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G_q-dependent signalling by the lysophosphatidic acid receptor LPA_3 in gastric smooth muscle: reciprocal regulation of MYPT1 phosphorylation by Rho kinase and cAMP-independent PKA

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Abstract

The present study characterized the signalling pathways initiated by the bioactive lipid, LPA (lysophosphatidic acid) in smooth muscle. Expression of LPA_3 receptors, but not LPA_1 and LPA_2, receptors was demonstrated by Western blot analysis. LPA stimulated phosphoinositide hydrolysis, PKC (protein kinase C) and Rho kinase (Rho-associated kinase) activities: stimulation of all three enzymes was inhibited by expression of the $G_{\alpha q}$, but not the $G_{\alpha i}$, minigene. Initial contraction and MLC_{20} (20 kDa regulatory light chain of myosin II) phosphorylation induced by LPA were abolished by inhibitors of PLC (phospholipase C)-β (U73122) or MLCK (myosin light-chain kinase; ML-9), but were not affected by inhibitors of PKC (bisindolylmaleimide) or Rho kinase (Y27632). In contrast, sustained contraction, and phosphorylation of MLC_{20} and CPI-17 (PKC-potentiated inhibitor 17 kDa protein) induced by LPA were abolished selectively by bisindolylmaleimide. LPA-induced activation of IKK2 {IκB [inhibitor of NF-κB (nuclear factor κB)] kinase 2} and PKA (protein kinase A; cAMP-dependent protein kinase), and degradation of I$\kappa B\alpha$ were blocked by the RhoA inhibitor (C3 exoenzyme) and in cells expressing dominant-negative mutants of IKK2(K44A) or RhoA(N19RhoA). Phosphorylation by Rho kinase of MYPT1 (myosin phosphatase targeting subunit 1) at Thr^{696} was masked by phosphorylation of MYPT1 at Ser^{695} by PKA derived from IκB degradation via RhoA, but unmasked in the presence of PKI (PKA inhibitor) or C3 exoenzyme and in cells expressing IKK2(K44A). We conclude that LPA induces initial contraction which involves activation of PLC-β and MLCK and phosphorylation of MLC_{20}, and sustained contraction which involves activation of PKC and phosphorylation of CPI-17 and MLC_{20}. Although Rho kinase was activated, phosphorylation of MYPT1 at Thr^{696} by Rho kinase was masked by phosphorylation of MYPT1 at Ser^{695} via cAMP-independent PKA derived from the NF-κB pathway.

Keywords

gastric muscle; muscle contraction; myosin light chain; nuclear factor κB (NF-κB) signalling; protein kinase C (PKC)

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INTRODUCTION

The bioactive lipids LPA (lysophosphatidic acid) and S1P (sphingosine 1-phosphate) are produced by *de novo* synthesis and during metabolism of membrane phospholipids, and have been implicated in a variety of biological processes, such as cell growth and differentiation, cell survival, regulation of actin cytoskeletons and cell migration [1–4]. LPA acts in an autocrine and paracrine fashion and signals via distinct G-protein-coupled LPA receptors ($LPA_{1–5}$) [1,2,5–9]. LPA_1 is widely expressed, with high levels in testis, brain, lung, heart, spleen and intestine, whereas LPA_2 and LPA_3, which shares ~60% sequence similarity with LPA_1, expression is more restricted with high levels of expression in testis and kidney and low levels of expression in heart and stomach. LPA_4 receptors, which are related to the purinergic receptor family, in contrast share only ~20% sequence similarity with LPA_1, LPA_2 and LPA_3 [10]. A recently identified LPA_5 receptor shares ~35% sequence similarity with the LPA_4 receptor and lower similarity with $LPA_{1–3}$ receptors [11].

LPA_1, LPA_2, LPA_3 and LPA_5 receptors are variously coupled to the G_i, G_q and G_{12} family of G-proteins [3,5,12–14]. LPA_4 receptors appear to couple to G_s [10]. Since many cell types express more than one LPA receptor, and each receptor can couple to multiple G-proteins, the responses to LPA are varied depending on the cell type and on the composition and expression levels of the receptor types and signalling proteins. Studies using LPA receptor knockout mice demonstrate that LPA_1 receptors are coupled to G_i and inhibition of adenylate cyclase, LPA_2 receptors are coupled to G_{12} and RhoA and cytoskeletal reorganization, and that LPA_3 receptors are coupled to G_q and stimulation of PLC (phospholipase C)-β activity [13,15,16].

Little is known of the expression of LPA receptors or the signal transduction pathways initiated by these receptors in visceral and vascular smooth muscle. In rabbit and cat tracheal smooth muscle rings, LPA had no effect on its own, but augmented the response to serotonin, substance P and the cholinergic agonist, methacholine [17]. In human myofibroblast and myometrial cells, and guinea-pig ileal longitudinal smooth muscle strips, LPA induced contraction; the response in myometrial cells and longitudinal muscle strips is mediated via the RhoA/Rho kinase (Rho-associated kinase) pathway, whereas in myofibroblasts it is mediated via both MLCK (myosin light-chain kinase) and Rho kinase pathways [18–21].

In the present study, we identified the signalling pathways initiated by LPA in gastric muscle cells. Selective G-protein minigene expression was used to identify the coupling of specific G-proteins to effector enzymes, and selective inhibitors were used to characterize the pathways involved in MLC_{20} (20 kDa regulatory light chain of myosin II) phosphorylation and muscle contraction. The results demonstrated the selective expression of LPA_3 and identified distinct signalling pathways to mediate initial and sustained contraction via $G_{\alpha q}$-dependent activation of PLC-β/IP_3 (inositol 1,4,5-trisphosphate)/Ca^{2+} and RhoA/PKC (protein kinase C)/CPI-17 (PKC-potentiated inhibitor 17 kDa protein) pathways respectively. LPA had no effect on cAMP levels, but induced activation of Rho kinase, PKC, NF-κB (nuclear factor κB) and PKA (protein kinase A; cAMP-dependent protein kinase). Although Rho kinase was activated, phosphorylation by Rho kinase of MYPT1 (myosin

phosphate targeting subunit 1) at the inhibitory site Thr^{696} was prevented by PKA-mediated phosphorylation of MYPT1 at Ser^{695}.

EXPERIMENTAL

Materials

LPA was obtained from Biomol Research Labs; [γ-^{32}P]ATP was from Amersham Pharmacia Biotech; *myo*-[^{3}H]inositol and [^{125}I]cAMP were from DuPont NEN; U73122, ML-9, Y27632 and bisindolylmaleimide were from Calbiochem; polyclonal antibodies to $G_{\alpha i1}$ $G_{\alpha i2}$, $G_{\alpha i3}$, $G_{\alpha 13}$, $G_{\alpha s}$, MLC_{20}, CPI-17, MYPT1, IκBα (inhibitor of NF-κB), LPA_1, LPA_2 and LPA_3, and phosphospecific antibodies to MLC_{20}, CPI-17, MYPT1 and IKK2 (IκB kinase 2) were from Santa Cruz Biotechnology. A phospho-PKA substrate antibody was from Cell Signaling Technologies. The phospho-PKA substrate antibody detects proteins containing a phospho-serine residue that is phosphorylated by PKA and does not cross-react with the non-phosphorylated PKA substrate motif. pGreen Lantern-1 and Lipofectamine™ plus reagent were from Life Technologies Gibco-BRL; VPC 32179 and OMPT were from Avanti Polar Lipids; and all other reagents were from Sigma. DNA sequencing was performed by the Virginia Commonwealth University Nucleic Acids Core Facility (Richmond, VA, U.S.A.).

Preparation of dispersed and cultured gastric smooth muscle cells

Smooth muscle cells were isolated from the circular muscle layer of rabbit distal stomach by sequential enzymatic digestion, filtration and centrifugation as previously described [6,22–24]. For permeabilization, dispersed smooth muscle cells were treated for 5 min with saponin (35 μg/ml) and resuspended in low Ca^{2+} (100 nM) medium (20 mM NaCl, 100 mM KCl, 5 mM $MgSO_4$, 1 mM NaH_2PO_4, 25 mM $NaHCO_3$, 0.34 mM $CaCl_2$, 1 mM EGTA and 1% BSA) as previously described [24]. In some experiments, the cells were cultured in DMEM (Dulbecco's modified Eagle's medium) containing 10% fetal bovine serum until they attained confluence and were then passaged once for use in various studies [24].

Minigene construction and transfection into cultured smooth muscle cells

Activation of specific G-protein subunits was blocked by the expression of cDNA encoding the last C-terminal 11 amino acids of $G_{\alpha i}$ and $G_{\alpha q}$ as described previously [24–27]. The cDNAs encoding the last C-terminal 11 amino acids of mouse $G_{\alpha q}$ and human $G_{\alpha i}$ were amplified by PCR under standard conditions using Taq DNA polymerase. The purified PCR products were subcloned into the mammalian expression vector pcDNA3.1(+). Cultured rabbit gastric smooth muscle cells were transiently transfected with minigene plasmid DNA using Effectene transfection reagent. Transfection efficiency was monitored by co-transfection of pGreen Lantern-1. Analysis by fluorescence microscopy showed that ~75% of the cells were transfected [25].

Expression of dominant-negative RhoA and IKK2 in cultured smooth muscle cells

Dominant negative RhoA (N19RhoA) or IKK2 [IKK2(K44A)] was subcloned into the multiple cloning site (EcoRI) of the eukaryotic expression vector pEXV. Recombinant plasmid DNAs (2 μg each) were transiently transfected into smooth muscle cells in primary

cultures using Effectene transfection reagent for 48 h and transfection efficiency was monitored by co-transfection of pGreen Lantern-1 [23,24].

Assay for PI (phosphoinositide) hydrolysis

PI hydrolysis was determined from the formation of total inositol phosphates using anion-exchange chromatography as described previously [6,24,25]. Muscle cells in culture were labelled for 24 h in inositol-free DMEM containing 0.5 μCi/well (6-well plate). The cells were washed with PBS and treated with LPA for 60 min and the reaction was terminated by addition of 940 μl of chloroform/methanol/HCl (50:100:1, by vol.). After extraction with 340 μl of chloroform and 340 μl of H_2O, the aqueous phase was applied to DOWEX AG-1 columns; [^{3}H]inositol phosphates were eluted and radioactivity was determined in a liquid scintillation counter.

Assay for Rho kinase activity

Rho kinase activity was determined using an immunokinase assay in cell extracts as described previously [6,23–25]. Kinase assays were initiated by the addition of 10 μCi of [γ-^{32}P]ATP (3000 Ci/mmol) and 20 μM ATP, followed by incubation for 10 min at 37°C. ^{32}P-labelled myelin basic protein was absorbed on to phosphocellulose discs, and free radioactivity was removed by repeated washings with 75 mM phosphoric acid. The amount of radioactivity on the discs was measured by liquid scintillation.

Assay for PKC activity

PKC activity was measured in the particulate fractions as described previously [23,28]. Muscle cells (3×10^6 cells/ml) were incubated with LPA for 5 min, and the reaction was terminated by rapid freezing. After homogenization, PKC activity was measured in the membrane pellet by phosphorylation of myelin basic protein, and the results are expressed as c.p.m. per mg of protein per min.

Assay for PKA activity

PKA activity was measured using the method of Jiang et al. [29] as described previously [30]. Muscle cells (3×10^6 cells/ml) were incubated with LPA for 5 min, and the reaction was terminated by rapid centrifugation (25000 ***g*** for 15 min at 4°C). After homogenization of the pellet, PKA activity in the supernatant was measured in a volume of 60 μl using [γ-^{32}P]ATP and 150 μM Kemptide (Leu-Arg-Arg-Ala-Ser-Leu-Gly). PKA activity was calculated as picomoles of phosphate transferred into Kemptide per min per mg of protein.

Measurement of MYPT1, CPI-17, MLC_{20} and IKK2 phosphorylation and IkBα degradation

Phosphorylated MLC_{20}, MYPT1, CPI-17 and IKK2 were determined by immunoblot analysis using a phospho-specific antibody, and degradation of IκBα was analysed using an IκBα antibody as described previously [6,25,28,31]. Cell lysate proteins were resolved by SDS/PAGE and electrophoretically transferred on to PVDF membranes. Membranes were incubated for 12 h with antibodies to MLC_{20} (Ser^{19}), MYPT1 (Thr^{696}), MYPT1 (Ser^{695}) and CPI-17(Thr^{38}), a phospho-substrate antibody specific for the PKA phosphorylation site, IKK2 ($Ser^{177/181}$) or IκBα, and then incubated for 1 h with HRP (horseradish peroxidase)-

conjugated secondary antibodies. The bands were identified by ECL (enhanced chemiluminescence). For control and experimental samples, cells were derived from the same culture and compared by electrophoresis on the same gel.

Measurement of muscle contraction

Muscle cell contraction was measured in freshly dispersed muscle cells by scanning micrometry as described previously [6,24,25,28,31]. A cell aliquot containing 10^4 muscle cells/ml was added to 0.1 ml of medium [25 mM Hepes, 120 mM NaCl, 4 mM KCl, 2.6 mM KH_2PO_4, 0.6 mM $MgCl_2$, 14 mM glucose and 2.1 (v/v) Eagles essential amino acid mixture] containing LPA and the reaction was terminated with 1% acrolein. The lengths of muscle cells treated with LPA were compared with the lengths of untreated cells, and contraction was expressed as the decrease in mean cell length from control.

RESULTS

Expression of LPA receptors in gastric smooth muscle

Western blot analysis using specific antibodies for LPA_1, LPA_2 or LPA_3 receptors demonstrated the selective expression of LPA_3 in extracts of dispersed smooth muscle cells derived from the circular muscle layer of the stomach. Similar results were obtained in extracts derived from cultured muscle cells (Figure 1A). All three LPA receptors were expressed in the brain (Figure 1A).

G_q-dependent activation of PLC-β

LPA stimulated a 4-fold increase in PI hydrolysis (PLC-β activity) above basal levels in cultured smooth muscle cells (basal: 586 ± 98 c.p.m./mg of protein). LPA-stimulated PLC-β activity was inhibited by 89 ± 6% in muscle cells expressing the $G_{\alpha q}$ minigene, but was not affected in cells expressing the $G_{\alpha i}$ minigene (Figure 1B). The effectiveness of the $G_{\alpha i}$ minigene was examined using the adenosine A_1 receptor agonist, CPA (cyclopentyl adenosine; 1 μM), in cells expressing $G_{\alpha i}$ and $G_{\alpha q}$ minigenes. CPA-stimulated PLC-β activity (2865 ± 296 c.p.m./mg of protein above basal levels of 532 ± 58 c.p.m./mg of protein) was blocked by 91 ± 6% in muscle cells expressing the $G_{\alpha i}$ minigene (692 ± 78 c.p.m./mg of protein, $n = 3$, $P < 0.001$), but was not affected in cells expressing the $G_{\alpha q}$ minigene (2638 ± 302 c.p.m./mg of protein, $n = 3$) consistent with the selective activation of $G_{\alpha i}$, but not $G_{\alpha q}$, by adenosine A_1 receptors in smooth muscle cells [32]. The selective LPA_3 receptor antagonist VPC 32179 (10 μM) inhibited LPA-induced PI hydrolysis by 86 ± 5% ($n = 4$, $P < 0.001$) in cultured muscle cells, but had no effect on basal PI hydrolysis. LPA also stimulated a 4-fold increase in PI hydrolysis above basal levels (653 ± 125 c.p.m./mg of protein, $n = 4$) in freshly dispersed muscle cells: stimulation was blocked by an inhibitor of PI hydrolysis, U73122, but not by pertussis toxin (results not shown). Consistent with activation of $G_{\alpha q}$-dependent activation of PI hydrolysis, LPA selectively induced an increase in the binding of [^{35}S]GTP[S] to $G_{\alpha q}$, but not $G_{\alpha i1}$, $G_{\alpha i2}$, $G_{\alpha i3}$, $G_{\alpha 12}$, $G_{\alpha 13}$ or $G_{\alpha s}$ (results not shown).

In permeabilized muscle cells, LPA (1 μM) stimulated Ca^{2+} release to the same extent as 1 μM IP_3 (33 ± 5% decrease in steady-state $^{45}Ca^{2+}$ content in 15 s by LPA and 32 ± 4%

decrease by IP_3, $n = 5$). The effect of LPA was abolished by VPC 32179 (4 ± 3% decrease, $n = 4$), U73122 (5 ± 5% decrease, $n = 6$) and xestospongin C (3 ± 5% decrease, $n = 4$), a blocker of IP_3 receptors.

In freshly dispersed gastric smooth muscle cells, LPA had neither increased basal cAMP levels (basal: 5.4 ± 0.6 pmol/mg of protein; LPA: 5.6 ± 0.8 pmol/mg of protein, $n = 3$) nor inhibited forskolin-stimulated cAMP levels (forskolin: 31.5 ± 2.6 pmol/mg of protein above basal level and forskolin plus LPA: 30.8 ± 3.2 c.p.m./mg of protein above basal levels, $n = 3$). In contrast, CPA inhibited forskolin-stimulated cAMP (14.8 ± 2.8 pmol/mg of protein) in freshly dispersed muscle cells by 53 ± 5% ($n = 3$, $P < 0.01$).

G_q-dependent activation of Rho kinase and PKC by LPA

Treatment of freshly dispersed muscle cells with LPA for 5 min caused a 2-fold stimulation of Rho kinase activity that was blocked by the Rho kinase inhibitor, Y27632 (LPA: 4397 ± 506 c.p.m./mg of protein above basal levels of 2135 ± 352 c.p.m./mg of protein; LPA plus Y27632: 565 ± 125 c.p.m./mg of protein above basal levels, $n = 4$). Similarly, treatment of cultured gastric muscle cells with LPA for 5 min caused an increase in Rho kinase activity that was abolished in cells expressing the $G_{\alpha q}$ minigene (Figure 1C), but was not affected in cells expressing either $G_{\alpha 12}$ or $G_{\alpha 13}$ minigenes (results not shown). LPA-induced Rho kinase activity in cultured muscle cells was blocked by 10 μM VPC 32179 (LPA: 4021 ± 567 c.p.m./mg of protein above basal levels of 1938 ± 302 c.p.m./mg of protein; LPA plus VPC 32179: 445 ± 107 c.p.m./mg of protein above basal levels, $n = 4$, $P < 0.001$).

A similar pattern of G_q-dependent activation of PKC was obtained with LPA. LPA-induced activation of PKC was abolished in cells expressing the $G_{\alpha q}$ minigene (Figure 1D), but was not affected in cells expressing the $G_{\alpha 12}$ or $G_{\alpha 13}$ minigene (results not shown). LPA-induced PKC activity was also abolished by the C3 exoenzyme, which ADP-ribosylates and specifically inhibits RhoA (LPA: 5145 ± 602 c.p.m./mg of protein; C3 exoenzyme + LPA: 359 ± 98 c.p.m./mg of protein above basal levels of 1125 ± 189 c.p.m./mg of protein, $n = 4$, $P < 0.001$). LPA-induced PKC activity in cultured muscle cells was blocked by 10 μM VPC 32179 (LPA: 4764 ± 654 c.p.m./mg of protein above basal levels of 986 ± 125 c.p.m./mg of protein; LPA plus VPC 32179: 445 ± 107 c.p.m./mg of protein above basal levels, $n = 4$, $P < 0.001$). VPC 32179 had no effect on basal Rho kinase or PKC activities.

Previous studies in smooth muscle cells have shown that sustained activation of PKC was largely dependent on RhoA-mediated activation of PLD (phospholipase D) [33]. LPA caused a 3-fold stimulation of PLD activity in freshly dispersed smooth muscle cells (2683 ± 302 c.p.m./mg of protein above basal levels of 668 ± 95 c.p.m./mg of protein, $n = 3$).

Phosphorylation of MLC_{20}, MYPT1, CPI-17 and muscle contraction in dispersed muscle cells by LPA

LPA caused contraction of dispersed gastric smooth muscle cells and the contraction was characterized by an initial transient phase followed by a sustained phase. The maximal peak response (31 ± 3% decrease in cell length, $n = 6$) and the sustained response (28 ± 2% decrease in cell length, $n = 6$) elicited by LPA were similar to those elicited by other receptor-coupled agonists [24,34]. The initial peak response at 30 s was used to construct

concentration–response curves. Muscle contraction was detectable at concentrations below 0.1 nM and was concentration-dependent with an EC_{50} of 1 nM (results not shown).

Pathways mediating LPA-induced initial muscle contraction and MLC_{20} phosphorylation

The initial (30 s) contraction induced by LPA was blocked by U73122 (89 ± 5% inhibition) and the MLCK inhibitor, ML-9 (85 ± 4% inhibition) (Figure 2A), but was not affected by the PKC inhibitor, bisindolylmaleimide, or the Rho kinase inhibitor, Y27632 (Figure 2A).

LPA caused phosphorylation of MLC_{20} at Ser^{19} and CPI-17 at Thr^{38} in dispersed gastric muscle cells. Phosphorylation of MLC_{20} at Ser^{19} was rapid (30 s) and sustained (5 min), whereas phosphorylation of CPI-17 at Thr^{38} was absent at 30 s and occurred at only 5 min (Figures 2A and 2B). The pattern of initial MLC_{20} phosphorylation induced by LPA and its inhibition by various agents closely paralleled that of initial contraction. Initial MLC_{20} phosphorylation was abolished by U73122 and ML-9, but was not affected by bisindolylmaleimide or Y27632 (Figure 2A).

Pathways mediating LPA-induced sustained muscle contraction and MLC_{20} phosphorylation

LPA-induced sustained contraction measured at 5 min was blocked by bisindolylmaleimide (Figure 2B). U73122, ML-9 or Y27632 had no effect on sustained contraction. Sustained phosphorylation of MLC_{20} and CPI-17 was blocked by bisindolylmaleimide, but was not affected by U73122, ML-9 or Y27632 (Figure 2B). The pattern of sustained phosphorylation of MLC_{20} and CPI-17 induced by LPA and inhibition by bisindolylmaleimide closely paralleled that of sustained muscle contraction. Despite activation of Rho kinase, LPA had no effect on MYPT1 phosphorylation at Thr^{696}.

LPA-induced initial (31 ± 2% decrease in cell length) and sustained (26 ± 3% decrease) contraction was blocked by VPC 32179 (10 μM) and mimicked by the selective LPA_3 receptor agonist 1 μM OMPT (initial contraction: 30 ± 3% decrease; sustained contraction: 28 ± 4% decrease in cell length, $n = 6$). VPC 32179 by itself had no effect on muscle cell length (control: 104 ± 4 μm; VPC 32179: 102 ± 5 μm, $n = 4$). A combination of LPA and OMPT did not cause a significant increase in muscle contraction over that of each agonist separately.

Activation of PKA by LPA blocks phosphorylation of MYPT1 at Thr^{696} by Rho kinase

Although LPA had no effect on cAMP formation, it stimulated PKA in both freshly dispersed and cultured muscle cells. LPA-induced activation of PKA was blocked by inhibitors of PKA [H-89, myristoylated PKI (PKA inhibitor)], proteasomal degradation (MG-132) and RhoA (C3 exoenzyme) in freshly dispersed cells, and in cells overexpressing dominant-negative mutants of IKK2 [IKK2(K44A)] or RhoA (N19RhoA) (Figures 3A and 3B). LPA also induced phosphorylation of MYPT1, measured using a phospho-substrate antibody specific for the PKA site (Figure 4). The pattern of LPA-induced phosphorylation of MYPT1 by PKA closely paralleled that of PKA activation: phosphorylation was blocked by H-89, myristoylated PKI, MG-132 and C3 exoenzyme in freshly dispersed cells, and in cells overexpressing IKK2(K44A) or N19RhoA (Figures 4A and 4B). These results

demonstrate that RhoA mediates LPA-induced phosphorylation of MYPT1 by cAMP-independent PKA derived from proteasome degradation of IκB [35]. Treatment of cells with forskolin, a direct activator of adenylate cyclase, to stimulate cAMP-dependent PKA also caused MYPT phosphorylation that was abolished by PKI (Figure 4, inset).

In the absence of PKA activation and MYPT1 phosphorylation by PKA, LPA induced phosphorylation of MYPT1 at Thr^{696} in freshly dispersed and cultured muscle cells. LPA-induced MYPT1 phosphorylation at Thr^{696} was unmasked by H-89, myristoylated PKI and MG-132 in freshly dispersed cells, and in cells overexpressing IKK2(K44A) (Figures 5A and 5B). The results indicate that concurrent activation of PKA prevents MYPT1 phosphorylation by Rho kinase and the phosphorylation by Rho kinase can be unmasked in the presence of PKI or by blocking proteasomal degradation of IκB. The results also imply that phosphorylation of MYPT1 by PKA and Rho kinase are reciprocally regulated and LPA, although it stimulates Rho kinase, preferentially phosphorylates MYPT1 via a PKA-dependent mechanism.

Phosphorylation of MYPT1 by PKA was also demonstrated using a phospho-specific antibody to Ser^{695}, a site selectively phosphorylated by cyclic-nucleotide-dependent kinases [36]. Forskolin is a direct activator of adenylate cyclase and of LPA-induced phosphorylation MYPT1 at Ser^{695}. LPA-induced phosphorylation of MYPT1 at Ser^{695} was blocked by H-89, myristoylated PKI, MG-132 and C3 exoenzyme, whereas forskolin-induced phosphorylation was selectively blocked by PKI (Figure 6A).

LPA induced IKK2 phosphorylation and IκBα degradation in both freshly dispersed muscle cells and cultured muscle cells (Figure 6B). C3 exoenzyme, but not PKI, blocked IKK2 phosphorylation and IκBα degradation, whereas MG-132 selectively blocked IκBα degradation (Figure 6B). LPA-induced IKK2 phosphorylation, IκBα degradation and MYPT1 phosphorylation at Ser^{695} were blocked in cultured cells expressing IKK2(K44A), suggesting that phosphorylation of MYPT1 at Ser^{695} mediated by PKA was derived from the NF-κB pathway (Figure 6C).

Consistent with the phosphorylation of MYPT1 by Rho kinase in the presence of PKI or MG132, blockade of IκB degradation and subsequent activation of PKA resulted in Rho-kinase-sensitive sustained contraction. Thus, in the presence of either MG132 or myristoylated PKI, the sustained contraction induced by LPA was partially inhibited by Y27632 and bisindolmaleimide and abolished by the combination of both inhibitors, suggesting participation of both Rho kinase and PKC in sustained contraction (Figure 7).

DISCUSSION

The present study demonstrates the expression of LPA_3, but not LPA_1 and LPA_2, receptors in gastric smooth muscle and their ability to induce initial Ca^{2+}-dependent and sustained Ca^{2+}-independent smooth muscle contraction and MLC_{20} phosphorylation via receptor coupling to G_q and RhoA. The initial Ca^{2+}-dependent phase reflected stimulation of PI hydrolysis via $G_{\alpha q}$ resulting in IP_3-dependent Ca^{2+} release, and MLCK-dependent MLC_{20} phosphorylation and muscle contraction. In contrast, sustained MLC_{20} phosphorylation and

muscle contraction reflected activation of RhoA via G_q resulting in CPI-17 phosphorylation by PKC, MLC_{20} phosphorylation and contraction. Silencing of LPA_3 receptors in mouse gastric muscle cells virtually abolished LPA-induced stimulation of PI hydrolysis, Rho kinase and PKC activities (K. S. Murthy, unpublished work).

The relative contribution of CPI-17 (PKC inhibitor) and MYPT1 (Rho kinase inhibitor) to inhibition of MLCP (myosin light-chain phosphatase) and MLC_{20} phosphorylation appears to be receptor-specific. G_q/G_{13}-coupled muscarinic m3 receptors, motilin, and $S1P_2$ receptors engaged both RhoA-dependent pathways. In contrast, G_q/G_{13}-coupled endothelin ET_A receptors engaged only the Rho kinase/MYPT1 pathway [6,24,25,28]. The second limb of the pathway involving PKC-mediated phosphorylation of CPI-17 and inhibition of MLCP was masked by the concurrent activation of p38 MAPK (mitogen-activated protein kinase)-mediated activation of protein phosphatase 2A and dephosphorylation of CPI-17 via ET_B receptors [28]. G_i-coupled adenosine A_1, somatostatin sst_3 or opioid δ receptors, although utilizing a similar Ca^{2+}-dependent MLCK pathway to mediate initial contraction, utilize a distinct pathway to mediate sustained contraction [31]. These receptors activated PI3K (PI 3-kinase) and ILK (integrin-linked kinase), but not RhoA [31]. Activation of ILK results in direct phosphorylation of MLC_{20} and an increase in MLC_{20} phosphorylation via inhibition of MLCP by ILK-mediated phosphorylation of CPI-17 [31,37–42]. In the present study, we provide evidence that LPA-induced sustained contraction and MLC_{20} phosphorylation were exclusively mediated by PKC. This is consistent with the phosphorylation of CPI-17 at Thr^{38}, but not MYPT1 at the inhibitory site Thr^{696}, and blockade sustained phosphorylation of MLC_{20} and CPI-17, and muscle contraction by bisindolylmaleimide but not Y27632. LPA-induced phosphorylation of MYPT1 at Thr^{696}, however, was unmasked in the presence of inhibitors of PKA or proteasome degradation or in cells expressing IKK2(K44A) implying that phosphorylation of MYPT1 at Thr^{696} by Rho kinase was blocked by cAMP-independent PKA derived from the NF-κB pathway. Recent studies have identified a novel cAMP-independent mechanism for activation of PKA by lipopolysaccahride, thrombin, endothelin and angiotensin II involving release of the PKA catalytic subunit from the IκB complex upon phosphorylation and degradation of IκBα [35].

We conclude that RhoA mediates LPA-induced MYPT1 phosphorylation by cAMP-independent PKA via proteasome degradation of IκBα, based on the following evidence: (i) LPA did not activate G_s and had no effect on cAMP levels, but induced activation of PKA and phosphorylation of MYPT1 at a PKA site, Ser^{695}; (ii) LPA-induced activation of PKA was abolished by a RhoA inhibitor C3 exoenzyme or a proteasome degradation inhibitor MG-132 in freshly dispersed muscle cells and a dominant-negative RhoA mutant (N19RhoA) or kinase-deficient IKK2 mutant [IKK2(K44A)] in cultured muscle cells; and (iii) LPA-induced MYPT1 phosphorylation by PKA was inhibited by two different PKA inhibitors, H-89 and PKI, by C3 exoenzyme or MG-132 in freshly dispersed muscle cells and by expression of N19RhoA or IKK2(K44A) in cultured muscle cells.

The absence of MYPT1 phosphorylation at Thr^{696} in the presence of PKA activation involves phosphorylation of MYPT1 at Ser^{695}, which prevents phosphorylation at Thr^{696}. Recent studies have identified MYPT1 as a target for PKA and PKG (protein kinase G) [36]. Both kinases directly phosphorylate MYPT1 at a site (Ser^{695}) adjacent to the Rho kinase site

(Thr^{696}). Phosphorylation of Ser^{695} by PKA or PKG prevented MYPT1 phosphorylation at Thr^{696} by Rho kinase and thus blocked Rhokinase-dependent inhibition of MLCP activity [36].

It is well established that activation of PKA in gastric smooth muscle cells inhibits muscle contraction, resulting from a decrease in $[Ca^{2+}]_i$ and MLC_{20} phosphorylation [37]. These effects of PKA are a consequence of the inhibitory action of PKA on multiple components of the $G_{\alpha q}$ signalling culminating in muscle relaxation. Myristolylated PKI or H-89 had no effect on LPA-induced PLC-β activity (results not shown), suggesting that activation of PKA had no effect on LPA-induced Ca^{2+} signalling. One possibility is that the stimulation of PLC-β precedes PKA activation as agonist-induced IP_3 generation is rapid and attains a peak within 30 s, whereas activation of RhoA is delayed and attains a peak within 5–10 min. Alternatively, localization of PKA derived from IκB degradation prevents its access to inhibit PLC-β activity as compartmentalization of PKA through association with anchoring proteins or scaffolding proteins ensures specificity of signal transduction [43].

In summary, gastric smooth muscle expresses LPA_3 receptors. LPA-induced initial Ca^{2+}-dependent MLC_{20} phosphorylation and muscle contraction are mediated by $G_{\alpha q}$-dependent stimulation of PLC-β. LPA-induced sustained MLC_{20} phosphorylation and muscle contraction are predominantly mediated by $G_{\alpha q}$-dependent stimulation of RhoA and CPI-17 phosphorylation and inhibition of MLCP by PKC derived from RhoA activation. Our results also provide evidence for activation of PKA by G_q-coupled agonists and reveal a novel mechanism for regulation of MYPT1 phosphorylation by Rho kinase via activation of a portion of PKA in a cAMP-independent fashion (Figure 8). These findings raise an interesting possibility for signal specificity via the interaction of ubiquitous signalling molecules and their activation in an agonist-specific fashion. Such signal specificity is probably the case in the PKA–MYPT1 interaction, in which PKA is ubiquitously expressed, and its activation and interaction with MYPT1, independent of cAMP, is agonist-specific. The present study also provides evidence for regulation of the contractile pathway via cAMP-independent PKA activated by G_q-coupled receptors.

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Abbreviations used

CPA	cyclopentyl adenosine
CPI-17	PKC-potentiated inhibitor 17-kDa protein
DMEM	Dulbecco's modified Eagle's medium
IκB	inhibitor of NF-κB
IKK2	IκB kinase 2
ILK	integrin-linked kinase

IP_3	inositol 1,4,5-trisphosphate
LPA	lysophosphatidic acid
MLC_{20}	20-kDa regulatory light chain of myosin II
MLCK	myosin light-chain kinase
MLCP	myosin light-chain phosphatase
MYPT1	myosin phosphatase targeting subunit 1
NF-κB	nuclear factor κB
PI	phosphoinositide
PKA	protein kinase A or cAMP-dependent protein kinase
PKC	protein kinase C
PKG	protein kinase G
PKI	PKA inhibitor
PLC	phospholipase C
PLD	phospholipase D
Rho kinase	Rho-associated kinase
S1P	sphingosine 1-phosphate

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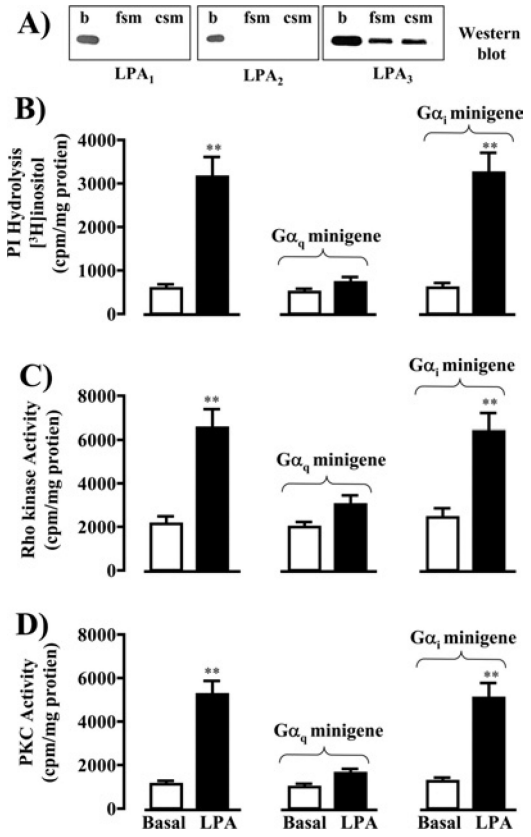

Figure 1. Selective expression of LPA_3 receptors and stimulation of $G_{\alpha q}$-mediated PI hydrolysis, Rho kinase and PKC by LPA in gastric smooth muscle

(**A**) Lysates were prepared from freshly dispersed smooth muscle cells (fsm), cultured smooth muscle cells (csm) and brain (b). The proteins were resolved by SDS/PAGE and transferred on to PVDF membranes and were detected with specific antibodies to LPA_1, LPA_2 or LPA_3 receptors. (**B**) Cultured muscle cells expressing the $G_{\alpha q}$ or $G_{\alpha i}$ minigene were treated with LPA for 60 s. In all experiments, the cells were prelabelled with *myo*-[^{3}H]inositol. (**C**) Rho kinase activity was measured by immunokinase assay as described in the Experimental section. (**D**) PKC activity was measured in membrane fractions using myelin basic protein as the substrate. Results were expressed as c.p.m./mg of protein. Values are means ± S.E.M. of four experiments. $^{**}P < 0.01$ significant stimulation of PI hydrolysis, Rho kinase or PKC by LPA.

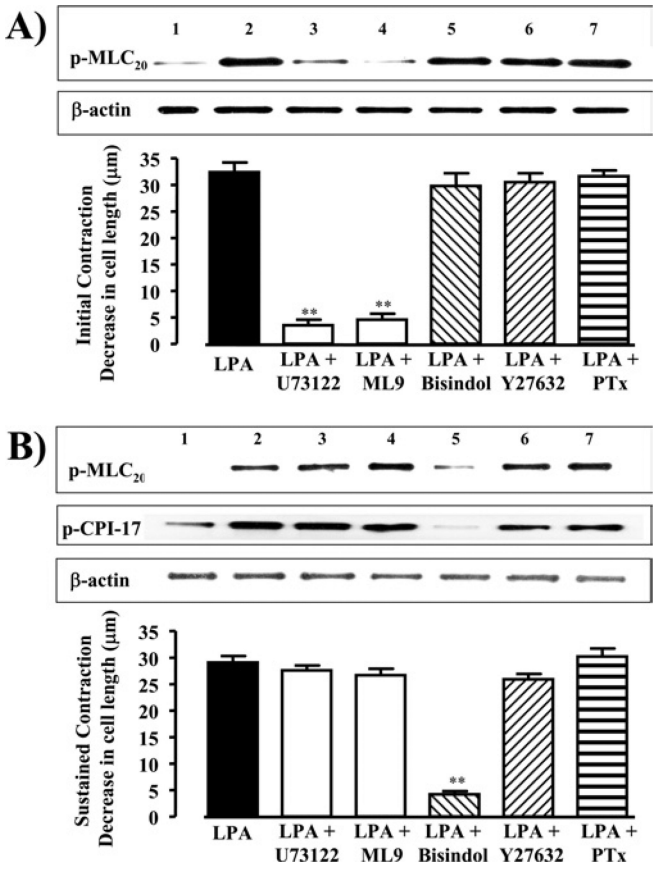

Figure 2. Inhibition of LPA-induced MLC_{20} and CPI-17 phosphorylation and muscle contraction

Freshly dispersed gastric smooth muscle cells were incubated with U73122 (1 μM), ML-9 (10 μM), bisindolylmaleimide (1 μM) or Y27632 (1 μM) for 10 min, or PTx (400 ng/ml) for 1 h and then treated with LPA (1 μM). (**A**) Effect of LPA on MLC_{20} phosphorylation and muscle contraction at 30 s (initial response). LPA had no effect on CPI-17 phosphorylation at 30 s. (**B**) Effect of LPA on MLC_{20} and CPI-17 phosphorylation and muscle contraction at 5 min (sustained response). MLC_{20} phosphorylation was measured using phospho-specific-Ser^{19} MLC_{20} antibody (p-MLC_{20}). CPI-17 phosphorylation was measured using a phospho-specific Thr^{38} CPI-17 antibody (p-CPI-17). Muscle contraction was measured by scanning micrometry and expressed in μm as the decrease in cell length from control (106 ± 5 μm). Values are means ± S.E.M. of three or four experiments. $^{**}P < 0.01$ significant inhibition of LPA-induced contraction. Legend for Western blot: lane 1, basal (untreated); lane 2, LPA alone; lane 3, LPA + U73122; lane 4, LPA + ML9; lane 5, LPA + bisindolylmaleimide; lane 6, LPA + Y27632; and lane 7, LPA + PTx, PTx. pertussis toxin.

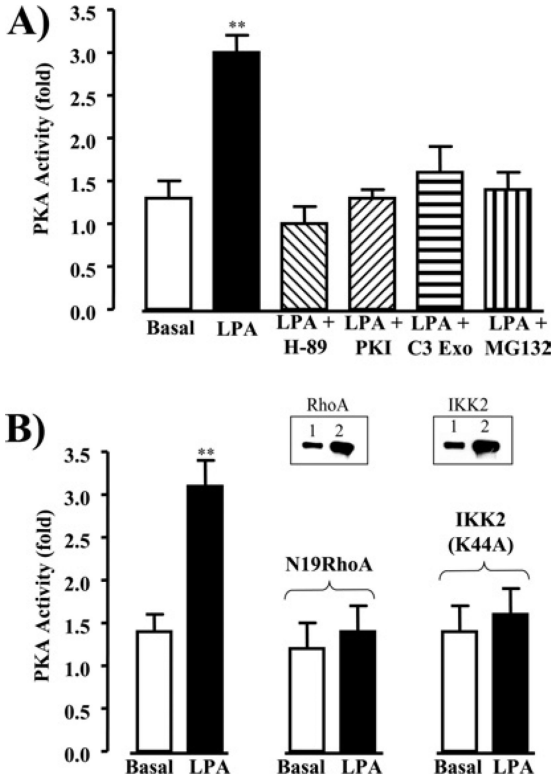

Figure 3. RhoA-mediated activation of cAMP-independent PKA by LPA via IκB degradation

(**A**) Dispersed muscle cells were incubated for 10 min with H-89 (1 μM), myristoylated PKI (1 μM), MG-132 (10 μM) or for 2 h with C3 exoenzyme (2 μg/ml) and then treated with LPA (1 μM) for 5 min. (**B**) Cultured muscle cells expressing control vector or dominant-negative mutants of RhoA (N19RhoA) or IKK2(K44A) were treated with LPA for 5 min. PKA activity was measured using Kemptide as described in the Experimental section. Inset: Western blot analysis of RhoA and IKK2 in cells expressing vector alone (lane 1) and overexpressing RhoA (N19RhoA) or IKK2(K44A) (lane 2). Values are means ± S.E.M. of four experiments. $^{**}P < 0.01$ significant activation of PKA.

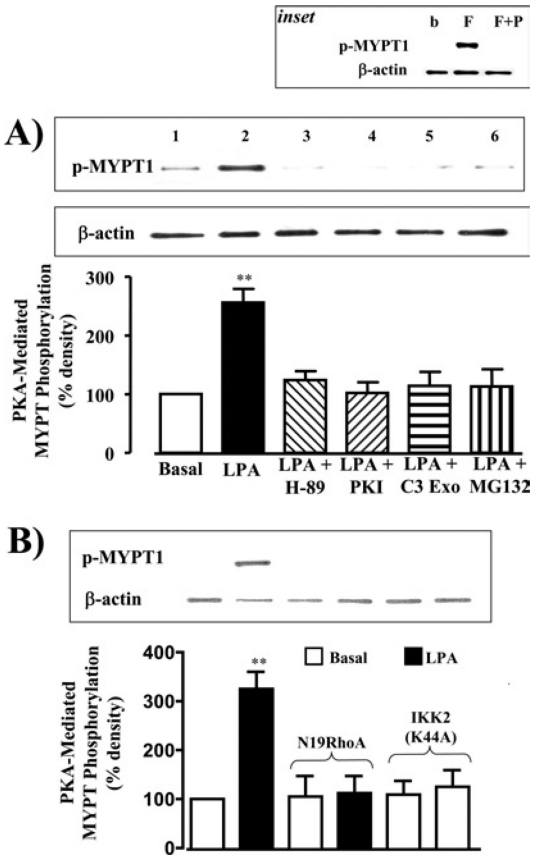

Figure 4. LPA-induced phosphorylation of MYPT1 by cAMP-independent PKA

(**A**) Dispersed muscle cells were incubated for 10 min with H-89 (1 μM), myristoylated PKI (1 μM), MG-132 (10 μM) or for 2 h with C3 exoenzyme (2 μg/ml) and then treated with LPA (1 μM) for 5 min. Legend for Western blot: lane 1, basal (untreated); lane 2, LPA alone; lane 3, LPA + H-89; lane 4, LPA + PKI; lane 5, LPA + C3 exoenzyme; and lane 6, LPA + MG132. Inset: MYPT1 phosphorylation in basal cells (b) and in cells treated with forskolin in the absence (F) or presence (F + P) of PKI. (**B**) Cultured muscle cells expressing control vector or dominant-negative mutants of RhoA (N19RhoA) or IKK2(K44A) were treated with LPA for 5 min. Phosphorylation of MYPT1 was measured using a phospho-substrate antibody specific for the PKA site. $^{**}P < 0.01$ significant phosphorylation of MYPT1.

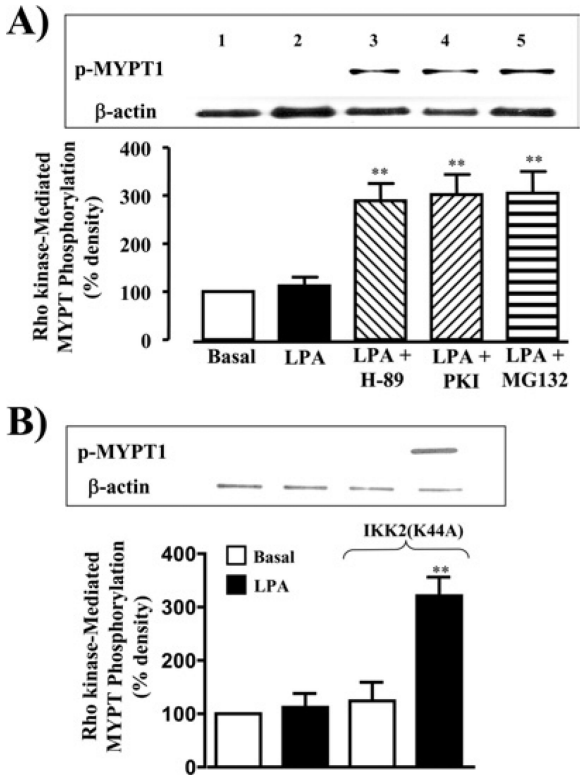

Figure 5. LPA-induced phosphorylation of MYPT1 by PKA masks MYPT1 phosphorylation at Thr^{696} by Rho kinase

(**A**) Dispersed muscle cells were incubated for 10 min with H-89 (1 μM), myristoylated PKI (1 μM) or MG-132 (10 μM) and then treated with LPA (1 μM) for 5 min. Legend for Western blot: lane 1, basal (untreated); lane 2, LPA alone; lane 3, LPA + H-89; lane 4, LPA + PKI; and lane 5, LPA + MG132. (**B**) Cultured muscle cells expressing control vector or the dominant-negative mutant of IKK2(K44A) were treated with LPA for 5 min. Phosphorylation of MYPT1 by Rho kinase was measured using a phospho-specific Thr^{696} antibody. $^{**}P < 0.01$ significant phosphorylation of MYPT1.

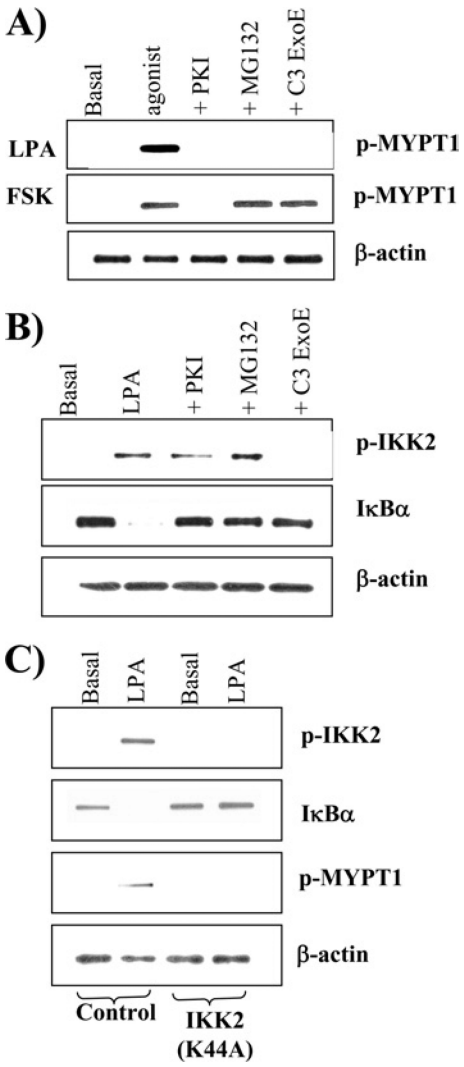

Figure 6. LPA-induced phosphorylation of MYPT1 at Ser^{695} and activation of the NF-κB pathway

Dispersed muscle cells were incubated for 10 min with H-89 (1 μM), myristoylated PKI (1 μM), MG-132 (10 μM) or for 2 h with C3 exoenzyme (2 μg/ml) and then treated with LPA (1 μM) or forskolin (FSK, 10 μM) for 5 min. (**A**) Phosphorylation of MYPT1 was measured using a phospho-specific Ser^{695} antibody. '+' indicates addition of inhibitors in the presence of LPA. (**B**) IKK2 phosphorylation was measured using a phospho-specific Ser$^{177/181}$ antibody and IκBα degradation was measured using an IκBα antibody. (**C**) Cultured muscle cells expressing control vector or IKK2(K44A) were treated with LPA for 5 min. Phosphorylation of MYPT1 and IKK2, and degradation of IκBα were measured as described above.

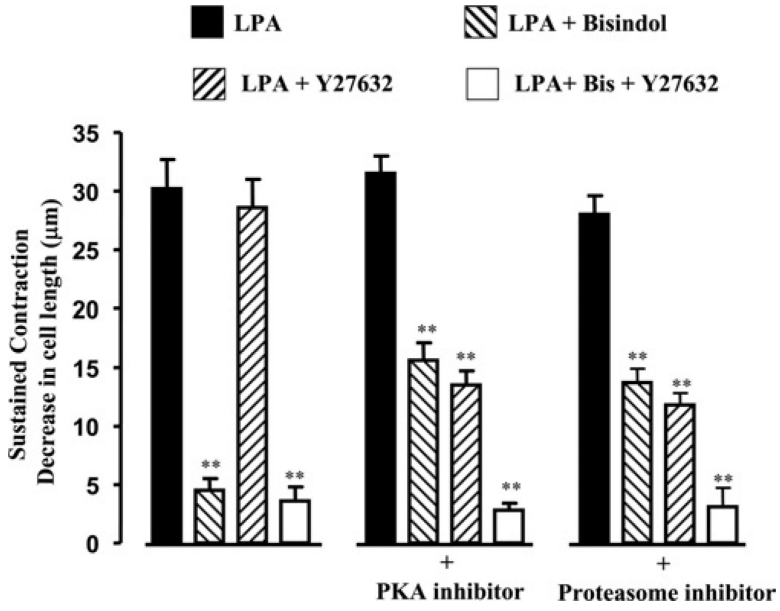

Figure 7. LPA induces Rho-kinase-dependent contraction after inactivation of NF-κB and PKA stimulation

LPA-induced sustained muscle contraction (response at 5 min) was measured in the presence or absence of bisindolylmaleimide (1 μM), Y27632 (1 μM) or bisindolmaleimide plus Y27632. Experiments were performed in the presence or absence of a PKA inhibitor (myristolylated PKI, 1 μM) or a proteasome degradation inhibitor (MG-132, 10 μM). Muscle contraction was measured by scanning micrometry and expressed in μm as the decrease in cell length from control (106 ± 5 μm). Values are means ± S.E.M. of four experiments. ** $P < 0.01$ significant inhibition of LPA-induced sustained contraction.

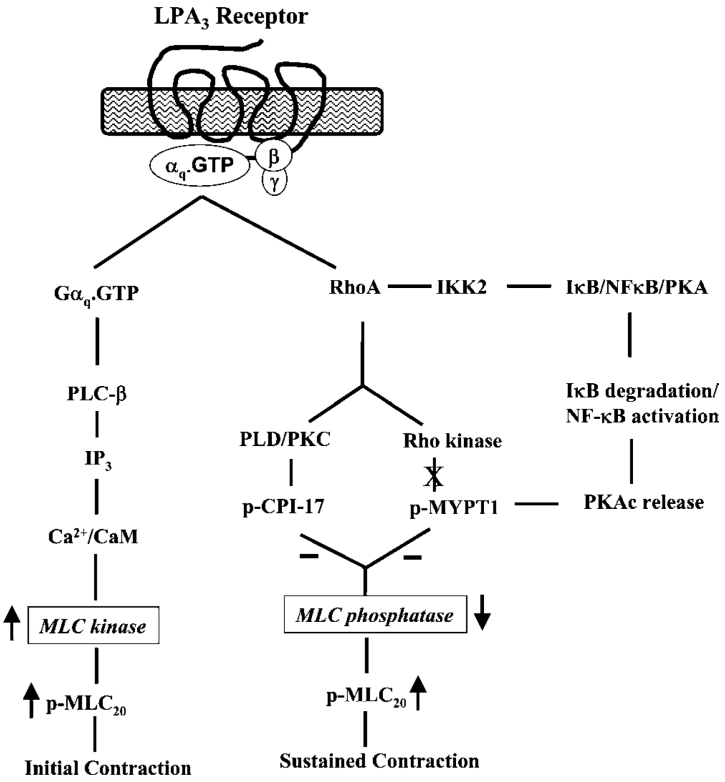

Figure 8. Pathways mediating initial and sustained contraction and MLC_{20} phosphorylation by LPA

Initial contraction and MLC_{20} phosphorylation induced by LPA involves G_q-dependent PLC-β activation, IP_3 generation, Ca^{2+} release and Ca^{2+}/calmodulin-dependent activation of MLCK. Sustained contraction and MLC_{20} phosphorylation involves G_q-dependent activation of RhoA and PKC, phosphorylation of CPI-17 and inhibition of MLCP. Although Rho kinase was activated, phosphorylation by Rho kinase of MYPT1 at the inhibitory site Thr^{696} was prevented by PKA-mediated phosphorylation of MYPT1 at Ser^{695}. The pathway involves canonical activation of NF-κB and release of the PKA catalytic subunit (PKAc) from its binding to the IκBα complex leading to phosphorylation of MYPT1 by PKA.