red labeling to the apical area. N: Nucleus; TJ: Tight Junction; AJ: Adherens Junction; D: Desmosomes; *Arrows*: ruthenium red marker. Scale bars: B: 0.8 μm and C: 1.2 μm

Figure 6. ROCK does not regulate actin cytoskeleton disorganization in calcium-depleted cells. Caco-2 cells were cultivated in NC medium, NC medium plus Toxin A or LC plus Y-27632, fixed and stained for actin filaments with TRICT-conjugated phalloidin. A: Representative confocal images showing the distribution of F-actin in X-Y optical sections close to the apical, medial (5 μm) or basal cell sides (9 μm). B: Images in X-Z plane. Note that Toxin A caused alteration of the actin distribution in the apical and medial regions and stress fiber reorientation. Y-27632 did not prevent the LC medium effect on the actin distribution on the apical, medial and basal sides of the cell volume; conversely it caused an enhancement of lamellipodia formation on the basal side. *Arrows*: stress fibers; *arrowheads*: lamellipodia. Bar, 20 μm.

Figure 7. Calcium depletion-induced membrane translocation of RhoA, Rac and RhoGDI in Caco-2 cells. Caco-2 cells cultured in NC medium were pretreated or not with forskolin or Toxin A and in LC medium in the presence or absence of H-89. After the respective treatments, cells were lysed and the membrane fraction was obtained as described in Materials and Methods. The expressions of RhoA (A), Rac (B) and RhoGDI (C) proteins in the membrane fraction were examined by Western blotting and the representative band images are shown in upper panels. Densitometric analysis of the bands is shown in the lower panels. Each bar represents means ± S.D value obtained from three independent experiments. Staining with Ponceu S carried out a control loading of the membrane fractions. * $p<0.05$

Figure 8. A Model for the regulation of AJC disassembly and actin cytoskeleton organization in calcium-depleted cells. Depletion of extracellular calcium directly causes disruption of homotypic E-cadherin interactions between neighbor cells inducing loss of cell polarity, and in parallel PKA is activated by an upstream-unknown mechanism E-cadherin-independent. **A**: Activated PKA could modulate AJC disassembly and cause Rho activation through its translocation to the membrane and subsequent recruitment of effector proteins. AJ disassembly could modulate Rho-ROCK I signaling, and TJ disassembly the recruitment of another effector protein for Rho (i.e. ROCK II). At the basal cell side, PKA inhibits RhoA-ROCK signaling through RhoGDI sequestration to the cytosol in order to disrupt stress fibers. **B**: At the apical domain, E-cadherin internalization might inhibit Rac through RhoGDI sequestration to the cytosol or another unknown mechanism. At the basal domain, PKA activation or Rho inhibition could activate Rac by translocating it to the membrane and form lamellipodia.

Figure 1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occludin
claudin- 1
ZO-1
NC
LC
LC + H89
LC + PD98
LC + PD15
LC + LY29

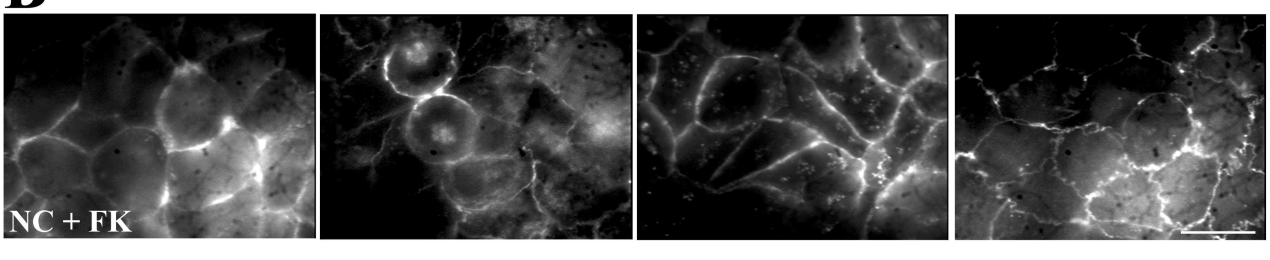
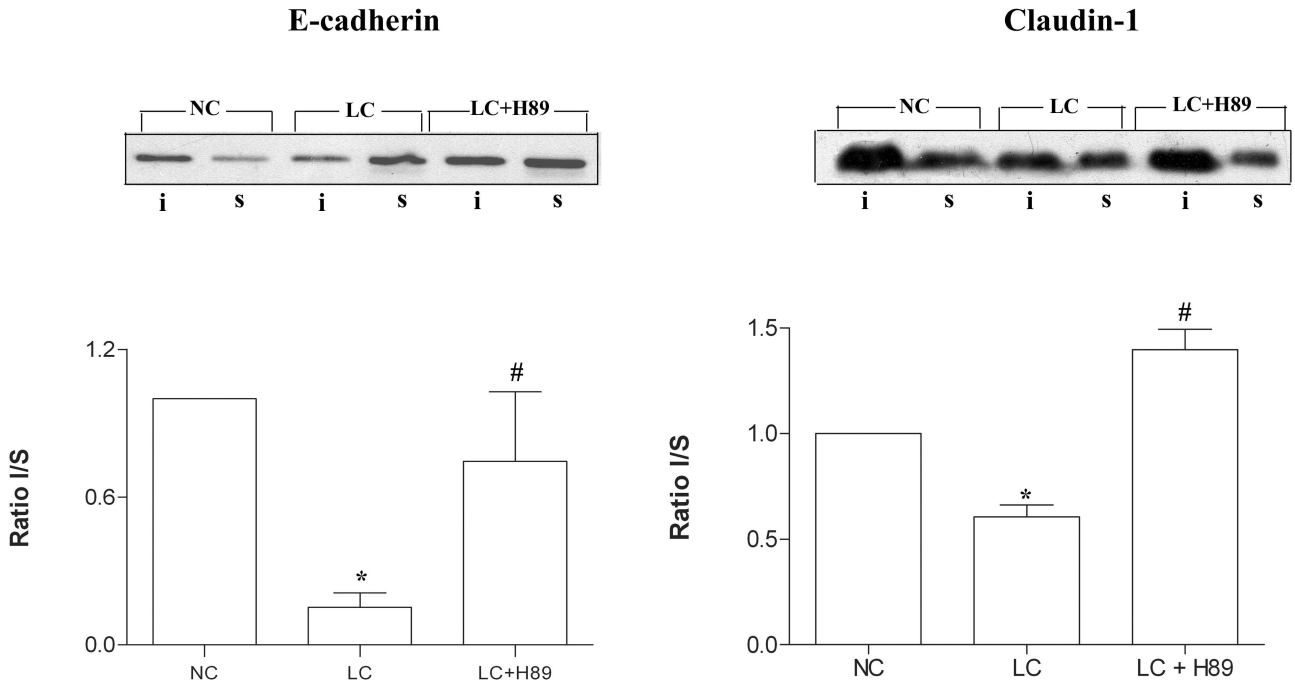

B
NC + FK

Figure 1

C


E-cadherin
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LC+H89
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1.2
0.6
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#
NC
LC
LC+H89
Claudin-1
NC
LC
LC+H89
i
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Ratio I/S
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1.0
0.5
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*
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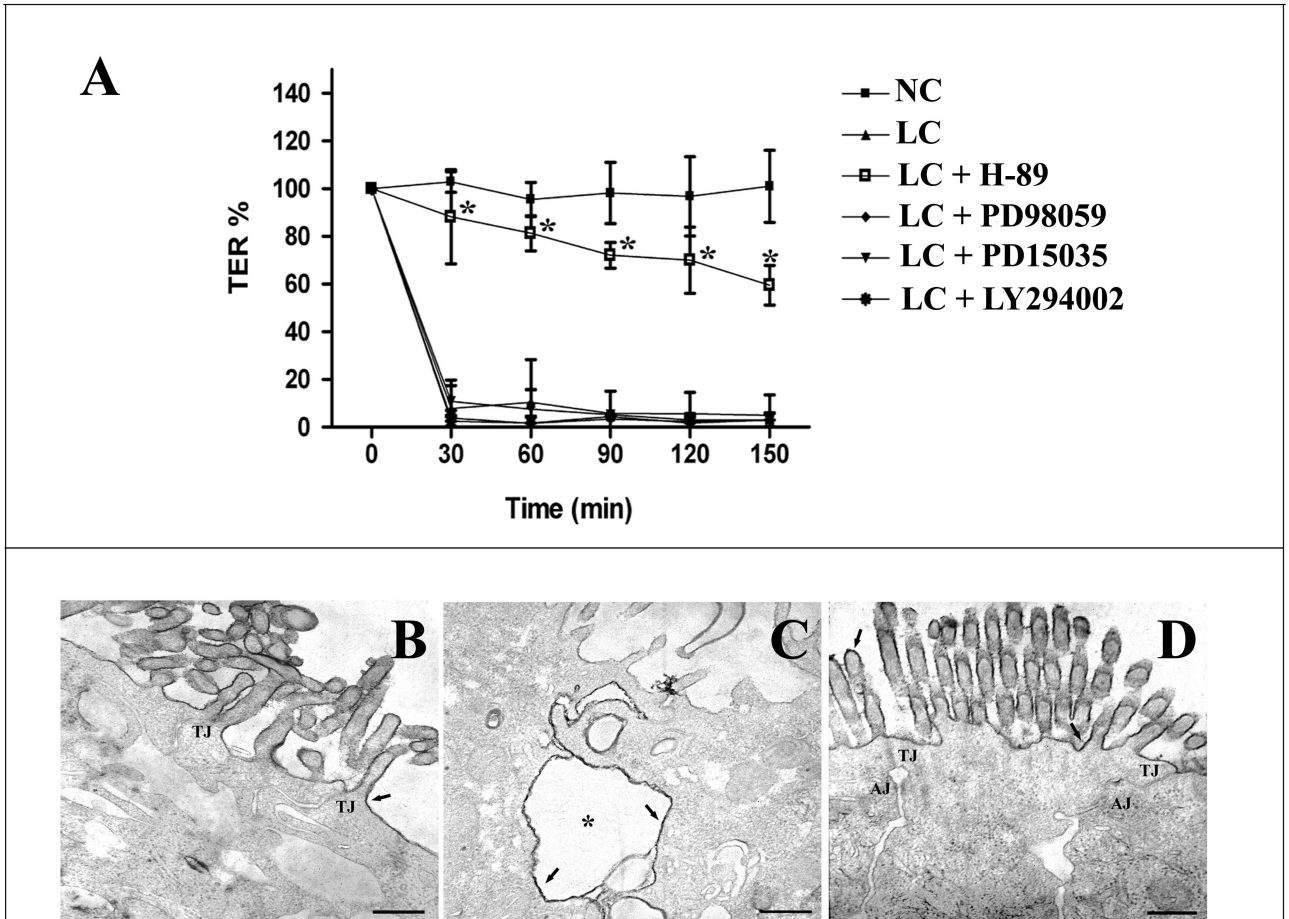
NC
LC
LC + H89

Figure 2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60
40
20
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0
30
60
90
120
150
Time (min)
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LC
LC + H-89
LC + PD98059
LC + PD15035
LC + LY294002
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TJ
C
*
D
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TJ
AJ
AJ

Figure 3

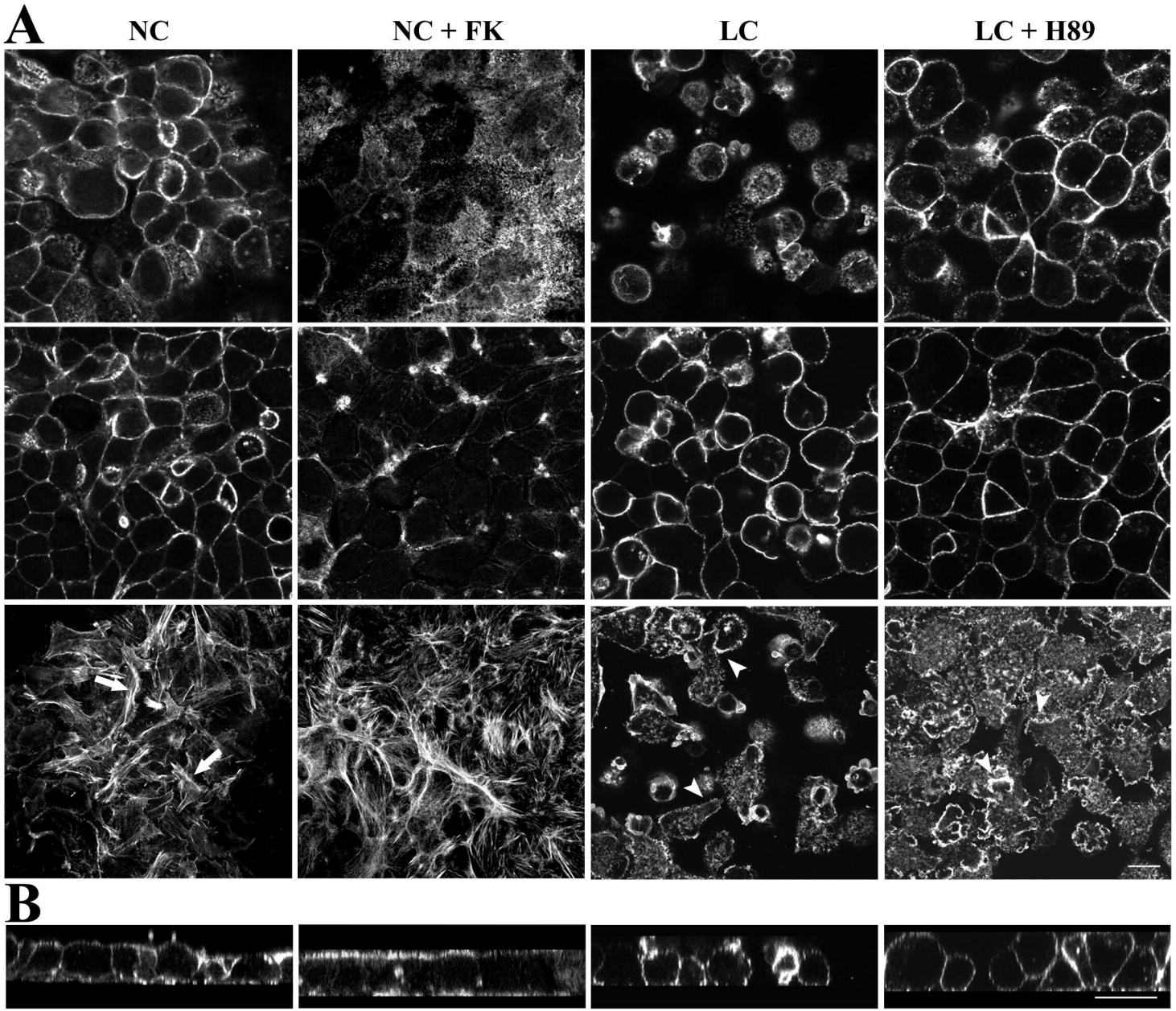
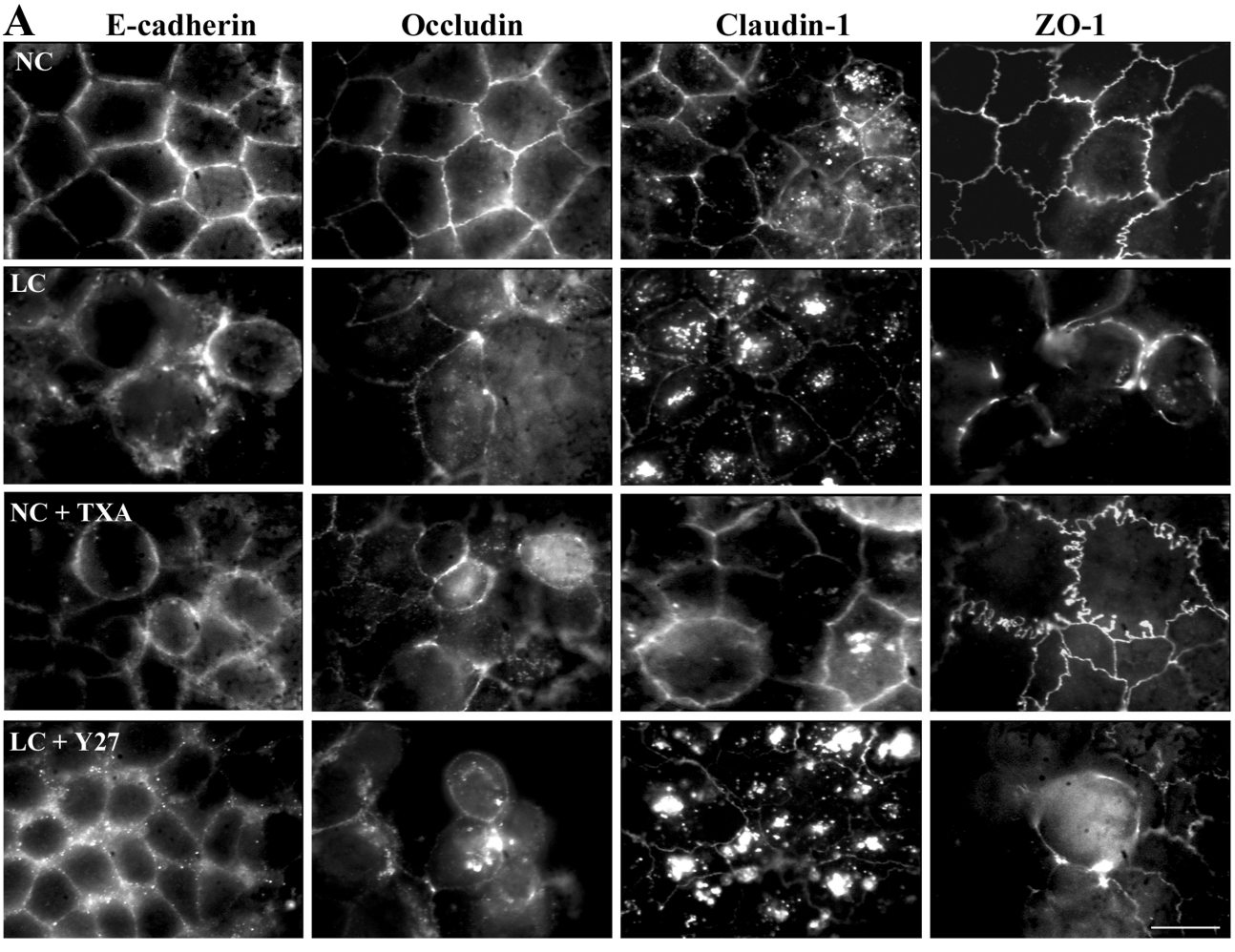


Figure 4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Occludin
Claudin-1
ZO-1
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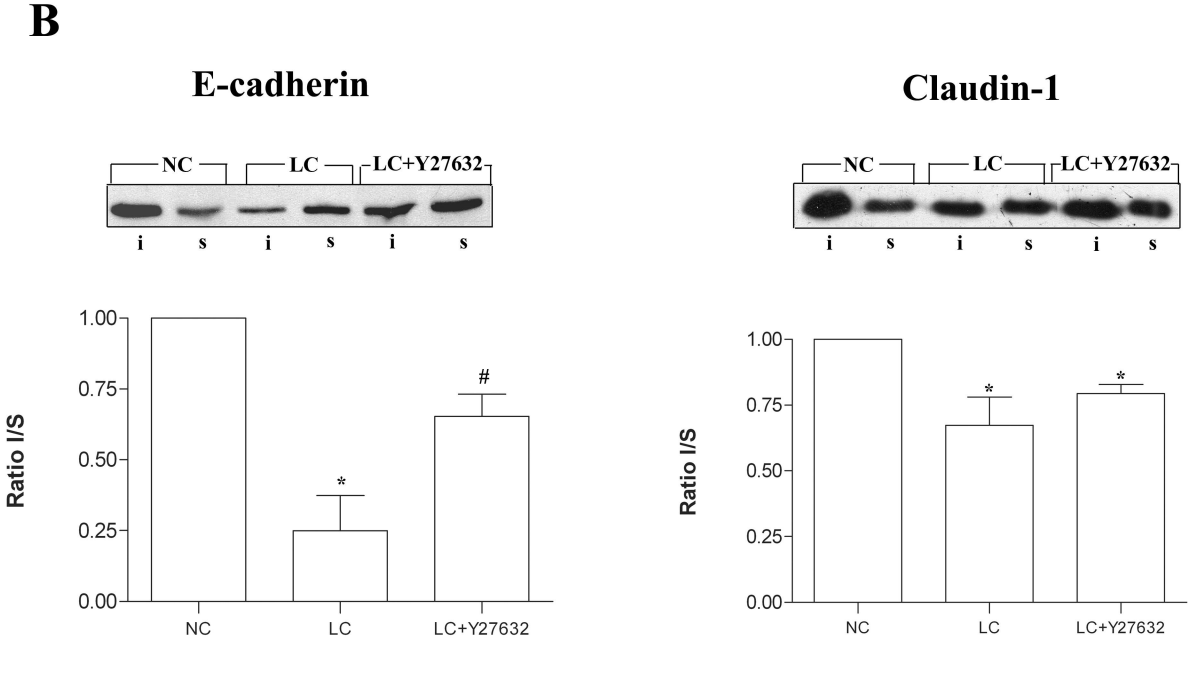
LC + Y27


B
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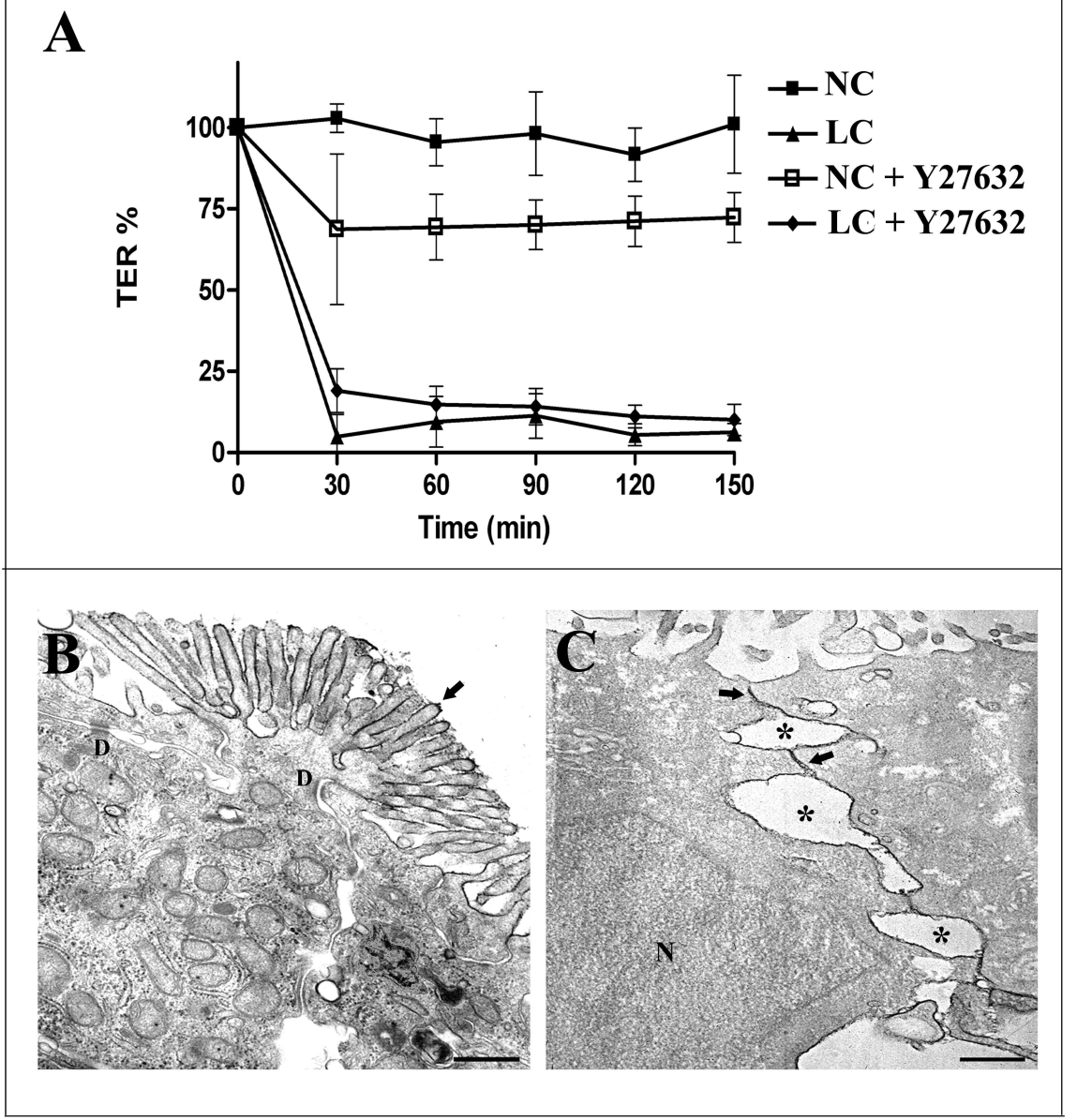
NC
LC
LC+Y27632

Figure 5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30
60
90
120
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Time (min)
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LC + Y27632
B
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*
*
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N

Figure 6

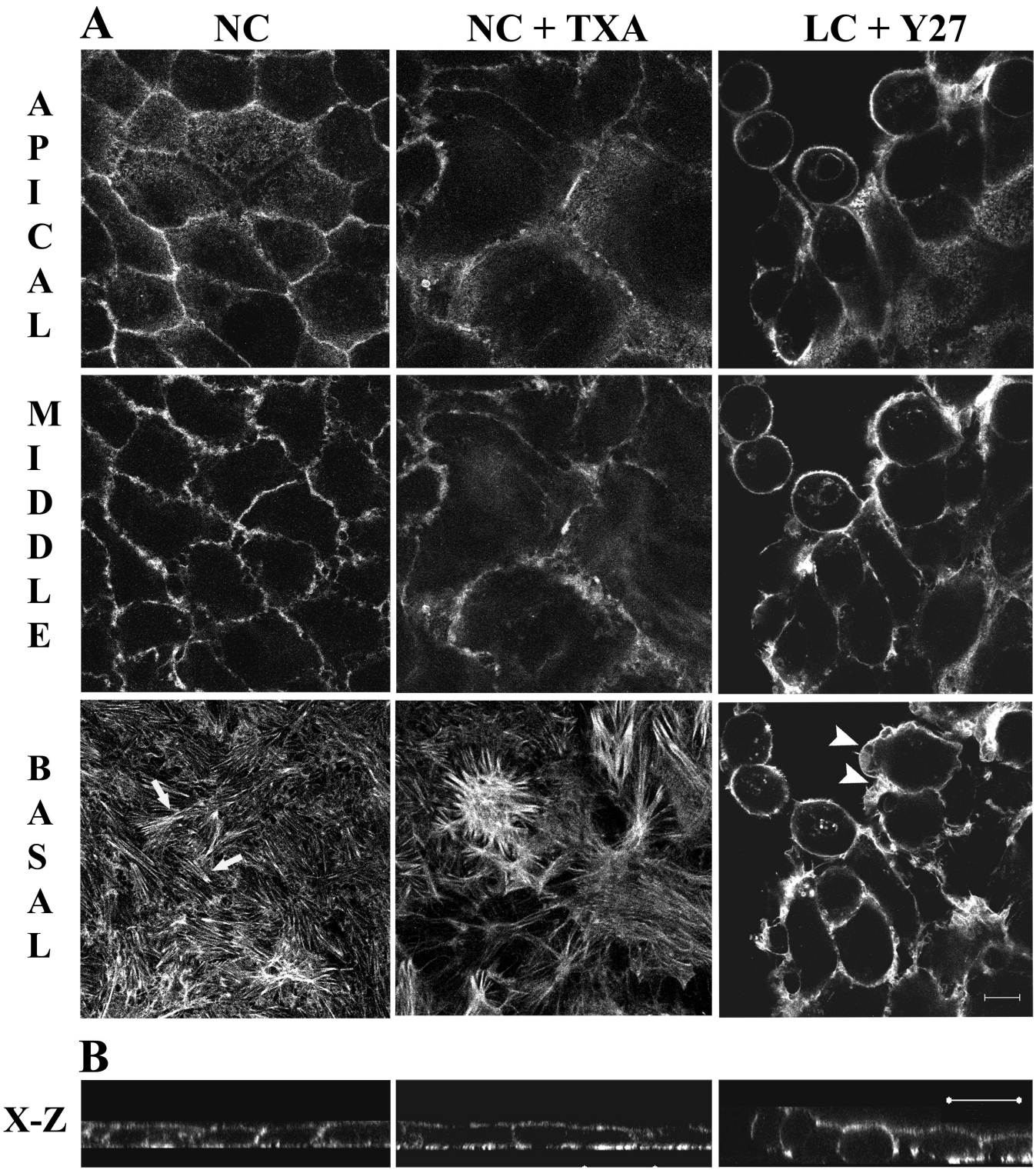
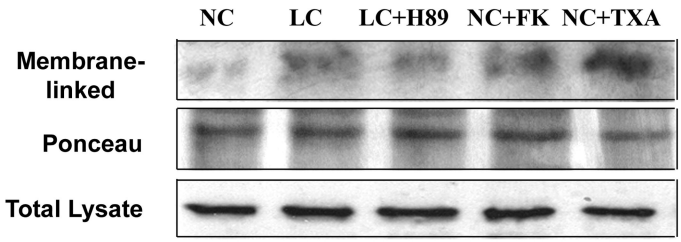
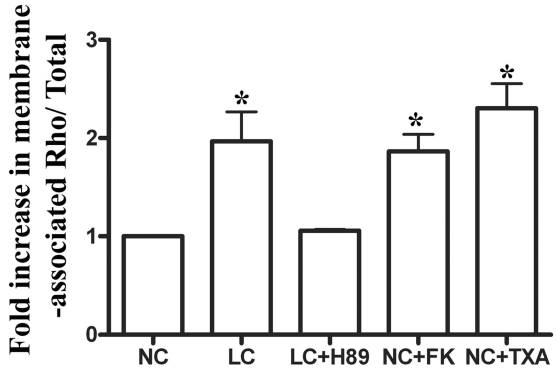


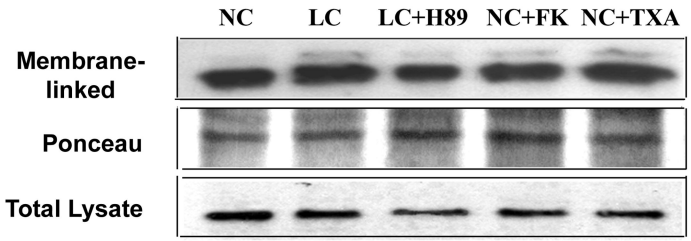
Figure 7

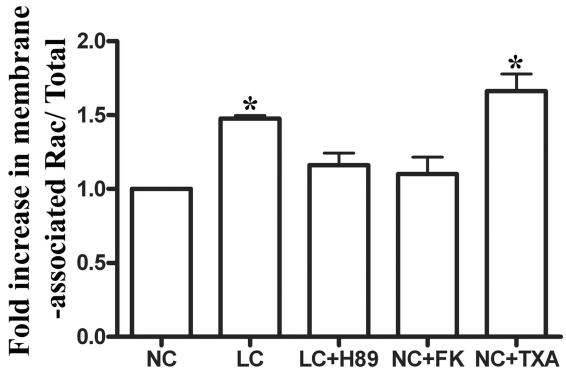
A Rho



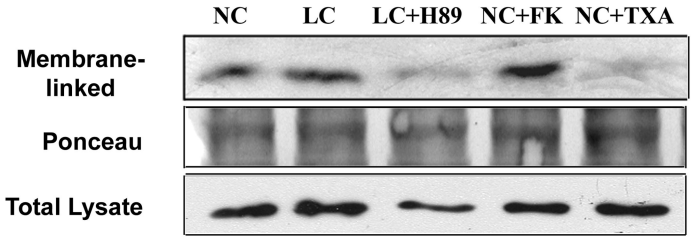


B Rac





C RhoGDI



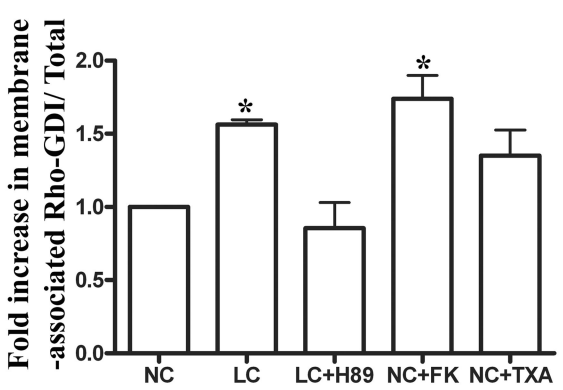
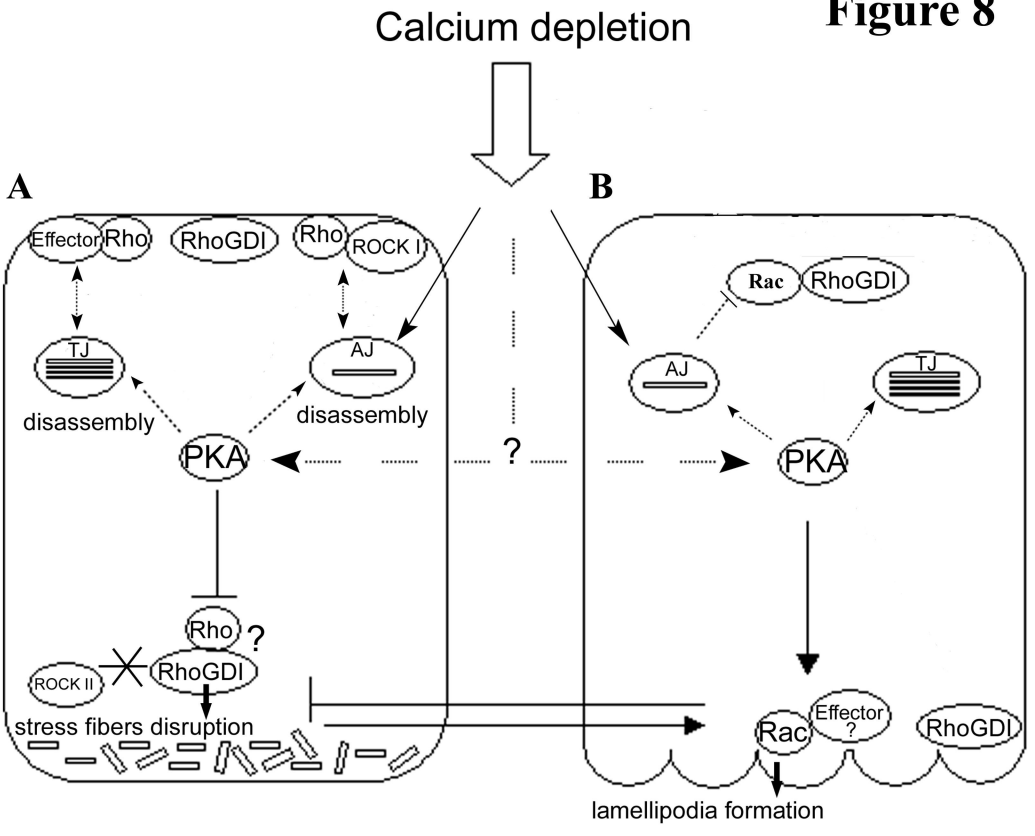


Figure 8


Calcium depletion
A
B
Effector
Rho
RhoGDI
Rho
ROCK I
TJ
AJ
disassembly
disassembly
PKA
?
Rho
?
ROCK II
RhoGDI
stress fibers disruption
Rac
RhoGDI
AJ
TJ
PKA
Rac
Effector
?
RhoGDI
lamellipodia formation