European Journal of Pharmacology 671 (2011) 7–17

Contents lists available at SciVerse ScienceDirect

European Journal of Pharmacology

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

Molecular and Cellular Pharmacology

Lysophosphatidic acid induces a migratory phenotype through a crosstalk between RhoA–Rock and Src–FAK signalling in colon cancer cells

Fernanda Leve, Taline Guimarães Corrêa Marcondes, Lilian Gonçalves R. Bastos, Sarah Vieira Rabello, Marcelo Neves Tanaka, José Andrés Morgado-Díaz *

Grupo de Biologia Estrutural, Divisão de Biologia Celular, Centro de Pesquisas, Instituto Nacional de Câncer —INCA, 37André Cavalcanti street, 5th floor, Rio de Janeiro, RJ, CEP 20231-050, Brazil

ARTICLE INFO

Article history:
Received 12 May 2011
Received in revised form 23 August 2011
Accepted 8 September 2011
Available online 21 September 2011

Keywords:
Lysophosphatidic acid
Adherent junction
Actin cytoskeleton
Cell migration
Colorectal cancer
Rho GTPase

ABSTRACT

Lysophosphatidic acid (LPA) acts as a potent stimulator of tumorigenesis. Cell–cell adhesion disassembly, actin cytoskeletal alterations, and increased migratory potential are initial steps of colorectal cancer progression. However, the role that LPA plays in these events in this cancer type is still unknown. We explored this question by using Caco-2 cells, as colon cancer model, and treatment with LPA or pretreatment with different cell signalling inhibitors. Changes in the location of adherent junction proteins were examined by immunofluorescence and immunoblotting. The actin cytoskeleton organisation and focal adhesion were analysed by confocal microscopy. Rho-GTPase activation was analysed by the pull-down assay, FAK and Src activation by immunoblotting, and cell migration by the wound healing technique. We show that LPA induced adherent junction disassembly, perijunctional actin cytoskeletal reorganisation, and increased cell migration. These events were dependent on Src, Rho and Rock because their chemical inhibitors PP2, toxin A and Y27632, respectively, abrogated the effects of LPA. Moreover, we showed that Src acts upstream of RhoA in this signalling cascade and that LPA induces focal adhesion formation and FAK redistribution and activation in confluent monolayers. Focal adhesion formation was also observed in the front of migrating cells in response to LPA, and Rock inhibitor abolished this effect. In conclusion, our findings show that LPA modulates adherent junction disassembly, actin cytoskeletal disorganisation, and focal adhesion formation, conferring a migratory phenotype in colon tumour cells. We suggest a functional regulatory cascade that integrates RhoA–Rock and Src–FAK signalling to control these events during colorectal cancer progression.

1. Introduction

Lysophosphatidic acid (LPA) is a natural phospholipid related to a broad range of cellular responses, such as cell proliferation, migration and survival. This bioactive lipid is present at high levels in the plasma and ascitic fluid of ovarian cancer patients (Erickson et al., 2001). Although high levels of lysophosphatidylcholine, a LPA precursor, were found in the plasma of colorectal patients (Zhao et al., 2007), few studies have focused on the role that LPA plays during the progression of this neoplasia.

LPA evokes its cellular responses through its G protein-coupled receptors, LPA1-6, which can stimulate multiple downstream signalling pathways. For example, LPA can activate Erk (Zhang et al., 2007), Src (Huang et al., 2008), PKA (Müller et al., 2010), Rho and Rac GTPases (Harper et al., 2010; Yamada et al., 2005), and transactivate EGFR (Mori et al., 2006). In colon cancer, the fact that LPA1-3 receptors are aberrantly expressed (Müller et al., 2010) implicates a role during tumour progression. For instance, in a model of colitis using Apc (min/+) mice, the absence of LPA2 attenuates tumour formation (Lin et al., 2009).

Epithelial adherent junctions encircle E-cadherin, which interacts with the neighbouring cell through its ectodomain and with the actin cytoskeleton through its cytoplasmic domain via p120-catenin, β-catenin and α-catenin (Huang et al., 2008; Leve et al., 2008). Various studies have shown that the downregulation of adherent junctions is related to cell migration, invasion and metastasis of epithelial tumour cells (Syed et al., 2008). Nevertheless, there are few studies showing that LPA can modulate adherent junction disassembly in colon cancer. It has been shown that long-term LPA treatment caused nuclear translocation of β-catenin, inducing the proliferation of colon cancer cells (Yang et al., 2005). Additionally, LPA-induced adherent junction dissociation leads to β-catenin and E-cadherin localisation at the perinuclear endocytic recycling compartment in invasive colon cancer cells (Kam and Quaranta, 2009). However, whether LPA induces adherent junction disruption to modulate actin cytoskeletal disorganisation in colon cancer cells and the cell signalling pathways underlying this process remain to be defined.

It is known that cell–cell adhesion, actin cytoskeletal dynamics (Leve et al., 2008), and cell migration (Pertz et al., 2006) are

* Corresponding author. Tel.: +55 21 3207 6533; fax: +55 21 3207 6587.
E-mail address: jmorgado@inca.gov.br (J.A. Morgado-Díaz).

0014-2999/$ – see front matter © 2011 Elsevier B.V. All rights reserved.
doi:10.1016/j.ejphar.2011.09.006

controlled by small Rho GTPases. Additionally, LPA also triggers events related to cell migration, such as stress fibre and focal adhesion formation at the cell–matrix adhesion, through the activation of Rho–Rock signalling in different cell lines (Ridley and Hall, 1992; Zhang et al., 2007). However, the molecular mechanism of LPA-induced cell migration in colon cancer remains poorly understood. In the present study, we demonstrate that the treatment of Caco-2 cells with LPA causes adherent junction disassembly and actin cytoskeletal disorganisation, leading to increased cell migration. Furthermore, we report that LPA modulates these events through a functional regulatory cascade that integrates RhoA–Rock and Src–FAK signalling to control colon cancer progression.

2. Materials and methods

2.1. Antibodies and reagents

Mouse monoclonal anti-E-cadherin (clone 36) was purchased from BD Biosciences (San Diego, CA, USA). L-α-lysophosphatidic acid (oleoyl sodium), mouse anti-p120-catenin (6H11), mouse anti-IQGAP1 and mouse anti-α-tubulin (Z022) were purchased from Invitrogen Corporation (Carlsbad, CA, USA). Rabbit anti-Rock II, monoclonal anti-Rac (23A8), rabbit anti-phospho-FAK (Tyr 397), rabbit anti-p125 FAK, and rabbit anti-β-catenin were obtained from Sigma-Aldrich (Saint Louis, MO, USA). Mouse anti-phospho-Src (Tyr 416, 100F9 rabbit) was obtained from Cell Signaling Technology, Inc. EZ-Detect TM Rho and Rac activation kits for pull-down assays, including anti-Rho and anti-Rac antibodies, were obtained from Pierce (Rockford, IL, USA). The Alexa 488-conjugated secondary antibody was obtained from Invitrogen Corporation. Horseradish peroxidase-conjugated goat anti-rabbit and anti-mouse IgG were obtained from Sigma-Aldrich. H-89 (N-[2-(p-bromocinnamylamino) ethyl]–5–isoquinolinesulfonamide) and LY294002 (2-(4-morpholinyl)-8-phenyl-4H-1-benzopyran-4-one) inhibitors were purchased from Biomol Research Laboratories Inc. (Plymouth Meeting, PA, USA). PD153035 (4-[(3-bromophenyl) amino]-6,7-dimethoxyquinazoline, Y-27632 ((R)-(+)-trans-N-(4-Pyridyl)-4-(1-aminoethyl)-cyclohexanecarboxamide), toxin A from *Clostridium difficile* and PP2 (4-amino-5-(4-chlorophenyl)-7-(t-butyl) pyrazolo[3,4-d]pyrimidine were purchased from Calbiochem (Darmstadt, Germany).

2.2. Cell culture and LPA treatment

The Caco-2 human colon adenocarcinoma cell line (ATCC, # HTB-37) was passaged weekly with 0.05% trypsin/0.02% EDTA in PBS solution. The cells were grown in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% foetal bovine serum (FBS), penicillin G (60 mg/l) and streptomycin (100 mg/l) at 37 °C in a humidified atmosphere of 5% CO2/air.

Cell cultures were switched to serum-free medium for 24 h prior to LPA treatment. We tested concentrations of 1, 10 or 20 μM of LPA and used confocal microscopy to find that 10 μM was the minimal concentration that induced stress fibre formation in our model. Thus, using this concentration, which was also used in previous studies (Shida et al., 2008; Yamada et al., 2005), we analysed the effect of LPA on Caco-2 cells at the indicated times.

2.3. Treatments with pharmacological inhibitors

Selective pharmacological inhibitors were added to the cell cultures 1 h before LPA treatment and were present throughout the treatment as indicated. The inhibitors were diluted in DMSO and stored at −20 °C. Each concentrated solution was diluted immediately before use to give final concentrations as follows: 100 nM PP2 (Src inhibitor), 20 μM H-89 (PKA), 100 nM PD153035 (EGFR), 10 μm LY294002 (PI3K), 10 μM toxin A from *Clostridium difficile* (Rho), and 10 μM Y-27632 (Rock).

2.4. Immunofluorescence and actin cytoskeleton detection

Caco-2 cell monolayers were grown on sterile glass coverslips and, after the treatments, washed in PBS, fixed in 4% paraformaldehyde, and incubated in NH_4Cl for 10 min at room temperature. The cells were then permeabilised with 0.5% Triton X-100 and blocked with 3% BSA in PBS for 2 h. Subsequently, the cells were incubated overnight at 4 °C in primary antibodies against E-cadherin (1:100), β-catenin (1:220), p120-catenin (1:200), RhoA (1:200), Rock II (1:500), Rac1 (1:60), IQGAP (1:50) and FAK (1:1000), followed by 1 h in the respective Alexa 488-conjugated secondary antibodies (1:500). The cells were washed and mounted using n-propyl-gallate, and the cell staining was detected using an Axiovert S-100 immunofluorescence microscope equipped with a CCD camera and the KS 300 image analyser programme (Carl Zeiss Inc., Germany).

For the visualisation of the F-actin distribution, cell monolayers were fixed and permeabilised as described above and incubated in 500 ng/mL TRITC-phalloidin for 40 min at room temperature. After washing, the stained monolayers were analysed using a Zeiss LSM510 Meta laser scanning confocal microscope with a 543-nm excitation laser. Individual images through the cell volume of similar confluent regions were collected, and optical sections near the apical, medial (5 μm) and basal (9 μm) planes from monolayers (X–Y plane) and their perpendicular planes (X–Z plane) were obtained. The images shown are representative of at least three independent experiments.

2.5. Cell extraction in Triton X-100 and western blotting

To evaluate the subcellular distribution of adherent junction proteins, the samples were rinsed in PBS and incubated for 20 min at 4 °C in CSK extraction buffer (50 mM NaCl, 10 mM Pipes, pH 6.8, 3 mM $MgCl_2$, 0.5% Triton X-100 (TX-100), 300 mM sucrose containing 1 mM orthovanadate, 20 mM NaF, and protease inhibitor cocktail (1:100, Sigma). The cells were scratched from the plates, homogenised, 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, and 1% SDS) and boiled at 100 °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 removed and stored at −20 °C for later analysis.

To obtain total cell lysates, the samples were rinsed and incubated for 20 min at 4 °C in extraction buffer (150 mM NaCl, 10 mM Hepes, pH 7.3, 0,2% sodium dodecyl sulphate, 1% TX-100, 0.5% deoxycholate, and 2 mM EDTA) containing 2 mM orthovanadate, 20 mM NaF, and protease inhibitor cocktail (1:100, Sigma). The cells were then scratched from the plates, homogenised and centrifuged at 11,000 *g* for 10 min at 4 °C. The supernatant was collected and stored at −20 °C.

Equal amounts of protein (30 μg/lane of cell fractions or 60 μg/lane for phosphorylated forms analysis) were electrophoretically separated by SDS-PAGE on 7.5% or 10% gels and transferred to nitrocellulose sheets. The membranes were blocked and incubated overnight in primary antibodies against E-cadherin (1:5000), β-catenin (1:4000), p120-catenin (1:2000), phospho-Src tyrosine 416 (1:500), phospho-FAK tyrosine 397 (1:1000) and α-tubulin (1:500). After washing, membranes were incubated for 1 h in peroxidase-conjugated goat anti-rabbit or anti-mouse secondary antibodies. Proteins were visualised using an enhanced chemiluminescence kit (GE Healthcare, Chalfont St. Giles, UK). Bands were quantified according to their optical densities using LabWorks 4.6 (Bio-Rad Laboratories, Hercules, CA).

2.6. Pull-down assay

The activities of the Rho and Rac proteins were determined using specific pull-down assays, following the manufacturer's instructions (Pierce, Rockford, IL, USA). Briefly, Rhotekin RBD and PAK-1 PBD bound to glutathione-agarose beads were used to precipitate GTP-bound Rho and Rac, respectively, from cell lysates. The active and total Rho and Rac were separated by 12% SDS-PAGE and visualised by western blotting using specific antibodies against Rho (1:500) and Rac (1:1000).

2.7. Scratch-healing assay

Caco-2 monolayers were serum-starved for 24 h, pre-treated for 1 h with kinase and GTPase inhibitors and scratched using a sterile pipette tip. For each dish, three wounds were manually made, and the three regular wound sites were verified under a microscope, selected and marked. After washing with PBS, fresh media containing the inhibitors and LPA was added to the cells, which were incubated at 37 °C. Untreated and treated cells were permitted to migrate into the denuded area and were photographed immediately after wounding (0 h), 6 h after wounding, and at the end of the experiment (24 h). The distance between the two edges of the injury was quantified from three independent experiments. The values are represented as percentages and plotted on the graph.

2.8. Cell proliferation

The relative viable cell numbers were determined by the crystal violet assay. Briefly, the same cell density used in the cell migration assay (1.5×10^4 cells) was seeded for 4 h in multi-well culture plates, incubated with cell culture medium with or without FBS and treated with LPA for 24 h. The cell culture medium was removed, and the surviving cells were then fixed with ethanol for 10 min and stained with 0.05% (w/v) crystal violet in 20% ethanol for 10 min at room temperature. The plates were washed extensively, air-dried, and solubilised with methanol, and the optical densities were measured at 595 nm using an ELISA reader (Spectra Max 190, Molecular Devices, Sunnyvale, CA, USA).

2.9. Statistical analysis

Statistical analyses were carried out using GraphPad Prism version 5.0 for Windows (GraphPad Software, San Diego, CA, USA). For each experiment, the data from triplicate samples were calculated and are expressed as the mean ± S.D. The statistics were performed using a two-tailed ANOVA with *post hoc* Dunnett tests. Differences were considered to be significant for P-values <0.05.

3. Results

3.1. LPA causes adherent junction protein redistribution

Initially, we used immunofluorescence to analyse the subcellular localisation of E-cadherin, β-catenin and p120-catenin after LPA treatment. Fig. 1A shows that the adherent junction proteins were preferentially localised at cell–cell contacts in untreated cells. After LPA treatment for 15, 30 and 60 min, cells in the monolayers dissociated from each other and exhibited a discontinuous staining pattern and internalisation of adherent junction proteins, but the attachment to the substratum was maintained. The same effect was observed at 6, 24 and 48 h of LPA treatment (Supplemental data, Fig. 1). Because junctional proteins are functional when they are linked to the cytoskeleton, we evaluated the subcellular distribution of E-cadherin, β-catenin and p120-catenin by performing western blotting on the TX-100-soluble and -insoluble cell fractions after treatment with LPA. Densitometric analysis of these proteins revealed a significant translocation from the insoluble to the soluble fraction when cells were incubated in LPA (Fig. 1B), but the expression of these proteins was not altered after 24 h of treatment (Fig. 1C). Together, these results indicate that LPA mediates adherent junction disassembly in Caco-2 cells.

3.2. LPA modulates perijunctional actin cytoskeleton organisation concomitantly to stress fibres and focal adhesion formation

Caco-2 monolayers were serum-starved for 24 h (control) or treated with 10 μM LPA for 15, 30 and 60 min, and the actin cytoskeletal organisation was analysed through the entire cell monolayer with time-frame confocal microscopy. Fig. 2A shows that, in the control group, the cells had punctate labelling in the apical region, reflecting the presence of actin at the microvillus and in the cell–cell contacts. At the medial level, actin was predominant at intercellular junctions, and few small stress fibres were observed on the basal side. LPA treatment altered the cell morphology at the analysed times, increasing the cell size. Apparently, LPA did not alter the actin distribution in the apical region, but reduced the continuous linear staining pattern in the medial region, indicating that LPA alters perijunctional actin, and as expected, induced the formation of longer stress fibres. Again, this effect was also observed at 6, 24 and 48 h of LPA treatment (Supplemental data, Fig. 2).

Because our results showed that LPA increased stress fibres formation, and since these structures is accompanied by the assembly of focal adhesions (Ridley and Hall, 1992), we decided to investigate the distribution of focal adhesion kinase (FAK), a focal adhesion protein, in response to LPA using cell monolayers and confocal microscopy analysis. Fig. 2B shows that FAK localises throughout cell monolayers at focal complexes as punctate spots in control cells. After 15 min of LPA treatment, aside from a drastic increase in actin bundles, the extent of FAK staining increased and became elongated, and FAK was localised to the termini of stress fibres, indicating focal adhesion formation. This effect was also observed at 30 min of LPA treatment but was reduced after 1 h (data not shown).

It is widely accepted that FAK autophosphorylation on tyrosine residue 397 recruits Src, which in turn phosphorylate other sites on FAK that are needed for maximal FAK-associated activity (Schlaepfer et al., 2004). This activated FAK–Src complex could promote multiple intracellular signalling cascades involved in the disassembly of cell–cell contacts and cell migration (Avizienyte et al., 2002; Jiang et al., 2006). To confirm that our immunofluorescence data correlated with the biochemical analysis of the FAK–Src complex, we examined the phosphorylation levels of FAK and Src. We used an anti-phospho-FAK antibody that detects phosphorylated Tyr 397 FAK and an anti-phospho-Src antibody that detects phosphorylated Tyr 416 Src. Fig. 2C shows that LPA stimulates the tyrosine phosphorylation of both FAK and Src, predominantly at 15 and 30 min of treatment.

3.3. Src and Rho–Rock signalling mediate LPA-induced adherent junction and actin cytoskeletal disassembly

Based on previous studies showing that LPA activates different signalling pathways (Noguchi et al., 2009), we decided to investigate which one LPA mediates to induce adherent junction disassembly in Caco-2 cells. Cell monolayers were preincubated for 1 h with different inhibitors of cell signalling, including H89 (PKA inhibitor), LY294002 (PI3 kinase inhibitor), PD153035 (EGFR inhibitor), PP2 (Src inhibitor), toxin A from *Clostridium difficile* (Rho inhibitor), and Y27632 (Rock inhibitor). The cells were then treated for 1 h with 10 μM LPA supplemented with the respective chemical inhibitors. Of the inhibitors, the Src inhibitor PP2 prevented the redistribution of p120-catenin but did not abrogate LPA-induced E-cadherin and β-catenin redistribution. Moreover, Rho and Rock inhibitors impaired LPA-induced adherent junction disassembly, as evidenced by the localisation of E-cadherin, β-catenin

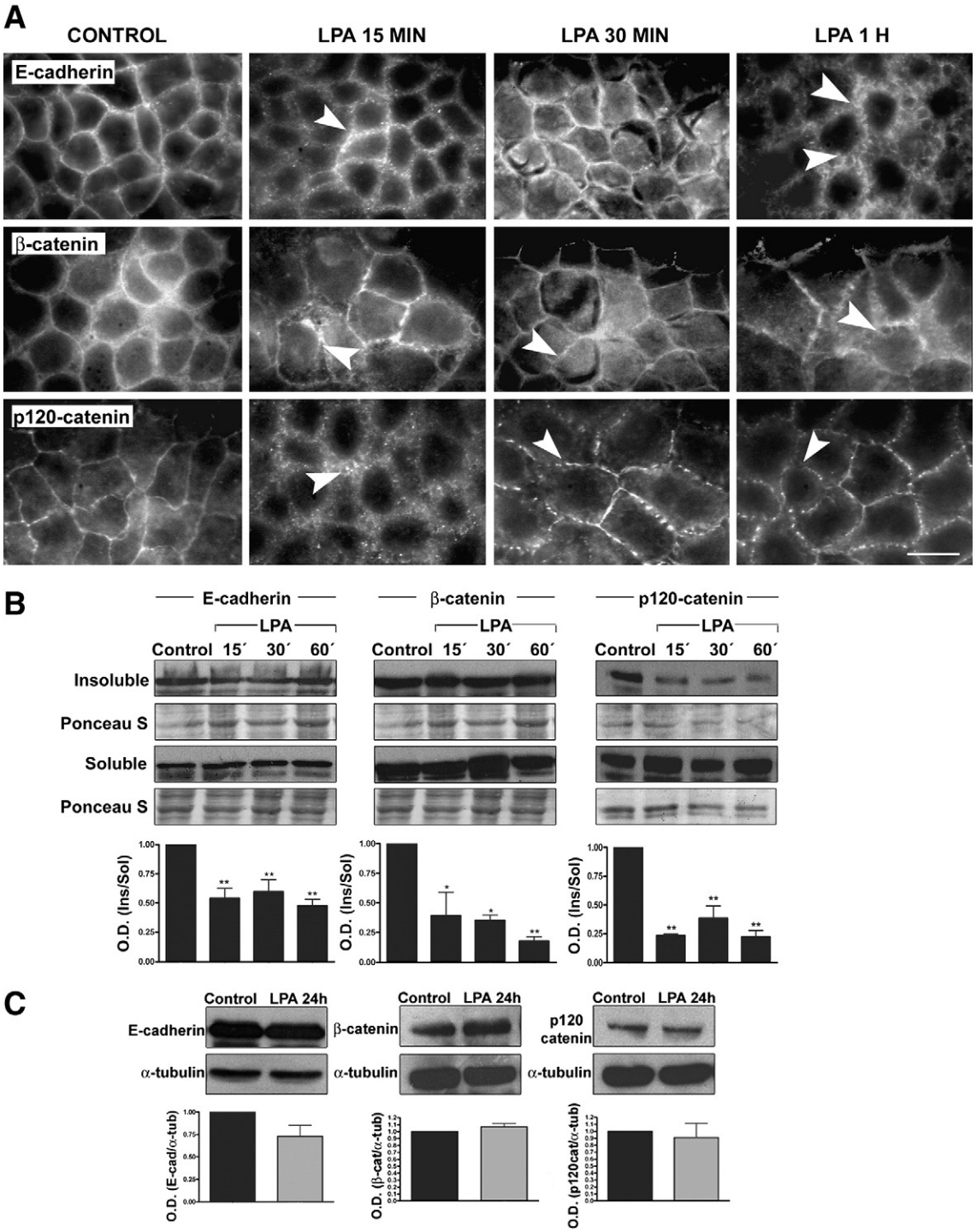

Fig. 1. LPA causes adherent junction protein redistribution in Caco-2 cells. (A) Cell monolayers were serum-starved overnight (control), incubated in medium containing 10 μM LPA for 15, 30 and 60 min, fixed and stained for E-cadherin, β-catenin and p120-catenin immunofluorescence. LPA caused adherent junction protein redistribution, indicating cell–cell contact disassembly (*Arrowheads*). Scale bar, 10 μm. (B) Representative western blotting and densitometric analyses for E-cadherin, β-catenin and p120-catenin in the Triton X-100 insoluble (Ins) and soluble (Sol) fractions of cells that were incubated with LPA at the indicated times. Note that LPA induced the redistribution of the proteins from the insoluble to the soluble fractions. In each case, the score was calculated using the following equation: arbitrary score = (amount of the protein in the insoluble fraction)/(amount of the protein in the soluble fraction). (C) Representative western blotting and densitometric analyses of cells treated with 10 μM LPA for 24 h. Cells were homogenised in lysis buffer, and E-cadherin, β-catenin and p120-catenin were analysed by western blotting. In each case, the score was calculated using the following equation: arbitrary score = (O.D. of the analysed protein)/(O.D. of α-tubulin). O.D., optical density; E-cad, E-cadherin; β-cat, β-catenin; p120, p120-catenin. The scores for the control group were normalised to 1 in each case. Average scores ± S.E.M. for three independent experiments are shown. Significantly different: *, $P<0.05$; **, $P<0.01$.

and p120-catenin at cell–cell contacts (Fig. 3A and Supplemental Fig. 3). This data, together with the finding that Src is required for focal adhesion formation, indicates that LPA-mediated Src activation modulates cell–cell and cell–substrate adhesions.

Because stress fibre and focal adhesion formation are well-known cellular responses to Rho activation in other cell models (Ridley and Hall, 1992) and as our observations showed that Rho inhibition prevented LPA-mediated adherent junction disassembly, we investigated

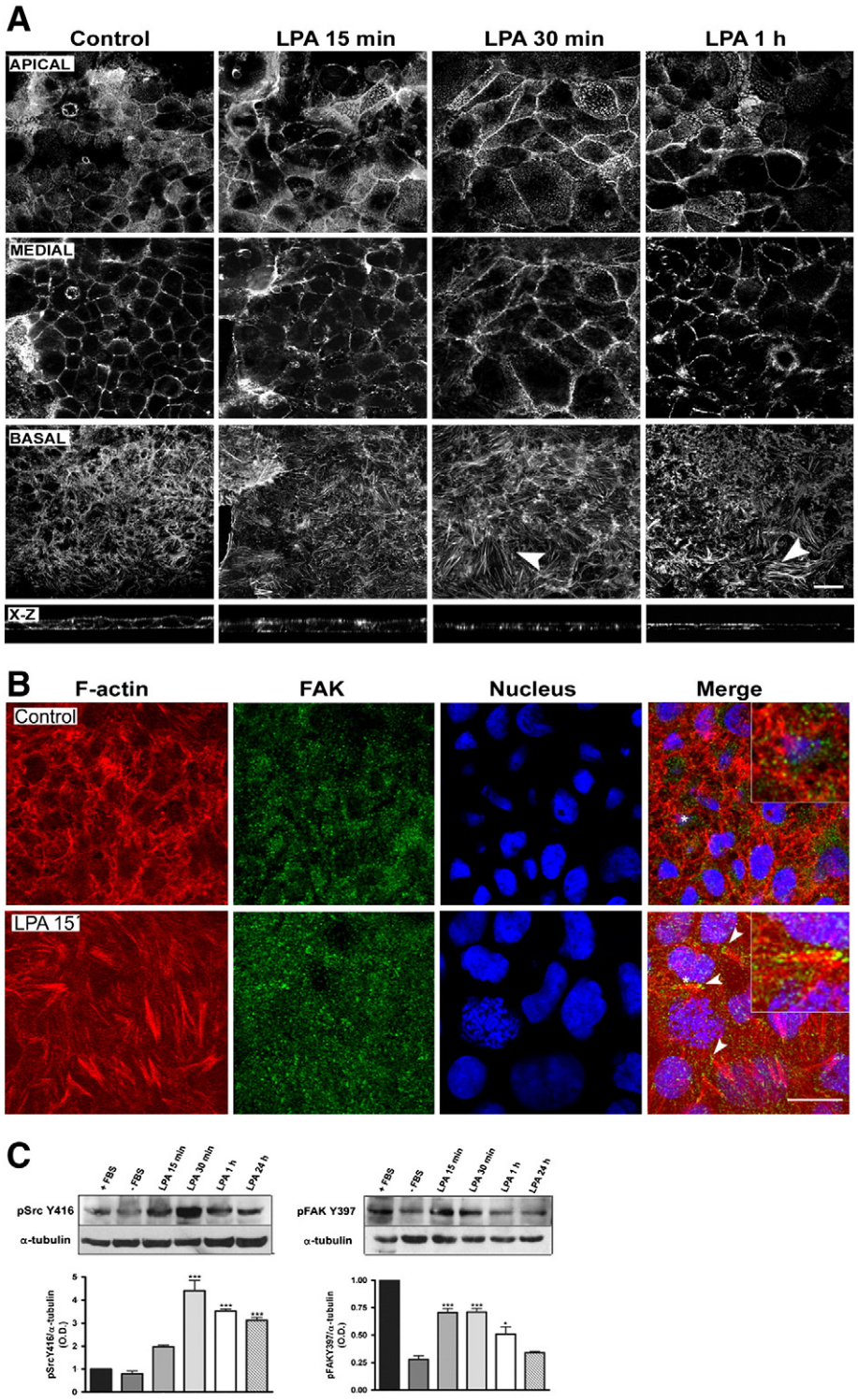

Fig. 2. LPA modulates actin cytoskeletal organisation and focal adhesion formation. A) Representative confocal images of serum-starved Caco-2 cells (control), incubated with 10 μM LPA for different times as indicated and probed with TRITC-conjugated phalloidin. Optical sections of the X–Y plane close to the apical, medial (5 μm), or basal (9 μm) cell sides and an X–Z plane. Note that LPA induced reorganisation of medial region at all analysed times. *Arrowheads*, stress fibres. Scale bar, 20 μm. B) Caco-2 cell monolayers were serum-starved overnight (control) and incubated in LPA-containing medium for 15 min. Cells were fixed, stained for F-actin and FAK, and analysed by confocal microscopy. LPA treatment increased actin bundle formation, and FAK was elongated and localised at the termini of stress fibres, indicating focal adhesion formation (*Arrowheads*). Insets on the superior-right part of each panel in the merge images indicate a higher magnification of the area marked with asterisks. Scale bar, 20 μm. C) Phosphorylation analysis of Src and FAK. Cell monolayers were treated with 10 μM LPA as indicated, and total lysates were obtained and prepared for western blotting using specific antibodies against the phosphorylated forms of Src at tyrosine 416 (p-Src) and FAK at tyrosine 397 (p-FAK). Band images were quantified by optical density in three independent experiments. The score was calculated using the ratio between the analysed protein and α-tubulin as protein loading control. The score for the control group was normalised as 1 in each case, and statistical analyses were carried out using ANOVA with a *post hoc* Dunnett test. *, $P<0.05$ and ***, $P<0.001$. O.D., optical density.

the activation of Rho and Rac GTPases after LPA treatment using a pull-down assay. Fig. 3B shows that confluent monolayers of Caco-2 cells have a basal level of activated Rho in the control group, which was increased after 1 h of LPA treatment. However, LPA did not alter the levels of activated Rac. We then used immunofluorescence to evaluate the cellular distribution of Rock II and IQGAP, effector

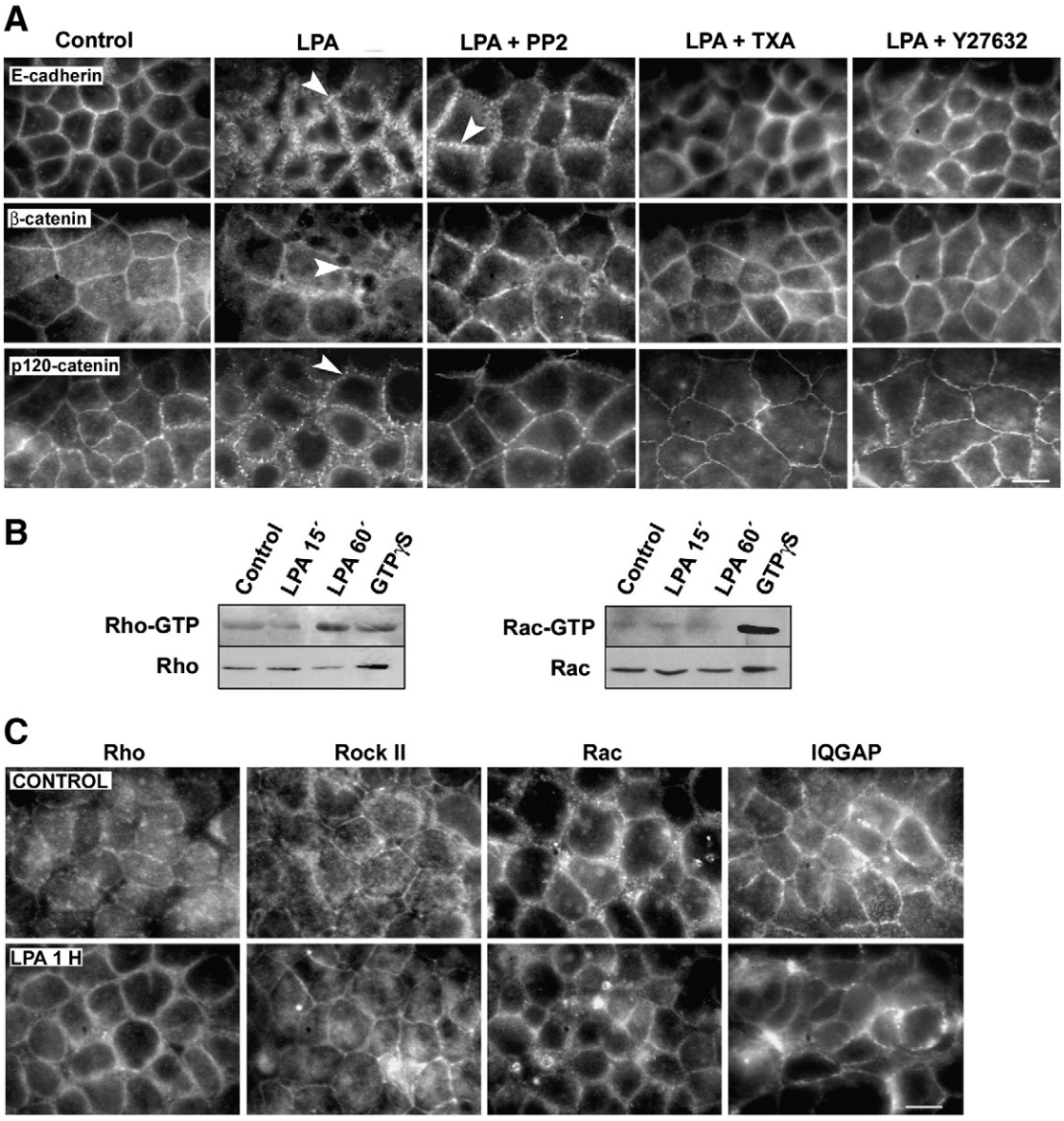

Fig. 3. Src and Rho–Rock signalling mediates LPA-induced adherent junction disassembly. A) Caco-2 cells were serum-depleted (control) and treated with LPA for 1 h or pretreated for 1 h with the inhibitors as indicated before incubation with LPA. Cells were fixed and stained for E-cadherin, β-catenin and p120-catenin. The Src inhibitor PP2 prevented LPA-induced p120-catenin redistribution but did not alter E-cadherin or β-catenin localisation. Rho and Rock inhibitors impaired adherent junction protein redistribution. *Arrowheads* indicate LPA-mediated protein redistribution. Scale bar, 10 μm. B) Rho and Rac activity using pull-down assays. Caco-2 cells were serum-starved (control) and treated with LPA as indicated. Fresh lysates were used to detect the relative amounts of Rho-GTP and Rac-GTP, respectively. The total levels of Rho and Rac were detected by western blotting in cell lysates and used as a protein loading control. C) Representative immunofluorescence images of RhoA, Rock II, Rac1 and IQGAP in cells treated with LPA for 1 h. LPA treatment increased RhoA and Rock II at cell–cell contacts but not Rac1 and IQGAP. Scale bar, 10 μm.

proteins of Rho and Rac, respectively. We observed that LPA treatment increased RhoA and Rock II at cell–cell contacts, but neither Rac1 nor IQGAP were redistributed (Fig. 3C). Because the predominant membrane-linked Rho GTPases are active, the labelling of Rac1 at cell–cell contacts visualised in the control group probably corresponds to the basal activity observed in the pull-down assay. Overall, these data suggest that LPA activates Rho–Rock, which in turn lead to cell–cell contacts disruption.

Next, we examined the signalling pathways triggered by LPA that are responsible for modulating the actin cytoskeletal redistribution based on the F-actin staining visualised by confocal microscopy. Cell monolayers were serum-starved for 24 h, pretreated with selective inhibitors prior to LPA treatment, and images were obtained in the apical, medial and basal regions and in the X–Z plane. As shown in Fig. 4, the Src inhibitor PP2 and the Rho inhibitor toxin A clearly restored the actin filament distribution in the medial and basal region. The Rock inhibitor Y27632 partially abrogated the effects in the medial region and, as expected, abrogated LPA-induced stress fibres (Fig. 4A). The inhibitors of PKA, PI3 kinase, and EGFR did not prevent the LPA effects throughout the cell volume (Supplemental data, Fig. 4). We also evaluated whether Src is required for LPA-mediated Rho activation. Cell monolayers were serum-starved for 24 h, pretreated with the Src inhibitor PP2 for 1 h, and then treated for 1 h with 10 μM LPA supplemented with PP2, and the levels of activated RhoA were measured using a pull-down assay. Fig. 4B shows that the Src inhibitor partially prevented LPA-induced RhoA activation, suggesting that Src acts upstream of Rho–Rock signalling.

3.4. LPA-induced cell migration depends on Src and Rho–Rock signalling

Previous studies have shown that LPA increases cell migration in different cell types (Shida et al., 2008; Xu et al., 2007). Here using the

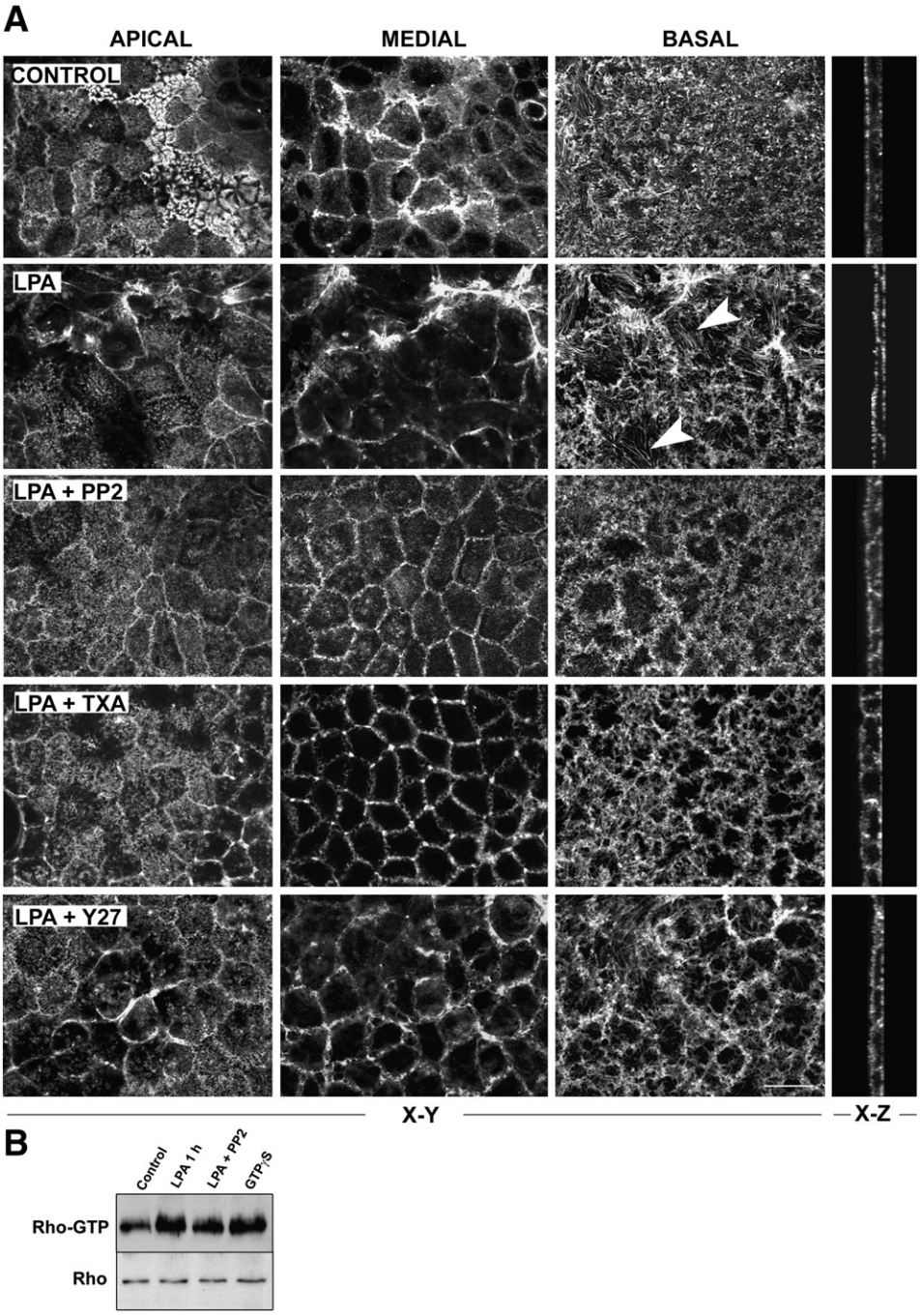

Fig. 4. Src and Rho–Rock signalling mediate LPA-induced actin cytoskeletal disruption. A) Caco-2 cell monolayers were serum-starved overnight (control), incubated with 10 μM LPA for 1 h or pretreated for 1 h with the inhibitors before incubation with LPA as indicated. Cells were fixed, probed with TRITC-conjugated phalloidin, and analysed by confocal microscopy. Representative optical sections in the X–Y plane close to the apical, medial (5 μm), or basal (9 μm) cell sides and an X–Z plane are shown. Src and Rho inhibitors restored the pattern of actin filament distribution in the medial region and the LPA-induced stress fibre organisation, effects partially abrogated by the Rock inhibitor. *Arrowheads* indicate longer stress fibres. Scale bar, 20 μm. B) Pull-down assay to detect Rho activity. Caco-2 cells were treated with LPA for 1 h or pretreated for 1 h with the Src inhibitor PP2. The Src inhibitor partially prevented LPA-induced RhoA activation.

wound-healing technique, we show that LPA promotes the migration of Caco-2 cells. In serum-starved control monolayers, the wounds remained open at 6 h and just started to close after 24 h in culture. However, LPA-treated cells migrated faster than the control cells, initiated healing at 6 h of treatment, and almost completely closed the wound at 24 h (Fig. 5A–B). To further investigate the signalling pathways involved in LPA-induced cell migration, the cells were pretreated with specific kinase inhibitors prior to the wound-healing assay. We observed that PP2, toxin A and Y27632, which inhibit Src, Rho and Rock respectively, prevented LPA-induced cell migration at 6 h of treatment. In addition, the Rock inhibitor completely abrogated LPA-induced wound closure even after 24 h in culture. Other kinase inhibitors, such as H89 for PKA, LY294002 for PI3 kinase, and PD153035 for EGFR, did not prevent the LPA-induced increases in cell migration (Supplemental data, Fig. 5). The relative viable cell numbers were determined to verify that cells were indeed migrating and not proliferating (Fig. 5C). This result indicates that the same signalling pathway that modulates cell–cell disassembly is also responsible for cell migration control.

We also investigated whether LPA-induced stress fibres accompanied focal adhesion formation at the migration front using subconfluent

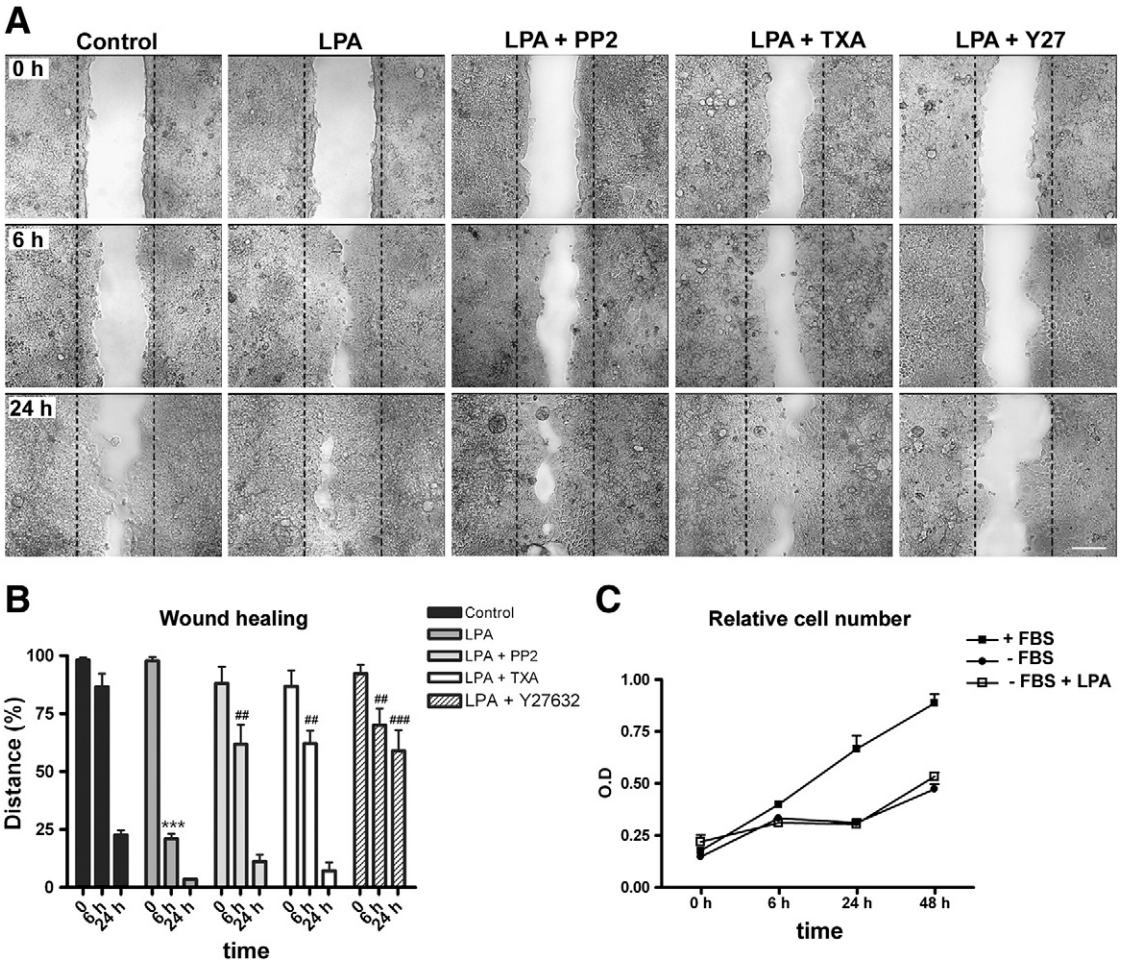

Fig. 5. LPA-induced cell migration depends on Src and Rho–Rock signalling. A) Cell migration was evaluated using the wound healing technique. Caco-2 monolayers were serum-starved (control), incubated with 10 μM LPA for 1 h or pre-treated for 1 h with the chemical inhibitors prior to LPA treatment. Cell migration was monitored at 6 and 24 h as indicated in the Materials and methods. All of the inhibitors prevented LPA-induced cell migration at 6 h of treatment, and the Rock inhibitor (but neither Src nor Rho inhibitors) completely abrogated the wound closure after 24 h of LPA treatment. Scale bar, 100 μm. B) The wound closures were measured and the migration rates were calculated as the difference between the two edges of the injury (Distance). The results are representative of three independent experiments. C) A proliferation assay was carried out after 48 h of treatment in parallel with the cell motility assay. Statistical analyses were carried out using ANOVA with a *post hoc* Dunnett test. ***, $P<0.001$ compared to the control group; ###, $P<0.001$ and ##, $P<0.01$ compared to the LPA-treated cells.

monolayers and confocal microscopy. Fig. 6A shows that control cells do not project membrane protrusions, and FAK staining had a punctate labelling pattern throughout the cells. At 1 h of LPA treatment, it was possible to observe large membrane protrusions, elongated focal adhesions, and longer stress fibres. At 15 and 30 min of LPA treatment, these structures were also observed (data not shown). Furthermore, Src and Rock inhibition abrogated this LPA-induced migratory phenotype, corroborating our cell migration results.

4. Discussion

The loss of cell–cell adhesion, actin cytoskeletal disorganisation, and the acquisition of a migratory phenotype are initial steps of epithelial cancer. However, there are few studies attempting to investigate the role that LPA plays on these events, particularly during colorectal cancer progression. Thus, the present study was designed to explore the cell transduction mechanisms by which LPA could promote the loss of cell–cell adhesion, actin cytoskeletal disorganisation conferring a migratory phenotype. Our results show for the first time that LPA can modulate both intercellular junctions and the actin cytoskeleton to induce a migratory phenotype in colon cancer cells. Furthermore, we show that a regulatory cascade involving a crosstalk between Rho–Rock and Src–FAK may play an important role in the regulation of these events.

We showed that LPA causes redistribution of the adherent junction proteins E-cadherin, β-catenin and p120-catenin in colon cancer Caco-2 cells (Fig. 1A). LPA-induced intercellular junction dispersal was also reported in ovarian cancer cells (Huang et al., 2008). Additionally, our results showing that LPA induces the disorganisation of the perijunctional actin cytoskeleton (Fig. 2A) suggests that LPA-induced adherent junction disassembly could be a consequence of actin contraction. A previous study using HCT-116 colon cancer cells analysed the long-term effects of LPA on junctional proteins and showed that this biolipid causes aberrant nuclear accumulation of β-catenin that promotes cell proliferation. We did not observe increased cell proliferation (Fig. 5C), but these contradictory results can be explained by the differences in the cell lines. Namely, Caco-2 cells are well differentiated, exhibit functional cell–cell contacts, and are less invasive than HCT-116 cells (de Freitas Junior et al., 2011). In most epithelial cancers, compromised E-cadherin function enhances the transition to metastasis (Carothers et al., 2006). Moreover, E-cadherin is regulated by its cytoplasmic binding partners, catenins. α- and β-catenins are associated with the actin cytoskeleton (Huang et al., 2008; Leve et al., 2008), whereas p120-catenin modulates E-cadherin stability at the cell surface (Davis et al., 2003). Thus, the fact that LPA

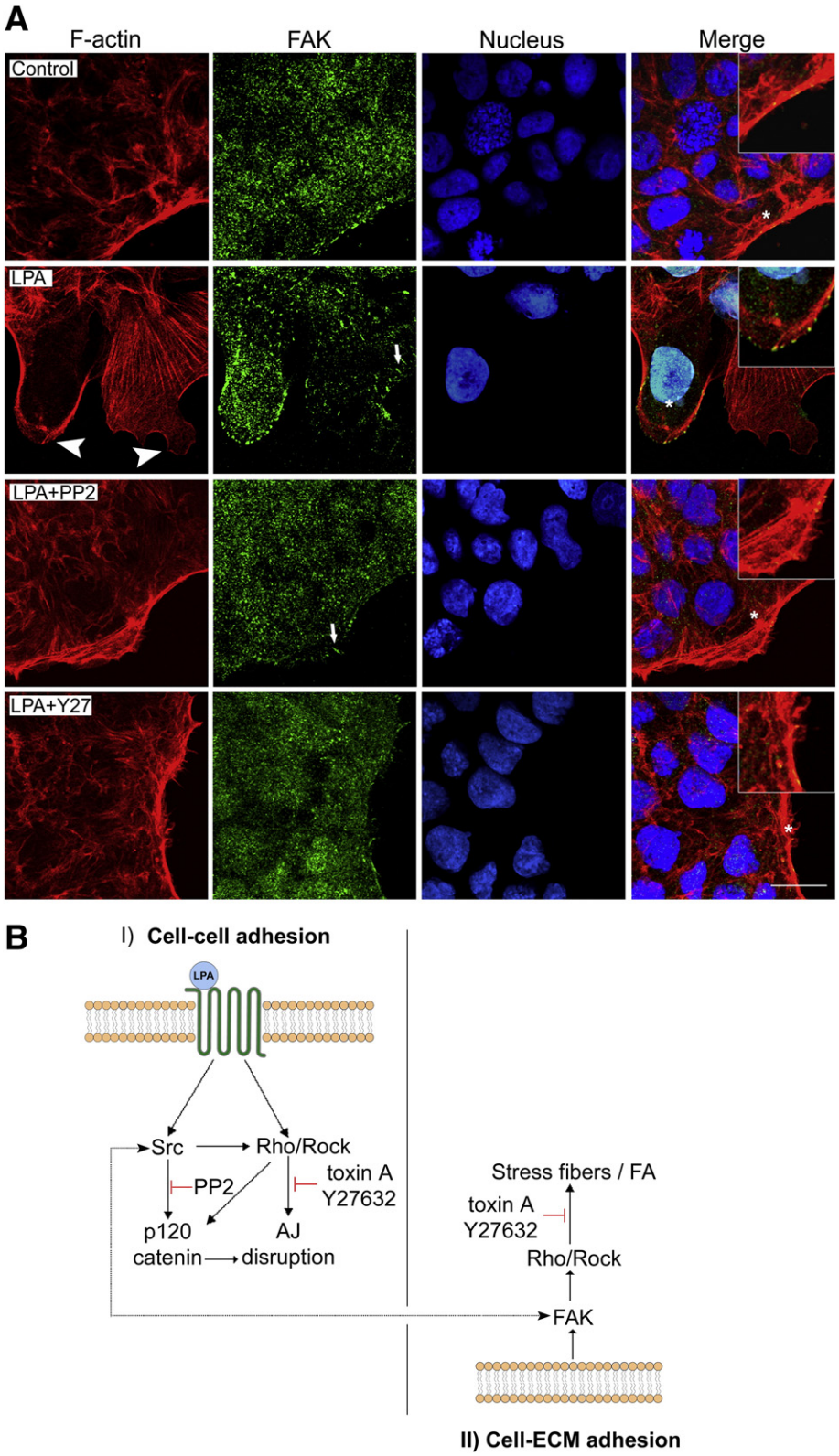

Fig. 6. Src and Rock control LPA-induced membrane protrusions in migrating cells. A) Subconfluent Caco-2 cell monolayers were serum-starved (control) and incubated in LPA for 1 h or pre-treated for 1 h with the chemical inhibitors of Src kinase (PP2) and Rock (Y27632) prior to LPA treatment. Cells were fixed and stained for F-actin and FAK and analysed using confocal microscopy. LPA treatment induced the formation of large membrane protrusions (*Arrowheads*) and elongated focal adhesion (*Arrows*), which were abrogated by Src and Rock inhibitors. Insets on the superior right part of each panel in merge images indicate a higher magnification of the area marked with asterisks. Scale bar, 20 μm. B) A model for the regulation of the LPA-induced migratory phenotype. (I) LPA, through its G protein-coupled receptors, induces adherent junction disassembly by a Src-dependent and/or -independent mechanism. Thus, Src activation can induce the redistribution of p120-catenin and activate Rho–Rock. Alternatively, can also directly activate Rho/Rock and induce adherent junction disruption. (II) LPA activates G protein-coupled receptors to trigger FAK/Src complex activation, which, in turn, recruits Rho/Rock to mediate focal adhesion (FA) and stress fibre formation. PP2: Src inhibitor; toxin A: Rho inhibitor; Y27632: Rock inhibitor. ECM, extracellular matrix; FAK, focal adhesion kinase.

induced β-catenin and p120-catenin redistribution from adherent junctions to the cytosol could explain the increased cell migration observed here (Fig. 5).

A study showed that LPA increases the interaction between activated Src and p120-catenin, leading to decreased α- and β-catenin interaction in ovarian cancer cells (Huang et al., 2008). We found that LPA-induced adherent junction and actin cytoskeletal disorganisation in colon cancer cells are Src- and Rho–Rock-dependent (Figs. 3A and 4), and that Src mainly participates in the redistribution of p120-catenin (Fig. 3A). Additionally, it was shown that LPA enhances colon cancer cell growth and apoptosis protection after 3 days of treatment, both in a MAPK- and PI3K-dependent manner (Sun et al., 2009). Given that EGFR, MAPK and PI3K did not reverse the effects of LPA in our study, it is possible that their activations are important late events that modulate cell growth, while Src activity is an initial cell signal that is necessary to trigger adherent junction and actin cytoskeletal disorganisation.

It is known that Rho GTPases modulate the function of adherent junctions, and that G protein-coupled receptors, such as those for LPA, act through a variety of α subunits to activate different signalling pathways. In this context, Rho could be activated through the Gα12 and Gα13 family (Bian et al., 2006). Interestingly, it has been shown in MDCK cells that Gα12 and Gα13 signalling disrupts adherent junctions via Src and Rock, respectively (Donato et al., 2009; Meyer et al., 2003). Therefore, our results suggest that LPA could activate RhoA–Rock directly through its G protein-coupled receptors or via Src to disrupt the adherent junctions. Also, we have previously shown that a low-calcium medium disrupts adherent junctions and the actin cytoskeleton through Rho-Rock signalling in Caco-2 cells (Leve et al., 2008). Here we showed that LPA activates RhoA (but not Rac1) and recruits Rho and Rock II to cell–cell contacts (Fig. 3B–C). Thus, because Rho and Rock inhibitors abrogated LPA-induced adherent junction and actin cytoskeletal disassembly (Figs. 3A and 4, respectively), and as nonmusclemyosin II inhibition impaired adherent junction formation in colon cells (Ivanov et al., 2007), we suggest that adherent junction disruption is mediated by actin–myosin contraction.

Coordinated remodelling of cell–cell and cell–substrate adhesions with assembly and disassembly of focal adhesions are key steps in cell migration. So, we investigated whether the same signalling pathway that modulates the reorganisation of cell–cell contacts could also modulate focal adhesions. It is known that Src localises to peripheral cell-substrate adhesions, where it regulates the turnover of focal adhesion through FAK phosphorylation (Lee et al., 2010; Webb et al., 2004). Our results showing that Src participates in the LPA effects on the p120-catenin redistribution, the longer stress fibres and focal adhesion formation, FAK activation, and cell migration suggests that reciprocal regulation between the two types of adhesions can occur during Src-mediated cell migration, as previously described (Avizienyte et al., 2002) and points LPA as a stimulus for Src activation in colon cancer cells.

FAK is a non-receptor tyrosine kinase located at focal adhesions and is crucial for cell movement. It is activated in response to G protein-coupled receptors, including those for LPA among others (Jiang et al., 2006), and its phosphorylation leads to the recruitment and activation of Src (Schlaepfer et al., 2004). Our results agree with previous studies showing that LPA induces FAK activation and cell migration in other cell types (Bian et al., 2006; Jiang et al., 2006). It has been suggested that FAK recruits proteins that contribute to the dynamic remodelling of adhesions through a Src- and Rho-dependent cascade (Webb et al., 2004). Our results showing that a) LPA activates Rho in a Src-dependent manner (Fig. 4B), b) LPA induces FAK activation through the phosphorylation of the tyrosine residue specific for Src (Fig. 2B–C), and c) Rho and Rock activities are required for the LPA-induced migration (Fig. 5) all support this hypothesis. Moreover, we showed that Src and Rock inhibitors in LPA-treated subconfluent cells prevent the wide membrane protrusions that are crucial for cell migration. Interestingly, mature focal adhesions were abrogated by Rock inhibition but not Src inhibition (Fig. 6A). This is consistent with studies showing that cells lacking Src family members are still able to assemble enlarged focal adhesions, but with a slower turnover (Lee et al., 2010; Webb et al., 2004). Together, these data suggest that Src and Rock inhibition prevent cell migration by impairing proper focal adhesion dynamics.

It was shown that the activation of tyrosine kinases by G protein-coupled receptors is required for the efficient stimulation of Rho signalling (Sah et al., 2000). Indeed, many G protein-coupled receptors are activators of FAK, which indicates a close link between FAK activation and Rho signalling (Bian et al., 2006) and suggests that LPA receptors could activate FAK. In agreement, we showed here that LPA induces activation of FAK–Src complex in Caco-2 cell monolayers (Fig. 2). Some studies suggested that Src is upstream of RhoA because Src inhibits RhoA in a p190RhoGAP-dependent pathway (Haskell et al., 2001). Also, Src phosphorylates RhoGDI, decreasing its ability to bind and inhibits RhoA, Rac and Cdc42 (DerMardirossian et al., 2006). Consistent with this latter possibility and with findings showing that RhoA is active in the leading edge of migrating cells (Pertz et al., 2006), our results indicate that active Src is upstream of RhoA activation (Fig. 4B) because Src inhibition decreased Rho activation, and because LPA induced Src activation at 15 min of treatment while Rho was activated at 1 h. It is also possible that Src phosphorylates Rock directly to reduce Rho–Rock mediated contraction force and control focal adhesion turnover and directional migration, as shown recently (Lee et al., 2010). However, whether Src activation occurs directly by any specific LPA receptor, by E-cadherin internalisation, or directly by FAK, remains to be determined. Some studies have shown that LPA activates Rac and induces lamellipodium formation (Jourquin et al., 2006), and that Src phosphorylates Tiam, a guanine exchange factor that activates Rac, leads to adherent junction disruption and cell migration (Woodcock et al., 2009). We observed cell protrusions formation in subconfluent Caco-2 cells (Fig. 6A), consistent with increased cell migration after LPA treatment (Fig. 5), but we did not detect active Rac (Fig. 3B). Consistent with this result, it has already been shown that lamellipodium can induce cell migration in a Rac-independent manner (Spaargaren and Bos, 1999; Yip et al., 2007).

In summary, we have shown that LPA modulates cell–cell adhesion disassembly, actin cytoskeletal remodelling, and focal adhesion turnover, three events important to induce cell migration. We suggest that LPA acts through a mechanism involving Src activation in the apical cell compartment (where it causes p120-catenin internalisation either directly or through Rho/Rock activation to modulate adherent junction disassembly) and in the basal cell compartment (where Src activity forms the Src–FAK complex to recruit Rho/Rock and induce stress fibre and focal adhesion formation) (Fig. 6B). We propose that a similar molecular event may regulate the initial steps of colorectal cancer progression.

Supplementary materials related to this article can be found online at doi:10.1016/j.ejphar.2011.09.006.

Acknowledgments

This study was sponsored by Conselho Nacional de Desenvolvimento Cientifico e Tecnologico (CNPq), Ministerio da Saúde — Brasil, and Fundação Carlos Chagas Filho de Amparo à Pesquisa do Estado de Rio de Janeiro (FAPERJ). We are grateful to the Programa de Cooperação INCA/FIOCRUZ for the use of its facility. This text was reviewed by American Journal Experts.

References

Avizienyte, E., Wyke, A.W., Jones, R.J., McLean, G.W., Westhoff, M.A., Brunton, V.G., Frame, M.C., 2002. Src-induced de-regulation of E-cadherin in colon cancer cells requires integrin signalling. Nat. Cell Biol. 4, 632–638.

Bian, D., Mahanivong, C., Yu, J., Frisch, S.M., Pan, Z.K., Ye, R.D., Huang, S., 2006. The G12/13-RhoA signaling pathway contributes to efficient lysophosphatidic acid-stimulated cell migration. Oncogene 25, 2234–2244.

Carothers, A.M., Javid, S.H., Moran, A.E., Hunt, D.H., Redston, M., Bertagnolli, M.M., 2006. Deficient E-cadherin adhesion in C57BL/6j-Min/+ mice is associated with increased tyrosine kinase activity and RhoA-dependent actomyosin contractility. Exp. Cell Res. 312, 387–400.

Davis, M.A., Ireton, R.C., Reynolds, A.B., 2003. A core function for p120-catenin in cadherin turnover. J. Cell Biol. 163, 525–534.

de Freitas Junior, J.C.M., D'aguilar Silva, B.D.R., de Souza, W.F., de Araújo, W.M., Abdelhay, E.S.F.W., Morgado-Díaz, J.A., 2011. Inhibition of N-linked glycosylation by tunicamycin induces E-cadherin-mediated cell–cell adhesion and inhibitors cell proliferation in undifferentiated human colon cancer cells. Cancer Chemother. Pharmacol. 68, 227–238.

DerMardirossian, C., Rocklin, G., Seo, J.Y., Bokoch, G.M., 2006. Phosphorylation of RhoGDI by Src regulates Rho GTPase binding and cytosol–membrane cycling. Mol. Biol. Cell 17, 4760–4768.

Donato, R., Wood, S.A., Saunders, I., Gundsambuu, B., Yan Mak, K., Abbott, C.A., Powell, B.C., 2009. Regulation of epithelial apical junctions and barrier function by Galpha13. Biochim. Biophys. Acta 1228–1235.

Erickson, A., Hasegawa, Y., Fang, X., Eder, A., Mao, M., Furui, T., Aoki, J., Morris, A., Mills, G.B., 2001. Lysophosphatidic acid and ovarian cancer: a paradigm for tumourigenesis and patient management. Prostaglandins 64, 63–81.

Harper, K., Arsenaul, D., Boulay-Jean, S., Lauzier, A., Lucien, F., Dubois, C.M., 2010. Autotaxin promotes cancer invasion via the lysophosphatidic acid receptor 4: participation of the cyclic AMP/EPAC/Rac1 signaling pathway in invadopodia formation. Cancer Res. 70, 4634–4643.

Haskell, M.D., Nickles, A.L., Agati, J.M., Su, L., Dukes, B.D., Parsons, S.J., 2001. Phosphorylation of p190 on Tyr1105 by c-Src is necessary but not sufficient for EGF-induced actin disassembly in C3H10T1/2 fibroblasts. J. Cell Sci. 114, 1699–1708.

Huang, R.Y.J., Wang, S.M., Hsieh, C.Y., Wu, J.C., 2008. Lysophosphatidic acid induces ovarian cancer cell dispersal by activating Fyn kinase with p120-catenin. Int. J. Cancer 123, 801–809.

Ivanov, A.I., Bachar, M., Babbin, B.A., Adelstein, R.S., Nusrat, A., Parkos, C.A., 2007. A unique role for nonmuscle myosin heavy chain IIA in regulation of epithelial apical junctions. PLoS One 2, e658.

Jiang, X., Jacamo, R., Zhukova, E., Sinnett-Smith, J., Rozengurt, E., 2006. RNA interference reveals a differential role of FAK and Pyk2 in cell migration, leading edge formation and increase in focal adhesions induced by LPA in intestinal epithelial cells. J. Cell. Physiol. 207, 816–828.

Jourquin, J., Yang, N., Kam, Y., Guess, C., Quaranta, V., 2006. Dispersal of epithelial cancer cell colonies by lysophosphatidic acid (LPA). J. Cell. Physiol. 206, 337–346.

Kam, Y., Quaranta, V., 2009. Cadherin-bound β-catenin feeds into the Wnt pathway upon adherens junctions dissociation: evidence for an intersection between β-catenin pools. PLoS One 4, e4580.

Lee, H.H., Tien, S.C., Jou, T.S., Chang, Y.C., Jhong, J.G., Chang, Z.F., 2010. Src-dependent phosphorylation of Rock participates of focal adhesion dynamics. J. Cell Sci. 123, 3368–3377.

Leve, F., de Souza, W., Morgado-Díaz, J.A., 2008. A cross-link between protein kinase A and Rho-family GTPases signaling mediates cell–cell adhesion and actin cytoskeleton organization in epithelial cancer cells. J. Pharmacol. Exp. Ther. 327, 777–788.

Lin, S., Wang, D., Iyer, S., Ghaleb, A.M., Shim, H., Yang, V.W., Chun, J., Yun, C.C., 2009. The absence of LPA_2 attenuates tumour formation in an experimental model of colitis-associated cancer. Gastroenterology 136, 1711–1720.

Meyer, T.N., Hunt, J., Schwesinger, C., Denker, B.M., 2003. Galpha12 regulates epithelial cell junctions through Src tyrosine kinases. Am. J. Physiol. Cell Physiol. 285, C1281–C1293.

Mori, K.M.D., Kitayama, J., Shida, D., Yamashita, H., Watanabe, T., Nagawa, H., 2006. Lysophosphatidic acid-induced effects in human colon carcinoma DLD1 cells are partially dependent on transactivation of epifermal growth factor receptor. J. Surg. Res. 132, 56–61.

Müller, R., Berliner, C., Leptin, J., Pörtner, D., Bialecki, W., Kleuser, B., Schumacher, U., Milićević, N.M., 2010. Expression of sphingosine-1-phosphate receptors and lysophosphatidic acid receptors on cultured and xenografted human colon, breast, melanoma, and lung tumour cells. Tumour Biol. 31, 341–349.

Noguchi, K., Herr, D., Mutoh, T., Jerold, C., 2009. Lysophosphatidic acid (LPA) and its receptors. Curr. Opin. Pharmacol. 9, 15–23.

Pertz, O., Hodgson, L., Klemke, R.L., Hahn, K.M., 2006. Spatiotemporal dynamics of RhoA activity in migrating cells. Nature 440, 1069–1072.

Ridley, A.J., Hall, A., 1992. The small GTP-binding protein rho regulates the assembly of focal adhesions and actin stress fibres in response to growth factors. Cell 70, 389–399.

Sah, V.P., Seasholtz, T.M., Sagi, S.A., Brown, J.H., 2000. The role of Rho in G protein-coupled receptor signal transduction. Annu. Rev. Pharmacol. Toxicol. 40, 459–489.

Schlaepfer, D.D., Satyajit, K.M., Ilic, D., 2004. Control of motile and invasive cell phenotypes by focal adhesion kinase. Biochim. Biophys. Acta 1692, 77–102.

Shida, D., Fang, X., Kordula, T., Takabe, K., Lépine, S., Alvarez, S.E., Milstien, S., Spiegel, S., 2008. Cross-talk between LPA1 and epidermal growth factor receptors mediates up-regulation of sphingosine kinase 1 to promote gastric cancer cell motility and invasion. Cancer Res. 68, 6569–6577.

Spaargaren, M., Bos, J.L., 1999. Rab5 induces Rac-independent lamellipodia formation and cell migration. Mol. Biol. Cell 10, 3239–3250.

Sun, H., Ren, J., Zhu, Q., Kong, F.Z., Wu, L., Pan, B.R., 2009. Effects of lysophosphatidic acid on human colon cancer cells and its mechanisms of action. World J. Gastroenterology 15, 4547–4555.

Syed, V., Mak, P., Balaji, K.C., 2008. β-catenin mediates alteration in cell proliferation, motility and invasion of prostate cancer cells by differential expression of E-cadherin and protein kinase D1. J. Cell. Biochem. 104, 82–95.

Webb, D.J., Donais, K., Whitmore, L.A., Thomas, S.M., Turner, C.E., Parsons, J.T., Horwitz, A.F., 2004. FAK–Src signalling through paxillin. ERK and MLCK regulates adhesion disassembly. Nat. Cell Biol. 6, 154–161.

Woodcock, S.A., Rooney, C., Liontos, M., Connolly, Y., Zoumpourlis, V., Whetton, A.D., Gorgoulis, V.G., Malliri, A., 2009. SRC-induced disassembly of adherens junctions requires localized phosphorylation and degradation of the Rac activator Tiam1. Mol. Cell 33, 639–653.

Xu, K.-P., Yin, J., Yu, F.-S.X., 2007. Lysophosphatidic acid promoting corneal epithelial wound healing by transactivation of epidermal growth factor receptor. Invest. Ophthalmol. Visual. Sci. 48, 636–643.

Yamada, T., Yoshiharu, O., Kogo, M., Inagaki, S., 2005. Physical and functional interactions of the lysophosphatidic acid receptors with PDZ domain-containing Rho guanine nucleotide exchange factors (RhoGEFs). J. Biol. Chem. 19, 19358–19363.

Yang, M., Zhong, W.W., Srivastava, N., Slavin, A., Yang, J., Hoey, T., An, S., 2005. G protein-coupled lysophosphatidic acid receptors stimulate proliferation of colon cancer cells through the β-catenin pathway. Proc. Natl. Acad. Sci. U.S.A. 102, 6027–6032.

Yip, S.-C., El-Sibai, M., Coniglio, S.J., Mouneimne, G., Eddy, R.J., Drees, B.E., Neilsen, P.O., Goswami, S., Symons, M., Condeelis, J.S., Backer, J.M., 2007. The distinct roles of Ras and Rac in PI 3-kinase dependent protrusion during EGF-stimulated cell migration. J. Cell Sci. 120, 3138–3146.

Zhang, H., Wang, D., Sun, H., Hall, R.A., Yun, C.C., 2007. MAGI-3 regulates LPA-induced activation of Erk and RhoA. Cell. Signal. 19, 261–268.

Zhao, Z., Xiao, Y., Elson, P., Tan, H., Plumier, S.J., Berk, M., Aung, P.P., Lavery, I.C., Achkar, J.P., Li, L., Casey, G., Xu, Y., 2007. Plasma lysophosphatidylcholine levels: potential biomarkers for colorectal cancer. J. Clin. Oncol. 25, 2696–2701.