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INSIGHTS INTO THE ROLES OF NON-MUSCLE MYOSIN IIA IN HUMAN KERATINOCYTE MIGRATION

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Abstract

Epidermal cell migration is a key factor in wound healing responses, regulated by the F-actin-myosin II systems. Previous reports have established the importance of non-muscle myosin II (NMII) in regulating cell migration. However, the role of NMII in primary human keratinocytes has not been investigated. In this study we used a microfabrication-based two-dimensional migration assay to examine the role of NMII in keratinocyte migration. We developed confluent cell islands of various sizes (0.025 – 0.25 mm²) and quantified migration as Fold Increase in island area over time. We report here that NMII was expressed and activated in migrating keratinocytes. Inhibition of NMIIA motor activity with blebbistatin increased migration significantly in all cell island sizes in six hours compared to control. Inhibition of Rho-kinase by Y-27632 did not alter migration while inhibition of myosin light chain kinase by ML-7 suppressed migration significantly in six hours. Both blebbistatin and Y-27632 induced formation of large membrane ruffles and elongated tails. In contrast, ML-7 blocked cell spreading, resulting in a rounded morphology. Taken together, these data suggest that NMIIA decreases migration in keratinocytes, but the mechanism may be differentially regulated by upstream kinases.

Keywords

Microfabrication; myosin light chain kinase; Rho-kinase; F-actin; lamellar extension; myosin regulatory light chain phosphorylation; soft lithography

INTRODUCTION

Epidermis, the outermost layer of skin is composed of epithelial cells, primarily keratinocytes associated with specialized cells such as Langerhans cells, melanocytes and Merkel cells¹³. In intact epidermis, a layer of basal keratinocytes adhere to the basement membrane via desmosomes and are non-migratory in nature. Above this basal layer are several additional stratified, differentiated layers of keratinocytes referred to as the granular, spinous, and cornified layers⁶. In wounded skin, the keratinocytes polarize rapidly, produce cytoplasmic protrusions (also known as lamellipodia) and migrate into the wound zone. In motile cells, the lamellipodia are formed by polymerized actin filaments¹⁶ and translocation of the cell body is accomplished by contractile forces generated in part by myosin II.

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The myosin family consists of actin-associated motor proteins that can be subdivided into 25 different classes⁴. In non-muscle cells such as fibroblasts, it has been shown that myosin II is maximally activated at the posterior region during migration, thus confirming the role of myosin in retraction of the rear edge²². However, further research indicates that non-muscle myosin II (NMII) is also active at the anterior end of fibroblasts, near the membrane ruffles¹⁵. NMII is known to be expressed as three different isoforms in mammalian cells: IIA, IIB and IIC^{12,21,9}. NMII activity is regulated by phosphorylation of its heavy chains and regulatory light chains (RLC). The effect of RLC phosphorylation by kinases such as myosin light chain kinase (MLCK), Rho-kinase, p21-activated kinase, citron kinase and leucine zipper interacting kinase is well documented in a number of cell lines^{8,1,25,27,17}. In fibroblasts, MLCK-mediated RLC phosphorylation was found to be predominant at the leading edge and cell periphery while Rho-kinase-mediated effect was predominant at the cell centre^{11,24}. These findings imply an intracellular site-specific control of myosin II activity by the regulatory kinases.

Interestingly, these effects seem to be strongly dependent on the type of cells studied. In a Boyden chamber assay, the Rho-kinase inhibitor Y-27632 prevented migration of smooth muscle cells, macrophages and endothelial cells, but not the migration of fibroblasts or HeLa cells¹⁸. The same study showed that Y-27632 treatment could enhance migration of the HeLa cells only in a two-dimensional (2-D) wound healing assay. In intestinal epithelial cell migration, it was found that NMII promotes migration on 2-D surfaces but suppresses invasion through 3-D matrices². Other recent studies investigating the role of NMII in cells of epithelial origin have found contradictory results. Inhibition of NMII by blebbistatin, a chemical inhibitor of myosin II ATPase activity resulted in decreased cell spreading of MDA-MB-231 breast cancer cells³. Pancreatic epithelial cells exhibited lower migration when treated with blebbistatin⁵. In contrast, blebbistatin increased migration of human fibroblasts and mouse embryonic stem cells substantially⁷. Thus, it seems apparent that while the significance of NMII is unambiguous in cell migration, its specific effect not only varies between cells of different origin, but also within those of same origin when evaluated in different assays.

Despite the importance of keratinocyte migration in wound healing responses, the role of myosin II has not been investigated in these cells. The goal of this study was to characterize the expression and activation of myosin II, specifically NMIIA, in primary human keratinocyte migration. The previous studies indicate that the NMIIA isoform is involved in epithelial cell spreading and migration, and is differentially regulated by MLCK and Rho-kinase. Here we provide data that NMIIA is expressed in keratinocytes and is recruited to the lamellar margins in migrating cells. We use a microfabrication-based migration assay to quantify keratinocyte migration on 2-D surfaces. Our data indicates that NMIIA is differentially involved in regulating cell shape and migration, via distinct pathways controlled by MLCK and Rho-kinase. The effect of MLCK inhibition is to suppress migration and lamellar protrusions by reducing NMIIA activation, while inhibition of Rho-kinase as well as inhibition of NMII motor activity results in increased migration and lamellar protrusion.

MATERIALS AND METHODS

Reagents

Anti-Myosin IIA and IIB (nonmuscle) antibodies were purchased from Sigma (St Louis, MO). Phospho-Myosin Light Chain 2 (Thr18/Ser19) antibody was purchased from Cell Signaling Technology Inc (Beverly, MA). Texas Red-X phalloidin and Alexa Fluor-488 conjugated goat anti-rabbit IgG (H+L) antibody were purchased from Invitrogen (Carlsbad, CA). TRITC-conjugated goat anti-rabbit antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The inhibitors used for migration assay: blebbistatin, Y-27632 and ML-7 were purchased from Calbiochem (La Jolla, CA). DAPI (4',6-diamidino-2-phenylindole,

dihydrochloride) was purchased from Invitrogen. Keratinocyte serum free media (KSFM) and human plasma fibronectin were purchased from Invitrogen.

Cell Culture

Human neonatal foreskin samples were obtained from Human Tissue Procurement Facility of the Cancer Center at the University Hospitals, Cleveland, OH. The procedure was approved by the Institutional Review Board at Case Western Reserve University, Cleveland, OH. Primary human keratinocytes were isolated from the foreskin samples using previously described procedure²⁰. Briefly, foreskin samples were kept overnight in 0.5% dispase II solution. Then the epidermis was separated from the dermis and incubated in 0.05% trypsin for 5 minutes. Bovine serum was added to the suspension and centrifuged. After removing the supernatant, keratinocytes were plated in KSFM and allowed to grow till confluent. Keratinocytes were routinely cultured in KSFM in a humidified atmosphere at 37°C and 5% CO₂. Media was changed once every 2–3 days and the cells were used between passages 1 and 2 in all the experimental studies.

Fabrication of PDMS membranes by soft lithography

Polydimethylsiloxane (PDMS) membranes were obtained using a standard combination of photolithography and soft lithography techniques. Briefly, AUTOCAD (Autodesk, Inc., San Rafael, CA) designs of microarrays (smallest feature: 158 µm) were used to obtain photomasks (Advance Reproductions, North Andover, MA). The array consisted of square shaped patterns in four different sizes: 0.025–0.25 sq.mm. Silicon wafers were spin-coated with a negative photoresist SU-8 2075 (Microchem Corp, Newton, MA). The photoresist was then processed with the photomask to produce a “master” template containing features of ~200 µm height. The polymer PDMS (Sylgard 184, Dow Corning, Midland, MI) was used to create molds of the master by spin-coating and curing at 80°C. The resulting membranes were about 100 µm thick. The membranes were attached to a 0.4 cm thick PDMS ring for easy handling and stability.

Development of cell islands

The membranes were sterilized in 200-proof ethanol (Fisher Scientific, Pittsburgh, PA) overnight, then transferred to a fresh petri dish (P-60 BD Falcon, Franklin Lakes, NJ) and allowed to attach. The membranes were wetted with 1X PBS under vacuum and blocked with 1% bovine serum albumin (Sigma Aldrich). 1.5×10^6 keratinocytes were seeded on the membrane and allowed to attach overnight or longer in order to form confluent “cell islands”. The PDMS membranes were then removed and cell islands washed with phosphate buffered saline (PBS, Invitrogen). For cell migration studies on fibronectin (20 µg/ml, diluted in KSFM), the following steps were added: (i) incubation of cell-contact surfaces before cell seeding with the fibronectin solution for 1 hour (hr) at 37°C for 1 hour at 37°C and 5% CO₂ and (ii) incubation of cell islands and migratory surfaces in the petri dish after membrane removal for 1 hr with the solution under the same conditions. The fibronectin solution was then aspirated out and fresh culture media added to the cells.

Cell migration assay

Two-dimensional cell migration from well-formed confluent cell islands was studied in 60 mm petri dishes for 6 hours. Islands were imaged at $t = 0$ hrs (initial time point) and at $t = 6$ hrs (end time point). We chose a 6 hour end time point as this represented the optimal time point that minimized cell proliferation effect on island expansion (since the doubling time for keratinocytes in culture is ~24 hours). For inhibitor migration assay, cell islands were exposed to the following concentration of inhibitors: blebbistatin (30 µM), ML-7 (10 µM) and Y-27632 (5 µM). The experimental endpoint was fixed at $t = 6$ hrs from the time of removal of the

membrane in all studies, so to maintain consistency, inhibitors were incubated for 5 hrs after the 1 hr fibronectin incubation. Phase contrast images of the cells islands were taken with an Olympus IX71 inverted microscope (Olympus, Japan) using UPlanFl 10× and LCPlanFl 20× objectives.

Immunocytochemistry and Confocal microscopy

Keratinocytes cell islands grown on BD Falcon 60 mm petri dishes were fixed with a fixing buffer of 4% paraformaldehyde, 2 mM MgCl₂ and 2 mM EGTA in PBS for 20 followed by permeabilization with 0.5% Triton X-100, 2 mM MgCl₂ and 2 mM EGTA for 10 minutes. The cells were washed with PBS and blocked with a buffer composed of 1% BSA in Tris buffered saline (TBS) with 0.1% Tween for 20 minutes. The primary antibodies were diluted in blocking buffer as follows: anti-NMIIA (1:500), anti-NMIIB (1:100) and anti-pRLC (1:100) and incubated for 2 hours. The secondary antibodies were diluted 1:1000 in blocking buffer and incubated with the cells for 1 hour. F-actin visualization was done by diluting phalloidin in PBS (1:40) and incubating with the cells for 20 minutes. The cell nuclei were labeled with DAPI at 300 nM final concentration for 10 minutes. Coverslips were mounted on the petri dishes with Vectashield mounting media (Vector Laboratories, Burlingame, CA). All procedures were performed at room temperature. Immunofluorescence microscopy images were obtained with an upright Leica TCS-SP-AOBS Spectral Laser Scanning confocal microscope (Leica Microsystems, GmbH, Wetzlar, Germany) using a HCX PL APO 63x oil objective.

Data Analysis

The ImagePro software (Media Cybernetics, Inc., Bethesda, MD) was used to quantify the initial and final island areas. The area occupied by each cell island was measured immediately after the removal of PDMS membrane (referred to as $t = 0$ hrs) and then after 6 hours of migration ($t = 6$ hrs). Migration was quantified as the area of island at any given time normalized to the initial area, with margins of cell islands tracked manually. This parameter was termed Fold Increase and expressed as mean $\pm$ SEM of at least 9 cell islands ($n = 9$) from three experiments. Island data for each size was obtained in triplicate from each experiment. Statistical analysis of the data was performed using Student's t -distribution. A p -value of <0.05 was considered as statistically significant.

RESULTS

Characterization of keratinocyte motility in microfabrication-based migration assay

Keratinocyte islands were established on fibronectin and uncoated surfaces. Fig. 1A shows that the soft-lithography method produced arrays of confluent cell islands. We were able to form four different square islands of sizes 0.025, 0.0625, 0.125, and 0.25 mm² using this technique (Fig. 1A and B). Fig. 1C shows the effect of fibronectin on 0.25 mm² island. The cells plated on uncoated surface (tissue culture plastic) migrated little while those cultured on fibronectin migrated considerably more in 6 hours. The cells on fibronectin spread out, formed lamellar extensions with distinctive leading and trailing edges while those on the untreated surface were rounded with minimal polarization. Fig. 2 shows quantitatively the effect of initial island area and fibronectin on keratinocyte migration. Cells attached to fibronectin showed significantly higher migration (defined as Fold Increase in island area at 6 hrs) from most islands compared to control (Fig. 2) ($p < 0.05$ for 0.0625–0.25 mm² islands). Cell islands with larger initial areas (0.125 and 0.25 mm²) showed greater increase in island area.

Expression, localization and activation of myosin isoforms in keratinocytes

To compare the distribution of NMIIA isoform in motile and non-motile cells, keratinocytes were either fixed after 6 hrs of migration or with the PDMS membranes *in situ* to ensure that no migration occurred. As expected, non-motile keratinocytes showed no lamellar spreading (Fig. 3A, top panel). Both NMIIA and F-actin expression was diffuse throughout the cytosol, with some enrichment at the edges of the cells but with no distinction of polarity, concomitant with the static nature of the cells. Similar results were obtained in cells grown on tissue culture plastic surfaces (data not shown). There was very limited RLC phosphorylation, consistent with the diffuse staining for NMIIA in non-migratory cells (Fig. 3A, bottom panel). In contrast, NMIIA was strongly recruited to the periphery of the migrating cells observed 6 hours after the removal of PDMS membranes (Fig. 3B, top panel). The cells also expressed RLC phosphorylation colocalized with NMIIA recruitment at the edges (Fig. 3B, bottom panel). Interestingly, the cells that were in contact with other cells showed very little RLC phosphorylation at the contact margins. Cells within the islands showed almost no phosphorylation at the contact margins while cells at the island periphery showed RLC phosphorylation at the free edges (Fig S1, supplementary data). We observed the above effects on both fibronectin-treated and untreated surfaces.

Since mammalian cells are known to express multiple isoforms of NMII, we stained keratinocytes for NMIIIB isoform as well. NMIIIB was expressed at the cell margins but to a lesser extent than NMIIA (Fig S2, supplementary data). Previous studies using epithelial cells have shown that NMIIA is the dominant isoform expressed in these cells, and given our observations, subsequent experiments were focused on determining NMIIA activity in keratinocytes migrating on fibronectin-treated surfaces.

Quantitative evaluation of myosin II, MLCK and Rho-kinase inhibition on keratinocyte migration

To determine the impact of NMII on keratinocyte migration, cells were treated with blebbistatin, an inhibitor of NMII ATPase activity. Treatment with blebbistatin (30 μ M) significantly increased migration (defined as Fold Increase in island area at 6 hrs) on fibronectin ($> \sim 3$ fold expansion compared to ~ 1.5 fold without blebbistatin) (Fig. 4A). The effect was consistently observed in islands of all sizes (Fig. 4B). Blebbistatin induced significant changes in keratinocyte morphology, creating large ruffled edges and long tails (Fig. 4A, 5B). Since NMII activity is regulated by the upstream kinases Rho-kinase and MLCK, keratinocytes were treated with the respective inhibitors Y-27632 and ML-7. The effect of Rho-kinase inhibitor Y-27632 (5 μ M) was morphologically similar to that of blebbistatin (Fig. 4A, 5D). Y-27632 induced ruffling at the cell edges and tail formation in some of the cells. Quantitatively, Y-27632-treated cells showed migration characteristics similar to control cells (Fig. 4B). In contrast, the effect of MLCK inhibitor ML-7 (10 μ M) was to suppress cell spreading and migration severely (Fig. 4B). In two of the island sizes (0.125 and 0.25 mm²), there was significant suppression of migration compared to control ($p < 0.05$). Cells treated with ML-7 did not form any lamellar protrusions, appearing nearly spherical in shape (Fig. 4A, 5C). All of the above quantitative effects were also observed on untreated surfaces (Fig S3, supplementary data).

Effect of blebbistatin on NMIIA localization

Immunocytochemical staining of keratinocytes (at $t = 6$ hrs) treated with blebbistatin revealed that the large membranous protrusions of the cells contained extensive F-actin filaments at the lamellipodia periphery, immediately followed by a NMIIA-rich zone and actomyosin fibers in the elongated tails (Fig. 5B). Blebbistatin treatment reduced NMIIA recruitment at the leading edge, but failed to suppress NMIIA localization at the trailing edge or in the central region of

the cells. In most cells RLC phosphorylation was not observed in the anterior ruffled edges, but some expression was retained at the lateral and posterior edges of the cells.

Effect of Rho-kinase and MLCK inhibition on NMIIA localization

Given that ML-7 strongly suppressed cell migration, the effect of MLCK inhibition on NMIIA distribution and RLC phosphorylation was analyzed. NMIIA was found diffusely localized in the keratinocytes. A thin band of F-actin was expressed around the periphery of the cells (Fig. 5C). Large lamellar protrusions were not observed in these cells. Correlating with the migration data, RLC phosphorylation was almost completely suppressed in the cells.

The Rho-kinase inhibitor Y-27632 has been previously shown to decrease RLC phosphorylation effectively, although its effect was more potent at the cell center as compared to cell edges²⁴. Y-27632 did not significantly affect migration in keratinocytes, but it induced formation of ruffled protrusions in some cells. This implied a negative control by Rho-kinase on migration, possibly by suppression of NMII activity at the anterior end of the cells, or by alterations of other lamellar pathways downstream of Rho kinase such as the LIM kinase/cofilin pathway²⁶. Y-27632 –treated cells were immunostained with NMIIA to determine its localization and distribution of RLC phosphorylation. Migrating keratinocytes treated with Y-27632 showed an overall reduction in NMIIA localization at the peripheral edges, although all of the cells depicted a diffuse distribution throughout the cytosol (Fig. 5D). RLC phosphorylation was reduced at the ruffled edges and was more localized at the cell centre as well as at the posterior edges of the cells. Overall, the suppression of RLC phosphorylation was less than that observed in ML-7 cells, suggesting that the MLCK may play a greater role in RLC activation than Rho kinase in this setting.

DISCUSSION

In this study, we focused on the role of NMIIA in regulating migration of keratinocytes on 2-D surfaces. Resting keratinocytes undergo a rapid change in cytoskeletal organization and expression of transmembrane integrins upon induction of migration. Migrating keratinocytes upregulate fibronectin and vitronectin binding integrins $\alpha 5\beta 1$, $\alpha v\beta 5$ and $\alpha v\beta 6$ in addition to the existing collagen and laminin binding integrins¹⁴. This study showed that keratinocytes migrate more and become morphologically polarized on fibronectin when compared to control. This was accompanied by both NMIIA and NMIIIB accumulation at the edges of the cells. Non-motile keratinocytes showed NMIIA randomly distributed in the cytoskeleton in inactive form, as demonstrated by the lack of RLC phosphorylation. In contrast, migrating cells both on tissue culture plastic and on fibronectin showed RLC phosphorylation at the periphery along with F-actin bundles. This localization was observed at both leading and trailing edges of cells.

The microfabrication-based migration assay highlighted the effect of cell-cell interaction on migration of keratinocytes. The larger cell islands migrated more than the smaller islands on fibronectin. Smaller cell islands have larger perimeter to area ratio and were expected to migrate more since migration occurs primarily from the periphery. However, epithelial cell migration is also known to be density dependent. Smaller islands have less cell-cell interaction than the larger islands. Therefore, this effect is possibly due to the characteristic epithelial migration observed in keratinocytes. Interestingly, RLC phosphorylation was limited in areas of cell-cell contact. Many of the islands depicted RLC phosphorylation only at the edges and not at the center of the island, where cell-cell contact is maximal. Correlating NMII activity with migration, it could be hypothesized that NMIIA-mediated cell migration is down-regulated by cell-cell contact in this migration model (or conversely, cell-cell contact down-regulates NMIIA).

NMIIA activity is known to either increase or decrease cell migration depending on the cell line being studied. Pharmacological inhibition of myosin II motor activity by blebbistatin increased migration of fibroblasts and fibrosarcoma cells^{7,19} but decreased 2-D migration of intestinal epithelial and breast cancer cells^{2,3}. In keratinocytes, migration on 2-D surfaces was significantly increased by blebbistatin. The migration was generally oriented outward from the island core. The morphological changes induced by blebbistatin in keratinocytes—flattened cell bodies, large membranous ruffles, and extremely long tails have also been observed in other cell lines. The localization of NMIIA shifted from the cell periphery to two distinct locations: the region behind the extended lamellipodia and at the posterior edges of the membrane ruffles, and in the protracted tails. Blebbistatin did not interfere with F-actin localization, as observed in the extensive microfilament formation at the anterior ruffles. The only regions of cells displaying significant RLC phosphorylation were the posterior edges of the cells. Taken together, this data suggests that NMII restricts migration as well as the formation of membrane protrusions in keratinocytes. Since blebbistatin inhibits both NMIIA and IIB, further studies with siRNA knockdown of IIA and IIB could determine whether the contribution of NMII is isoform-specific in keratinocytes. Previous studies have used siRNA depletion to show that NMIIA-depleted cells exhibit enhanced migration in human fibroblasts and lung carcinoma (A549) cells while NMIIIB-depleted cells either did not migrate at higher velocities or showed reduced migration^{7,23}. Cell morphology is also differentially regulated by the two isoforms, with NMIIA-depleted breast cancer and lung cancer cells showing increased cell spreading while NMIIIB-depleted cells showed a rounded phenotype^{3,23}. Since both isoforms are expressed in keratinocytes, it is possible that they may have specific roles in determining migration and cell spreading.

The inhibition of MLCK, a kinase that regulates myosin II function by phosphorylating RLC, resulted in suppression of migration. Treatment of the cells with ML-7 induced a rounded phenotype with no lamellar extensions. Concurrently, RLC phosphorylation was strongly suppressed in a large majority of the cells. NMIIA localization studies revealed a diffuse distribution in the cells. These findings are consistent with studies showing the loss of polarity caused by ML-7¹⁰ as well as the decrease in migration in multiple cell types^{3,19}. We conclude that MLCK has key roles in keratinocyte migration, particularly in allowing lamellar protrusion and spreading to occur.

Rho-kinase inhibitor Y-27632 did not change migration significantly but it promoted the formation of large lamellar protrusions in keratinocytes. However, the change in cell morphology was not as dramatic as that induced by blebbistatin. Immunostaining showed that Y-27632 reduced NMIIA recruitment at the cell periphery. RLC phosphorylation was also greatly reduced at the cell periphery, consistent with the notion that Y-27632 induces formation of broad lamellipodial protrusions that are generally devoid of significant amounts of NMII. Thus, we can conclude that Rho-kinase limits membrane protrusion in keratinocytes but does not alter migration significantly in a 2-D assay.

In summary, NMIIA is involved in controlling migration and cell spreading in keratinocytes, and its activity is regulated by both Rho-kinase and MLCK pathways, though in contradictory manner. MLCK-mediated NMIIA activity is essential in promoting lamellipodia formation as well as migration. Rho-kinase activity and/or full levels of NMIIA motor activity tend to favor static, non-migratory behavior, with reduced levels of membrane protrusions.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.

Keratinocyte island migration on plastic on fibronectin (Fn). (A) Keratinocyte cell island array at $t = 0$ hrs. The islands shown here are square islands of size 0.025 mm^2 . (B) Individual square islands of sizes 0.0625 mm^2 , 0.125 mm^2 and 0.25 mm^2 at $t = 0$ hrs. (C) A typical 0.25 mm^2 cell island after 6 hrs of migration on untreated (control) and fibronectin - coated (Fn) surface. Scale bar: $100\mu\text{m}$.

Figure 2.

Quantification of island migration on untreated surfaces and fibronectin (Fn). Migration is defined as Fold Increase in island area (at $t = 6$ hrs) normalized to the initial island area (at $t = 0$ hrs). Cells on Fn migrated significantly more compared to control. Data is presented as mean Fold Increase in island area $\pm$ SEM for $n = 9$ islands from 3 experiments.

Figure 3.

Expression and activation of Non-Muscle Myosin IIA in non-migratory and migratory keratinocytes. (A) Expression of NMIIA and F-actin in non-migrating keratinocytes. Cell islands developed on Fn were fixed with PDMS membranes *in situ* (at $t = 0$ hrs). The cells were labeled with the following: NMIIA (green)/phalloidin (red) or pRLC (green)/phalloidin (red). Top: NMIIA was distributed diffusely in the cytosol. Bottom: pRLC expression was not found in these cells. F-actin distribution is also diffuse in the cells. (B) Expression of NMIIA and F-actin in migrating keratinocytes. After 6 hrs of migration on Fn, cells were fixed and labeled with NMIIA (green)/phalloidin (red) or pRLC (green)/phalloidin (red). Top: NMIIA was recruited to the edges in migrating cells (block arrow). Bottom: pRLC expression

(indicated by block arrow) was observed specifically at the cellular edges not in contact with other cells (cell-cell contact area indicated by line arrow). F-actin is localized at the cellular edges. Cell nuclei were labeled with DAPI (blue). Scale bar: 20 μ m.

Figure 4.

Effect of inhibitors on island migration. (A) Confluent cell islands were treated with blebbistatin (30 μ M), ML-7 (10 μ M) or Y-27632 (5 μ M) as described in Materials and Methods. Migration of a typical 0.25 mm² cell island on Fn at 6 hrs is shown here for each condition. Fn (control) indicates cells grown on fibronectin without any inhibitors. Scale bar: 100 μ m. (B) Quantification of island migration (at t = 6 hrs) on Fn induced by inhibitors. Migration is shown as normalized Fold Increase in island area after 6 hrs. Blebbistatin induced significant increase in keratinocytes migration (* Indicates $p < 0.05$ compared to control).

Figure 5.

Impact of inhibitors on NMIIA localization and phosphorylation (t = 6 hrs). The left panels show visualization of F-actin (red), NMIIA (green) and DAPI (blue). The right panels show pRLC (green) and DAPI. (A) Control cells grown on Fn without any inhibitors. (B) Blebbistatin-treated cells showed large F-actin rich ruffles (block arrow) followed by polarized NMIIA bands (*) and actomyosin-rich tails (line arrow). pRLC expression was observed at the cell edges (arrowhead). (C) ML-7- treated cells showed narrow F-actin bands at the cell edges and diffuse NMIIA staining. pRLC expression was very low. (D) Y-27632 treated cells showed smaller F-actin- rich membrane ruffles (block arrow) with NMIIA distributed through the cell

body (*) and some polarization toward the posterior edge of the cell. pRLC expression was strongly localized at the cell centre and trailing edges of the cells (arrowhead). Scale bar: 20µm.