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Muscarinic m2 Receptor Mediated Actin Polymerization via PI3 Kinase γ and Integrin-Linked Kinase in Gastric Smooth Muscle

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Abstract

BACKGROUND: Actin polymerization plays an important role in smooth muscle contraction. Integrin-linked kinase (ILK) was shown to mediate actin polymerization in airway smooth muscle. The role of ILK in actin polymerization in response to m2 receptor activation was not examined in gastric smooth muscle.

METHODS: Phosphorylation of paxillin, N-WASp, and association of paxillin with GEF proteins (Cool2/ α Pix (Cool2/PAK interacting exchange factor alpha), Cool1/ β Pix (Cool1/PAK interacting exchange factor beta) and DOCK 180 (Dedicator of cytokinesis)), and N-WASP with Arp2/3 complex were measured by western blot. Activation of Cdc42 was determined using an antibody for activated Cdc42. Actin polymerization was measured as an increase in F-actin/G-actin ratio.

RESULTS: Phosphorylation of paxillin, an association of paxillin with GEF proteins, Cdc42 activity, and actin polymerization were increased in response to m2 receptor activation in gastric smooth muscle cells. The increases in paxillin phosphorylation, Cdc42 activity, and actin polymerization were inhibited by a PI3K γ inhibitor (AS-605240). ILK siRNA and ILK dominant negative mutant [ILK(R211)]. Increase in actin polymerization was also inhibited by Cdc42 dominant negative mutant (Cdc42 [T17N]). Increases in the association of paxillin with GEF proteins and phosphorylation of N-WASP and its association with Arp2/3 complex were inhibited by ILK (R211).

CONCLUSION: In gastric smooth muscle cells, activation of PI3K γ by muscarinic m2 receptors causes ILK-dependent phosphorylation of paxillin, an association of paxillin with Cdc42 GEF proteins and activation of Cdc42, which, in turn, causes phosphorylation of N-WASP and its association with Arp2/3 complex leading to actin polymerization.

Keywords

ILK; PI3 kinase; m2 receptors; smooth muscle; actin polymerization

INTRODUCTION

In gastrointestinal (GI) circular smooth muscle, initial and sustained contraction is mediated by phosphorylation of MLC_{20} via receptor coupled to Gq/G13 , Gi or both. Phosphorylation of MLC_{20} is regulated by two enzymes myosin light chain kinase (MLCK) and myosin light chain phosphatase (MLCP) [1–5]. Gq -coupled receptors activate phospholipase $\text{C-}\beta 1$ ($\text{PLC-}\beta 1$), whereas Gi -coupled receptors activate phospholipase $\text{C-}\beta 3$ ($\text{PLC-}\beta 3$) (1, 6). Both $\text{PLC-}\beta 1$ and $\text{PLC-}\beta 3$ preferentially hydrolyze phosphatidylinositol 4,5-bisphosphate (PIP_2) to yield diacylglycerol, and inositol 1,4,5-trisphosphate (IP_3). IP_3 -mediated increase in Ca^{2+} release causes activation of Ca^{2+} -calmodulin-dependent MLCK to initiate MLC_{20} phosphorylation and muscle contraction. The initial Ca^{2+} -dependent phase is terminated by the decrease in Ca^{2+} uptake into Ca^{2+} stores via the sarcoendoplasmic reticulum Ca^{2+} ATPase, SERCA_2 (1–5).

Sustained contraction by G13 -coupled receptors is mediated by two RhoA dependent pathways; one pathway involves phosphorylation of MYPT1 (regulatory subunit of MLCP) via Rho kinase and another pathway involves phosphorylation of CPI-17 (endogenous inhibitor of MLCP) via protein kinase C. Both lead to inhibition of MLCP and increase in MLC_{20} phosphorylation [1–5, 7]. Sustained contraction by Gi -coupled receptors, in contrast, is mediated by activation of phosphatidylinositol-3 kinase (PI3K) and integrin-linked kinase (ILK) pathway via $\text{G}\beta\gamma$. ILK localized in myofilaments of smooth muscle cells promotes muscle contraction by mediating phosphorylation of MLC_{20} and CPI-17 (8–10). The studies of the airway and vascular smooth muscle cells show that cytoskeletal reorganization and actin polymerization play an important role in the force transmission [11–17]. Several studies have shown that actin polymerization is regulated by interaction of ILK with α and β parvin and paxillin.

After agonist stimulation, ILK, particularly interesting new cysteine histidine rich protein (PINCH), and α -parvin complex are recruited to the plasma membrane and activates a variety of tyrosine kinases to phosphorylate paxillin [18–23]. This leads to activation of the actin nucleation-promoting protein N-WASp, and its association with the actin polymerization catalyst Arp2/3 complex, and the polymerization of cortical actin [24, 25].

Our previous studies in gastrointestinal smooth muscle showed expression of muscarinic m_3 and m_2 receptors (26). Expression of m_2 receptors is more abundant than m_3 receptors [6, 26]. Our previous studies using 4-DAMP as selective m_3 receptor antagonist and methoctramine as selective m_2 receptor antagonist showed that acetylcholine (ACh)-induced contraction is selectively mediated by Gq/13 coupled m_3 receptors but not Gi3 -coupled m_2 receptors because activation of m_2 receptors also causes activation of p21-activated kinase and p38 MAP kinase that inhibit MLCK and ILK, respectively. Suppression of PAK and p38 MAP kinase unmasks MLCK and ILK activities to mediate initial and sustained contraction, respectively (6, 8) in response to m_2 receptor activation.

Our recent studies in gastric muscle showed that activation of m_3 receptors also caused actin polymerization via a RhoA -dependent pathway involving paxillin/Cdc42/N-WASp/Arp2/3 pathway. In the present study we have examined whether activation of m_2 receptors are

linked to actin polymerization via ILK. Our results demonstrate that activation of m2 receptors increases paxillin phosphorylation via PI3 kinase γ (PI3K γ) and ILK. Binding of ILK to PIP3 is essential for paxillin phosphorylation and assembly of proteins involved in the stimulation of Cdc42 activity and N-WASP-mediated actin polymerization.

MATERIALS AND METHODS

Materials:

Cdc42 and F-actin/G-actin ratio assay kits were purchased from Cytoskeleton (Denver, CO), PI3K γ inhibitor AS-605240 was purchased from Cellagen Technology (San Diego, CA), Phosphorylated Paxillin(Tyr¹¹⁸) antibody, ILK siRNA and transfection reagents were purchased from Thermo Fisher (Rockford, IL), Arp2/3, N-WASP antibodies were purchased from Abcam (Cambridge, MA), Cdc42 and Paxillin antibodies were purchased from BD Biosciences (San Jose, CA), Cool1/ β -Pix, Cool2/ α -Pix, Dock 180, antibodies were purchased from Cell Signaling Technologies (Danvers, MA). Arp2/3 complex inhibitor CK666 was purchased from EMD Millipore (Billerica, MA), western blot supplies were purchased from BioRad (Hercules, CA). Cdc42-DN was a gift from Dr Lee Slice (Department of Medicine, CURE: Digestive Diseases Research Center, University of California, Los Angeles, CA, U.S.A.). 4-Diphenylacetoxy-N-methyl-piperidine methiodide (4-DAMP) and all other chemicals were purchased from Sigma-Aldrich (St. Louis, MO).

Methods:

Preparation of dispersed and cultured gastric smooth muscle cells.—The antrum of rabbit was separated from the rest of the stomach and the mucosal layer was removed by sharp dissection from both fundus and antrum. Smooth muscle cells from the circular muscle layer of the antrum were isolated by sequential enzymatic digestion of muscle strips, filtration, and centrifugation as described previously [27–29]. First, antrum was cut into thin slices using a Stadie-Riggs tissue slicer and then the slices were incubated at 31°C for 30 min in a smooth muscle buffer (NaCl 120 mM, KCl 4 mM, KH₂PO₄ 2.6 mM, CaCl₂ 2.0 mM, MgCl₂ 0.6 mM, HEPES (N-2-hydroxyethylpiperazine-N' 2-ethanesulfonic acid) 25 mM, glucose 14 mM, and essential amino acid mixture 2.1% (pH7.4)) with 0.1% collagenase II (300 U/ml) and 0.01% soybean trypsin inhibitor (w/v). The partly digested tissues were washed twice with 50-ml of collagenase-free smooth muscle buffer and the muscle cells were allowed to disperse spontaneously for 30 min in collagenase-free medium. Cells were harvested by filtration through 500 μ m Nitex and centrifuged twice at 350 *g* for 10 min to eliminate broken cells and organelles [27–29].

Dispersed muscle cells isolated from the stomach were resuspended in DMEM containing penicillin (200 U/ml), streptomycin (200 μ g/ml), gentamycin (100 μ g/ml), amphotericin B (2.5 μ g/ml) and 10% fetal bovine serum (DMEM-10). The muscle cells were plated at a concentration of 5 X 10⁵ cells/ml and incubated at 37°C in a CO₂ incubator. DMEM-10 medium was replaced every three days for 2–3 weeks until confluence was attained. All experiments were done on cells in the first passage [27–29].

Transfection of ILK dominant negative mutant (ILK [R211A], ILK siRNA and Cdc42 dominant negative mutant (Cdc42[T17N]) in cultured smooth-muscle cells: Inactive ILK mutant protein (ILK [R211A]) and inactive Cdc42 mutant protein (Cdc42[T17N]) were subcloned into the multiple cloning site (EcoRI) of the eukaryotic expression vector pcDNA3. Recombinant plasmid DNAs (2 µg each) or ILK siRNA (40 pmol) were transiently transfected into smooth muscle cells in primary culture using LIPOFECTAMINE 2000 reagent for 48 h. The cells were co-transfected with 1 µg of pGreen Lantern-1 to monitor transfection efficiency. Control cells were co-transfected with 2 µg of vector (pcDNA3) and 1 µg of pGreen Lantern-1 DNA. Transfection efficiency (approx. 85% of the cells) was monitored by the expression of green fluorescent protein using FITC filters [28].

Phosphorylation of paxillin and N-WASp: Phosphorylation of paxillin and N-WASp was measured by western blot and Immuno histochemistry using phospho-specific antibodies. Cultured gastric smooth muscle cells (2×10^6 cell/ml) were treated with acetylcholine (ACh, 1 µM) in the presence of m3 receptor antagonist 4-DAMP (1 µM) and solubilized on ice for 1 h in medium containing 20 mM Tris-HCl (pH 8.0), 1 mM DTT, 100 mM NaCl, 0.5% sodiumdodecyl sulfate, 0.75% deoxycholate, 1 mM PMSF, 10mg/ml of leupeptin and 100mg/ml of aprotinin. The proteins were separated by SDS/PAGE and electrophoretically transferred onto PVDF membranes. The membranes were incubated for 12 h with phospho-specific paxillin (Tyr¹¹⁸) antibody (1:1000) or phospho-specific N-WASp (Tyr²⁵⁶) antibody (1:10000), and then for 1h with horse-radish peroxidase-conjugated secondary antibody (1:5000). The protein bands were identified by enhanced chemiluminescence reagent [28]

Rabbit gastric smooth muscle cells were cultured in 8-well chamber slide for 2 days and treated with acetylcholine in the presence of 1 µM of 4-DAMP and in the presence or absence of PI3Kγ inhibitor AS605240. Cells were fixed in 4% paraformaldehyde for 30 min and incubated with blocking solution containing 5% normal donkey serum in PBS for 1 h followed by incubation with phospho-specific paxillin (Tyr¹¹⁸) (1:200) for 2 h at room temperature. Cells were rinsed twice in PBS and then incubated with fluorochrome-conjugated antibody for 1 h at room temperature. Cells were rinsed twice in PBS, once in water, then mounted on glass microscope slides and analyzed under a Zesis fluorescent photomicroscope (Carl Zeiss Microscopy, LLC (Thornwood, NY). Immunostaining in the absence of primary or secondary antibody was assessed for background evaluation.

Protein-Protein Association (paxillin:cool2/α pix, paxillin:cool1/β pix, paxillin:DOCK 180 and N-WASP:Arp2): Associations between paxillin and cool2/α-Pix, paxillin and cool1/β-Pix, paxillin and DOCK 180 were determined by immunoprecipitation with paxillin and immunoblot with cool2/α-Pix, cool1/β-Pix or DOCK-180 antibodies. N-WASP and Arp2/3 association was determined by immunoprecipitation with N-WASP and immunoblot with Arp2/3 antibody. Cultured gastric smooth muscle cells (2×10^6 cell/ml) were treated with 1 µM ACh for 5 min in the presence of m3 receptor antagonist 4-DAMP (1 µM) and then solubilized on ice for 1 h in medium containing 20 mM Tris-HCl (pH 8.0), 1 mM DTT, 100 mM NaCl, 0.5% SDS, 0.75%

deoxycholate, 1 mM PMSF, 10 mg/mL of leupeptin, and 100 mg/mL aprotinin. The immunoprecipitated proteins were separated by SDS-PAGE, and electrophoretically transferred onto polyvinylidene difluoride membranes. The membranes were incubated for 12 h with primary antibody (1:1000), and then for 1 h with horse-radish-peroxidase-conjugated secondary antibody (1:5000). The protein bands were identified by enhanced chemiluminescence reagent. [28].

Determination of Cdc42 Activation: The Cdc42 activation was determined by using a pull-down assay for activated Cdc42. Muscle cells were solubilized in lysis buffer plus protease and phosphatase inhibitors containing 1% NP-40, 50 mM Tris-HCl, (pH 7.4), 150 mM NaCl, 10% glycerol, 2 mM EDTA, 2 mM benzamidine, 0.5 mM aprotinin, 1 mM phenylmethylsulfonyl fluoride, 2 mM sodium orthovanadate, 2 mM molybdate, and 2 mM sodium pyrophosphate for 5 min at 4°C. For the analysis of cdc42 activation, the extracted proteins were reacted with GST-p21activated kinase (PAK) binding domain (PBD). Activated GTP-bound cdc42 was affinity-precipitated by glutathione beads and analyzed by western blot [16].

Analysis of F-Actin and G-Actin: The relative proportions of F-actin and G-actin in cultured gastric smooth muscle cells were analyzed by F-actin/G-actin assay kit from Cytoskeleton (Denver, CO). Cultured gastric smooth muscle cells were homogenized in 200 µl of F-actin stabilization buffer containing 50 mM PIPES, pH 6.9, 50 mM NaCl, 5 mM MgCl₂, 5 mM EGTA, 5% glycerol, 0.1% Triton X-100, 0.1% Nonidet P-40, 0.1% Tween 20, 0.1% β-mercaptoethanol, 0.001% antifoam, 1 mM ATP, 1 µg/ml pepstatin, 1 µg/ml leupeptin, 10 µg/ml benzamidine, and 500 µg/ml TAME. Supernatants of the protein extracts were collected after centrifugation at 150,000 X g for 60 min at 37 °C. The pellets were resuspended in 200 µl of F-actin depolymerization buffer containing 10 µM cytochalasin D and then incubated on ice for 1 h to depolymerize F-actin. The resuspended pellets were gently mixed every 15 min. Twenty microliters of supernatant (G-actin) and two microliter of pellet (F-actin) fractions were subjected to immunoblot analysis using anti-actin antibody. The relative amounts of F-actin and G-actin were determined using densitometry [16].

Statistical analysis.—The results were expressed as means ± SE of *n* experiments and analyzed for statistical significance by Student's *t*-test for paired and unpaired values. Differences among groups were tested by ANOVA and checked for significance via Fisher's protected least significant difference test. *P* < 0.05 was considered significant.

RESULTS

Increase of paxillin phosphorylation via PI3Kγ and ILK:

Our previous studies have shown that muscarinic m2 receptors activate PI3K via Gβγ₁₃ (6, 8). Several studies show that ILK activity is mediated by PI3K activation [8, 30]. Phosphorylation of paxillin in response to m2 receptor activation was measured by western blot and immunocytochemistry. Treatment of cultured gastric smooth muscle cells with 1 µM of acetylcholine (ACh) in the presence of 1 µM of m3 receptor antagonist 4-DAMP for 5 min caused a significant increase in paxillin phosphorylation at tyrosine 118 (80±10%

increase) (Figs. 1A and 1B). The increase in phosphorylation of paxillin was significantly inhibited by PI3K γ inhibitor ($70\pm 8\%$ decrease) (Figs. 1A and 1B). Increase in paxillin phosphorylation in response to m2 receptor activation was also inhibited in cells transfected with ILK siRNA ($68\pm 7\%$ decrease) (Fig. 2A) or ILK dominant negative mutant (ILK (R211A)) ($72\pm 10\%$ decrease) (Fig. 2B) compared to response in control cells. These results suggest that phosphorylation of paxillin in response to m2 receptor activation is dependent on ILK via PI3K γ .

Increase of paxillin association with GEFs via ILK:

Consistent with the increase in phosphorylation of paxillin, treatment of the cultured gastric smooth muscle cells with $1\text{ }\mu\text{M}$ of ACh in the presence of $1\text{ }\mu\text{M}$ of 4-DAMP for 5 min caused a significant increase in the association of paxillin with cool2/ α -Pix ($50\pm 5\%$ increase) (Fig. 3A), cool2/ β -Pix ($49\pm 6\%$ increase) (Fig. 3B) and DOCK-180 ($65\pm 8\%$ increase) (Fig. 3C). The increase in association of paxillin with cool2/ α -Pix, cool2/ β -Pix or DOCK-180 was inhibited in cells expressing ILK (R211A) ($52\pm 5\%$, $53\pm 6\%$ and ($60\pm 6\%$ decrease, respectively). These results suggest that an increase in association of paxillin with cool2/ α -Pix, cool2/ β -Pix and DOCK-180 in response to m2 receptor activation is dependent on ILK.

Stimulation of Cdc42 activity, N-WASp phosphorylation and increase in the association of N-WASp and Arp2/3 complex.

Activation of N-WASp which is an actin regulatory protein, is directly regulated by small GTPase, Cdc42 (16, 17). Treatment of cultured gastric smooth muscle cells with $1\text{ }\mu\text{M}$ of ACh in the presence of $1\text{ }\mu\text{M}$ of 4-DAMP for 5 min caused a significant increase in Cdc42 activation ($60\pm 6\%$ increase) (Fig. 4A). The increase in Cdc42 activation was inhibited by PI3K inhibitor (AS605240) ($38\pm 5\%$ decrease) (Fig. 4A) and in cells transfected with ILK dominant negative mutant (ILK (R211A)) ($36\pm 4\%$ decrease) (Fig. 4B) or ILK siRNA ($44\pm 5\%$ decrease) (Fig. 4C). Consistent with the increase in Cdc42 activation, treatment of cultured gastric smooth muscle cells with $1\text{ }\mu\text{M}$ of ACh in the presence of $1\text{ }\mu\text{M}$ of 4-DAMP for 5 min caused a significant increase in phosphorylation of N-WASP at tyrosine 256 ($55\pm 6\%$ increase) (Fig. 5A) and the association of N-WASp with Arp2/3 ($150\pm 20\%$ increase) (Fig. 5B). The increase in N-WASp phosphorylation ($60\pm 8\%$ decrease) (Fig. 5A) and its association with Arp2/3 was inhibited ($60\pm 8\%$ decrease and $65\pm 7\%$ decrease respectively) in cells expressing ILK dominant negative mutant (ILK(R211A)) (Figs. 5A and 5B). These results suggested that the stimulation of Cdc42 activity and increase in phosphorylation of N-WASP, and its association with Arp2/3 in response to m2 receptor activation is dependent on ILK.

Increase of F-actin/G-actin ratio via PI3K/ILK/Cdc42 by acetylcholine:

Treatment of cultured gastric smooth muscle cells with $1\text{ }\mu\text{M}$ of ACh in the presence of $1\text{ }\mu\text{M}$ of 4-DAMP for 5 min caused a significant increase in F-actin/G-actin ratio reflecting an increase in actin polymerization, which is consistent with the increase in activation of Cdc42, phosphorylation of N-WASp and association of N-WASp with Arp2/3. The increase in actin polymerization was inhibited by AS-605240 ($54\pm 5\%$ decrease) and actin related protein inhibitor CK-666 ($52\pm 5\%$ decrease) (Fig. 6) and in cells transfected with ILK

dominant negative mutant (ILK (R211A)) ($50 \pm 6\%$ decrease) (Fig. 7A) or ILK siRNA ($52 \pm 5\%$ decrease) (Fig. 7B). Increase in actin polymerization was inhibited in cells transfected with Cdc42 dominant negative mutant (Cdc42 [T17N]) ($55 \pm 7\%$ decrease) (Fig. 8) suggesting that actin polymerization is dependent on Cdc42 activation. These results suggested that increase in actin polymerization in response to m2 receptor activation is mediated by stimulation of Cdc42 activity via PI3K γ and ILK.

DISCUSSION

The results of our study demonstrate that adhesion complex protein ILK regulates the actin polymerization via PI3K γ by m2 receptors in gastric smooth muscle. This is the first demonstration that ILK mediates signaling pathways that regulate actin polymerization in gastric smooth muscle cells. ILK is a multi-domain scaffolding protein and is bound to cytoplasmic domains $\beta 1$ and $\beta 3$ integrins (31). The N terminal domain of ILK binds to the N-terminal domain of the particularly interesting new cysteine histidine-rich protein (PINCH), whereas the C-terminal domain of ILK binds to several adaptor proteins including paxillin and parvin. The central pleckstrin homology (PH)-like domain of ILK binds with phosphatidylinositol (3–5)-trisphosphate (PIP3) (18, 32, 33). We and others showed that ILK is a downstream effector of PI3K through PIP3 (6, 8, 21).

The assembly of ILK, PINCH and parvin complex (ILK.PINCH. α parvin) occurs in the cytoplasm and recruited to integrin adhesions (34). A focal adhesion protein kindlin-2, directly, binds to β integrin tails and also interacts with ILK to the recruitment of ILK/paxillin complex, to focal adhesions (35). Several studies show that phosphorylation of paxillin at Tyr118 is mediated by several kinases including Src kinase (36–37). We have also shown that Gi-coupled m2 receptors activate PI3K and c-Src (38). In airway and vascular smooth muscle, in response to the contractile agonist recruitment of ILK.PINCH. α -parvin complex to membrane adhesion complexes is required for cytoskeletal organization and contraction [25, 30, 32, 39].

The adaptor protein, paxillin is important for ILK complex recruitment and actin polymerization (16, 35). In airway smooth muscle, paxillin is bound in the cytosol to the scaffolding protein, vinculin, and mediates the recruitment of the paxillin-vinculin complex to focal adhesions in response to contractile stimulation. Phosphorylation of vinculin at Tyr1065 promotes an open conformation that enables the head domain of vinculin to bind the integrin-binding cytoskeletal protein, talin, and its tail domain to bind α -actinin and filamentous actin, thereby creating a link for force transmission between integrin-associated focal adhesions and sliding actomyosin filaments. Phosphorylation of paxillin at Tyr31 and Tyr118 facilitates its interaction with adaptor proteins Crk and GIT1/2 (G protein receptor kinase interacting tyrosine phosphorylated) and enables them to bind, respectively, to DOCK180, a member of the ‘dedicator of cytokinesis’ family, and PIX (PAK-interacting exchange factor); both DOCK180 and PIX act as GEFs (guanosine exchange factors) for the small GTPase, Cdc42 [16, 17]. Activated Cdc42 binds directly to the CRIB (Cdc42 and Rac-interactive binding) domain of the nucleation-promoting factor, N-WASp (neuronal Wiskott-Aldrich syndrome protein). Activated N-WASp, phosphorylated at Tyr256, couples to the

actin-related proteins, Arp2/3, greatly enhancing their ability to nucleate the assembly of branched actin filaments [40–43].

The present studies showed that activation of Gi-coupled m2 receptors causes phosphorylation of paxillin at Tyr118 via PI3K γ and phosphorylation is dependent on presence and recruitment of ILK. Phosphorylation of paxillin was inhibited upon inhibition of PI3K γ by AS-605240 and also upon suppression of ILK expression or expression of dominant negative ILK mutant that lacks the ability to bind to PIP3 indicating that recruitment of ILK to PIP3 is involved in paxillin phosphorylation. ILK R211A is a mutant of the PH domain of ILK which makes it deficient in membrane PIP3, and provides it resistance to PI3K-dependent activation (44). Blockade of paxillin phosphorylation by ILKR211A or ILK siRNA suggests that binding of ILK is essential for paxillin phosphorylation. We also found that phosphorylation of paxillin increases its association with Cdc42 GEF proteins such as Cool2/ α -Pix, Cool1/ β -Pix, and DOCK-180 to increase Cdc42 activity. The increase in association of paxillin with Cool2/ α -Pix, Cool1/ β -Pix and DOCK-180 and Cdc42 activity were inhibited in cells transfected with ILK siRNA or ILK dominant negative (ILK R211A). Cdc42 activity was also inhibited by AS-60520. These results imply that Cdc42 activation is downstream of PI3K γ activation and dependent on ILK recruitment to PIP3.

Cdc42 activity is necessary for activation (phosphorylation) of N-WASp, actin polymerization, and muscle contraction. Phosphorylation of N-WASp at Tyr256 increases its association with Arp2/3 and actin polymerization. The increase in phosphorylation of N-WASp, its association with Arp2/3 complex and actin polymerization in response to m2 receptor activation was inhibited in cells transfected with ILK dominant negative ILK[R211A]. Actin polymerization was also inhibited by AS-605240 and Arp2/3 inhibitor CK666 and also by Cdc42 dominant negative mutant (Cdc42 [T17N]), suggesting that N-WASp/Arp2/3 complex-mediated actin polymerization are dependent on ILK binding to PIP3 and activation of Cdc42.

Previous studies in gastrointestinal smooth muscle have shown that activation of Gi3 coupled m2 receptors lead to activation of PI3K γ and c-Src via G β γ i3. The present studies demonstrate the underlying mechanism of actin polymerization in response to activation of m2 receptors in gastric smooth muscle cells. The pathways involve activation of PI3K γ to generate PIP3 and recruitment of ILK/paxillin complex to PIP3 leading to phosphorylation of paxillin, presumably via c-Src. Phosphorylation of paxillin increases its association with the Cdc42GEF proteins and causes activation of Cdc42, which in turn facilitates N-WASp phosphorylation and its association with the Arp2/3 complex to mediate actin polymerization.

In contrast to muscarinic m2 receptors, Gq coupled m3 receptors cause actin polymerization and muscle contraction via Ca²⁺ dependent phosphorylation of proline-rich tyrosine kinase 2 (Pyk2) at Tyr402 and Ca²⁺ independent activation of RhoA and phosphorylation of FAK at Tyr397. Both phosphorylations of Pyk2 and FAK phosphorylates paxillin at Tyr118. Both pathways lead to an increase in association of paxillin with Cdc42GEF proteins leading to

increased Cdc42 activity, phosphorylation of N-WASp, an association of N-WASp with Arp 2/3 complex, actin polymerization, and muscle contraction.

Previous studies showed that contraction in response to ACh was abolished by 4-DAMP suggesting that contraction is mainly mediated by m3 receptors (6, 26). Activation of muscarinic m2 receptor did not induce muscle contraction despite an increase in actin polymerization suggesting that increase actin polymerization alone is not sufficient to induce contraction. Phosphorylation of MLC₂₀ by MLCK or ILK appears to be necessary for muscle contraction. Increase in MLC₂₀ phosphorylation in response to m2 receptor activation was prevented by activation of PAK and p38 MAP kinase, which inhibits MLCK and ILK activity, respectively (6). MLC₂₀ phosphorylation and contraction, however, can be restored when PAK and p38 MAP kinase were inhibited (8). Our results also suggest that binding of ILK to PIP3 is necessary for actin polymerization and as shown before ILK activity is necessary for MLC₂₀ phosphorylation (8). Increase in actin polymerization was shown to play an important role in the regulation of contractile protein expression (45) and thus the physiological significance of m2 receptor-induced actin polymerization may be associated with its role in the regulation of contractile phenotype.

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KEY POINTS

- Actin polymerization plays a role in the maintenance of smooth muscle contractile phenotype.
- In gastric smooth muscle cells, activation of m2 receptors causes phosphorylation of paxillin and stimulation of Cdc42 activity leading to phosphorylation of N-WASp and actin polymerization.
- Phosphorylation of paxillin and N-WASp, Cdc42 activity and actin polymerization were blocked by PI3 kinase γ inhibitor, ILK siRNA and ILK mutant that is deficient in PIP3 binding.
- The results suggest that m2 receptor-induced actin polymerization is dependent on PI3 kinase activity and ILK binding to PIP3.

Figure 1. PI3K γ dependent phosphorylation of paxillin by m2 receptor activation.

Cultured smooth muscle cells were treated for 5 min with ACh (1 μ M) plus m3 receptor antagonist (4-DAMP, 1 μ M) in the presence or absence of PI3K γ inhibitor (AS605240, 25 nM). 4-DAMP and AS-605240 were added 10 min prior to the addition of ACh.

Phosphorylation of paxillin (p-Paxillin) was measured by immunocytochemistry (**A**) and western blot (**B**). Values are means $\pm$ SE of 3–4 experiments. ** P <0.001

Figure 2. ILK dependent phosphorylation of paxillin by m2 receptor activation.

Cultured smooth muscle cells transfected with vector alone or dominant negative mutant ILK(R211A) (A) or siRNA for ILK (B) were treated for 5 min with ACh (1 μ M) plus 4-DAMP (1 μ M). Phosphorylation of paxillin (p-Paxillin) was measured by western blot. Values are means $\pm$ SE of 3 experiments. ** $P < 0.001$

Figure 3. Association of p-paxillin with Cdc42 GEFs by m2 receptor activation.

Cultured smooth muscle cells transfected with vector alone or dominant negative mutant ILK(R211A) (A) or siRNA for ILK (B) were treated for 5 min with ACh (1 μ M) plus 4-DAMP (1 μ M). Association of paxillin with Cdc42 GEFs by m2 receptor activation. Values are means $\pm$ SE of 3 experiments. ** $P < 0.001$

Figure 4. PI3K γ - and ILK-dependent Cdc42 activity by m2 receptor activation.

Cultured smooth muscle cells were treated for 5 min with ACh (1 μ M) plus m3 receptor antagonist (4-DAMP, 1 μ M) in the presence or absence of PI3K γ inhibitor (AS605240, 25 nM). 4-DAMP and AS-605240 were added 10 min prior to the addition of ACh (**A**). Cultured smooth muscle cells transfected with vector alone or dominant negative mutant ILK(R211A) (**B**) or transfected with siRNA for ILK (**C**) were treated for 5 min with ACh (1 μ M) plus 4-DAMP (1 μ M). Cdc42 activity was determined by using pull-down assay for activated Cdc42. Activated GTP-bound Cdc42 was affinity-precipitated by glutathione beads and quantitated by western blot. Values are means $\pm$ SE of 3–4 experiments. ** P <0.001

Figure 5. ILK-dependent phosphorylation of N-WASp and association of N-WASp with Arp2/3 by m2 receptor activation.

(A) Cultured smooth muscle cells transfected with vector alone, dominant negative mutant ILK(R211A) were treated for 5 min with ACh (1 μ M) plus 4-DAMP (1 μ M). Phosphorylation of N-WASp (pNWASp) was measured by western blot. Values are means $\pm$ SE of 3–4 experiments. (B) Cultured smooth muscle cells transfected with vector alone or dominant negative mutant ILK(R211A) were treated for 5 min with ACh (1 μ M) plus 4-DAMP (1 μ M). Association of N-WASp with Arp2/3 was measured by immunoprecipitation followed by western blot. Values are means $\pm$ SE of 3 experiments. ** P <0.001

Figure 6. PI3K γ -dependent actin polymerization by m2 receptor activation.

Cultured smooth muscle cells were treated for 5 min with ACh (1 μ M) plus m3 receptor antagonist (4-DAMP, 1 μ M) in the presence or absence of PI3K γ inhibitor (AS605240, 25 nM) or Arp2/3 inhibitor (CK-666, 200 μ M). 4-DAMP and AS-605240 were added 10 min and CK-666 was added 1h prior to the addition of ACh. Actin polymerization was measured by western blot. Values are means $\pm$ SE of 3 experiments. . ** P <0.001

Figure 7. ILK-dependent actin polymerization by m2 receptor activation.

Cultured smooth muscle cells transfected with vector alone or dominant negative mutant ILK(R211A) (A) or transfected with siRNA for ILK (B) were treated for 5 min with ACh (1 μ M) plus 4-DAMP (1 μ M). Actin polymerization was measured by western blot. Values are means $\pm$ SE of 5–6 experiments. ** P <0.001

Figure 8. Cdc42-dependent actin polymerization by m2 receptor activation. Cultured smooth muscle cells transfected with vector alone or dominant negative Cdc42 [T17N] were treated for 5 min with ACh (1 μ M) plus 4-DAMP (1 μ M). Actin polymerization was measured by western blot. Values are means $\pm$ SE of 3 experiments. ** $P < 0.001$

Figure 9. Signaling mechanism of actin polymerization by m2 receptor activation.

Activation of muscarinic m2 receptors causes sequential activation of $PI3K\gamma$ via $G\beta\gamma_{13}$ and recruitment of ILK/paxillin complex to PIP3. This results in an increase in phosphorylation of paxillin and its association with Cdc42 GEF proteins and Cdc42 activity, which in turn causes an increase in phosphorylation of N-WASP and its association with Arp2/3 complex to mediate actin polymerization.