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Extracellular acidosis induces Rho Kinase/myosin-II-mediated vacuolarization of the apical plasma membrane and formation of intracytoplasmic apical vacuoles

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ABSTRACT

The apical surface domain of epithelial cells is a highly specialized membrane that allows organs to interact with the external environment. The accumulation of apical membranes within the cytoplasm has been observed in (hypoxic) epithelial tumors and in epithelial cells of patients diagnosed with microvillus inclusion disease. In this study we have investigated the hypothesis that a common microenvironmental factor, i.e. acidosis, contributes to this phenotype. Using live cell imaging, we demonstrate that extracellular and cytoplasmic acidosis induces thinning/stretching and extensive vacuolarization of the apical plasma membrane, while the basolateral plasma membrane remains unaffected. The invaginated membranes contain functional apical plasma membrane resident proteins but not components of tight and adherens junctions, and are lined with filamentous actin. Internalized apical membranes can fuse with each other and give rise to larger cytoplasmic vacuolar compartments. Comparable apical membrane vacuolarization is observed upon treatment of the cells with compounds that interfere with actin filament dynamics. Moreover, acidosis-induced vacuolarization is inhibited with Y-27632 and blebbistatin, inhibitors of Rho kinase and myosin-II ATPase activity, respectively. Together, the data demonstrate that acidosis induces a selective retrieval of the apical plasma membrane and formation of intracytoplasmic apical vacuoles via Rho kinase/myosin-II-dependent membrane vacuolarization.
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INTRODUCTION

The apical plasma membrane of polarized epithelial cells is a structurally, compositionally, and functionally distinct cell surface domain, separated from a basolateral cell surface domain by tight junctions, and specialized in the interaction with an external environment that dramatically differs from the interstitium. For instance, the apical surface of enterocytes in the intestine selectively processes and retrieves nutrients and other molecules from the gut lumen. The apical surface of hepatocytes in the liver expels bile components and detoxified waste product in the bile canaliculi. Not surprisingly, the loss of apical plasma membrane identity (e.g. atrophy of apical microvilli and/or reduced apical expression of apical resident proteins) and, consequently, loss of apical plasma membrane function has been associated with several, often lethal disease conditions.

One of the most striking and peculiar apical surface abnormalities is the accumulation of vacuoles with typical structural, compositional, and functional characteristics of the apical surface in the cytoplasm (see below). These vacuoles were reported for the first time in the 1970s and are since known by different names, including intracellular lumina (Remy, 1986), microvillus inclusions (Sherman et al., 2004; Ruemmele et al., 2006; Iancu et al., 2007), or vacuolar apical compartments (VACs; Vega-Salas et al., 1988; Gilbert et al., 1991; Brignoni et al., 1993; Vega-Salas et al., 1993; Low et al., 2000). We will refer to these structures as vacuolar apical compartments, or VACs, from hereon. The origin and (patho) physiological relevance of VACs remains unclear. VACs have been proposed to originate from the biosynthetic protein transport pathway, in which impaired or delayed fusion of apical transport vesicles with the cell surface may allow homotypic fusion of these vesicles in the cytoplasm (Vega-Salas et al., 1993). Alternatively, VACs have also been proposed to be the result of apical plasma membrane phagocytosis in which large parts of the apical surface are internalized (Reinshagen et al., 2002). VACs have been proposed to represent a transient stage in epithelial cell dedifferentiation and possibly
epithelial-to-mesenchyme transition, and in the process of epithelial cell polarization and differentiation (Martin-Belmonte and Mostov, 2008; Martin-Belmonte et al., 2008).

VACs are the diagnostic hallmark of a rare but fatal enteropathy called microvillus inclusion disease (MVID), hence its name. MVID is clinically characterized by severe secretory diarrhea, malabsorption and metabolic acidosis and, at the morphological level, by microvillus atrophy and the accumulation of apical proteins and VACs in the cytoplasm. In addition to MVID, VACs have been reported in (hypoxic) epithelial tumor cells of various origins (Remy, 1986), including medullary carcinoma of the thyroid gland (Kinjo et al., 2003), Hürthle cell tumor (Ambu et al., 2000), epithelioid ependymomas (Kawano et al., 2000), fibrolamellar carcinoma (an hepatocellular carcinoma) (An et al., 1983; Caballero et al., 1985; Sato et al., 1997), and ocular neoplasm (Spinak and Dembitzer, 1978).

In this study we have tested the hypothesis that microenvironmental factors play a role in the formation of VACs. Given that an acidic extracellular environment is a shared feature in MVID (due to metabolic acidosis and low duodenum pH) and solid tumors (due to hypoxia), we have investigated the effects of a reduced extracellular pH on the dynamics of the epithelial apical plasma membrane. We find that exposure of epithelial cells to low extracellular pH induces a selective vacuolarization of the apical plasma membrane resulting in the formation of intracytoplasmic VACs, and identify perturbed actin dynamics and the activity of Rho kinase and myosin-II as part of the underlying mechanism.

MATERIALS AND METHODS

Cell culture

Human hepatoma cell line HepG2 were cultured in high-glucose (4500 mg/L) DMEM (Gibco, Invitrogen), containing 10% heat-inactivated fetal calf serum (56°C, 30 min.), 2mM L-glutamine and penicillin (100 U/ml)/streptomycin (100µg/ml) antibiotics, at
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37°C in a 5% CO₂-humidified incubator. The stably transfected cells HepG2-EGFP-MDR1 and HepG2-EGFP-GPI were cultured in the same medium supplemented with selection antibiotic G418 (potency 0.8mg/ml). The cells were passaged every 3-4 days, using trypsin.

Acid media

Acidic medium was prepared with cell culture medium supplemented with 4mM phosphoric acid and 15mM HEPES; pH was adjusted with HCl and NaOH. The medium was sterilized using 0.2μm-pore filters.

Cytosol acidification using ammonium prepulse technique involved washing the cells with a buffer 1 composed of 135mM NaCl, 5mM KCl, 0.8mM MgSO₄, 1.2mM CaCl₂, 0.8mM NaHPO₄, 10mM HEPES, 5mM glucose. During the 10 minutes pre-incubation with loading buffer, (composed as buffer 1 with 20mM NaCl replaced by 20mM NH₄Cl), the cytosol was loaded with NH₄⁺ ions that establishes an equilibrium with NH₃. Subsequently the cells were incubated for 2 hours with the acidification buffer, prepared as buffer 1, but with 135mM NaCl replaced by 135mM cholinechloride. The removal of the NH₄Cl from the medium causes rapid efflux of NH₃ from cytosol, leaving the membrane impermeable NH₄⁺ behind what results in acidification of the cytosol to pH 6.0 (c.f. Sandvig et al., 1987). The endogenous Na⁺/H⁺ antiporters, which usually become activated by cytosol acidification, were effectively blocked by lack of Na⁺ in the external acidification medium (Roos & Boron, 1981).

Immunofluorescence

For experiments cells were cultured for 1-3 days on glass ethanol-sterilized coverslips. Cells were fixed with 4% PFA, methanol, ethanol, or acetone, permeabilized by incubation in 0.5% TritonX-100 for 5 minutes, and blocked with 1% FCS for 30-60 minutes. Primary antibodies were diluted in 1% FCS to the concentration recommended by the manufacturer.
Primary antibodies: monoclonal anti-ZO-1 (Zymed), monoclonal anti-B-catenin (BD Transduction Laboratories), monoclonal anti-5’ nucleotidase (Aït Slimane et al., 2003), anti-MRP2 (/ABCC2, Alexis), monoclonal anti-MDR1 (C219, Abcam).

Secondary antibody: anti-mouse and anti-rabbit antibodies labeled with Alexa Fluor -488, -546 and -594 (Molecular Probes) together with TRITC-phalloidin (Sigma) and DAPI and/or DRAQ5, for actin and nuclei labeling respectively.

Samples were analyzed and photographed using an Olympus fluorescence microscope (Provis AX70, New Hyde Park, NY) and analySIS® (Soft Imaging System) or with Leica SP2 confocal laser scanning microscope (Leica Confocal Software Light), and processed in Adobe Photoshop v.8.0.

Live imaging

Cells were plated in LabTek II-chambered Coverglass (Nagle Nunc Int.) at a concentration of 1*10^5 cells per dish and cultured for 2.5 days. Images were taken every 5 minutes with a 60x glycerol magnification objective using a confocal fluorescence microscope with a Solamere NIPKOW Spinning Disc Camera system. Data were processed using ImageJ software.

Determination of the TJ “barrier” function

Parental HepG2 cells were subjected to acidification at 37°C for 1.5 hours in pH 5.5 and ammonia prepulse. Then 5-carboxyfluorescein diacetate (CFDA, 0.5 µM, Sigma) was added to the media for 30 minutes to allow its internalization, removal of the diacetate ester, and subsequent translocation of the CF^- into the BC lumen by the MRP2 ABC transporter. After extensive washes the capacity of the BC to maintain the fluorescent CF^- anion was analyzed with an Olympus fluorescence microscope (Provis AX70, New Hyde Park, NY).

Determination of TJ “fence” function

To determine the “fence” function of tight junctions we used C6-NBD-Glc-CER
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fluorescent lipids as previously described by van der Wouden (2002). After acidification HepG2 cells were cooled to 4°C by washing with ice-cold HBSS, and incubated with 4μM C₆-NBD-Glc-CER for 30 minutes at 4°C to label the basolateral plasma membrane while preventing endocytosis. Following extensive washes with ice-cold HBSS, the cells were analyzed under a fluorescence microscope to detect leakage of lipids from the basolateral membrane to the apical membrane. As a positive control, the HepG2 cells kept at pH 7.4 were incubated with C₆-NBD-GlcCer for 30 minutes at 4°C in the presence of 2mM EDTA, which destroys tight junctions and allows leakage of lipids to the apical plasma membrane (see results).

Inhibitors and active compounds
Compounds used that affect actin dynamics: jasplakinolide (200 nM; Calbiochem), latrunculin B (10μM; Sigma) and cytochalasin D (10μg/mL; Sigma); microtubule dynamics: nocodazole (33μM; Sigma), colchicine (10μM). Inhibitors that affect the myosin-II function: the myosin-II inhibitor - blebbistatin (0.1μM; Toronto Research Chemicals Inc.) and Y27632 an inhibitor of Rho Kinase (2.5μM; Calbiochem).

Counting
HepG2-EGFP-MDR1 were cultured for 3 days on glass coverslips, subjected to acidosis in the presence or absence of compounds, fixed in MetOH (5 minutes, -20°C), rehydrated with HBSS and stained for actin and nuclei with TRITC-phalloidin and DAPI respectively. The slides were analyzed using a Leica SP2 confocal laser scanning microscope (Leica Confocal Software Light), where the normal BC, invaginating BC and VACs were counted. The graphs represent the mean +/- standard deviation of at least 3 separate experiments, for minimum 300 cells at each condition. The differences between groups were determined by the Student t-test, and considered significant for the p value lower then 0.05 (*), p<0.01 (**) and p<0.001 (***).
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RESULTS

Extracellular acidification results in the appearance of apical-like vacuolar structures in close proximity to the apical bile canalicular plasma membrane of HepG2 cells

In order to investigate the effects of extracellular acidosis on apical plasma membrane dynamics, we employed a polarized human hepatocyte cell line, HepG2. Hepatocytes in culture have the advantage that their apical-basal polarity axis is orientated parallel to the substratum, where the apical plasma membrane domains of two adjacent cells form an intercellular apical lumen. This typical orientation of cell surface polarity allows high resolution imaging of their apical surface in the x-y plane. HepG2 cells stably expressing a fluorescently tagged bile canalicular plasma membrane polytopic ABC transporter EGFP-MDR1(/ABCB1) (Äit Slimane et al., 2003) were incubated in culture medium of different pH ranging from 7.4 to 5.0 (reported in solid tumors), for 2 hours at 37°C, fixed and stained for F-actin and nuclei (See Materials and Methods). As shown in Figure 1A-C, cells exposed to an extracellular pH of 7.4 displayed well-organized EGFP-MDR1 (/ABCB1)-positive and filamentous actin-lined apical surface domains in which numerous microvilli can be distinguished. By contrast, exposing the cells to an acidic environment (pH 5.5; Fig. 1D-F) resulted in a much less organized apical plasma membrane domain, and in a prominent appearance of EGFP-MDR1(/ABCB1)- and actin filament-positive (arrows) vacuole-like structures near the apical plasma membrane domain. In addition to EGFP-MDR1 (/ABCB1), these vacuoles also contained other endogenous resident bile canalicular proteins such as the glycosylphosphatidylinositol-anchored 5’nucleotidase and another ABC transporter MRP2 (/ABCC2) (data not shown). Thirty to fifty percent of all apical lumens revealed these vacuole-like structures following exposure to an acidic environment of pH 5.5 (Fig. 1J).

High concentrations of extracellular protons may activate proton-sensitive plasma membrane signaling receptors (Holzer, 2003) or result in the accumulation of protons
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in the cell cytoplasm. We determined whether a direct acidification of the cells’ cytosol resulted in a similar phenotype. For this, we employed a well-established technique, which involves acidification of the cytoplasm with NH₄⁺ ions (see Materials and Methods). The level of acidification is correlated to the concentration of NH₄Cl, with visible results obtained for 20mM and higher. It was previously reported that this technique reduces the cytosolic pH to approximately 6.0 (Sandvig et al., 1987). As shown in Figure 1 G-I and J, cytoplasmic acidification similarly resulted in the appearance of EGFP-MDR1 (/ABCB1)- and F-actin-positive (arrows) vacuole-like structures near the apical plasma membrane domains, and this was observed in ~50% of all apical surface domains. The percentage of polarized cells (i.e. the ratio of apical lumens/100 cells) did not change within the time span of the experiment (data not shown).

The acidosis-induced formation of apical VACs was temperature-dependent (Suppl. Fig. 1, Appendix 2), indicative of an active process. The remaining apical lumens did not show an aberrant morphology when compared to control cells (pH 7.4; Fig. 1J), indicating heterogeneity within the cell population. Exposure of the cells to acidic media under the conditions of our experiments did not negatively affect cell viability as evidenced by trypan blue exclusion assays (data not shown).

In conclusion, extracellular and cytoplasmic acidosis induces the appearance of apical-like vacuolar structures (i.e. containing apical plasma membrane resident proteins and lined with actin filaments) surrounding the apical plasma membrane domain.

Acidosis-induced vacuoles originate from the apical plasma membrane

The vacuolar structures could originate from the existing apical plasma membrane or, alternatively, represent newly synthesized apical cargo carrying vesicles that failed to efficiently fuse with the apical plasma membrane (i.e. biosynthetic origin). In order to conclusively determine the origin of the apical-like vacuolar structures, we performed live cell imaging of HepG2-EGFP-MDR1 (/ABCB1) cells incubated in culture.
Figure 1. Acidosis induces the formation of apical protein-positive vacuoles in the cytoplasm of HepG2-MDR1-EGFP cells. HepG2 cells stably expressing apical MDR1-EGFP were cultured for 72 hours on glass coverslips, followed by 2 hours incubation in neutral medium (pH 7.4) (A-C), acidic medium (pH 5.5) (D-F), or treatment with ammonia prepulse (G-I). Cells were fixed and stained for F-actin (phalloidin-TRITC) and nuclei (DAPI) and analyzed with a fluorescence microscope. Note that the acid-induced MDR1-positive vacuoles are decorated with F-actin (E,H; arrows). Scale bar 5µm. (J) HepG2-MDR1-EGFP cells were exposed to media of different pH or treated with ammonia prepulse for 2 hours. Note the significant increase in number of apical vacuoles in pH 5.5 and 5.0, and with the ammonium prepulse acidification when compared to control (neutral pH) conditions.
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Figure 2. Acidosis causes thinning and vacuolarization of the apical plasma membrane. HepG2-MDR1-EGFP cells were cultured for 60 hours on LabTek Chambers and then translocated to the life imaging microscope and cultured for another 16 hours in 37°C and 5% CO2 in normal (A) or acidic (B, E) medium and photographed every 5 minutes. The acidic environment causes thinning (B, arrow) mostly followed by vacuolarization of apical membrane (C, arrow), resulting in the formation of vacuoles which can detach from the apical membrane (D, arrow) and fuse with each other (E, box) forming larger vacuoles. Scale bar 5µm.

medium of different pH. As shown in Figure 2 and supplementary movies (see Suppl. Movies A-E, Appendix 2), apical plasma membrane domains of cells exposed to an extracellular pH of 7.4 (Fig. 2A, Suppl. Movie A, Appendix 2) appeared rather stable as a function of time, while being dynamic as evidenced by their pulsating behavior,
consistent with previous reports and most likely reflecting actin-myosin-mediated canalicular contractions (Phillips et al., 1982; Watanabe et al., 1991; St-Pierre et al., 1997).

In striking contrast, the apical plasma membrane of cells exposed to an acidic extracellular pH did not show pulsating behavior, but showed extensive thinning or stretching (Fig. 2B arrow, Suppl. Movie B, Appendix 2) and vacuolarization (Fig. 2C arrow, Suppl. Movie C, Appendix 2), resulting in the formation of vacuolar structures of which some appeared to remain connected with the apical plasma membrane while others detached (Fig. 2D arrow, Suppl. Movie D, Appendix 2). Interestingly, the

Figure 3. Acidosis does not affect β-catenin localization. HepG2 cells stably expressing MDR1-EGFP were cultured on coverslips for 72h in normal conditions, and incubated for 2h in medium with a pH of 7.4 (A-C), 5.5 (D-F) or treated with ammonia prepulse (G-I). Cells were then fixed, stained for β-catenin (monoclonal antibody) and nucleus (DAPI), analyzed and photographed under fluorescent microscope. Note the lateral localization of β-catenin (arrows). Scale bar 5µm.

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Fusion of EGFP-MDR1(/ABCB1)-positive vacuolar structures in the apical cytoplasm was also observed, giving rise to larger apical-like intracytoplasmic vacuoles (Fig. 2E box, Suppl. Movie E, Appendix 2).

Thinning/stretching and inward expansion of the apical plasma membrane frequently preceded the vacuolarization, while in some cases only apical plasma membrane thinning was observed. The onset of apical plasma membrane deformation (thinning) occurred between 0.5 and 6 hours following exposure to the acidic environment, with most vacuolarizations starting around 2 hours. The thinning and

![Figure 4. Acidosis does not perturb the localization of the tight junction protein ZO-1. HepG2 cells stably expressing MDR1-EGFP were cultured on glass coverslips for 72 hours in normal conditions, and incubated for 2 hours in acidic medium (pH 5.5) or treated with ammonia prepulse. Cells were then fixed, and the tight junction protein ZO-1 was immunolabeled and the nucleus was stained with DAPI. Cells were then analyzed using a fluorescence microscope and photographed. Note that the acid-induced vacuoles do not contain the ZO-1 protein (F,I, arrows). Scale bar 5µm.](image-url)
subsequent vacuolarization of the apical plasma membrane appeared as a relatively slow process taking 3-5 hours after initiation.

Taken together, these data demonstrate that extracellular acidosis prevents canalicular contractions and induces apical plasma membrane deformation and vacuolarization, resulting in the appearance of apical vacuoles that, when pinched off, can fuse to form larger intracytoplasmic apical vacuoles. The apical plasma membrane as the origin of the vacuolar structures is consistent with our observation that treatment of the cells with the protein synthesis inhibitor cycloheximide (20µg/ml) did not prevent the acidosis-induced apical plasma membrane vacuolarization (data not shown).

**Acidosis-induced apical plasma membrane dynamics does not perturb apical adhesion junctions**

We next investigated whether the observed acidosis-induced deformations and vacuolarization of the apical plasma membrane also affected apical cell-cell adhesion junctions or the barrier function of tight junctions, both of which are important for maintaining a polarized epithelial architecture. As shown in Figures 3 and 4, exposure of the cells to an acidic environment did not visibly change the immunofluorescent staining pattern of the adherens junction-associated beta-catenin or the tight junction-associated ZO-1 respectively. Thus, beta-catenin was predominantly localized at the lateral surface in between the cells (Fig. 3D-I) and ZO-1 was observed as a ring surrounding the apical plasma membrane domain (Fig. 4D-I), both comparable to control situations (Fig. 3A-C and Fig. 4A-C; cf. van der Wouden et al., 2003; Théard et al., 2007). These apical junction proteins also did not colocalize with the vacuolar structures.

To test whether tight junctions remained capable to restrict paracellular transport, i.e. separate apical and basal extracellular compartments (barrier function), cells that were exposed to a neutral or acidic environment were incubated with CFDA (5-carboxyfluorescein diacetate, 0.5µM), membrane-permeable substrate for the apically
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Figure 5. Acidosis does not perturb the barrier function of the tight junctions. HepG2 cells were cultured on glass coverslips for 72 hours in normal conditions, followed by 2 hours incubation in neutral medium (pH 7.4), acidic medium (pH 5.5) or treated with ammonia prepulse. During the last 30 minutes of acidification, the membrane permeable CFDA (0.5 µM) was added. The ability of live cells to retain CF in the apical lumen was subsequently analyzed using a fluorescence microscope. Note that the acidified cells retained CFDA in the apical lumens. Scale bar 5µm.

located MRP2 ABC-cassette transporter. After being processed by intracellular esterases in the cytoplasm the resultant green fluorescent and membrane-impermeable anion CF is translocated into the apical lumens. In both control and acid treated cells, CF fluorescence was retained in the apical lumens (Fig. 5), in the vacuolarizations that appeared continuous with the apical membrane (arrow), and in the intracytoplasmic apical vacuoles. These data suggest that the barrier function of tight junction as well as the MRP2 activity and topology is not inhibited by exposure to an acidic environment. The “fence” function of ZO-1 was tested with fluorescently labeled sphingolipids (C6-NBD-GlcCer) (van der Wouden et al., 2002) (see Materials and Methods). Lipids that were inserted into the outer leaflet of the basolateral plasma membrane domain did not diffuse to the apical plasma membrane domain in cells exposed to either neutral or acidic medium (Fig. 6A, B) or in cells treated with the prepulse technique; Fig. 6C), indicative of an intact fence function of the tight junctions. As a positive control we treated the cells with 2mM EDTA in pH 7.4 which resulted in a pronounced lateral diffusion of NBD-lipids to the apical plasma membrane. These data indicate that acidosis-induced deformation and vacuolarization
Figure 6. Acidosis does not perturb the barrier function of the tight junctions. HepG2 cells were cultured on glass coverslips for 72h in normal conditions, followed by 2h acidification in pH 5.5 or ammonia prepulse. Cells were then incubated for 30 minutes at 4°C with the fluorescently labeled lipid C6-NBD-GlcCer to allow its incorporation in the outer leaflet of basal plasma membrane. In normal conditions the tight junctions prevent diffusion of the lipids to the apical domain, as seen in pH 7.4 (A), as well as in acidic conditions (B, C) (the BC is encircled with dashed line). For comparison, the presence of 2mM EDTA disrupts the tight junction fence what results in diffusion of lipids from basolateral to the apical plasma membrane (D). Scale bar 5μm.
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of the apical plasma membrane does not affect the ability of the cells to maintain cell-cell contact as well as distinct apical and basal compartments.

Agents that perturb actin filament dynamics stimulate the formation of apical vacuoles

Because cortical actin filaments play an important role in apical plasma membrane dynamics and are associated with the acidosis-induced apical vacuoles, we investigated the involvement of actin filament dynamics in their formation. For this, cells in a neutral or acidic environment were treated for 2 hours with either of three agents known to disrupt actin filaments: jasplakinolide (Fig. 7), cytochalasin D, and latrunculin B (see Suppl. Fig.2, Appendix 2). The viability of the cells was not affected by these concentrations of the compounds, as evidenced by trypan blue exclusion assays (data not shown). The presence of jasplakinolide in neutral medium induced formation of apical vacuoles that were similar to those observed in cells exposed to an acidic environment (Fig. 7B arrows, G; cf. Fig. 1) and promoted the formation of apical vacuoles while added to acidic media (Fig. 7 C-G). By contrast, treatment of the cells with microtubule-disrupting agents nocodazole (33 μM) or colchicine (1μM) did not affect apical membrane structure in cells exposed to neutral pH neither changed the acid-induced internalization in pH 5.5 and prepulse (data not shown). These data show that disruption of functional actin filaments similarly induces the formation of apical vacuoles and exaggerates the apical-vacuole-inducing effect of extracellular acidosis, and therefore implicate the involvement of altered actin filament dynamics.

Acidosis-induced formation of apical vacuoles requires Rho kinase and myosin-II activity

The activity of Rho kinase and its target protein myosin-II are well-known for regulating actin filament dynamics and apical plasma membrane morphogenesis (Utech et al., 2005; Herrema et al., 2006; Ferrari et al., 2008). In order to provide
further evidence for the involvement of actin filament dynamics in acidosis-induced apical vacuole formation, we employed an inhibitor of Rho kinase activity, Y27632

**Figure 7.** The actin stabilizing agent, jasplakinolide, enhances the apical membrane internalization in neutral pH 7.4 and in acidified conditions. The HepG2-MDR1-EGFP cells were cultured on glass coverslips for 72 hours, followed by 2 hours acidification in pH 5.5 and ammonia prepulse in the presence of jasplakinolide (200 nM). Cells were then fixed and stained for nucleus (DAPI) and analyzed under fluorescent microscope. Jasplakinolide, a compound that affects actin dynamics (as well as latrunculin B and cytochalasin D, see Suppl. Fig. 2, Appendix 2) enhances the internalization in acidic conditions: pH 5.5 (D) and prepulse (F), and was able to induce the apical membrane internalization in control pH 7.4 (B). Scale bar 5 µm. (G) The presence of jasplakinolide significantly enhances the level of apical membrane internalization in comparison to cells without this compound, in both: neutral pH 7.4 and acidified conditions (pH 5.5 and prepulse).
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and an inhibitor of myosin-II ATPase activity, blebbistatin. Treatment of the cells with either of these compounds resulted in significant decrease of acidosis-induced apical vacuolarizations to a level comparable to the control condition (Fig. 8A, B). These data indicate that Rho kinase and myosin-II ATPase activity are required for the formation of apical vacuoles in response to extracellular acidosis, and support the involvement of actin filament dynamics in this process.

DISCUSSION

The data reported in this study demonstrate that an extracellular stress factor such as acidosis can induce thinning and deformation of the apical plasma membrane of epithelial cells resulting in the vacuolarization and actin-filament-coated intracytoplasmic accumulation of large parts of the apical plasma membrane. Intracytoplasmic apical vacuoles were observed to fuse and form larger vacuolar apical compartments, reminiscent of those previously reported (VACs).

The formation of VACs could be mimicked by three agents known to disrupt actin filaments. Jasplakinolide stabilizes actin filaments and promotes actin polarization by enhancing the rate of actin filament nucleation, resulting in disruption of functional fibers (Bubb et al., 2000). Cytochalasin D binds actin filaments and inhibits polymerization and elongation, whereas latrunculin B binds actin monomers, preventing them from polymerizing into filaments. These data clearly demonstrate that the regulated polymerization of actin filaments is important for the dynamics and shape of the apical plasma membrane. Actin filament dynamics is regulated by signaling proteins that include, among others, small GTPase Rho proteins and their effector proteins. Our data demonstrate that acidosis-induced vacuolarization of the apical plasma membrane correlates with the activity of Rho kinase and its target myosin-II ATPase, the latter of which has been implicated in the regulation of actin filament polymerization (Isik et al., 2008). Our Western blot analyses did not reveal a
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statistically significant increase in Rho kinase or myosin-II ATPase activity (i.e. increased myosin-II phosphorylation) between cells exposed to a neutral or acidic environment (our unpublished observations). It is possible that acidosis-induced formation of apical plasma membrane vacuolarizations requires a basal activity level of these enzymes. Alternatively, the heterogeneity with regard to the cells’ response to acidosis (i.e. 40-50% of the apical lumens showed deformations/vacuolarizations) may have masked changes in the myosin-II phosphorylation status (the analysis of

Figure 8. The inhibition of myosin-II significantly decreases acid-induced internalization of apical membrane. The HepG2-MDR1-EGFP cells were cultured on glass coverslips for 72 hours in normal conditions, followed by 2 hour-long acidification in pH 5.5 and ammonia prepulse, in the presence of Rho-kinase inhibitor (Y27632, 2.5 µM) (A) and blebbistatin (0.1 µM) (B). Cells were then fixed, stained for actin (TRITC-phalloidin) and nucleus (DAPI) and counted under fluorescence microscope.
which is based on a lysate of the entire cell population). Indeed, more stress fibers and actin spikes, which can be induced by Rho kinase activity (Sato et al., 2003), were observed in acid-exposed cells that show apical plasma membrane vacuolarization (Suppl. Fig. 3, Appendix 2). Because signaling pathways that regulate actin filament dynamics are involved in acidosis-mediated VACs formation, while VACs formation, in turn, is mimicked by agents that disrupt actin filament dynamics, it is conceivable that an acidosis-mediated disruption of actin filament dynamics destabilizes the apical surface and promotes apical plasma membrane vacuolarization. In support of this, the RhoA/ROCK pathway of activating myosin-II has been shown to be pH-sensitive. Thus, an increased concentration of cytosolic protons was shown to elevate the concentration of Ca\(^{2+}\) resulting in the activation of myosin-II light chain kinase, actin rearrangement, and contraction (Huang et.al. 1994; Huang and Cheung 1994; Stock and Schwab, 2009).

The observed vacuolarization of the apical plasma membrane resembles membrane blebbing at the morphological and mechanistical (i.e. mediated by Rho kinase and myosin II, and induced by cytochalsin D and latrunculin) level (reviewed in Charras, 2008). Membrane blebbing is a physiological process that involves local disruption of membrane–actin cortex interactions, leading to rapid protrusion of the PM into the extracellular environment as a result of the cell internal hydrostatic pressure (Charras et al., 2005). The inward vacuolarization of the apical surface (as opposed to the typically outward-directed blebs) observed in this study may be driven by a relatively higher hydrostatic pressure in the apical lumen in comparison to that in the cell. Along this line of reasoning, it is thus conceivable that exposure of cells to extracellular acidosis, via a mechanism that involves Rho kinase, myosin II and F-actin dynamics, results in an extracellular hydrostatic pressure-driven vacuolarization of the apical plasma membrane and resultant shedding of fusogenic apical plasma membranes into the cytoplasm. If one assumes that plasma membrane–actin cortex attachment is homogeneously fragilized throughout the polarized cell (Charras et al., 2008), pressure differences between the cytosol and the apical extracellular space on
the one hand and the cytosol and the basolateral extracellular space on the other may explain why blebbing of the basolateral plasma membrane was never observed in our experiments. Alternatively, extracellular acidosis may preferentially affect apical plasma membrane–actin cortex interactions.

Long term (>38 hours) treatment of intestinal epithelial cells with interferon (IFN)-gamma has been reported to result in the formation of large actin-coated vacuoles that originated from the apical plasma membrane resembling the vacuolar apical compartment (VAC) (Utech et al., 2005) and the vacuoles reported in this study. Similar as in our study, the IFN-gamma dependent formation of VACs required ATPase activity of a myosin-II motor. However, the IFN-gamma induced VACs also contained tight junction proteins, disrupting the barrier function (Utech et al., 2005). This is in contrast to our data because we did not observe tight junction proteins associated with acidosis-induced VACs and because acidosis did not inhibit the barrier and fence function of tight junctions. IFN-gamma and acidosis therefore induce VACs with distinct composition and via distinct mechanisms which may share common elements (e.g. requirement for Rho kinase and myosin-II).

We postulate that acidification of the extracellular environment, as occurs in hypoxic tumors and in microvillus inclusion disease (MVID; Wilson et al., 2001; Ukarapol et al., 2001), may contribute to the formation of intracytoplasmic VACs by stimulating the vacuolarization of apical plasma membranes, possibly driven by a local high hydrostatic pressure. Large apical plasma membrane invaginations have been observed in MVID enterocytes (Reinshagen et al., 2002). In addition, thinning of the apical plasma membrane can be observed in MVID enterocytes (Ameen and Salas, 2000; Wilson et al., 2001), and appears to precede VAC formation in response to acidosis (Fig. 2). MVID has recently been associated with mutations in the MYO5B gene, encoding the actin filament-based motor protein myosin Vb that regulates the intracellular transport of - among others - newly synthesized apical proteins and apical recycling proteins (Müller et al., 2008; Erickson et al., 2008; our unpublished data). Despite the identification of MYO5B mutations in MVID patients (several of which
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are predicted to express no myosin Vb protein), it remains uncertain whether formation of VACs can be directly linked to intracellular protein transport pathways that are regulated by myosin Vb. For instance, the number of MVID enterocytes that actually contain VACs significantly varies between patients (sometimes <1% of all enterocytes), and VACs or clear apical plasma membrane defects are not typically observed in other organs of MVID patients such as the kidney (our unpublished data). In addition, RNAi-mediated knockdown of myosin Vb or the overexpression of a myosin Vb tail fragment that interferes with endogenous myosin Vb function in cultured epithelial cells of various origin does not typically induce the appearance of VACs (Wakabayashi et al., 2005; Swiatecka-Urban et al., 2007; Nedvetsky et al., 2007).

In the body, location/organ-specific differences/changes in extracellular pH (which locally, e.g. in the duodenum or solid tumor microenvironment, can reach extremely low values) exist. In combination with a frequently elevated intraluminal hydrostatic pressure in tumors (due to tumor-induced duct obstructions) or MVID (due to fluid accumulation and diarrhea), VACs may therefore not be disease-specific but rather reflect a cellular response to external stress factors.