Polyamines are required for phospholipase C-γ1 expression promoting intestinal epithelial restitution after wounding

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Rao JN, Liu L, Zou T, Marasa BS, Boneva D, Wang SR, Malone DL, Turner DJ, Wang JY. Polyamines are required for phospholipase C-γ1 expression promoting intestinal epithelial restitution after wounding. Am J Physiol Gastrointest Liver Physiol 292: G335–G343, 2007. First published September 14, 2006; doi:10.1152/ajpgi.00282.2006.—Intestinal mucosal restitution occurs by epithelial cell migration, rather than by proliferation, to reseal superficial wounds after injury. Polyamines are essential for the stimulation of intestinal epithelial cell (IEC) migration during restitution in association with their ability to regulate Ca2+ homeostasis, but the exact mechanism by which polyamines induce cytosolic free Ca2+ concentration ([Ca2+]cyt) is remains unclear. Phospholipase C (PLC)-γ1 catalyzes the formation of inositol (1,4,5)-trisphosphate (IP3), which is implicated in the regulation of [Ca2+]cyt by modulating Ca2+ store mobilization and Ca2+ influx. The present study tested the hypothesis that polyamines are involved in PLC-γ1 activity, regulating [Ca2+]cyt and cell migration after wounding. Depletion of cellular polyamines by α-difluoromethylornithine inhibited PLC-γ1 expression in differentiated IECs (stable Cdx2-transfected IEC-6 cells), as indicated by substantial decreases in levels of PLC-γ1 mRNA and protein and its enzyme product IP3. Polyamine-deficient cells also displayed decreased [Ca2+]cyt and inhibited cell migration. Decreased levels of PLC-γ1 by treatment with U-73122 or transfection with short interfering RNA specifically targeting PLC-γ1 also decreased IP3, reduced resting [Ca2+]cyt and Ca2+ influx after store depletion, and suppressed cell migration in control cells. In contrast, stimulation of PLC-γ1 by 2,4,6-trimethyl-N-(meta-3-fluoromethylphenyl)benzenesulfonamide increased IP3, increased [Ca2+]cyt, and promoted cell migration in polyamine-deficient cells. These results indicate that polyamines are absolutely required for PLC-γ1 expression in IECs and that polyamine-mediated PLC-γ1 signaling stimulates cell migration during restitution as a result of increased [Ca2+]cyt.

mucosal injury; early mucosal repair; cell migration; capacitative Ca2+ entry; Ca2+ influx; Cdx2 gene; intestinal epithelium

EARLY EPITHELIAL RESTITUTION is an important repair modality in the gastrointestinal mucosa and occurs as a consequence of epithelial cell migration over the damaged area after superficial injury, a process that is independent of cell proliferation (6, 8, 20, 39, 48). Defective regulation of this process underlies various critical pathological states such as mucosal bleeding and ulcers, disruption of epithelial integrity, and gut barrier dysfunction. This rapid reepithelialization is a complex process that is highly regulated by numerous extracellular and intracellular factors, but its exact mechanism are still unclear.

Polyamines, including spermidine, spermine, and their precursor putrescine, are organic cations found in all eukaryotic cells and have been intimately implicated in a wide variety of distinct biological functions (16, 42). Polyamines have been shown to stimulate early mucosal repair of gastric and duodenal injury in vivo (50, 51) and enhance epithelial cell migration in an in vitro model (18, 27, 52) that mimics the early cell division-independent stage of epithelial restitution. Studies from our laboratory (29, 30, 53) have further demonstrated that cellular polyamines stimulate epithelial cell migration during restitution primarily by controlling the cytosolic free Ca2+ concentration ([Ca2+]cyt). However, little is known about the exact process by which polyamines modulate [Ca2+]cyt in intestinal epithelial cells (IECs) except that these compounds are involved in the activation of voltage-gated K+ (Kv) channels (29).

Phospholipase C (PLC) is an important regulatory enzyme that catalyzes hydrolysis of the phospholipid phosphatidylinositol (4,5)-bisphosphate to generate diacylglycerol (DAG) and inositol (1,4,5)-trisphosphate (IP3), both of which are implicated in the regulation of a variety of cellular processes (3, 11, 17, 54, 56). It is well known that DAG functions as a PKC activator (15) and that IP3 acts as a Ca2+-mobilizing messenger, resulting in the release of Ca2+ from IP3-sensitive intracellular Ca2+ stores and activation of Ca2+ influx via plasma membrane Ca2+-permeable channels (21, 47). To date, three isoforms of PLC have been identified in mammalian cells (β, γ, and δ), but their expression is cell type dependent in various tissues. Activity of these PLC isoforms is regulated through different signaling pathways and has distinct biological roles in the signal transduction cascade. Although the activation mechanism of PLC-δ is unknown, PLC-β isoenzymes are activated by agonists whose receptors are coupled to heterotrimeric G proteins, whereas regulation of PLC-γ activity is implicated in its activation with, and phosphorylation by, receptor tyrosine kinases (12, 19). Among the PLC-γ isoenzymes, the PLC-γ1 is expressed ubiquitously, whereas PLC-γ2 is expressed commonly in cells of hematopoietic origin (36, 38). Several studies (1, 55) have shown that treatment with growth factor induces PLC-γ1 activation, resulting in the enhancement of cell motility. In contrast, pharmacological inhibition of PLC-γ1 activity represses cell migration and reduces cell invasiveness in breast, prostate, and glioblastoma multiforme cancer cell lines (25, 45). Piccolo et al. (24) reported that EGF induces a phosphoinosi-
tide 3-kinase-dependent translocation of PLC-γ1 at the leading edge of migrating cells in a wound healing assay, suggesting that induced PLC-γ1 is relevant in cell migration during epithelial repair.

Receptor-operated (ROC) and store-operated cation channel (SOC) Ca2+ influx pathways have been described in nonexcitable cells including IECs for many years, but the functional properties and molecular identities of channels supporting ROC and SOC Ca2+ influx have remained elusive and are the focus of intensive investigations. Our previous studies (29, 33) have demonstrated that canonical transient receptor potential (TRPC)1 protein is highly expressed in IECs and functions as SOC channels mediating capacitative Ca2+ entry (CCE) after store depletion. Recently, it has also been found that PLC-γ1 is necessary for the activation of TRPC channels in human keratinocytes and is implicated in the regulation of SOC-mediated Ca2+ influx (46). The present study determined whether polyamines regulate [Ca2+]s cyt by altering PLC-γ1 activity and if polyamine-induced PLC-γ1 plays a role in intestinal epithelial restitution after wounding. The data presented herein demonstrate that polyamines are absolutely required for PLC-γ1 expression in IECs and that induced PLC-γ1 signaling stimulates cell migration during epithelial restitution as a result of increased [Ca2+]s cyt through CCE. Some of these data have been published previously in abstract form (32).

**MATERIALS AND METHODS**

Chemicals and supplies. Disposable culture ware was purchased from Corning Glass Works (Corning, NY). Tissue culture media, isopropyl-β-D-thiogalactopyranoside (IPTG), LipofectAMINE 2000, and dialyzed FBS (dFBS) were obtained from Invitrogen (Carlsbad, CA), and biochemicals were from Sigma (St. Louis, MO). The primary antibody, an affinity-purified mouse monoclonal antibody against PLC-γ1, PLC-γ2, or PLC-B1, was purchased from Upstate Biotechnology (Lake Placid, NY). U-73122 was purchased from BioMol Research Laboratories (Plymouth Meeting, PA), whereas 2,4,6-trimethyl-N-(meta-trifluoromethylphenyl)-benzenesulfonamide (m-3M3FBS) was obtained from Calbiochem (San Diego, CA). The IP3 (3H) Biotak assay kit was purchased from Amersham Biosciences (Arlington Heights, IL). D(-)-Difluoromethyloxime (DFMO) was purchased from Ilex Oncology (San Antonio, TX).

Cell culture. Stable Cdx2-transfected IEC-6 cells were developed and characterized by Suh and Traber (41) and were a kind gift from Dr. Peter G. Traber (Baylor College of Medicine, Houston, TX). The expression vector, the LacSwitch System (Stratagene, La Jolla, CA), was used for directing the conditional expression of the Cdx2 gene, and IPTG served as the inducer for gene expression (43). IEC-6 cells, derived from normal rat intestinal crypts, were transfected with pORPSVCdx2 by electroporation techniques, and clones resistant to selection medium containing 0.6 mg/ml G418 and 0.3 mg/ml hygromycin B were isolated and screened for Cdx2 expression by Northern blot, RNase protection assays, and electrophoretic mobility shift assay. Stock-stable Cdx2-transfected IEC-6 (IEC-Cdx2L1) cells were grown in DMEM supplemented with 5% heat-inactivated FBS, 10 μg/ml insulin, and 50 μg/ml gentamicin sulfate. Before experiments, IEC-Cdx2L1 cells were grown in DMEM containing 4 mM IPTG for 16 days to induce cell differentiation as described in our earlier studies (27–29, 33).

**RNA interference.** Short interfering (si)RNA that was designed to specifically cleave PLC-γ1 mRNA (siPLC-γ1) was synthesized and purchased from Dharmacon (Lafayette, CO). Scrambled control siRNA (C-siRNA), which had no 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 siPLC-γ1 or C-siRNA were mixed with 500 μl of Opti-MEM (Invitrogen). This mixture was gently added to a solution containing 6 μl of LipofectAMINE 2000 in 500 μl of Opti-MEM. 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 a 24- or 48-h incubation.

**RT-PCR.** Total RNA was isolated by using the RNaseasy Mini Kit (Qiagen, Valencia, CA). Equal amounts of total RNA (5 μg) were transcribed to synthesize single-strand cDNA with a RT-PCR kit (Invitrogen). The specific sense and antisense primers for PLC-γ1 included 5’-ACACCGTGTCTTTTGGC-3’ and 5’-CCCTGTAGTC- GAAGGAGG-3’, and the expected size of the PLC-γ1 fragments was 672 bp. RT-PCR was performed as described in our earlier studies (29, 33). To quantify the PCR products (the amounts of mRNA) of PLC-γ1, an invariant mRNA of β-actin was used as an internal control. Optical density (OD) values for each band on the gel were measured by a gel documentation system (UVP, Upland, CA), and their signals were normalized to OD values in the β-actin signals.

**Western blot analysis.** Cell samples, placed in SDS sample buffer [50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1 mM DTT, 0.5 mM EDTA, 1.0% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 2 mM PMSF, 20 μg/ml aprotinin, 2 μg/ml leupeptin, and 2 mM sodium orthovanadate], were sonicated and centrifuged (12,000 rpm) at 4°C for 15 min. The supernatant from cell samples was boiled for 5 min and then subjected to electrophoresis on 7.5% SDS-PAGE gels according to Laemmli (13). After the transfer of protein onto nitrocellulose filters, filters were incubated for 1 h in 5% nonfat dry milk in 1× PBS-Tween 20 [PBS-T; 15 mM NaH2PO4, 80 mM Na2HPO4, 1.5 mM NaCl (pH 7.5), and 0.5% (vol/vol) Tween 20]. Immunological evaluation was then performed for 1 h in 1% BSA-PBS-T buffer containing 1 μg/ml of specific antibody against PLC-γ1 protein. Filters were subsequently washed with 1× PBS-T and incubated for 1 h with the second antibody conjugated with horseradish peroxidase for 1 h at room temperature. Immunocomplexes on the membranes were reacted for 1 min with enhanced chemiluminescence reagent (NEL-100, DuPont NEN).

**Measurement of cellular IP3.** Cellular IP3 levels were measured by using a Biotak assay system purchased from Amersham Biosciences. After different treatments, cells were rapidly mixed with ice-cold 20% perchloric acid and kept on ice for 20 min. Preparations were centrifuged (2,000 rpm) at 4°C for 15 min, and supernatants were removed and neutralized with KOH (10 M) to pH 7.5. Preparations were centrifuged again, and supernatants were collected and utilized for the IP3 assay. Levels of IP3 were measured by the competitive binding assay system with highly isomeric specificity. Assays were assessed for their linearity with respect to various incubation conditions, and the results were expressed as picomoles per milligram of protein.

**Measurement of [Ca2+]s cyt.** Details of the digital imaging methods employed for measuring [Ca2+]s cyt have been described in our previous studies (28, 29, 33, 53). Briefly, cells were plated on 25-mm coverslips and incubated in culture medium containing 3.5 μM fura-2 AM for 30–40 min at room temperature (22–24°C) under an atmosphere of 10% CO2 in air. Fura-2 AM-loaded cells were then superimposed with standard bath solution for 30–30 min at 22–24°C to wash away extracellular dye and permit intracellular esterases to cleave cytoxic 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 (Amperex XX1381, Opelco, Washington, DC) coupled by fiber optics to a Pulnix charge-coupled device video camera (Stanford Photonics, Stanford, CA). Image acquisition and analysis were performed with a Metamorph Imaging System (Universal Imaging). The ratio imaging of [Ca2+]s cyt was obtained from fura-2 fluorescent emission excited at 380 and 340 nm (17, 46).

**Measurement of cell migration.** Migration assays were carried out as described in our earlier studies (27–29, 33, 53). Cells were plated
at 6.25 × 10⁴ cells/cm² in DMEM plus dFBS on 60-mm dishes thinly coated with Matrigel according to the manufacturer’s instructions and were incubated as described for stock cultures. Cells were fed on day 2, and migration was tested on day 4. To initiate migration, the cell layer was scratched with a single-edge razor blade to ~27 mm in length. The scratch was made over the diameter of the dish and extended over an 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 triplicate, and results were reported as numbers of migrating cells per millimeter of scratch. 

**Polyamine analysis.** The cellular polyamine content was analyzed by HPLC analysis as previously described (18, 49). Briefly, after cells had been washed three times with ice-cold Dulbecco’s phosphate buffer saline (D-PBS), 0.5 M perchloric acid was added, and cells were frozen at −80°C until ready for extraction, dannelization, and HPLC analysis. The standard curve encompassed 0.31–10 µM. Values that fell >25% below the curve were considered undetectable. Results are expressed as nanomoles of polyamines per milligram of protein.

**Statistical analysis.** All data are expressed as means ± SE from six dishes. PCR 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 Duncan’s multiple-range test (10).

## RESULTS

**Changes in PLC-γ₁ expression and its enzyme product IP₃ following polyamine depletion.** Induced expression of the Cdx2 gene by treatment of stable IEC-Cdx2L1 cells with 4 mM IPTG for 16 days resulted in a significant development of the differentiated phenotype. These differentiated IEC-Cdx2L1 cells exhibited multiple morphological and molecular characteristics of intestinal epithelial differentiation as indicated by polarization, development of lateral membrane interdigitations and microvilli at the apical pole, and expression of brush-border enzymes such as sucrase-isomaltase (data not shown). Because these differentiated IEC-Cdx2L1 cells migrate over the wounded edge much faster than undifferentiated parental IEC-6 cells after injury (27, 29, 31), they provided an excellent model for the present study.

**Fig. 1.** Changes in phospholipase C (PLC)-γ₁ expression and inositol (1,4,5)-triphosphate (IP₃) levels in control differentiated IEC-Cdx2L1 cells and cells treated with either α-difluoromethylornithine (DFMO) alone or DFMO plus putrescine (PUT). Before experiments, stable IEC-Cdx2L1 cells were grown in DMEM containing 4 mM isopropyl β-D-thiogalactopyranoside (IPTG) for 16 days to induce cell differentiation. These differentiated IEC-Cdx2L1 cells were grown in DMEM containing either DFMO (5 mM) alone or DFMO plus PUT (10 µM) for 4 and 6 days, and then total RNA and whole cell lysates were harvested for various measurements. A: changes in PLC-γ₁ expression. a, mRNA levels as measured by RT-PCR analysis. First-strand cDNAs, synthesized from total cellular RNA, were amplified with specific sense and antisense primers, and PCR-amplified products are displayed in agarose gel for PLC-γ₁ (−627 bp) and β-actin (−244 bp). b, Representative immunoblots of Western blot analysis in cells described in A.a. Twenty micrograms of total protein were applied to each lane, and immunoblots were hybridized with the antibody specific for PLC-γ₁ (−135 kDa). After the blot was stripped, actin (−42 kDa) immunoblotting was performed as an internal control for equal loading. c, Quantitative analysis derived from densitometric scans of immunoblots of PLC-γ₁ as described in A.b. Values are means ± SE of data from 3 separate experiments; relative levels of PLC-γ₁ protein were corrected for loading as measured by the densitometry of actin. B: levels of IP₃ in cells described in A. Values are means ± SE of data from 6 dishes. *P < 0.05 compared with control and DFMO + PUT. C, Representative immunoblots of Western blot analysis for PLC-γ₂ and PLC-β₁ proteins in cells described in A. Three experiments were performed, which showed similar results.
4 days to totally deplete spermidine and substantially decreased spermine (by ~60%) (data not shown). Similar results have been published in our previous studies (27, 29, 48, 49). The results shown in Fig. 1 show that depletion of cellular polyamines by DFMO significantly inhibited PLC-γ1 expression in differentiated IEC-Cdx2L1 cells. Levels of PLC-γ1 mRNA in cells treated with DFMO for 4 and 6 days were decreased by ~80% (Fig. 1A,a), which were paralleled by decreases in PLC-γ1 protein (Fig. 1A,b). Levels of PLC-γ1 protein in cells exposed to DFMO for 4 and 6 days were decreased by ~75%. Consistently, the decreased levels of PLC-γ1 protein in polyamine-deficient cells were associated with a reduction of its enzyme product IP3 (Fig. 1B). Levels of IP3 were decreased by ~60% in cells exposed to DFMO for 4 and 6 days. In the presence of DFMO, the addition of exogenous putrescine (10 μM) to cultures not only prevented the decreased levels of PLC-γ1 mRNA and protein but also restored IP3 levels to near normal. Spermidine (5 μM) had an effect equal to putrescine on levels of PLC-γ1 when it was added to cultures that contained DFMO (data not shown). We also examined the changes in other mammalian PLC isozymes, including PLC-γ2 and PLC-β1, in the presence or absence of cellular polyamines and demonstrated that there were no significant differences in levels of these PLC proteins between control cells and cells exposed to DFMO alone or DFMO plus putrescine for 4 and 6 days (Fig. 1C). These results clearly indicate that polyamines are required for PLC-γ1 expression and that decreasing cellular polyamines inhibits PLC-γ1 formation, thus leading to the reduction of IP3 in IECs.

Polyamine depletion-mediated reduction of IP3 was associated with decreases in [Ca2+]cyt and cell migration. As shown in Fig. 2, reduced levels of IP3 following polyamine depletion decreased the resting [Ca2+]cyt and inhibited Ca2+ influx after Ca2+ store depletion induced by cyclopiazonic acid (CPA). Exposure to CPA resulted in an initial transient increase in [Ca2+]cyt in the absence of extracellular Ca2+, which was apparently due to Ca2+ mobilization from intracellular Ca2+ stores. The addition of extracellular Ca2+ to the cell superfusate, when the CPA-induced transient rise in [Ca2+]cyt returned to the basal level, caused a sustained increase in [Ca2+]cyt because of CCE. In polyamine-deficient cells, levels of resting [Ca2+]cyt and store depletion-induced Ca2+ influx were decreased by ~50%. These decreased levels of [Ca2+]cyt were also accompanied by a significant inhibition of cell migration after wounding (Fig. 2C). The numbers of cells migrating over the wounded edge were decreased by ~70% in DFMO-treated cells. Restoration of IP3 by exogenous putrescine given together with DFMO not only returned the resting [Ca2+]cyt and store depletion-induced Ca2+ influx to near-normal levels but also abolished the inhibition of cell migration in polyamine-deficient cells.

Effect of PLC-γ1 inhibition on [Ca2+]cyt and cell migration. To elucidate the exact relationship between PLC-γ1 and intestinal epithelial restitution, the following two experiments were carried out. First, we examined the effects of decreased levels of PLC-γ1 by treatment with its specific chemical inhibitor U-73122 on [Ca2+]cyt and cell migration. Results shown in Fig. 3A show that exposure of control differentiated IEC-Cdx2L1 cells (without DFMO) to U-73122 dose dependently decreased the levels of PLC-γ1 protein, which were also associated with a significant decrease in IP3 (Fig. 3B). When U-73122 at different concentrations was added to the medium, levels of IP3 were decreased by ~17% at 1 μM, ~68% at 2 μM, and ~75% at 5 μM, respectively. The reduced levels of IP3 by U-73122

![Fig. 2. Changes in resting free cytosolic Ca2+ concentration ([Ca2+]cyt), Ca2+ influx after cyclopiazonic acid (CPA)-induced Ca2+ store depletion, and cell migration in differentiated IEC-Cdx2L1 cells described in Fig. 1. A: representative records of [Ca2+]cyt, changes measured in peripheral areas of control cells and cells exposed to DFMO or DFMO plus PUT for 4 days. Ca2+ stores were depleted by treatment with CPA in the absence of extracellular Ca2+ (0 Ca2+). B: 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 = 20. C: changes in cell migration after wounding in cells described in A. Cell migration was assayed 6 h after part of the monolayer was removed, as described in MATERIALS AND METHODS. Values are means ± SE of data from 6 dishes. *P < 0.05 compared with control cells and cells exposed to DFMO plus PUT.](http://ajpgi.physiology.org/doi/10.1152/ajpgi.00971.2006)
decreased resting \([Ca^{2+}]_{cyt}\) and inhibited store depletion-induced \(Ca^{2+}\) influx (Fig. 3C). Levels of resting \([Ca^{2+}]_{cyt}\) in cells exposed to U-73122 were decreased by \(\sim 25\%\), whereas \(Ca^{2+}\) influx after \(Ca^{2+}\) store depletion was decreased by \(\sim 50\%\). Treatment with U-73122 also inhibited cell migration after wounding (Fig. 3D). In U-73122-treated cells, the numbers of cells migrating over the wounded edge were decreased by \(\sim 21\%\) at 1 \(\mu M\), \(\sim 66\%\) at 2 \(\mu M\), and \(\sim 80\%\) at 5 \(\mu M\), respectively.

Second, we examined the changes in levels of \([Ca^{2+}]_{cyt}\) and cell migration after inhibition of PLC-\(\gamma_1\) expression by siPLC-\(\gamma_1\). These specific siPLC-\(\gamma_1\) nucleotides were designed to cleave rat PLC-\(\gamma_1\) mRNA by activating endogenous RNase H and to have a unique combination of specificity, efficacy, and reduced toxicity (33). Initially, we determined the transfection efficiency of the siRNA nucleotides in differentiated IEC-Cdx2L1 cells and demonstrated that \(>95\%\) of cells were positive when they were transfected with fluorescent FITC-conjugated siPLC-\(\gamma_1\) for 24 h (data not shown). As shown in Fig. 4A, transfection with siPLC-\(\gamma_1\) inhibited expression of PLC-\(\gamma_1\) in differentiated IEC-Cdx2L1 cells. Levels of PLC-\(\gamma_1\) protein were decreased by \(\sim 70\%\) at 24 h and \(\sim 85\%\) at 48 h after the transfection. To determine the specificity of siPLC-\(\gamma_1\) used in this study, we probed the membrane with anti-PLC-\(\beta_1\) antibody and showed that levels of PLC-\(\beta_1\) protein were not affected when cells were transfected with siPLC-\(\gamma_1\) (Fig. 4A,d). Inhibition of PLC-\(\gamma_1\) expression by siPLC-\(\gamma_1\) also decreased IP3, and its levels were decreased by \(\sim 70\%\) compared with those observed in control cells and cells transfected with C-siRNA. Decreased levels of IP3 by siPLC-\(\gamma_1\) reduced resting \([Ca^{2+}]_{cyt}\) and inhibited \(Ca^{2+}\) influx after \(Ca^{2+}\) store depletion (Fig. 5, A and B). Levels of resting \([Ca^{2+}]_{cyt}\) were decreased by \(\sim 40\%\), whereas store depletion-induced \(Ca^{2+}\) influx was decreased by \(\sim 50\%\) in differentiated IEC-Cdx2L1 cells transfected with siPLC-\(\gamma_1\) for 48 h. Furthermore, inhibition of PLC-\(\gamma_1\) expression and the subsequent decrease in \([Ca^{2+}]_{cyt}\) by siPLC-\(\gamma_1\) suppressed cell migration after wounding (Fig. 5C). The rate of cell migration was decreased by \(\sim 36\%\) in cells transfected with the siPLC-\(\gamma_1\) for 48 h (Fig. 5D). Transfection with C-siRNA at the same concentrations showed no inhibitory effects on PLC-\(\gamma_1\) expression, \([Ca^{2+}]_{cyt}\), and cell migration. In addition, neither siPLC-\(\gamma_1\) nor C-siRNA affected cell viability as measured by trypan blue staining (data not shown). These findings indicate that inhibition of PLC-\(\gamma_1\) expression decreases \([Ca^{2+}]_{cyt}\) and represses cell migration during restitution after wounding.

**Effect of increased PLC-\(\gamma_1\) on \([Ca^{2+}]_{cyt}\) and cell migration in polyamine-deficient cells.** In this study, the synthetic compound m-3M3FBS, which has been shown to specifically increase PLC-\(\gamma\) (2), was used to stimulate PLC-\(\gamma_1\) expression in polyamine-deficient cells. Results shown in Fig. 6 show that stimulation of PLC-\(\gamma_1\) by treatment with m-3M3FBS not only...
prevented the decrease in store depletion-induced Ca\(^{2+}\) influx but also stimulated cell migration in polyamine-deficient cells. When different concentrations of m-3M3FBS were added to the culture medium containing DFMO, they dose dependently increased levels of PLC-\(\gamma_1\) and IP3. Levels of IP3 in polyamine-deficient cells were increased by \(\sim 25\%\), whereas store depletion-induced Ca\(^{2+}\) influx was increased by \(\sim 65\%\). Furthermore, treatment with m-3M3FBS increased cell migration after wounding in polyamine-deficient cells (Fig. 6D). The numbers of cells migrating

![Fig. 4.](image)

Fig. 4. Effect of treatment with short interfering (si)RNA targeting the PLC-\(\gamma_1\) mRNA coding region (siPLC-\(\gamma_1\)) on levels of PLC-\(\gamma_1\) protein and IP3 in differentiated IEC-Cdx2L1 cells. A: changes in PLC-\(\gamma_1\) and PLC-\(\beta_1\) expression. a, Representative immunoblots of Western blot analysis for PLC-\(\gamma_1\) and PLC-\(\beta_1\) proteins; b, quantitative analysis of immunoblots of PLC-\(\gamma_1\) by densitometry from A.a. Cells were transfected with either control siRNA (C-siRNA) or siPLC-\(\gamma_1\) by the LipofectAMINE technique. Whole cell lysates were harvested 24 and 48 h after transfection, and levels of PLC-\(\gamma_1\) and PLC-\(\beta_1\) proteins were measured by Western immunoblot analysis. Actin immunoblotting was performed as an internal control for equal loading. Values are means \(\pm\) SE of data from 3 separate experiments. B: levels of IP3 in cells described in A. Values are means \(\pm\) SE of data from 6 dishes. *\(P < 0.05\) compared with C-siRNA.

![Fig. 5.](image)

Fig. 5. Changes in [Ca\(^{2+}\)]\(_{\text{cyt}}\) and cell migration in differentiated IEC-Cdx2L1 cells described in Fig. 4. A: representative records showing the time course of [Ca\(^{2+}\)]\(_{\text{cyt}}\) changes induced by exposure to CPA in the absence (0 Ca\(^{2+}\)) or presence of extracellular Ca\(^{2+}\). a, Control cells; b, cells transfected with C-siRNA for 48 h; c, cells transfected with siPLC-\(\gamma_1\) for 48 h. B: summarized data showing resting [Ca\(^{2+}\)]\(_{\text{cyt}}\) (left) and the amplitude of CPA-induced Ca\(^{2+}\) influx (right) from cells described in A. Values are means \(\pm\) SE; \(n = 25\). *\(P < 0.05\) compared with control cells and cells transfected with C-siRNA. C: images of cell migration 6 h after wounding by removal of part of the monolayer. a, Control cells; b, cells transfected with C-siRNA; c, cells transfected with siPLC-\(\gamma_1\). After cells were transfected for 48 h, cell migration was assayed 6 h after wounding. D: summarized data showing rates of cell migration after wounding in cells described in C. Values are means \(\pm\) SE of data from 6 dishes. *\(P < 0.05\) compared with controls and cells transfected with C-siRNA.
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over the wounded edge were increased by ~30% at 5 μM, ~45% at 10 μM, and ~68% at 25 μM. These results indicate that polyamine-induced PLC-γ₁ expression increases [Ca²⁺]ᵣᵣ and promotes IEC migration after wounding.

**DISCUSSION**

Epithelial cell migration is a primary process during early rapid mucosal repair after superficial wounds in the gastrointestinal tract, which absolutely requires cellular polyamines. Our previous studies (29, 30, 33, 53) have demonstrated that polyamines enhance epithelial cell migration, at least partially, by regulating [Ca²⁺]ᵣᵣ, because decreased levels of cellular polyamines reduced [Ca²⁺]ᵣᵣ and inhibited cell migration after wounding. The present study supports and extends our previous observations by demonstrating that polyamines are necessary for PLC-γ₁ expression and that induced PLC-γ₁ plays a critical role in the regulation of Ca²⁺ homeostasis during intestinal epithelial restitution. Decreased expression of PLC-γ₁ by polyamine depletion with DFMO decreased the formation of IP₃ (Fig. 1), which was associated with significant decreases in resting [Ca²⁺]ᵣᵣ and Ca²⁺ influx through CCE (Fig. 2). Furthermore, inhibition of PLC-γ₁ signaling in normal IEC-Cdx2L1 cells (without DFMO) by either treatment with its chemical inhibitor U-73122 (Fig. 3) or transfection with si-PLC-γ₁ also decreased [Ca²⁺]ᵣᵣ and repressed cell migration (Fig. 5). In contrast, increased levels of PLC-γ₁ protein in DFMO-treated cells by its specific chemical activator, m-3M3FBS, increased [Ca²⁺]ᵣᵣ and promoted cell migration in the absence of cellular polyamines (Fig. 6).

The findings reported herein clearly show that depletion of cellular polyamines inhibits expression of PLC-γ₁ in differentiated IECs. To provide insight into the molecular basis for PLC-γ₁ inhibition after polyamine depletion, the results shown in Fig. 1A indicate that levels of PLC-γ₁ mRNA decreased significantly in cells treated with DFMO for 4 and 6 days, which was paralleled by a reduction of PLC-γ₁ protein. This inhibition of PLC-γ₁ expression in DFMO-treated cells was completely prevented by the addition of exogenous putrescine, indicating that the observed changes in PLC-γ₁ expression must be related to polyamine depletion rather than to a non-specific effect of DFMO. This inhibitory effect of polyamine depletion on PLC-γ₁ expression is specific, because there were no significant differences in levels of other mammalian PLC isoforms such as PLC-γ₂ and PLC-β₁ between control cells and cells exposed to DFMO alone or DFMO plus putrescine for 4 and 6 days (Fig. 1C). Although the exact mechanism by which polyamine depletion decreases PLC-γ₁ mRNA remains unknown, the present study suggests that the regulation of PLC-γ₁ expression by polyamines appears to occur at the transcriptional level. In support of this possibility, our previous studies (14, 23, 57) and other studies (4, 5) have shown that polyamines are implicated in both transcription and posttranscription of various genes encoding different cellular signaling proteins and that decreases in mRNAs following polyamine depletion result predominantly from the inhibition of their gene transcription. On the other hand, decreasing polyamines increases cellular signaling factors primarily by stabilizing their mRNAs and proteins (57, 58). For example, polyamine depletion decreases c-Myc and c-Jun mRNAs in IECs by repressing their gene transcription but failing to affect their mRNA.

Fig. 6. Effect of induction of PLC-γ₁ by treatment with 2,4,6-trimethyl-N-(meta-3 trifluoromethylphenyl)-benzenesulfonamide (m-3M3FBS) on levels of IP₃, [Ca²⁺]ᵣᵣ, and cell migration in polyamine-deficient IEC-Cdx2L1 cells. A: representative immunoblots of Western blot analysis for PLC-γ₁ protein. Differentiated IEC-Cdx2L1 cells were grown in culture containing 5 mM DFMO for 4 days and then exposed to different concentrations of m-3M3FBS for 6 h. Levels of PLC-γ₁ protein were measured by Western immunoblot analysis, whereas actin immunoblotting was performed as an internal control for equal loading. B: levels of IP₃ in cells described in A. Values are means ± SE of data from 6 dishes. *P < 0.05 compared with cells treated without m-3M3FBS (control). C: representative records showing the time course of [Ca²⁺]ᵣᵣ changes after cells had been exposed to CPA in the absence (0 Ca²⁺) or presence of extracellular Ca²⁺. a, Cells treated with DFMO alone; b, cells treated with DFMO and then exposed to m-3M3FBS (25 μM). Three separate experiments were performed, which showed similar results. D: summarized data showing rates of cell migration 6 h after wounding in cells described in A. Values are means ± SE of data from 6 dishes. *P < 0.05 compared with controls.
stability (23), whereas decreasing cellular polyamines increases levels of p53 and JunD by stabilizing their mRNAs without an effect on gene transcription (14, 57). Clearly, further studies are needed to define the molecular process by which polyamines regulate the transcription of the PLC-γ1 gene in IECs.

The data from the present study also show that polyamine-modulated PLC-γ1 plays a critical role in the regulation of [Ca^{2+}]_{cyt}, at least in part, through IP_{3}-sensitive signaling pathway in IECs. Inhibition of PLC-γ1 expression by polyamine depletion decreased the level of IP_{3}, which was associated with a decrease in [Ca^{2+}]_{cyt} due to the reduction of CCE (Fig. 2). Consistently, inhibition of PLC-γ1 expression in normal IEC-Cdx2L1 cells by treatment with U-73122 (Fig. 3) or transfection with siPLC-γ1 also decreases IP_{3} and reduced [Ca^{2+}]_{cyt} (Fig. 5), whereas stimulation of PLC-γ1 by m-3M3FBs in polyamine-deficient cells increased IP_{3} and promoted Ca^{2+} influx through CCE (Fig. 6). These findings are consistent with results from others (21, 22, 26), who have demonstrated that IP_{3} triggers the release of Ca^{2+} from intracellular Ca^{2+} store through binding to IP_{3} receptors and results in the activation of Ca^{2+} influx via SOC channels. However, it also has been reported that PLC-γ1 augments Ca^{2+} entry induced by either a G protein-coupled receptor agonist or Ca^{2+} store depletion through its direct interaction with other signaling molecules such as TRPC3 and TRPC4, but independent of its lipase activity (17, 44). Several studies (9, 35, 46) have further shown that the interaction of PLC-γ1 with TRPC3 requires the partial pleckstrin homology (PH) domain and that the partial PH domain of PLC-γ1 interacts with a complementary partial PH-like domain in TRPC3 to elicit lipid binding and cell surface expression of TRPC3. Our previous studies have demonstrated that IECs do not express TRPC3 but highly express TRPC1, which functions as SOC channels mediating Ca^{2+} influx after store depletion. Interestingly, Tu et al. (44) recently found that PLC-γ1 activates SOC channels in human keratinocytes by interacting with TRPC1 but not with TRPC4. It is unclear at present whether polyamine-modulated PLC-γ1 directly binds to and regulates TRPC1 channels in IECs.

It is of physiological significance that polyamines regulate the expression of PLC-γ1 in IECs, because inhibition of PLC-γ1 signaling by polyamine depletion (Fig. 2) or siPLC-γ1 (Fig. 5) decreased [Ca^{2+}]_{cyt} and inhibited IEC migration after wounding. Under biological conditions, the pool of intracellular polyamines is dynamically regulated by polyamine biosynthesis, uptake, and degradation (40). Cellular levels of polyamines are changed rapidly, either increased or decreased, in response to various physiological and pathological stimuli, leading to the activation or inactivation of different cellular signaling pathways. It has been shown that levels of tissue polyamines in the damaged gastrointestinal mucosa are dramatically increased, which stimulates early rapid mucosal restitution in rats (50, 51). As reported in our previous studies (28–30, 53), elevated [Ca^{2+}]_{cyt} is a major mediator for the stimulation of IEC migration following an increase in cellular polyamines, but the exact mechanisms by which polyamines regulate Ca^{2+} influx and store Ca^{2+} release remain largely unknown. A series of studies from our laboratory (28, 34, 53) has demonstrated that polyamines regulate [Ca^{2+}]_{cyt} partially by governing membrane potential through control of K_{c} channel expression in IECs. Depletion of cellular polyamines inhibits K_{c} channel activity, as indicated by a decrease in K_{c} currents and membrane depolarization, contributing to the reduction of [Ca^{2+}]_{cyt} through shrinkage of the driving force for Ca^{2+} influx. The present study provides strong evidence for the role of polyamine-modulated PLC-γ1 signaling in the control of [Ca^{2+}]_{cyt} concentration during epithelial restitution after injury.

In summary, these results indicate that polyamines are necessary for PLC-γ1 expression and that induced PLC-γ1 is implicated in the signaling pathway of control of intracellular Ca^{2+} homeostasis during epithelial restitution after wounding. Depletion of cellular polyamines inhibits PLC-γ1 expression, reduces levels of IP_{3}, and decreases [Ca^{2+}]_{cyt}, thereby repressing IEC migration. In addition, inhibition of PLC-γ1 expression by treatment with its chemical inhibitor or transfection with siPLC-γ1 mRNA in normal IECs also decreases [Ca^{2+}]_{cyt} and causes the inhibition of cell migration. In contrast, increased PLC-γ1 by m-3M3FBs increases [Ca^{2+}]_{cyt} and promotes cell migration in polyamine-deficient cells. These findings suggest that PLC-γ1 is a biological regulator for control of [Ca^{2+}]_{cyt} in IECs under physiological and pathological conditions and plays a major role in polyamine-dependent IEC migration during restitution.

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