Published in final edited form as:

J Soc Gynecol Investig. 2006 December ; 13(8): 579–591.

Non-muscle myosin-II-B filament regulation of paracellular resistance in cervical epithelial cells is associated with modulation of the cortical acto-myosin

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Abstract

Objective— To understand myosin regulation of epithelial permeability.

Methods— Experimental study, using human cervical epithelial cells CaSki. Endpoints were paracellular permeability (determined in terms of transepithelial electrical resistance); non-muscle myosin-II-B (NMM-II-B) cellular localization; NMM-II-B phosphorylation status; NMM-II-B – actin interaction (determined in-vitro by the immunoprecipitation-immunoreactivity method); and NMM-II-B filamentation (determined in-vitro using purified NMM-II-B filaments in terms of filaments disassembly / assembly ratios.

Results— Treatment of cells with the ROCK inhibitor Y-27632 or with the phosphatase inhibitor okadaic acid decreased the Resistance of the Lateral Intercellular Space (R_{LIS}), and increased phosphorylation of non-muscle myosin-II-B (NMM-II-B) on threonine and serine residues. Y-27632 induced disorganization of the cortical acto-myosin and decreased co-immunoprecipitation of actin with NMM-II-B. Homodimerization assays using NMM-II-B filaments from cells treated with Y-27632 or okadaic acid revealed decreased filamentation compared to control cells. However, okadaic acid blocked Y-27632 decreased filamentation. Treatment with DRB, CK2 inhibitor, induced opposing effects to those of Y-27632 and okadaic acid. Treatment with DRB did not involve modulation of actin depolymerization, suggesting that NMM-II-B regulation of the R_{LIS} was independent of actin polymerization status. Exposure of NMM-II-B filaments to CK2 increased filamentation, regardless of prior treatments in-vivo with Y-27632, okadaic acid, or DRB.

Conclusions— The results suggest that NMM-II-B filaments are in steady-state equilibrium of phosphorylation-dephosphorylation mediated by CK2 and by ROCK-regulated myosin heavy chain phosphatase, respectively. Increased phosphorylation would tend to inhibit assembly of NMM-II-B filaments and lead to decreased actin-myosin interaction, which would tend to decrease the R_{LIS} and increase the paracellular permeability.

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Précis

In CaSki cells, phosphorylation status of NMM-II-B filaments is mediated by CK2 and ROCK-regulated myosin heavy chain phosphatase, and determines homodimerization characteristics of the filaments.

The study was supported by NIH grants HD29924 and AG15955 (GIG).

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Keywords

Cytoskeleton; dynamic; permeability; actin; human; cervical; vaginal

INTRODUCTION

Epithelial cells of the uterine cervix regulate secretion of fluid that lubricates the cervical and vaginal canals. The product of transcervical transport, cervical plasma, is important for human reproduction and for woman's health. Cervical epithelia, like other types of secretory epithelia, are organized as layers of confluent cells, where plasma membranes of neighboring cells come into close contact and functionally occlude the intercellular space. Molecules can move across epithelia either through the cells (transcellular route), or via the intercellular space (paracellular route). Human cervical cells form relatively leaky types of epithelia, and their overall permeability properties are determined by the paracellular route. Subsequently, the paracellular (intercellular) pathway is the major route of transcervical epithelial transport.

Free movement of water and solutes in the paracellular pathway is restricted by the Resistance of the Tight Junctions (R_{TJ}) and by the Resistance of the Lateral Intercellular Space (R_{LIS}), in series [1,2]. In most epithelia, the R_{TJ} determines the overall paracellular resistance, but in leaky epithelia such as the vaginal and cervical epithelia, the contributions of the R_{LIS} to the total resistance can be significant [3].

The R_{TJ} is determined by the tight junctional complexes that are usually located at apical regions of the cells (facing the lumen) above the zonula adherence and desmosomes. The tight-junctions extend in a belt-like manner around the perimeter of each epithelial cell and connect neighboring cells, thereby effectively occluding the intercellular space. The resistance to the free movement of molecules that is associated with the tight junctions is considered a high-resistive element and is termed the R_{TJ} . The second mechanism that gates the intercellular (paracellular) space is a resistance that is determined the proximity of neighboring epithelial cells. Thus, cells that are closer to each other result in formation of a lesser lateral intercellular space, while cells that are pulled apart result in formation of a greater lateral intercellular space. The resistance of the lateral intercellular space (R_{LIS}) is considered a low-resistive element, and it can be modeled by Poiseuille's law as series of narrow tubes so that the resistance of each tube depends on the length of the intercellular space from the tight junctions to the basal lamina (height of the epithelial cells), and reciprocally on the fourth power of the radius of the intercellular width. Mechanistically, the latter is determined by the proximity of the plasma membranes of neighboring cells such that even minor changes in cell volume can affect the lateral intercellular space and markedly affect the R_{LIS} .

The geometry of the intercellular space is determined reciprocally by the width and shape of the epithelial cells that define this space. The shape of epithelial cells depends mainly on the elasticity of the actin cytoskeleton: cells expressing a rigid cytoskeleton will change their shape less readily in response to stimuli than cells expressing a dynamic cytoskeleton. The actin cytoskeleton can be modulated by regulation of actin polymerization. Formation of G-actin – dominated cytoskeleton decreases the R_{LIS} , while actin polymerization (F-actin) produces rigid cytoskeleton and increases R_{LIS} [4,5].

The actin cytoskeleton can be also modulated by regulation of myosin filaments [6]. Myosins are proteins that interact with actin and hydrolyze ATP to generate force. Most of the knowledge about myosin-actin interaction has been derived from studies in muscle cells [7]. Studies in non-muscle cells showed that myosin-II, the prototypical two-headed myosin regulates actin organization into filaments that maintain the shape of cellular structures or

coordinate contraction for locomotion and cell division [7]. The non-muscle myosin-II, a subclass of myosin-II [8], was shown to play a role in epithelial cell attachment and intercellular communications [9]. In the human, the state of myosin-II activation regulates paracellular permeability in kidney cells [10,11], and abrogation of paracellular resistance leads to backleak of glomerular filtrate [7]. In patients with inflammatory bowel disease, colonic epithelial myosin light-chain kinase (MLCK) expression and enzymatic activity were found to be increased, suggesting that MLCK upregulation may contribute to barrier dysfunction and the pathogenesis inflammatory bowel disease [12]. These reports suggest a role for the cytoskeletal acto-myosin cortex in the regulation of epithelial cell shape and paracellular resistance. However, relatively little is known about the cellular and molecular mechanisms involved; very few studies researched the field of myosin regulation of epithelial permeability, and most mechanistic studies in the field were published more than decade ago.

The objective of the present study was to better understand how the acto-myosin regulates the rigidity of the cytoskeleton in epithelial cells, and hence the epithelial paracellular resistance. The experiments focused on the hexameric non-muscle myosin-II types A and B that are composed of two pairs of light chains that associate with two pairs of heavy chains, and which are most abundant in epithelial cells [13,14]. The experimental model was the cultured human epithelium CaSki, which has been previously characterized as relatively leaky type of epithelium [15,16]. The results suggest that non-muscle myosin-II B (NMM-II-B) filaments are constitutively and directly phosphorylated by casein kinase-II (CK2), and de-phosphorylated through the concerted actions of Rho-associated kinase (ROCK) – regulated myosin heavy chain phosphatase. Increased phosphorylation is associated with decreased filamentation of NMM-II-B filaments in-vitro and with lesser myosin-actin interaction. The results also suggest that decreased myosin-actin interaction may lead to formation of a dynamic cytoskeleton, resulting in a decrease in the R_{LIS} .

METHODS

Cell-Cultures

Experiments utilized the previously characterized cultured CaSki cell model, which is a stable line of transformed cervical epithelial cells [15,16]. CaSki cells were grown and subcultured in culture dish in RPMI-1640 supplemented with 8% fetal calf serum (FCS), 0.2% NaHCO_3 , nonessential amino acids, L-glutamine (2 mM), penicillin (100 U/ml), streptomycin (100 mg/ml) and gentamycin (50 $\mu\text{g}/\text{ml}$) at 37°C in 91% O_2 /9% CO_2 humidified incubator, and routinely tested for mycoplasma. Experiments were done on cells grown on cover-slips or on filters. For filter experiments cells were plated on Anocell (Anocell™-10) filters (Oxon, UK, obtained through Sigma Chemicals, St. Louis, MO), which are ceramic-base filters, pore size of 0.02 μm width and 50 μm depth. Filters were coated on their upper (luminal) surface with 3–5 $\mu\text{g}/\text{cm}^2$ collagen type IV. Filter experiments involved treatments with drugs to both the luminal and subluminal solutions.

Changes in Paracellular Resistance were determined in terms of changes in the Transepithelial Electrical Resistance (R_{TE}) as described [17]. Briefly, cells on filters were shifted to a modified Ringer buffer and mounted in a modified Ussing chamber. Drugs were added to both the luminal and subluminal solutions, and changes in paracellular permeability were determined in terms of changes in R_{TE} from successive measurements of the transepithelial potential difference (ΔPD , lumen negative), and the transepithelial electrical current (ΔI , obtained by measuring the current necessary to clamp the offset potential to zero, and normalized to the 0.6 cm^2 surface area of the filter) as $R_{TE} = \Delta\text{PD}/\Delta\text{I}$. Changes in the tight junctional resistance (R_{TJ}) were determined in terms of changes in the relative mobilities of Na^+ and Cl^- in the intercellular space ($U_{\text{Cl}}/U_{\text{Na}}$) from measurements of the dilution potentials. The experimental design of the electrophysiological measurements, including calibrations and

controls, the significance of the ΔPD and ΔI , and the conditions for optimal determinations of R_{TE} across low resistance epithelia, e.g. CaSki, were described and discussed [17].

Immunostaining, light microscopy and fluorescence microscopy were described [18].

Briefly, following treatments, cells attached on cover-slips or on filters were washed with cold PBS and fixed in methanol; blocked with 3% BSA / PBS / 0.2% Triton X-100 for 30 minutes; incubated with primary antibodies overnight at 4°C; washed three times with PBS, and incubated with secondary antibodies attached with Alexa Fluor 488 (donkey anti-rabbit IgG) or Alexa Fluor 594 (goat anti-mouse). Nuclei were stained with DAPI (Vector, Burlington, CA). In some experiments cells were incubated with HRP-labeled goat anti-rabbit IgG, heavy and light chain peroxidase, and the reaction was visualized by Fast-Red (Dako, <http://www.dakocytomation.com>).

Cell-fractionation by the Freeze / Thaw Method and **Western immunoblotting** were described [18]. Confirmation of the separation efficiency of the membrane-enriched fraction was determined by blotting with anti-occludin antibody (tight junction plasma-membrane protein). Likewise, confirmation of separation efficiency of the cytosolic fraction was determined by blotting with anti-GAPDH antibody. Aliquots, normalized to 15 μ g protein, were fractionated by 6–10% SDS-polyacrylamide gel electrophoresis and blotted by Western analysis, and bands were analyzed semi-quantitatively by densitometry.

Immunoprecipitation / immunoblotting assays utilized standard techniques that were recently described [18].

Phosphorylation Assays were described [18]. Briefly, cells on filters were shifted to phosphate-free DMEM containing 10mM HEPES, pH 7.4 at 37°C, and treated with 100 μ Ci/ml [32 P]orthophosphate (PerkinElmer Life Sciences, Boston MA) plus 1 μ g/ml microcystin L-R (Calbiochem, San Diego, CA). After treatments, cells were washed with ice-cold PBS, lysed in lysis buffer and processed by immunoprecipitation with anti non-muscle myosin-II heavy-chain-B (NMM-II-HC-B) antibody. Samples containing equal amounts of protein were resolved on 10% polyacrylamide gels and dried under vacuum. Radioactive bands were visualized by PhosphorImager (Molecular Dynamics [Amersham], Piscataway, NJ) and by exposure to x-ray film.

Extraction and purification of NMM-II-B filaments were done using a modification of existing methods [19,20]. All steps were done at 4°C. Cells pooled from 5–7 filter inserts were used to generate membrane-enriched and cytosolic fractions. Fractions were incubated overnight with anti non-muscle myosin-II heavy-chain-A (NMM-II-HC-A) antibody to immunoprecipitate the non-muscle myosin-II-A (NMM-II-A) filaments. Pansorbin was added to the immunoprecipitates for 2 hrs and the Pansorbin – anti NMM-II-HC-A antibody – NMM-II-A complexes were pelleted by centrifugation at 25,000g for 15 min. Since the amount of NMM-II-B remaining in the pellets was minimal (<5%, not shown), the pellets were discarded. The supernatants were washed three times with buffer containing 0.5 M NaCl, 50 mM MOPS, pH 7.4, and 0.1 mM EGTA to disassociate oligomerized filaments and to remove nonspecifically bound proteins. Supernatants were resuspended in 0.2 ml of wash buffer containing 50-fold molar excess of the peptide used to generate the anti human NMM-II-A antibody (GKADGAEAKPAE, corresponding to the C-terminus of the human NMM-II-HC-A), and after 2 hrs of incubation on ice, mixtures were spun at 35,000g for 15 min and the supernatants containing the released NMM-II-B were dialyzed against several changes of wash buffer and concentrated to a volume of about 250 μ l by washing in Centricon 100 (Amicon, Houston, TX).

Dynamic disassembly / assembly of NMM-II-B filaments was determined in real-time by measuring turbidity at 340 nm. Aliquots of purified NMM-II-B filaments extracted from

membrane and cytosolic fractions (25 μ l of 5 μ M) were transferred to a cuvette of 2 mm width that was placed in a spectrometer, containing 0.3 ml of solution composed of 10 mM imidazole-HCl (pH 7.5), 2 mM EGTA, 10 mM NaCl and 2.5 mM Mg^{2+} at room temperature. Under these conditions NMM-II-B filaments assemble into homodimers, and the absorbance at 340 nm is close to 1.0 [21,22]. Disassembly of NMM-II-B filaments into monomers was induced by increasing Ca^{2+} concentration in the solution to 1.2 mM by adding aliquots from a concentrated $CaCl_2$ solution. The endpoint was the half time ($t_{1/2}$) of the decrease in absorbance from 1.0 to 0 upon adding Ca^{2+} , calculated by fitting the data to an exponential. The reversibility of the effect (i.e. filaments assembly) was determined by adding EGTA to chelate Ca^{2+} .

In-vitro NMM-II-B filament assembly assay

Purified NMM-II-B filaments at concentrations of 1–500 μ g/ml (about 20 nM – 10 μ M) were incubated with 2.5 mM Mg^{2+} and 10 mM NaCl at 4°C in the presence of 10 mM imidazole-HCl (pH 7.5) and 0.1 mM Ca^{2+} to induce filament assembly. At the completion of incubations samples were centrifuged at 25,000g for 30 min at 4°C, and the protein content in the supernatant (i.e. non-dimerized NMM-II-B) was measured and expressed per total protein measured before centrifugation as the % non-dimerized NMM-II-B. To ascertain complete recovery of non-dimerized filaments, aliquots from the supernatant were centrifuged at 90,000g for 20 min. Assays where sediment was found, indicating inappropriate separation of the monomers from the oligomers, were omitted from analysis (less than 5% of assays). The degree of NMM-II-B filament assembly was expressed in terms of the critical concentration of NMM-II-B necessary to induce dimerization from the curve of [% non-dimerized NMM-II-B] Vs. [Added Protein]. The concentration of NMM-II-B that induced maximal dimerization was defined as the *critical concentration* of NMM-II-B necessary for dimerization. Some experiments studied the direct effect of CK2 on NMM-II-B filament assembly. In those experiments purified NMM-II-B filaments were exposed to 0.2 μ g per 100 μ l of purified CK2 from rat liver (Sigma, dissolved in PBS), or to heat-inactivated (65°C) CK2.

Cellular G-actin content was determined by the DNase-I Inhibition Assay as previously described [4,5]. Briefly, cells on filters were lysed in situ and DNase-I activity in the lysate was assayed by measuring DNase-I-dependent degradation of DNA. Total actin was measured by the guanidine-HCl method after depolymerization of F-actin to monomeric G-actin.

Cellular DNA and Total Protein were measured as described [23].

Antibodies

Mouse monoclonal antihuman β -actin antibody was from Zymed Laboratory Inc., (San Francisco, CA). Rabbit polyclonal antihuman NMM-II-HC-A antibody (PRB-440P) and its antigenic peptide, and the antihuman non-muscle myosin-II heavy-chain-B (NMM-II-HC-B) antibody (PRB-445P) were from Covance Research Products (Berkeley, CA). Mouse monoclonal antihuman phosphoserine, phosphothreonine, and phosphotyrosine PY20 and P99 antibodies were from Transduction Laboratories (BD Biosciences, San Jose, CA). The antibodies were used according to the manufacturers' instructions.

Chemicals and supplies

All chemicals, unless specified otherwise, were obtained from Sigma Chemical (St. Louis, MO). 5,6-dichloro-1- β -(D)-ribofuranosylbenzimidazole (DRB) and Y-27632 were obtained from Calbiochem, La-Jolla, CA).

Statistical analysis of the data

Data are presented as means ($\pm$ S.D.) and significance of differences among means was estimated by ANOVA using GB-STAT (Dynamic Microsystems Inc., Silver Spring, MD).

RESULTS

Modulation of R_{TE}

Baseline R_{TE} across CaSki cells was about $20 \Omega \cdot \text{cm}^2$ (Fig. 1, Table 1), and is in the range considered characteristic of “leaky” epithelia. To understand to what degree the stability of the cytoskeletal acto-myosin determines the paracellular resistance, CaSki cells were treated with drugs previously reported to affect actin status and phosphorylation of non-muscle myosin filaments in other types of cells. Drugs were used at doses that produced submaximal effects, and lengths of treatments were chosen so that upon removal of the drugs (by washes) R_{TE} levels returned to baseline within 1–24 hrs (not shown). The reversibility of the effects assured that observed changes in R_{TE} were not the result of toxic effects of the drugs.

The effects of actin modulators are shown in Fig. 1 and summarized in Table 1. Treatment with phalloidin increased R_{TE} to $50 \Omega \cdot \text{cm}^2$ (Fig. 1, Table 1). Treatment with cytochalasin-D decreased R_{TE} to $3 \Omega \cdot \text{cm}^2$, and in the presence of phalloidin the effect of cytochalasin-D was attenuated, R_{TE} decreased only to $10 \Omega \cdot \text{cm}^2$ (Fig. 1, Table 1). These results confirm previous studies [4,5] that modulation of actin polymerization affects the paracellular permeability.

One of the objectives of the study was to understand how myosin regulates the paracellular permeability. Unpublished results from the lab showed that CaSki cells express myosin light chain kinase, and that treatments with Ca^{2+} -calmodulin modulators change phosphorylation level of the regulatory component of myosin light chain kinase (not shown). In the present study treatment with the calmodulin inhibitor W-7 or with the Ca^{2+} /calmodulin-dependent protein kinase-II (CaMK-II) inhibitor KN-93 produced only small and short-lived (< 1 min) transient changes in R_{TE} (not shown), suggesting that in CaSki cells phosphorylation of the regulatory component of myosin light chain by the calcium-calmodulin-dependent myosin light chain kinase [8,24] does not significantly modulate the R_{TE} .

Similar to W-7 and KN-93, other protein kinase inhibitors, including tyrphostin 25, genistein, and SB203580 had no significant effect on R_{TE} (not shown). In contrast, DRB, Y-27632 and okadaic acid significantly modulated the resistance. Treatment with DRB, a cell permeable inhibitor of casein kinase II (CK2) [25], increased R_{TE} to $35 \Omega \cdot \text{cm}^2$ (Fig. 1, Table 1). Treatment with Y-27632, an inhibitor of the Rho-associated kinase (ROCK) [26], decreased R_{TE} to $10 \Omega \cdot \text{cm}^2$ (Fig. 1, Table 1). Treatment with okadaic acid, a non-specific inhibitor of serine / threonine protein phosphatase activity [27] decreased R_{TE} to $12 \Omega \cdot \text{cm}^2$ (Fig. 1). Combined treatments of okadaic acid plus Y-27632 produced changes in R_{TE} suggestive of additive effects (Fig. 1). Neither okadaic acid, Y-27632 or DRB affected phalloidin (10 ng/ml) increase in R_{TE} , and DRB did not affect cytochalasin-D decrease in R_{TE} ; however, both okadaic acid and Y-27632 inhibited the DRB-induced increases in R_{TE} (Fig. 1).

Cellular distribution of non-muscle myosins

Lack of an effect by W-7 and KN-93, together with the changes in R_{TE} induced by DRB Y-27632 and okadaic acid suggested modulation of phosphorylation of myosin heavy chains [28]. Since relatively little was known about the myosin composition in CaSki cells, we studied expression of myosins with emphasis on NMM-II-A and NMM-II-B filaments, the prototypical two-headed myosins in non-muscle cells. Immunostaining of CaSki cells attached on filters with anti NMM-II-HC-A or NMM-II-HC-B antibodies revealed different cellular distribution patterns of the two myosins. NMM-II-A was found predominantly in the cytoplasm and

perinuclear regions (Fig. 2, Control, second column), while NMM-II-B was found predominantly in submembranous areas (Fig. 2, Control, fifth column, and Fig. 3). Western immunoblots confirmed the differential cellular distribution of NMM-II-A and NMM-II-B: NMM-II-A was found mainly in the cytosolic fraction while NMM-II-B was found predominantly in the membrane-enriched fraction (Fig. 4A).

To determine whether NMM-II-A and NMM-II-B interact with actin, two experiments were done. The first experiment used the co-immunostaining technique for actin and the myosins. Fig. 2 (Control, columns one and four) and Fig. 3 show β -actin immunoreactivity decorating the cortical cytoskeleton frame, and a more diffuse staining in the cytoplasm and perinuclear regions. Co-staining with anti β -actin plus anti NMM-II-HC-A antibodies revealed some interaction between actin and NMM-II-A in the cytoplasm and perinuclear regions (Fig. 2, Control, column three). Co-staining with the anti β -actin plus the anti NMM-II-HC-B antibodies, on the other hand, revealed a pronounced interaction between actin and NMM-II-B in the cortical cytoskeleton frame (Fig. 2, Control, column six). The second experiment used the technique of co-immunoprecipitation and immunoblotting. Total homogenates and cell fractions of CaSki cells were immunoprecipitated with anti β -actin antibody and immunoblotted with anti NMM-II-HC-A or anti NMM-II-HC-B antibodies. NMM-II-A immunoreactivity was found predominantly in the cytosolic fraction (Fig. 4B) and NMM-II-B immunoreactivity in the plasma-membrane – enriched fraction (Fig. 4C). Both types of myosins associated in-vitro with actin (Figs. 4B, C). Collectively, the results in Figs. 2–4 suggest that in CaSki cells the cortical cytoskeleton frame is composed predominantly of NMM-II-B, and that NMM-II-B associates with actin.

Modulation of the cortical acto-myosin frame

Effects of phalloidin and cytochalasin-D—Treatment with phalloidin did not significantly affect the cellular distribution and content of actin (Figs. 2 and 5), NMM-II-A (Fig. 2), or NMM-II-B (Figs. 2, 3, and 5). Treatment with cytochalasin-D, on the other hand, resulted in disorganization of the cortical cytoskeleton frame, and the actin and NMM-II-B immunoreactivities in the cortical frame showed speckled, granular staining and breakage points at submembranous regions (Figs. 2, 3b, 3c, 3e, and 3f). Cytochalasin-D had little effect on the cellular content of actin (Fig. 5) and NMM-II-B (Figs. 4C and 5), but it decreased NMM-II-A content (Fig. 4B). Cytochalasin-D also decreased the actin – NMM-II-A co-immunoprecipitated product in total homogenates and in the cytosol, probably reflecting the decreased content of cellular NMM-II-A (Fig. 4B). In contrast, cytochalasin-D decreased the actin – NMM-II-B co-immunoprecipitated product in the membrane-enriched fraction, and increased it in the cytosolic fraction (Fig. 4C). Co-treatment with phalloidin did not significantly modulate cytochalasin-D effects (Figs. 3g–h, 4B, 4C, and 5).

Effects of DRB, Y-27632 and okadaic acid—Treatment with DRB had no significant effect on the cellular distribution of actin, NMM-II-A, or NMM-II-B (Figs. 2, 3i–j, and 5), but it increased the actin – NMM-II-B co-immunoprecipitated product (Fig. 5). Treatment with Y-27632 induced disorganization of cortical actin (Figs. 2 and 3m) and NMM-II-B (Figs. 2 and 3n), but had no significant effect on cellular NMM-II-A (Fig. 2) or total actin (Fig. 5). Treatment with Y-27632 decreased the actin – NMM-II-B co-immunoprecipitated product in total cells homogenates (Fig. 5). Co-treatment with DRB attenuated the effects of Y-27632 (Figs. 2, 3q–r, and 5). Treatment with okadaic acid had no significant effect on the cortical cytoskeletal frame (Fig. 2).

Multi-drugs effects—Co-treatment with DRB did not modulate cytochalasin-D effects on actin and NMM-II-B (Fig. 2). In contrast, Y-27632 effects were inhibited by co-treatment with phalloidin (Figs. 2, 3q–r, and 5).

Collectively, the results in Figs. 2–5 indicate that cytochalasin-D and Y-27632 induced disorganization of the cortical cytoskeletal frame and decreased the actin – NMM-II-B co-immunoprecipitation. Phalloidin and DRB attenuated the effects of Y-27632, but neither phalloidin nor DRB did modulate the effects of cytochalasin-D.

In view of the findings that in CaSki cells NMM-II-B is the predominant myosin of the cortical cytoskeleton frame, all subsequent experiments focused on the NMM-II-B.

Phosphorylation of NMM-II-B

The Y-27632 and DRB data in Figs. 1–5 suggested the involvement of ROCK and CK2, respectively, in the modulation of NMM-II-B. One of the mechanisms by which ROCK and CK2 modulate NMM-II-B function is through phosphorylation of myosin heavy chains [28]. To better understand how Y-27632 and DRB modulate NMM-II-B phosphorylation, the intracellular ATP pool of CaSki cells was labeled by incubation of cells with [³²P] orthophosphate, and cell lysates were immunoprecipitated with the anti NMM-II-HC-B antibody. Autoradiography revealed that NMM-II-B is phosphorylated at baseline conditions, and that treatment with DRB inhibited baseline NMM-II-B phosphorylation while treatments with Y-27632 and okadaic acid increased baseline NMM-II-B phosphorylation (Fig. 6A). Since none of the treatments had a significant effect on the cellular content of NMM-II-B, (Figs. 5 and 6), it was possible to evaluate the changes in NMM-II-B phosphorylation status semi-quantitatively using densitometry (Fig. 6B). Thus, DRB inhibited NMM-II-B phosphorylation by about two-thirds, while Y-27632 and okadaic acid increased NMM-II-B phosphorylation 5–6 fold. Co-treatment with DRB had no significant effect on the phosphorylation of NMM-II-B induced by Y-27632 or okadaic acid (Figs. 6A, 6B).

To determine what residues of the myosin are phosphorylated, cell lysates were immunoprecipitated with anti-phospho-antibodies, and immunoblotted with the anti NMM-II-HC-B antibody. Treatments with Y-27632 and okadaic acid increased phosphoserine and phosphothreonine immunoreactivities compared to baseline conditions, while treatment with DRB decreased the phosphoserine and phosphothreonine immunoreactivities (Fig. 7). Co-treatment with DRB had little effect on the effects of Y-27632 and okadaic acid (Fig. 7).

Treatment with phalloidin had no effect on baseline phosphorylation of NMM-II-B, and co-treatment with phalloidin had no effect on the increase in phosphorylation induced by Y-27632. Treatment with cytochalasin-D increased baseline phosphorylation of NMM-II-B by about a half (Figs. 6A, 6B).

Collectively, the results in Figs. 6 and 7 show that Y-27632 and okadaic acid increased, and DRB decreased NMM-II-B phosphorylation at serine and threonine sites.

Modulation of NMM-II-B homodimerization: Treatments in-vivo

Optimal binding of NMM-II-B to actin requires the myosin in its homodimeric form [20–22, 29]. To understand whether the effects of Y-27632 and DRB shown in Figs. 1–7 involve modulation of NMM-II-B oligomerization, NMM-II-B filaments extracted from cells treated with these drugs were purified and assayed for homodimerization in-vitro.

Fig. 8 shows the methodology used in the present study. The extraction and purification procedure described in Methods yielded a single band which immunoreacted with the anti NMM-II-HC-B antibody, but not with the anti NMM-II-HC-A antibody (Fig. 8A). Fig. 8B shows an example of real-time changes in NMM-II-B oligomerization: Ca²⁺ (high-salt) – induced disassembly, and low-Ca²⁺ – induced assembly (homodimerization) of NMM-II-B filaments. Table 2 summarizes results using that technique with preparations obtained from cells treated in-vivo with Y-27632 and DRB. Ca²⁺-induced disassembly of NMM-II-B

filaments was faster, and low- Ca^{2+} – induced assembly was slower in preparations obtained from cells treated with Y-27632 compared to control cells. DRB tended to inhibit disassembly and enhance assembly of NMM-II-B filaments, while co-treatment with DRB inhibited the effects of Y-27632 (Table 2).

The experiments in Fig. 8C and Table 3 used a secondary method to determine modulation of NMM-II-B filaments assembly, in terms of the critical concentration of NMM-II-B filaments necessary to induce homodimerization in-vitro. The critical concentration of NMM-II-B filaments was higher in preparations obtained from cells treated in-vivo with Y-27632 or okadaic acid, and lower in preparations obtained from cells treated in-vivo with DRB, compared to control cells (Table 3). These results resemble those in Fig. 8B and Table 2, and indicate that treatments with Y-27632 and okadaic acid decrease NMM-II-B filamentation, while DRB increases NMM-II-B filamentation.

Co-treatments in-vivo with Y-27632 plus DRB, with Y-27632 plus okadaic acid, or with DRB plus okadaic acid resulted in critical concentrations levels of NMM-II-B filaments that were similar to those in control cells (Table 3), suggestive additive effects.

One of the conditions for optimal myosin-actin interaction in striated and smooth muscle cells is the presence of actin in its polymerized form (i.e. F-actin) [30]. To test whether in the epithelial CaSki cells actin polymerization status affects NMM-II-B filamentation, the above experiments were repeated using preparations obtained from cells treated with phalloidin, to stimulate actin polymerization [5], or with sodium nitroprusside (SNP), to stimulate actin depolymerization [4]. The results in Tables 2 and 3 show that the assembly and disassembly rates of NMM-II-B filaments were not significantly affected by prior treatments with phalloidin or SNP.

Modulation of NMM-II-B homodimerization: Treatments in-vitro

The results in Tables 2 and 3 indicate that treatment with the CK2 inhibitor DRB enhanced NMM-II-B filamentation, suggesting a role for CK2 as inducer of disassembly of NMM-II-B filaments in-vivo. To better understand the effects of CK2 on NMM-II-B filamentation, extracted NMM-II-B filaments were exposed in-vitro to CK2, and the critical concentration of NMM-II-B filaments was determined. Exposure to CK2 in-vitro, but not to heat-inactivated CK2, increased the critical concentration of NMM-II-B filaments. Exposure in-vitro to DRB, okadaic acid or Y-27632 had no significant effect, but co-administration of DRB blocked CK2 effect. Neither Y-27632 or okadaic acid had any additional effect to that of CK2 (Table 4). These results suggest that CK2 directly inhibits NMM-II-B filamentation.

The combined effects of the in-vivo and in-vitro treatments are shown in Fig. 9. The data of the in-vivo treatments were similar to, and confirmed the data in Tables 2 and 3. Exposure of extracted NMM-II-B filaments to CK2 in-vitro resulted in higher critical concentrations of NMM-II-B filaments, irrespective of the degree of filamentation that was determined by prior treatments of the cells in-vivo. These results support the hypothesis that CK2 inhibits directly NMM-II-B filamentation.

Actin-dependent vs. myosin-dependent modulation of the R_{LIS}

One of our objectives was also to better understand how modulation of the NMM-II-B affects the paracellular resistance. Changes in the paracellular resistance can be the result of modulation of the Resistance of the Tight Junctions (R_{TJ}) or the Resistance of the Lateral Intercellular Space (R_{LIS}). Neither phalloidin, Y-27632, or DRB had any significant effect on the relative mobilities of Cl^- to Na^+ across the epithelium ($u_{\text{Cl}}/u_{\text{Na}}$, Table 1). The positive controls for those experiments were increases in R_{TE} induced by ATP and decreases in R_{TE}

induced by lowering extracellular calcium with EGTA, which as previously reported [17] were associated with decreases, and increases, respectively in the ratio of u_{Cl}/u_{Na} (Table 1). Therefore, the resistance effects of phalloidin Y-27632 and DRB do not involve changes in R_{TJ} , and most likely involve modulation of the R_{LIS} . The cytochalasin-D – induced decrease in resistance, on the other hand, was associated with an increase in u_{Cl}/u_{Na} (Table 1), suggesting abrogation of the R_{TJ} .

The mechanism of phalloidin regulation of the R_{LIS} through inhibition of F-actin de-polymerization is well established [5]. The experiment in Fig. 1 showed that both phalloidin and DRB produced a non-additive increase in resistance, and suggested initially that phalloidin and DRB utilize a common cellular mechanism. To gain better understanding in the matter, the experiment was repeated using non-saturating doses of phalloidin. Co-treatment with near maximal concentration of DRB (10 μ M) plus phalloidin at the low concentration of 1 ng/ml produced greater increases in resistance than each of the drugs alone (Fig. 10A). In contrast, co-treatment with a maximal concentration of DRB plus phalloidin at a concentration of 10 ng/ml produced non-additive effect, similar to that described in Fig. 1. Co-treatment with ATP produced additional increases in resistance, regardless of the concentrations of phalloidin used (Fig. 10A), indicating that the effect of ATP is additive to that of DRB and phalloidin.

The result in Fig. 10A suggested that the effects of DRB and phalloidin are mediated by different mechanisms. To test this hypothesis more directly, the effects of DRB on resistance were compared with those of Sodium-nitroprusside. Sodium-nitroprusside (SNP) reportedly decreases the R_{LIS} through by de-polymerization of F-actin to G-actin [4]. The results in Fig. 10B show that treatment with SNP decreased the resistance independent of the increase in resistance induced by DRB. Fig. 10B also confirmed [4,5] that treatment with SNP increased cellular G-actin; in contrast, DRB had no effect on cellular G-actin.

DISCUSSION

The main objective of the study was to understand how myosin regulates the rigidity of the cytoskeleton in epithelial cells. The results in CaSki cells, similar to findings in other non-epithelial non-muscle cells [31–34], showed that the cortical acto-myosin is composed predominantly of NMM-II-B. NMM-II-B was constitutively phosphorylated, and the degree of phosphorylation could be modulated by treatments with DRB, Y-27632, and okadaic acid. Treatment with the ROCK inhibitor Y-27632 induced effects that correlated in time and were reversible upon withdrawal of treatment (not shown). Those included a decrease in R_{LIS} , disorganization of the cortical acto-myosin, decreased co-immunoprecipitation of NMM-II-B with actin, increased phosphorylation of NMM-II-B at threonine and serine residues, and decreased NMM-II-B filamentation in-vitro. It is possible that these are unrelated epiphenomena. On the other hand it is possible that these effects are related, and represent a novel cellular cascade by which increased phosphorylation of NMM-II-B results in decreased filamentation, decreased interaction with actin, and de-stabilization of the cortical acto-myosin frame. These effects may lead to formation of a dynamic cytoskeleton and subsequently to decreased R_{LIS} .

One of the objectives of the study was to understand the mechanisms that regulate NMM-II-B filamentation. Both Y-27632 and the non-specific phosphatase inhibitor okadaic acid induced an increase in the phosphorylation of NMM-II-B at threonine and serine residues and inhibited NMM-II-B filamentation. However, okadaic acid blocked Y-27632 inhibition of filamentation, suggesting that the effects of Y-27632 depended on the action of a phosphatase. The Y-27632 and okadaic acid effects in the epithelial CaSki cells resemble effects in smooth muscle cells where calcium-dependent phosphorylation of myosin-II filaments involves ROCK – mediated inhibition of myosin heavy chain phosphatase [35,36]. Since activation of

Rho-related proteins modulates the cytoskeleton even in low organisms such as protozoa [37,38], the present results suggest that ROCK-related modulation of myosin heavy chain phosphatase is a general mechanism that determines the rigidity of the cytoskeleton among species and cell types. According to this hypothesis myosin heavy chain phosphatase de-phosphorylates the NMM-II-HC-B directly, while ROCK effect is secondary, through phosphorylation and inhibition of the myosin heavy chain phosphatase.

The present results also suggest that CK2 modulates NMM-II-B filamentation directly. This conclusion is supported by the findings that treatment with the CK2 inhibitor DRB decreased the phosphorylation of NMM-II-B at threonine and serine residues. DRB increased NMM-II-B filamentation, NMM-II-B – actin co-immunoprecipitation, and the R_{LIS} , and it attenuated Y-27632 effects. CK2 blocked NMM-II-B filamentation in-vitro, and the effect could be blocked by co-administration of DRB, possibly by competing with CK2 for target binding sites. Furthermore, the administration of CK2 in-vitro to NNM-II-B preparations of NNM-II-B decreased filamentation irrespective of the degree of filamentation determined by prior treatments of the cells in-vivo. Collectively, the data indicate that CK2 inhibits NMM-II-B filamentation, and that the effect involves direct interaction of the CK2 with the myosin. CK2 is a ubiquitous, pleiotropic and multifunctional constitutively active serine / threonine protein kinase that is expressed in most eukaryotic cells [39,40], including in CaSki cells. Although there are more than 300 documented substrates for CK2, most are involved in gene expression, protein synthesis and signaling, but only few in the regulation of the cytoskeleton [40], and only two reports documented the involvement of myosin II in smooth muscle cells [41,42]. NMM-II-HC-B contain phosphorylation sites for CK2 [21,43], and the filamentation effect of CK2 is possibly mediated by a direct increase in phosphorylation of NNM-II-B. The present data show for the first time that NMM-II-B of epithelial cells is a CK2 substrate. This conclusion is of interest given the known role of CK2 in cell survival, cell growth and proliferation [40], which require modulation of the cytoskeleton into a dynamic structure.

An additional objective of the study was to understand how modulation of NMM-II-B regulates the paracellular resistance. The results showed that DRB and Y-27632 modulated the R_{LIS} , and that their effects were distinct from those of phalloidin and SNP which modulate the R_{LIS} through modulation of cellular F-actin / G-actin status [5]. The present data suggest that NMM-II-B regulation of the R_{LIS} utilizes a different mechanism. This statement is supported by the following data: a. Both DRB and phalloidin increased the resistance, and the combined effects of DRB plus low concentrations of phalloidin were additive; b. SNP decreased the resistance, and the combined effects of DRB plus SNP were additive; c. Treatment with DRB did not affect cellular G-actin; d. Co-treatment with phalloidin had no significant effect on the increases in NMM-II-B phosphorylation induced by Y-27632 or okadaic acid.

The present and our previous data [17] therefore suggest three different modes of paracellular resistance regulation: Regulation of R_{TJ} , e.g. by ATP; actin regulation of the R_{LIS} , e.g. by phalloidin; and NMM-II-B regulation of the R_{LIS} , e.g. by CK2. Hierarchically and quantitatively R_{TJ} regulation of the R_{TE} is dominant over the R_{LIS} effects of actin and NMM-II-B. Thus, ATP Phase-II effect increased R_{TE} by about $75 \Omega \cdot \text{cm}^2$, compared to phalloidin increase in R_{TE} by about $37 \Omega \cdot \text{cm}^2$, and to DRB increase in R_{TE} by about $15 \Omega \cdot \text{cm}^2$ (Fig. 10B). While the R_{TJ} effects of ATP were additive to those of phalloidin and DRB R_{LIS} effects, increases in R_{LIS} were limited to about $60 \Omega \cdot \text{cm}^2$, which in the present study was the upper limit of R_{LIS} that the CaSki epithelium could generate through the combined effects of phalloidin and DRB. Likewise, the upper limit of total paracellular resistance (R_{TE}) that the CaSki epithelium could generate (i.e. $R_{LIS} + R_{TJ}$) was about $100 \Omega \cdot \text{cm}^2$.

The cytochalasin-D data demonstrated that significant perturbations of the cytoskeletal actomyosin frame can modify both the R_{LIS} and the R_{TJ} . Cytochalasin-D abrogated the R_{LIS} by

stimulating degradation of F-actin [44,45], inducing breakage of the cortical cytoskeleton (Figs. 2 and 3), and by decreasing the interaction of actin with NMM-II-B (Fig. 6). The structural changes in the cytoskeletal acto-myosin frame could also affect the structure of the tight junctions and the proximity of tight junctions of neighboring cells, and thereby decrease the R_{TJ} (Fig. 1). Phalloidin, an inhibitor of F-actin depolymerization [44] did not block cytochalasin-D decrease in R_{TE} , suggesting that the effect of the F-actin-disrupting agent cytochalasin-D dominates over the effect of the F-actin depolymerization-inhibitor phalloidin. A possible explanation is that in the presence of cytochalasin-D, actin filament nucleation sites might be blocked, or that actin monomers might be sequestered in a non-polymerizable form [44,46,47].

The cellular mechanism by which NMM-II-B regulates the stability of the cytoskeletal acto-myosin frame in epithelial cells is unknown. In muscle cells and in other types of non-muscle cells myosins regulate actin organization into filaments through the interaction of myosin heavy chains containing the motor domain and MgATPase with actin [22]. NMM-II-B heavy chains have potential phosphorylation sites within their tail regions [13,48,49], and in-vivo phosphorylation of NMM-II-HC-B [11,50,51] may inhibit assembly of NMM-II-B filaments and abrogate myosin-actin interaction [20,26,52]. The present results support this model also in the epithelial CaSki cells. Accordingly, NMM-II-B filaments contribute to the rigidity of the cytoskeleton via interactions of the myosin filaments with the actin filaments; this will allow binding of the myosin-MgATPase to actin and generation of force that stabilizes the acto-myosin structure. Phosphorylation of NMM-II-HC-B will block the effect through inhibition of assembly of NMM-II-B filaments and will prevent the myosin-actin interaction. The role of actin in modulation of myosin is also unclear, because phalloidin inhibited Y-27632 – modulation of NMM-II-B and attenuated Y-27632 – inhibition of the actin – NMM-II-B interaction. Therefore, the presence of F-actin overcame, to a degree, the effects of increased phosphorylation of NMM-II-B. However, in contrast to protozoa or muscle cells [30], the present data in the epithelial CaSki cells ruled out the requirement that optimal myosin-actin interactions depend on F-actin, because NMM-II-B assembly was similar in F-actin and G-actin dominated conditions.

In summary, the present results suggest that in CaSki cells NMM-II-B filaments are in steady-state equilibrium of phosphorylation-dephosphorylation mediated constitutively by CK2 and by ROCK-regulated myosin heavy chain phosphatase, respectively. Increased phosphorylation would tend to inhibit assembly of NMM-II-B filaments, while dephosphorylation would have the opposite effect. The results further suggest that the rigidity of the cytoskeleton is determined by the concerted effects of actin polymerization and actin – NMM-II-B interactions. Abrogation of NMM-II-B filamentation would lead to decreased actin-myosin interaction and to the formation of a dynamic cytoskeleton. The net effect would be decreased R_{LIS} and increased paracellular permeability.

Acknowledgements

The technical support of Kimberley Frieden, Brian De-Santis and Dipika Pal is acknowledged.

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Figure 1.

Modulation of R_{TE} . CaSki cells on filters were treated with one or more of the indicated drugs: C – control (vehicle only); Ph – phalloidin (10 ng/ml, 60 min); CD – cytochalasin-D (10 $\mu\text{g}/\text{ml}$, 20 min); DRB (10 μM , 6 hrs); Y-2 – Y-27632 (5 μM , 6 hrs); OA – okadaic acid (10 μM , 6 hrs). At the completion of treatments filters were mounted in the Ussing chamber for determinations of R_{TE} . Shown are means ($\pm$ SD) of 3–5 filters in each category. * - $p < 0.01$ compared to C. ** - $p < 0.01$ – 0.03 compared to C and CD. *** - $p < 0.01$ compared to C, DRB, OA, and Y-2.

Figure 2.

Co-immunostaining for actin plus NMM-II-A (left three columns) and for actin plus NMM-II-B (right three columns) in CaSki cells. Cells on filters were treated with the vehicle (Control) or with one of indicated drugs, as described in Fig. 1. At the completion of treatments immunostaining was done using anti- β -actin plus anti-NMM-II-HC-A antibodies (left three columns), or anti- β -actin plus anti-NMM-II-HC-B antibodies (right three columns). Columns three and six show also nuclear staining. Cellular distribution of actin (columns one and four) is shown in red-brown. Cellular distributions of NMM-II-A or NMM-II-B (columns two and five) are shown in green. Co-distribution of actin plus NMM-II-A and actin plus NMM-II-B are shown in columns three and six, respectively. The experiments were repeated twice with similar trends (x 40).

Figure 3.

Cellular distribution of actin and NMM-II-B. CaSki cells on filters were treated with the indicated drugs, alone or in combination, as described in Figs. 1. At the completion of treatments immunostaining was done using either the anti- β -actin or the anti-NMM-II-HC-B antibodies. The experiments were repeated twice with similar trends (x 40).

Figure 4.

Expression of NMM-II-HC-A and NMM-II-HC-B. **A**, Western blots using anti NMM-II-HC-A and NMM-II-HC-B antibodies on CaSki total cell homogenates (TH), plasma-membrane enriched fractions (MF), and cytosolic fractions (Cytosol). **B** and **C**, CaSki cells on filters were treated with phalloidin or cytochalasin-D, alone or in combination (as in Fig. 1). At the completion of treatments immunoprecipitation (IP, with anti β -actin antibody) and immunoblotting (IB, with anti NMM-II-HC-A or NMM-II-HC-B antibodies) were carried out on TH, MF, and cytosolic fractions. The experiments were repeated twice with similar trends.

Figure 5.

Expression of NMM-II-HC-B. CaSki cells on filters were treated with the indicated drugs, alone or in combination (as in Fig. 1). Cell lysates were fractionated on gel electrophoresis, immunoprecipitated (IP) with anti β -actin antibody and immunoblotted (IB) with the anti NMM-II-HC-B antibody (upper panel). Experiments were repeated for Western immunoblotting of cell lysates with anti β -actin antibody (middle panel) and with anti NMM-II-HC-B antibody (lower panel). The experiments were repeated twice with similar trends.

Figure 6. Phosphorylation of NMM-II-HC-B. **A.** CaSki cells on filters were labeled with [^{32}P] orthophosphate and treated with one of the indicated drugs, alone or in combination (as in Fig. 1). Cell lysates were fractionated on gel electrophoresis and immunoprecipitated (IP) with the anti NMM-II-HC-B antibody. Lower panel shows Western immunoblotting (IB) of cell lysates with anti NMM-II-HC-B antibody. The experiments were repeated twice with similar trends. **B.** Means (two experiments) of densitometry of [^{32}P]-labeled NMM-II-HC-B. A.U. – artificial units.

Figure 7.

Phosphorylation of NMM-II-HC-B. Experiments were done as in Fig. 4, except that cell lysates were immunoprecipitated with anti phosphoserine (PS), phosphothreonine (PT), and phosphotyrosine PY20 or P99 (PY20/PY99) antibodies, and immunoblotted (IB) with anti NMM-II-HC-B antibody. Lower panel shows Western immunoblotting (IB) of cell lysates with anti NMM-II-HC-B antibody. The experiments were repeated twice with similar trends.

Figure 8.

Modulation of homodimerization of NMM-II-B filaments. **A.** Extraction and purification of NMM-II-B filaments. Lane 1: Coomassie stain of total CaSki cell extract (bar is 200KDa). Lane 2: Coomassie stain of purified NMM-II-B filaments from the membrane fraction. Lane 3: Immunoblot of purified NMM-II-B filaments from the membrane-enriched fraction stained with the anti NMM-II-HC-B antibody. Lane 4: Immunoblot of purified NMM-II-B filaments from the membrane-enriched fraction stained with the anti NMM-II-HC-A antibody. All samples were resolved on 6% polyacrylamide gels. **B.** Real-time changes of Ca^{2+} -induced disassembly and low- Ca^{2+} – induced assembly of NMM-II-B filaments prepared from control cells. The experiments are described in Methods and Results, and data are summarized in Table 2. Readings were made about every 10 seconds. **C.** Changes in NNM-II-B filaments assembly determined in terms of the critical concentration of NMM-II-B filaments preparations necessary to induce oligomerization in-vitro. The experiments are described in Methods and Results, and data are summarized in Table 3.

Figure 9.

Modulation of homodimerization of NMM-II-B filaments. CaSki cells on filters were treated in-vivo with one or more of the indicated agents (as in Fig. 1) (filled bars). At the completion of treatments NMM-II-B filaments were extracted, warmed to 30°C, and exposed in-vitro for 6 hrs at 30°C to CK2 (0.2 µg / 100 µl). Assembly of NMM-II-B filaments was determined in terms of the critical concentration of filaments necessary for homodimerization as described in Methods and Fig. 8C (empty bars). * - $p < 0.01$ compared to Control. All values of the critical concentration of NNM-II-B in the in-vitro category (empty bars) were significantly higher ($p < 0.01$) compared to the corresponding in-vivo treatments (filled bars).

Figure 10.

A. Modulation of R_{TE} , using drugs combinations treatments. **B.** Effects of DRB and SNP (1 mM, 20 min) on R_{TE} and cellular G-actin content. Experiments were done as described in Fig. 1 with the indicated drugs concentrations.

Table 1

Modulation of the relative Cl^- and Na^+ mobilities ($u_{\text{Cl}}/u_{\text{Na}}$) and R_{TE} ($\Omega\cdot\text{cm}^{-2}$) across CaSki cultures.

	$u_{\text{Cl}}/u_{\text{Na}}$	R_{TE}	n
Baseline	1.31 ± 0.04	20 ± 2	3
+ Phalloidin	1.31 ± 0.03	$49 \pm 7^*$	4
+ CD	$1.40 \pm 0.04^*$	$3 \pm 2^*$	3
+ DRB	1.32 ± 0.04	$33 \pm 3^*$	3
+ Y-2	1.31 ± 0.04	11 ± 3	3
+ ATP (Phase-II)	$1.21 \pm 0.04^*$	$52 \pm 8^*$	3
+ EGTA	$1.41 \pm 0.03^*$	$3 \pm 2^*$	3

CaSki cells on filters were treated with the indicated drugs as described in Fig. 1. ATP was added at 10 μM and determinations were made after 10 min, at the peak of the increase in R_{TE} (Phase-II response) (15). EGTA was added at 0.6 mM to lower free Ca^{2+} in the extracellular buffer to 0.6 mM, and determinations were made after 10 min, at the trough of the decrease in R_{TE} (15).

* - $p < 0.05$ – 0.01 compared to Baseline.

Table 2
Modulation of NMM-II-B oligomerization ($t_{1/2}$ [min] of the reactions).

	Disassembly	Assembly	n
Control	5.3 ± 0.4	3.3 ± 0.4	4
+ Y-2	3.4 ± 0.4 *	4.6 ± 0.6 *	5
+ DRB	6.2 ± 0.5	2.6 ± 0.6	4
+ DRB + Y-2	4.9 ± 0.6	3.4 ± 0.4	4
+ Ph	5.2 ± 0.6	3.4 ± 0.4	4
+ SNP	5.4 ± 0.7	3.5 ± 0.5	4

CaSki cells on filters were treated with the indicated drugs as described in Fig. 1. NMM-II-B filaments were extracted, purified, and used for dynamic Disassembly / Assembly assays (see Methods).

* - $p < 0.05$ – 0.01 compared to Control.

Table 3
Modulation of NMM-II-B Assembly – Treatments In-Vivo.

	NMM-II-B (µg/ml) Critical Concentration	n
Control	65 ± 5	4
+ Y-2	89 ± 7 *	5
+ DRB	57 ± 4 *	5
+ OA	74 ± 4 *	4
+ Y-2 + DRB	62 ± 6	4
+ Y-2 + OA	62 ± 5	4
+ DRB + OA	59 ± 6	4
+ Ph	67 ± 11	4
+ SNP	65 ± 9	4

CaSki cells on filters were treated with the indicated drugs as described in Fig. 1. NMM-II-B filaments were extracted, and assembly of NMM-II-B filaments was determined in terms of the critical concentration of filaments necessary for homodimerization (see Methods).

* - p<0.05–0.01 compared to Control.

Table 4
Modulation of NMM-II-B Assembly – Treatments In-Vitro.

	NMM-II-B (µg/ml) Critical Concentration	n
Control	69 ± 4	3
+ CK2	103 ± 5 *	3
+ HI-CK2	72 ± 5	3
+ DRB	65 ± 6	3
+ CK2 + DRB	77 ± 8	3
+ OA	63 ± 6	3
+ CK2 + OA	68 ± 6	3
+ Y-2	70 ± 6	3
+ CK2 + Y-2	71 ± 6	3

NMM-II-B filaments were extracted from CaSki cells, warmed to 30°C, and exposed in-vitro for 6 hrs at 30°C to one of the following agents, alone or in combination: 0.2 µg/100 µl of CK2 (or to heat-inactivated CK2 [HI-CK2]); 50 µM DRB, Y-27632 (Y-2, 25 µM); and okadaic acid (OA, 25 µM). Assembly of NMM-II-B filaments was determined in terms of the critical concentration of filaments necessary for homodimerization.

* - p<0.01 compared to Control.