RhoA enhances store-operated $\text{Ca}^{2+}$ entry and intestinal epithelial restitution by interacting with TRPC1 after wounding

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Chung HK, Rathor N, Wang SR, Wang JY, Rao JN. RhoA enhances store-operated $\text{Ca}^{2+}$ entry and intestinal epithelial restitution by interacting with TRPC1 after wounding. Am J Physiol Gastrointest Liver Physiol 309: G759–G767, 2015. First published September 3, 2015; doi:10.1152/ajpgi.00185.2015.—Early mucosal restitution occurs as a consequence of epithelial cell migration to rescaling of superficial wounds after injury. Our previous studies show that canonical transient receptor potential-1 (TRPC1) functions as a store-operated $\text{Ca}^{2+}$ channel (SOC) in intestinal epithelial cells (IECs) and plays an important role in early epithelial restitution by increasing $\text{Ca}^{2+}$ influx. Here we further reported that RhoA, a small GTP-binding protein, interacts with and regulates TRPC1, thus enhancing SOC-mediated $\text{Ca}^{2+}$ entry (SOCE) and epithelial restitution after wounding. RhoA physically associated with TRPC1 and formed the RhoA/TRPC1 complexes, and this interaction increased in stable TRPC1-transfected IEC-6 cells (IEC-TRPC1). Inactivation of RhoA by treating IEC-TRPC1 cells with exoenzyme C3 transferase (C3) or ectopic expression of dominant negative RhoA (DNMRhoA) reduced RhoA/TRPC1 complexes and inhibited $\text{Ca}^{2+}$ influx after store depletion, which was paralleled by an inhibition of cell migration over the wounded area. In contrast, ectopic expression of wild-type (WT)-RhoA increased the levels of RhoA/TRPC1 complexes, induced $\text{Ca}^{2+}$ influx through activation of SOCE, and promoted cell migration after wounding. TRPC1 silencing by transfecting stable WT RhoA-transfected cells with siRNA targeting TRPC1 (siTRPC1) reduced SOCE and repressed epithelial restitution. Moreover, ectopic overexpression of WT-RhoA in polyamine-deficient cells rescued the inhibition of $\text{Ca}^{2+}$ influx and cell migration induced by polyamine depletion. These findings indicate that RhoA interacts with and activates TRPC1 and thus stimulates rapid epithelial restitution after injury by inducing $\text{Ca}^{2+}$ signaling.

$\text{Ca}^{2+}$ influx; epithelial restitution; cyclopiazonic acid; GTP-binding proteins; polyamines

The gastrointestinal (GI) epithelium is exposed to a wide array of luminal noxious substances, and acute mucosal injury occurs commonly during critical pathologic conditions (12, 13, 21, 42). Early mucosal restitution occurs as a consequence of intestinal epithelial cell (IEC) migration to reseal superficial wounds, a process not requiring cell proliferation; however, its exact mechanism is still unclear (5, 21, 38, 39). This early rapid reepithelialization after injury is a primary repair modality in the gastrointestinal tract and is regulated by multiple factors (25, 33, 49). Although the cytosolic free $\text{Ca}^{2+}$ ([Ca$^{2+}$]$_{\text{cyt}}$) plays a critical role in the regulation of epithelial restitution after injury (29–32, 34–36), the precise mechanisms regulating the $\text{Ca}^{2+}$ influx remain to be fully elucidated. $\text{Ca}^{2+}$ entry due to store depletion is often called store-operated $\text{Ca}^{2+}$ entry (SOCE) that is mediated by $\text{Ca}^{2+}$-permeable channels, termed store-operated $\text{Ca}^{2+}$ channels (SOCs) (1, 2, 22). SOCs contribute to the sustained increase in [Ca$^{2+}$]$_{\text{cyt}}$ and the refilling of $\text{Ca}^{2+}$ into the stores (2, 22). The voltage-gated K$^+$ (Kv) channels regulate $\text{Ca}^{2+}$ influx by regulating the membrane potential (E$_m$) that governs the driving force for $\text{Ca}^{2+}$ influx in IECs (27, 46, 50). Although the molecular identity of SOCs mediating SOCE is not completely defined, the canonical transient receptor potential-1 (TRPC1) protein is highly expressed in IECs and appears to be an excellent candidate for SOCs that [Ca$^{2+}$]$_{\text{cyt}}$ homeostasis after store depletion (29, 32). Recently, we have shown that Caveolin 1 (Cav1) regulates [Ca$^{2+}$]$_{\text{cyt}}$ by interacting with TRPC1 channels during intestinal epithelial restitution after wounding (35).

Rho proteins are members of the Ras superfamily of small GTP-binding proteins and their activation depends on the GTP/GDP exchange cycle (18, 26, 52). Activated Rho proteins exert distinct actions through their interaction with various target proteins or effectors to regulate a signal transduction pathway linking surface receptors to the formation of actomyosin stress fibers and focal adhesions (10, 17, 37, 45). Rho proteins regulate different cellular events involving cytoskeletal dynamics, myosin light chain phosphorylation, vesicle trafficking, and intracellular $\text{Ca}^{2+}$ homeostasis (37, 38). Our previous study shows that Rac1, another member of the Rho family GTPases, promotes IEC restitution after wounding by increasing $\text{Ca}^{2+}$ influx as a result of its interaction with PLC-$\gamma$1 (31). Although RhoA also affects the interaction of inositol 1,4,5-trisphosphate receptor (IP$_{3}$R) with TRPC1 at the plasma membrane (PM) of endothelial cells and triggers $\text{Ca}^{2+}$ influx after store depletion in endothelial cells (19), its exact role in modulating TRPC1/$\text{Ca}^{2+}$ signaling in migrating IECs after wounding remains elusive.

The purpose of this study was to test the possibility that RhoA protein interacts with TRPC1 and enhances $\text{Ca}^{2+}$ influx through SOCE, thus resulting in a stimulation of cell migration after wounding. First, we investigated whether the expression pattern of RhoA and its physical interaction with TRPC1 were changed in stable TRPC1-transfected cells (IEC-TRPC1). Second, we examined whether RhoA silencing decreased RhoA/TRPC1 complexes, altered SOCE, and inhibited cell migration in IEC-TRPC1 cells. Third, we investigated whether ectopic overexpression of wild-type (WT)-RhoA increased RhoA/TRPC1 complexes, SOCE, and promoted cell migration after wounding. Finally, we determined whether RhoA overexpres-
sion overcame the inhibition of SOC-induced Ca^{2+} influx and cell migration in polyamine-deficient cells. Results reported here clearly indicate that RhoA directly interacts with TRPC1 in IECs and that induced RhoA/TRPC1 association increases Ca^{2+} entry through SOCE, thus enhancing cell migration after wounding.

**MATERIALS AND METHODS**

**Chemicals and cell culture.** Disposable culture ware was purchased from Corning Glass Works (Corning, NY). Tissue culture media, LipofectAMINE 2000, and dialyzed fetal bovine serum (dFBS) were obtained from Invitrogen (Carlsbad, CA), and biochemicals were obtained from Sigma (St. Louis, MO). The affinity-purified rabbit polyclonal antibody against TRPC1 was purchased from Alomone Laboratories (Jerusalem, Israel), and the antibody against RhoA was from Santa Cruz Biotechnology (Santa Cruz, CA). The actin antibody was obtained from Genzyme (Cambridge, MA).

The IEC-6 cell line was purchased from the American Type Culture Collection (ATCC) at passage 13. IEC-6 cells were derived from normal rat intestinal crypt cells and were developed and characterized by Quaroni et al. (24). Stock cells were maintained in T-150 flasks in Dulbecco’s modified Eagle medium (DMEM) supplemented with 5% heat-inactivated FBS, 10 μg/ml insulin, and 50 μg/ml gentamicin sulfate. Flasks were incubated at 37°C in a humidified atmosphere of 90% air-10% CO2, and passages 15–20 were used in the experiments. The stable TRPC1-transfected IEC-6 cells (IEC-TRPC1) were developed and characterized as described in our recent publications (29, 32, 35) and cultured in DMEM medium used for growing IEC-6 cells.

**Plasmid construction and transfection.** The transfection grade eukaryotic expression vector pUSEamp(+), containing the full-length wild type cDNA of human RhoA gene, was purchased from Millipore. To construct the RhoA expression vector, WT-RhoA cDNA was subcloned into the Xho1 and HindIII sites of an expression vector pcDNA3.1(+) (Invitrogen) with the cytomegalovirus immediate-early promoter, and resulting clones were sequenced for the confirmation of successful subcloning of WT-RhoA cDNA. The IEC-6 cells were transfected with the WT-RhoA expression vector or control vector containing no RhoA cDNA (Null) by using LipofectAMINE 2000 and performed as recommended by the manufacturer (Invitrogen). After the 5-h period of incubation, the transfection medium was replaced by the standard growth medium containing 5% FBS for 2 days before exposure to the selection medium. These transfected cells were selected for RhoA integration by incubation with the selection medium containing 0.6 mg/ml of G418, and clones resistant to the selection medium were isolated, cultured, and screened for RhoA expression by Western blot analysis with the specific anti-RhoA antibody.

**Recombinant adenovirus construction and infection.** Adenoviral vectors were constructed using the Adeno-X Expression system (Clontech) according to the protocol recommended by the manufacturer and used previously (28). Briefly, the cDNA of human dominant negative mutant RhoA (DNMRhoA) was cloned into the pShuttle by digesting the pUSEamp (+)/DNMRhoA (T19N) with EcoRI/XhoI and ligating the resulting fragments into the Xho1 site of the pShuttle vector. pAdeno-X/DNMRhoA (AdDNMRhoA) was constructed by digesting pShuttle constructs with PI-Scel-M-Ceu and ligating the resulting fragments into the PI-Scel-M-Ceu sites of the pAdeno-X adenoviral vector. Recombinant adenoviral plasmids were packaged into infectious adenoviral particles by transfecting human embryonic kidney (HEK) 293 cells by using lipofectAMINE 2000 reagent. The adenoviral particles were propagated in HEK293 cells and purified on cesium chloride ultracentrifugation. Titters of the adenoviral stock were determined by standard plaque assay. Recombinant adenoviruses were screened for expression of the introduced genes by fluorescent microscopy and Western blot analysis using anti-RhoA antibody. pAdeno-X, the recombinant replication-incompetent adenovirus carrying no cDNA insert (AdNull), was grown and purified as described above and served as a control adenovirus. Cells were infected by AdDNMRhoA or AdNull (2 pfu/cell) (26) and cell samples were collected for various assays 72 h after the infection.

**RNA interference.** The siRNA that was designed to specifically cleave TRPC1 mRNA (siTRPC1) was synthesized and purchased from Dharmacon (Lafayette, CO). Scrambled control siRNA (C-siRNA), without the sequence homology to any known genes, was used as the control. For each 60-mm cell culture dish, 20 μl of the 5 μM stock siTRPC1 or C-siRNA were mixed with 500 μl of Opti-MEM medium (Invitrogen). This mixture was gently added to a solution containing 6 μl of LipofectAMINE 2000 in 500 μl of Opti-MEM medium. The solution was incubated for 15 min at room temperature and gently overlaid onto monolayers of cells in 3 ml of medium, and cells were harvested for various assays after 48-h incubation.

**Immunoprecipitation and western blotting analysis.** Cell samples, dissolved in ice-cold RIPA-buffer, were sonicated and centrifuged at 4°C, and then the supernatants were collected for immunoprecipitation (IP). Equal amounts of proteins (500 μg) for each sample were incubated with the specific antibody against TRPC1 or RhoA (4 μg) at 4°C for 3 h, and protein A/G-PLUS-Agarose was added and incubated overnight at 4°C. The precipitates were washed five times with ice-cold Tris-buffered saline (TBS), and the beads were resuspended in SDS sample buffer. For immunoblotting, samples were subjected to electrophoresis on PAGE gels described previously (34–36). Briefly, after the transfer of protein onto nitrocellulose membranes, the membranes were incubated for 1 h in 5% nonfat dry milk in 1× TBS-T buffer (0.1% Tween-20). Immunologic evaluation was then performed overnight at 4°C in 5% nonfat dry milk/TBS-T buffer containing a specific antibody against TRPC1 or RhoA. The membranes were subsequently washed with 1× TBS-T and incubated with the secondary antibodies conjugated with horseradish peroxidase for 1 h at room temperature. The immunocomplexes on the membranes were reacted for 1 min with Chemiluminescence Reagent (NEL-100 DuPont NEN).

**Measurement of [Ca^{2+}]_{cyt}**. Detailed digital imaging methods employed for measuring [Ca^{2+}]_{cyt} were described in our previous publications (29–32, 35). Briefly, cells were plated on 25-mm coverslips and incubated with the culture medium containing 3.3 mM fura-2 AM for 30 min under an atmosphere of 10% CO2 in air. The fura-2 AM-loaded cells were then superfused with standard bath solution for 20–30 min at 22–24°C to wash away extracellular dye and permit intracellular esterases to cleave cytosolic fura-2 AM into active fura-2. Fura-2 fluorescence from the cells and background fluorescence were imaged using a Nikon Diaphot microscope equipped for epifluorescence. Fluorescent images were obtained using a microchannel plate image intensifier (Opelco, Washington, DC) coupled by fiber optics to a Pulnix charge-coupled device video camera (Stanford Photonics, Stanford, CA). Image acquisition and analyses were performed with a Metamorph Imaging System (Universal Imaging). The ratio imaging of [Ca^{2+}]_{cyt} analyses were performed with a Metamorph Imaging System (Universal Imaging). The ratio imaging of [Ca^{2+}]_{cyt} was obtained from fura-2 fluorescence emission excited at 380 and 340 nm as described in our previous publications (31, 32).

**Measurement of cell migration.** Migration assays were carried out as described in our earlier publications (25–32, 34–36). Cells were plated at 6.25 × 10^4/cm^2 in DMEM containing FBS on 60-mm dishes thinly coated with Matrigel according to the manufacturer’s instructions (BD Biosciences, Bedford, MA) and were incubated as described for stock cultures. Cells were fed on day 2, and cell migration was assayed on day 4. To initiate migration, the cell layer was scratched with a single edge razor blade cut to ~27 mm in length. The scratch was made over the diameter of the dish and extended over an

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area 7–10 mm wide. The migrating cells in six contiguous 0.1-mm squares were counted at ×100 magnification beginning at the scratch line and extending as far out as the cells had migrated. All experiments were carried out in triplicates, and the results were reported as number of migrating cells per millimeter of scratch.

Statistical analysis. All data are expressed as means ± SE from six dishes. IP and immunoblotting results were repeated three times. The significance of the difference between means was determined by ANOVA. The level of significance was determined using the Duncan’s multiple-range test (14), and values of P < 0.05 were considered significant.

RESULTS

Induced RhoA association with TRPC1 in stable IEC-TRPC1 cells. To determine if RhoA regulates Ca\(^{2+}\) influx through its interaction with TRPC1, basal levels of RhoA and its interactions with TRPC1 were examined in stable IEC-TRPC1. As shown in Fig. 1A, levels of TRPC1 and RhoA proteins increased in two clones (Cs) of IEC-TRPC1 cells compared with those observed in parental IEC-6 cells transfected with an empty vector (Null). The level of RhoA protein in C1 and C2 of IEC-TRPC1 cells was approximately threefold of the value of null cells. To test the association of RhoA with TRPC1, whole cell lysates were IP with either the anti-RhoA or anti-TRPC1 antibody, and these precipitates were examined by Western blot analysis using the antibody against TRPC1 or RhoA. IP of RhoA or TRPC1 resulted in co-IP of TRPC1 and RhoA in null and stable IEC-TRPC1 cells, but the levels of RhoA/TRPC1 complexes in IEC-TRPC1 cells were higher than those observed in null cells (Fig. 1, C and D), which was associated with an increase in Ca\(^{2+}\) influx after store depletion as reported previously (29, 32). We also used IgG as a negative control in IP assays and found that incubation with IgG in the same conditions did not pull down either RhoA or TRPC1 (data not shown). These results indicate that RhoA physically interacts with TRPC1 in IECs.

RhoA silencing decreases RhoA/TRPC1 complex and Ca\(^{2+}\) influx through SOCE. To further determine the function of RhoA/TRPC1 association, the effects of RhoA inhibition by ectopic overexpression of the DNMRhoA or treatment with C3 on SOCE and cell migration were examined in stable IEC-TRPC1 cells. The adenoviral vector containing the DNMRhoA cDNA was used in this study, because adenoviral vectors are shown to infect a variety of cultured rat and human epithelial cells with >90% efficiency (28). We demonstrated that >95% of stable IEC-TRPC1 cells were positive when they were infected with the adenoviral vector encoding GFP served as the marker for 24 h (data not shown). As shown in Fig. 2A, reduction in RhoA by infection of stable IEC-TRPC1 cells with the AdDNMRhoA for 72 h or treatment with C3 at the concentration of 10 μg/ml for 6 h remarkably decreased RhoA/TRPC1 complexes as measured by IP assays (Fig. 2B). An adenovirus that lacked exogenous DNMRhoA cDNA (AdNull) was used as a negative control and did not alter levels of the RhoA/TRPC1 complex. Next, we examined if reduced RhoA/TRPC1 association by AdDNMRhoA or C3 affected SOCE and cell migration after wounding. AdDNMRhoA or C3 treatment not only decreased the levels of resting [Ca\(^{2+}\)]\(_{cyt}\) but also inhibited SOCE induced by cyclopiazonic acid (CPA), an inhibitor of Ca\(^{2+}\)-Mg\(^{2+}\)-ATPase in the endoplasmic reticulum and sarcoplasmic reticulum (Fig. 2, C and D). Exposure to CPA in control cells resulted in an initial transient increase in [Ca\(^{2+}\)]\(_{cyt}\) in the absence of extracellular Ca\(^{2+}\), which was apparently due to Ca\(^{2+}\) mobilization from intracellular Ca\(^{2+}\) stores. Addition of extracellular Ca\(^{2+}\) to the cell superfusate after store depletion by CPA caused a sustained increase in [Ca\(^{2+}\)]\(_{cyt}\) because of the SOCE. In AdDNMRhoA-treated cells, however, the level of resting [Ca\(^{2+}\)]\(_{cyt}\) was reduced by ∼40%, and Ca\(^{2+}\) influx due to SOCE was decreased by ∼50% compared with those observed in AdNull cells. Importantly, decreased RhoA/TRPC1 association and the subsequent reduction in [Ca\(^{2+}\)]\(_{cyt}\) by AdDNMRhoA or C3 suppressed cell migration after wounding (Fig. 3, A and B). The numbers of cells migrating over the wounded edge in AdDNMRhoA-infected cells were decreased by ∼40%, and they were decreased by ∼50% in cells treated with C3. In addition, cells exposed to either with AdDNMRhoA or C3 did not affect cell viability as measured by Trypan blue staining (data not shown). These findings indicate that RhoA/TRPC1 association is necessary for the activation of TRPC1/Ca\(^{2+}\) signaling and cell migration after wounding.

Ectopic overexpression of WT-RhoA increases RhoA/TRPC1 association and promotes cell migration by enhancing SOCE. To further define the role of RhoA/TRPC1 association in Ca\(^{2+}\)
inhibitor on RhoA interaction with TRPC1 and Ca\(^{2+}\) influx in the absence (0Ca\(^{2+}\)) in stable IECs, WT-RhoA cells were not significantly due to clonal variation, since identical results were observed when two independently transfected clones (C1 and C2) were analyzed. These results indicate that ectopic RhoA overexpression increases RhoA/TRPC1 association and promotes epithelial restitution after wounding by activating TRPC1-mediated Ca\(^{2+}\) signaling.

TRPC1 silencing decreases Ca\(^{2+}\) influx and represses restitution in stable WT-RhoA cells. To determine the effect of TRPC1 silencing on Ca\(^{2+}\) influx in stable WT-RhoA cells, siTRPC1 was used to specifically block endogenous TRPC1. Initially, we determined the transfection efficiency of the siRNA nucleotides and demonstrated that >95% of stable WT-RhoA cells were positive when they were transfected with a fluorescent FITC-conjugated C-siRNA for 48 h (data not shown). Transfection with siTRPC1 for 48 h decreased RhoA protein levels by ~85%, but it did not affect RhoA protein content (Fig. 5A). Transfection with C-siRNA at the same concentrations showed no significant effect on TRPC1 level. TRPC1 silencing had no effect on the levels of resting [Ca\(^{2+}\)]\(_{cyt}\) but it inhibited CPA-induced Ca\(^{2+}\) influx in stable WT-RhoA cells (Fig. 5, B and C). The level of CPA-induced Ca\(^{2+}\) influx was decreased by ~60% in TRPC1-silenced cells compared with that observed in cells transfected with C-siRNA. TRPC1 silencing also impaired epithelial restitution after wounding in stable WT-RhoA cells (Fig. 5D). The number of cells migrating over the denuded area 6 h after wounding was increased by ~30% after TRPC1 silencing. We also examined the effect of TRPC1 silencing on Ca\(^{2+}\) influx and epithelial restitution in other lines of IECs such as differentiated IEC-Cdx2L1 and stable IEC-STIM1 cells and demonstrated that decreased levels of TRPC1 by transfection with siTRPC1 also inhibited SOCE and repressed cell migration after wounding (data not shown). In addition, neither siTRPC1 nor C-siRNA affected cell viability as measured by Trypan blue staining (data not shown). These findings indicate that RhoA-induced Ca\(^{2+}\) signaling is mediated by activating TRPC1, thus inducing epithelial restitution after wounding.

Ectopic RhoA overexpression prevents inhibition of Ca\(^{2+}\) influx and cell migration induced by polyamine depletion. Our previous studies demonstrate that polyamine depletion by treatment with DFMO decreased RhoA levels and inactivated Ca\(^{2+}\) influx and cell migration induced by polyamine depletion. In the present study, we examined the effect of polyamine depletion on [Ca\(^{2+}\)]\(_{cyt}\) and cell migration in stable WT-RhoA cells. After parental IEC-6 (Null) and stable WT-RhoA cells were exposed to DFMO for 4 days, SOCE and cell migration were examined. Ectopic overexpression of the RhoA gene rescued polyamine depletion induced decrease in resting [Ca\(^{2+}\)]\(_{cyt}\) and Ca\(^{2+}\) influx due to SOCE,
because there were no significant differences in the levels of resting [Ca\(^{2+}\)]\(_{cyt}\) and SOCE in stable WT-RhoA cells treated with or without DFMO (Fig. 6, A and C). Furthermore, RhoA overexpression also prevented the inhibition of cell migration after wounding in polyamine-deficient cells (Fig. 6D). These results strongly suggest that polyamines enhance cell migration after wounding, at least partially, by activating TRPC1/Ca\(^{2+}\) signaling as a result of the stimulation of RhoA/TRPC1 association.

**DISCUSSION**

Early epithelial restitution in the gastrointestinal mucosa occurs by sloughing the damaged epithelial cells and migration of remaining viable cells from areas adjacent to, or just beneath, the injured surface to cover the wounded area in vivo (15, 42, 48). Although precise regulation of epithelial restitution to reseal superficial wounds is crucial for the maintenance of mucosal integrity under physiological and pathological conditions, the exact mechanism underlying this primary repair modality remains poorly understood. Our previous studies have demonstrated that TRPC1 functions as a SOC in IECs and regulates cell migration after injury (29). The current study provides new evidence that RhoA directly interacts with and activates TRPC1, thus stimulating Ca\(^{2+}\) influx through SOCE and enhancing epithelial restitution after wounding.

Results reported here clearly indicate that the increased RhoA levels in stable IEC-TRPC1 cells were associated with increases in SOCE. RhoA physically interacted with TRPC1 and formed the RhoA/TRPC1 complex, whereas RhoA silencing reduced RhoA/TRPC1 association and decreased SOCE in stable IEC-TRPC1 cells. Rho GTPases are important intracellular signal transducers linking cell surface signals to multiple intracellular responses and regulate actin polymerization and actomyosin contractility during cell motility (6, 53). A recent study shows that RhoA and its effectors such as ROCK1 and mDia1 modulate the downstream deformation-induced motogenic signaling by which cyclic strain stimulates IEC migration (4). Moreover, it has also been shown that the RhoA activation increases intracellular Ca\(^{2+}\) concentration via TRPC channels and this induction contributes to changes in cell contraction, adhesion, and cell shape (41). Our current results reveal that specific inhibition of RhoA by DNMRhoA or C\(_3\) reduced RhoA/TRPC1 complexes, decreased SOCE, and repressed cell migration after wounding. These results are consistent with our
previous studies (26, 28) and others (19, 45) showing that reduced levels of \([\text{Ca}^{2+}]_{\text{cyt}}\) prevent the stimulation of IEC migration over the wounded area after injury.

An increasing body of evidence indicates that SOCE is critical for maintaining sustained increases in \([\text{Ca}^{2+}]_{\text{cyt}}\) and in refilling \(\text{Ca}^{2+}\) into the store in nonexcitable cells including IECs (29). The most significant finding reported in this study, however, is that RhoA/TRPC1 association plays an important role in TRPC1 activation and SOCE during rapid restitution after wounding. Ectopic overexpression of RhoA increased RhoA/TRPC1 complexes, induced \([\text{Ca}^{2+}]_{\text{cyt}}\), and enhanced epithelial restitution, which were completely prevented by TRPC1 silencing. Consistent with the current findings, several studies have shown that elevated \([\text{Ca}^{2+}]_{\text{cyt}}\) is a major mediator for the stimulation of IEC migration (49, 50). On the other hand, it was also reported that RhoA augments \(\text{Ca}^{2+}\) entry induced by the G protein-coupled receptor agonist or \(\text{Ca}^{2+}\) store depletion through its interaction with TRPM or L-type \(\text{Ca}^{2+}\) channels (7, 9, 43, 44, 52). Studies from Urena and Lopez-Barneo (47) show that calcium channel induced \(\text{Ca}^{2+}\) release (CCICR) plays a major role in the metabotropic \(\text{Ca}^{2+}\) release during tonic vascular smooth muscle contractile activation and this process is also regulated by RhoA. RhoA was also found to bind to and regulate TRPC6 channels in several types of cells (16, 43, 51). Although, IECs also express TRPC5 and other TRPC proteins (27, 29), their functions as SOCs and interactions with RhoA remain unknown.

Our recent studies have demonstrated that induced STIM1 translocation to the PM promotes IEC migration after wounding by enhancing TRPC1-mediated \(\text{Ca}^{2+}\) signaling (32, 34). Although translocation of STIM1 from the ER/SR to the PM is a prerequisite for activation of SOCE, a little is known about
altering myosin light chain phosphorylation and formation of actin stress fibers in human endothelial cells (41). We have recently demonstrated that Cav1 also regulates TRPC1-mediated Ca2+ signaling through Src-dependent Cav1 phosphorylation (36), but it remains unknown if Cav1

the mechanism underlying STIM1 translocation, specifically the exact role of RhoA in this process. STIM1 is shown to couple the thrombin receptor to activate RhoA, thereby altering myosin light chain phosphorylation and formation of actin stress fibers in human endothelial cells (41). We have recently demonstrated that Cav1 also regulates TRPC1-mediated Ca2+ signaling through Src-dependent Cav1 phosphorylation (36), but it remains unknown if Cav1

Fig. 5. Effect of TRPC1 silencing on Ca2+ influx and cell migration in stable WT-RhoA transfected cells. A: representative immunoblots of TRPC1 and RhoA. Cells were transfected with control siRNA (C-siRNA) or siTRPC1 by LipofectAMINE 2000, and whole cell lysates were harvested 48 h thereafter (top). Quantitative analysis of TRPC1 immunoblots by densitometry that were corrected for actin loading from cells described above. Values are means ± SE; *P < 0.05, compared with C-siRNA transfected cells (bottom). B: representative records of Ca2+ influx after store depletion by CPA: a: control WT-RhoA cells; b: WT-RhoA cells transfected with C-siRNA; c: WT-RhoA cells transfected with siTRPC1. C: summarized data showing resting [Ca2+]cyt (left) and the amplitude of CPA-induced Ca2+ influx (right) from cells described in B. Values are means ± SE; n = 25. *P < 0.05, compared with cells transfected with C-siRNA. D: summarized data showing cell migration 6 h after wounding in cells described in A. Values are means ± SE from 6 dishes. *P < 0.05, compared with cells transfected with C-siRNA.

Fig. 6. Changes in the levels of RhoA, Ca2+ influx, and cell migration in stable WT-RhoA transfected IECs after polyamine depletion. Cells were grown in control cultures and cultures containing 5 mM 1-α-difluoromethylornithine (DFMO) for 4 days. A: representative immunoblots for RhoA as measured by Western immunoblot analysis. B: quantitative analysis of Western immunoblots by densitometry that were corrected for actin loading from cells described in A. Values are means ± SE; *P < 0.05 compared with control IEC-6 cells (bottom). C: summarized data showing resting [Ca2+]cyt (left) and the amplitude of CPA-induced Ca2+ influx (right) from cells described in A. Values are means ± SE; n = 25. *P < 0.05, compared with control IEC-6 cells. D: summarized data showing cell migration 6 h after wounding in cells described in A. Values are means ± SE from 6 dishes. *P < 0.05 vs. parental control IEC-6 cells.
interacts with and colocalized with RhoA in IECs during epithelial restitution after wounding.

Polyamines are potent regulators of mucosal regeneration (3, 11, 40), whereas depletion of cellular polyamines represses mucosal repair by inactivating TRPC1/Ca\(^{2+}\) signaling (29, 34). Polyamines, including spermidine, spermine, and their precursor putrescine, are intimately implicated in a wide variety of distinct biological functions (40) and shown to enhance early mucosal restitution (46, 48). Polyamines stimulate epithelial cell migration during restitution primarily by controlling [Ca\(^{2+}\)]\(_{cyt}\) (26, 30, 31, 34, 46), but the exact process by which polyamines regulate [Ca\(^{2+}\)]\(_{cyt}\) remains unclear. Depletion of cellular polyamines inhibits Kv channel activity, causes membrane depolarization, and attenuates agonist-mediated increase in [Ca\(^{2+}\)]\(_{cyt}\) (27, 50). The current study provides new additional evidence that the RhoA is necessary for the stimulatory effect of polyamines on Ca\(^{2+}\) influx, since forced overexpression of WT-RhoA restored SOCE and cell migration to near normal in polyamine-deficient IECs.

In summary, our results indicate that RhoA physically interacts with TRPC1 and forms RhoA/TRPC1 complexes and that this association is essential for the activation of TRPC1-mediated Ca\(^{2+}\) influx after injury. Specific inhibition of RhoA prevents RhoA/TRPC1 association and decreases SOCE, thus repressing cell migration after wounding. In contrast, ectopic RhoA overexpression increases RhoA/TRPC1 complex, induces SOCE, and stimulates epithelial restitution. These findings also suggest that RhoA/TRPC1/Ca\(^{2+}\) is crucial for maintaining normal intestinal epithelial integrity under biological and various pathological conditions.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS


REFERENCES


