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Upregulation of RGS4 and downregulation of CPI-17 mediate inhibition of colonic muscle contraction by interleukin-1 β

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Abstract

The pro-inflammatory cytokine IL-1 β contributes to the reduced contractile responses of gut smooth muscle observed in both animal colitis models and human inflammatory bowel diseases. However, the mechanisms are not well understood. The effects of IL-1 β on the signaling targets mediating acetylcholine (ACh)-induced initial and sustained contraction were examined using rabbit colonic circular muscle strips and cultured muscle cells. The contraction was assessed through cell length decrease, myosin light chain (MLC₂₀) phosphorylation, and activation of PLC- β and Rho kinase. Expression levels of the signaling targets were determined by Western blot analysis and real-time RT-PCR. Short interfering RNAs (siRNAs) for regulator of G protein signaling 4 (RGS4) were used to silence endogenous RGS4 in muscle strips or cultured muscle cells. IL-1 β treatment of muscle strips inhibited both initial and sustained contraction and MLC₂₀ phosphorylation in isolated muscle cells. IL-1 β treatment increased RGS4 expression but had no effect on muscarinic receptor binding or G α_q expression. In contrast, IL-1 β decreased the expression and phosphorylation of CPI-17 but had no effect on RhoA expression or ACh-induced Rho kinase activity. Upregulation of RGS4 and downregulation of CPI-17 by IL-1 β in muscle strips were corroborated in cultured muscle cells. Knockdown of RGS4 by siRNA in both muscle strips and cultured muscle cells blocked the inhibitory effect of IL-1 β on initial contraction and PLC- β activation, whereas overexpression of RGS4 inhibited PLC- β activation. These data suggest that IL-1 β upregulates RGS4 expression, resulting in the inhibition of initial contraction and downregulation of CPI-17 expression during sustained contraction in colonic smooth muscle.

Keywords

rabbit; short interfering RNA; acetylcholine; phospholipase C- β ; Rho kinase; regulator of G protein signaling 4

In chronic diseases such as inflammatory bowel diseases (IBD), the inflammatory response is persistent and recurrent. Such persistent inflammation may involve positive feedback regulation of inflammatory mediators including cytokines, chemokines, growth factors, and cell adhesion molecules that contribute to the maintenance and resolution of the inflamma-

tory response in the gut. These mediators are generated from multiple cell types including mucosal epithelium, smooth muscle cells, glial cells, and various leukocytes. Smooth muscle cells act as both sources and targets of these mediators, setting up an intricate, dynamic balance that maintains the inflammatory response (54, 58).

The role of cytokines as key mediators of inflammation is well established, and their blockade has generated new forms of therapy for many inflammatory diseases (40, 60). Intestinal inflammation in humans and animals has been widely shown to be associated with changes in contractility (26, 54). Recent studies have shown that inflammatory stimuli elicit distinct patterns of inflammatory mediators that either decrease or increase smooth muscle cell contractility (26, 44). A pattern involving time-dependent release of IL-1 β , TNF- α , IL-6, and IL-8 is accompanied by decreases in the response of smooth muscle to excitatory neurotransmitters [acetylcholine (ACh), neurokinin A, etc.] (45, 50, 54, 57), whereas the pattern observed with helminth infection involves transient activation of IL-4 and IL-13, resulting in initial hypercontractility followed by sustained expression of tumor growth factor- β_1 and cyclooxygenase-2, leading to persistent hypercontractility (1–4, 26, 63). The specific steps in the signaling pathways mediating contraction or relaxation that are affected by these cytokine patterns have not been identified.

Our previous study (35) characterized the signal transduction pathways for initial and sustained contraction in gut smooth muscle cells. The initial contraction reflects stimulation of PLC- β activity, resulting in Ca²⁺/calmodulin-dependent activation of myosin light chain (MLC) kinase (MLCK) and transient MLC₂₀ phosphorylation and contraction. Inhibition of phosphoinositide (PI) hydrolysis or MLCK activity blocks only the initial contraction. In contrast, the sustained contraction reflects activation of the monomeric G protein RhoA, resulting in inhibition of MLC phosphatase (MLCP) via Rho kinase-mediated phosphorylation of MYPT1, a regulatory subunit of MLCP, and PKC-mediated phosphorylation of CPI-17, an endogenous inhibitor of MLCP. Phosphorylation of both MYPT1 and CPI-17 leads to inhibition of MLCP activity, resulting in sustained MLC₂₀ phosphorylation and contraction. Inactivation of RhoA or inhibition of Rho kinase or PKC inhibits sustained contraction.

IL-1 β is a major proinflammatory cytokine produced by various cell types in the gut. Increased production of IL-1 β has been detected at both mRNA and protein levels in human IBD and animal colitis models (25, 30, 41, 61). Both IL-1 β antibody and IL-1 receptor antagonist have been shown to attenuate the development of experimental colitis (14, 31). IL-1 β has been widely shown to inhibit the contractile response of gut smooth muscle both in vivo and in vitro (5, 6, 9, 11, 16, 24, 25, 30, 41, 61). However, the mechanisms have not been fully understood. Indirect neural regulation by modulation of the release of ACh, norepinephrine, and substance P has been demonstrated (5, 6, 8, 41). Recently, direct cellular regulation or modification has been increasingly recognized (9, 11, 45, 57). The presence of IL-1 β receptors on smooth muscle cells implies a possibility of direct action by IL-1 β (62). Signaling pathways for smooth muscle relaxation (inhibition of contraction) such as inducible nitric oxide (NO) synthase-NO/cGMP signaling have been shown to mediate the inhibitory effects of cytokines (28, 61). PGE₂, a potent smooth muscle relaxant generated from IL-1 β -stimulated cyclooxygenase-2, has been shown to mediate the IL-1 β -

induced reduction in the tone of the lower esophageal sphincter (7). However, the effects of cytokines on the signaling targets mediating initial and sustained contraction of smooth muscle cells are not known. Ohama et al. reported that IL-1 β inhibits carbachol- or high-potassium-stimulated contractile responses of rat ileal circular muscle strips through decreasing CPI-17 expression (42, 45, 46). In the present study, we characterize the effect of IL-1 β on key targets in the signaling pathways mediating initial and sustained muscle contraction and MLC₂₀ phosphorylation in rabbit colonic circular smooth muscle cells. We show that IL-1 β treatment significantly decreases CPI-17 expression and ACh-induced CPI-17 phosphorylation but has no effect on RhoA expression or ACh-induced Rho kinase activity. In addition, IL-1 β treatment significantly increased the expression of regulator of G protein signaling 4 (RGS4) but had no effect on muscarinic receptor binding or G α_q expression. Increased RGS4 expression is responsible for the IL-1 β -induced inhibition of PLC- β activation, since knockdown of RGS4 by short interfering (si)RNA blocks the inhibitory effect of IL-1 β on ACh-stimulated initial contraction and PLC- β activation, whereas over-expression of RGS4 inhibited PLC- β activation.

MATERIALS AND METHODS

Preparation and culture of smooth muscle strips

A New Zealand White rabbit (2~2.5 kg) was euthanized with an overdose of pentobarbital. The distal colon was removed and placed in HEPES-buffered smooth muscle media (SMM) containing (in mM) 25 HEPES, 120 NaCl, 4 KCl, 2.6 KH₂PO₄, 0.6 MgCl₂, and 14 glucose with a 2.1% Eagle's essential amino acid mixture. The circular smooth muscle layer was dissected from the mucosa and longitudinal muscle layer and cut into small strips (1~2 mm wide). After being washed several times, circular strips were cultured in six-well plates with DMEM supplemented with 1% antibiotics and antimycotics (Invitrogen, Carlsbad, CA). After 1 h of incubation, experiments were performed.

Isolation and culture of smooth muscle cells

Smooth muscle cells were isolated from circular muscle strips by sequential enzymatic digestion, filtration, and centrifugation as previously described (19, 37). In brief, muscle strips were incubated for 30 min at 31°C in 15 ml SMM containing 0.1% collagenase (type II) and 0.1% soybean trypsin inhibitor. The partly digested tissues were washed with enzyme-free SMM and incubated at 31°C to allow spontaneous dispersion of muscle cells. Cells were harvested by filtration through 500- μ m Nitex and centrifuged twice at 350 g for 10 min.

Cells were cultured in DMEM containing 10% FBS and 1% antibiotics and antimycotics until they attained confluence and were then passaged once for use in various experiments.

Conventional and real-time RT-PCR

Total RNA was isolated from rabbit colonic smooth muscle cells with TRIzol reagent (Invitrogen) and treated with TURBO DNase (Ambion, Austin, TX). RNA (2 μ g) was used to synthesize cDNA using SuperScript II reverse transcriptase (Invitrogen) with random hexanucleotide primers. Degenerative primers for RGS4 and CPI-17 were designed based

on highly homologous sequences available from various species such as human, mouse, and rat. Conventional PCR was performed on cDNA from rabbit smooth muscle cells. PCR products were purified and cloned into the pCR2.1 T-A vector for confirmation by sequencing.

Quantitative real-time PCR analysis was carried out on the ABI Prism 7300 Sequence Detection System (Applied Biosystems, Foster, CA). Expression of rabbit RGS4 was analyzed using the TaqMan PCR Master Mix Reagents Kit (Applied Biosystems). The TaqMan probe and primers for rabbit RGS4 designed using the Primer Express 2.0 version were as follows: forward, 5'-tccacagcaagaaggacaaa-3'; reverse, 5'-ttcgcccatttctgactt-3'; and probe, 5'-tgactcaccctctggcaacaacca-3'. The probe was labeled at the 5'-end with 6-carboxyfluoresceine and at the 3'-end with 6-carboxytetramethylrhodamine. The optimized concentrations were 0.4 μ M for both primers and 0.2 μ M for probe and 5 ng cDNA in 20- μ l reaction volume. PCRs without reverse transcription were included to control for contamination by genomic DNA. Expression of rabbit CPI-17 was analyzed using SYBR green PCR mix (SuperArray Bioscience, Frederick, MD) containing 5 ng cDNA and 0.4 μ M each primer: forward 5'-ctggacgtggagaagtggatc-3' and reverse 5'-agctcctgatgaagtcctc-3' for CPI-17. Rabbit GAPDH primers (forward 5'-cgcttgagaaagctgctaa-3' and reverse 5'-cgacctgtcctcggttag-3') were used as the internal control. Each sample was tested in triplicate, and the mRNA level was normalized to that of GAPDH. Real-time PCR data were analyzed using the cycle threshold relative quantification method.

Preparation and validation of RGS4 siRNA

Lentiviral vectors encoding enhanced green fluorescent protein (EGFP) as an internal marker together with siRNA for RGS4 were generated as previously described (19). Briefly, two siRNA expression cassettes targeting nucleotides 280–299 and 681–699 of rabbit RGS4 (accession no. DQ120011) were generated through two consequential rounds of PCR and individually cloned into the pLL3.7 lentiviral vector via *Xba*I/ *Xho*I cloning sites. The sequence of each siRNA cassette was confirmed by restriction enzyme digestion with *Bam*HI/*Eco*RI and DNA sequencing. Silencing efficiency and specificity of these siRNA constructs were determined by Western blot and RT-PCR analysis in cultured colonic smooth muscle cells. The DNA sequence for the most effective RGS4 siRNA construct, RGS4A, was 5'-gaggaagtcaagaatgggc-3', which shares 100% homology with human RGS4.

The synthesized siRNA for RGS4A validated above and FAM-labeled negative scrambled siRNA were obtained from Ambion.

Transfection of cultured colonic smooth muscle cells and strips

Confluent smooth muscle cells in the first passage on six-well plates were transiently transfected with the pLL3.7 vector encoding RGS4 siRNA or pcDNA3 vector encoding hemagglutinin (HA)-tagged RGS4 using Lipofectamine 2000 according to the manufacturer's instructions (Invitrogen). Briefly, 2 μ g of the vector in 125 μ l Opti-MEM medium were mixed with 5 μ l Lipofectamine 2000 in 125 μ l Opti-MEM. The mixture was incubated at room temperature for 20 min and added to wells containing 1.5 ml DMEM with 10% FBS for 1 day. The medium was then replaced with DMEM with 10% FBS plus

antibiotics for 2 days. Cells were maintained for a final 24 h in DMEM without FBS before experiments were started. Fluorescence analysis of EGFP or immunocytochemical staining with anti-HA antibody showed a transfection efficiency of 60–70%.

About 20 pieces of colonic circular muscle strips were transfected with 100 pmol synthesized RGS4A siRNA or scrambled control siRNA using Lipofectamine 2000 according to the manufacturer's instructions (Invitrogen). Transfection efficiency (>70%) was monitored by the fluorescence of FAM-labeled siRNA, which was taken up by smooth muscle cells as early as 4 h.

Western blot analysis

Western blot analysis was performed as previously described (19). Briefly, freshly dispersed or cultured smooth muscle cells were solubilized in Triton X-100-based lysis buffer plus protease and phosphatase inhibitors. After centrifugation of the lysates at 20,000 *g* for 10 min at 4°C, protein concentrations of the supernatant were determined with the DC Protein Assay kit from Bio-Rad (Hercules, CA). Equal amounts of proteins were fractionated by SDS-PAGE and transferred to nitrocellulose membranes. Blots were blocked in 5% nonfat dry milk with Tris-buffered saline (TBS; pH 7.6) plus 0.1% Tween 20 (TBS-T) for 1 h and then incubated overnight at 4°C with various primary antibodies in TBS-T plus 1% milk. After an incubation for 1 h with horseradish peroxidase-conjugated corresponding secondary antibody (1:2,000, 10 µg/ml, Pierce) in TBS-T plus 1% milk, immunoreactive proteins were visualized using SuperSignal Femto maximum sensitivity substrate kit (Pierce). All washing steps were performed with TBS-T.

Radioligand binding assay

Binding of [³H]scopolamine to dispersed colonic smooth muscle cells was done as previously described (36, 37). Muscle cells were suspended in HEPES medium containing 1% BSA. Triplicate 0.5-ml aliquots (10⁶ cells/ml) were incubated for 15 min with 1 nM [³H]scopolamine alone or with ACh. Bound and free radioligands were separated by rapid filtration under reduced pressure through 5-µm polycarbonate Nucleopore filters followed by repeated washing (4 times) with 3 ml of ice-cold HEPES medium containing 0.2% BSA. Nonspecific binding (26 ± 5%) was calculated as the amount of radioactivity in the presence of 10 µM ACh. [³H]scopolamine binding was measured in control cells and cells obtained from muscle strips treated with IL-1β for 2 days.

Assay for PLC-β activity

PLC-β activity was determined in freshly dispersed or cultured smooth muscle cells by measuring the formation of inositol phosphates using ion-exchange chromatography as previously described (38). Cultured (at full confluence after transfection) or freshly dispersed smooth muscle cells labeled with myo-[³H]inositol (0.5 µCi/ml) for 24 h in inositol-free DMEM without FBS were washed with PBS and treated with ACh (0.1 µM) plus methoctramine (0.1 µM) (37, 39) for 0.5 min in 1 ml HEPES-buffered solution (pH 7.4). The reaction was terminated with 940 µl chloroform-methanol-HCl (50:100:1). The aqueous phase after extraction and centrifugation was applied to a DOWEX AG-1 column, and [³H]inositol phosphates were eluted with 0.8 M ammonium formamate and 0.1 M

formic acid. Radioactivity was determined by liquid scintillation and expressed as counts per minute (cpm).

Assay for Rho kinase activity

Rho kinase activity was determined in cell extracts by immunokinase assay as previously described (39). Cultured (at confluence after transfection) or freshly dispersed smooth muscle cells were treated with ACh (0.1 μ M) plus methoctramine (0.1 μ M) for 5 min and solubilized with lysis buffer containing 50 mM Tris-HCl (pH 7.5), 0.1% SDS, 0.5% sodium deoxycholate, 1% Nonidet P-40, 150 mM NaCl, 1 mM PMSF, 10 μ g/ml aprotinin, 10 μ g/ml pepstatin A, and 10 μ g/ml leupeptin. Equal amounts of protein extracts were incubated with Rho kinase-2 antibody or IgG control plus protein A/G agarose overnight at 4°C. Immunoprecipitates were washed twice in medium containing 10 mM MgCl₂ and 40 mM HEPES (pH 7.4) and then incubated for 5 min on ice with 5 μ g myelin basic protein as a substrate for Rho kinase. The kinase reaction was initiated by the addition of 10 μ Ci of [γ -³²P]ATP (3,000 Ci/mmol) and 20 μ M ATP, followed by an incubation for 10 min at 37°C. ³²P-labeled myelin basic protein was absorbed onto phosphocellulose disks, and repeated washings with 75 mM phosphoric acid removed free radioactivity. The amount of radioactivity on the disks was measured by liquid scintillation.

Measurement of contraction in dispersed smooth muscle cells

Contraction in freshly dispersed muscle cells from colonic circular muscle strips after incubation with IL-1 β or transfection with siRNA was measured by scanning microscopy as previously described (32). Cell aliquots containing $\sim 10^4$ muscle cells/ml were treated with ACh (0.1 μ M) plus methoctramine (0.1 μ M) for various time periods and fixed with 1% acrolein. The lengths of muscle cells treated with ACh were compared with the lengths of untreated cells, and contraction was expressed as the percent decrease in cell length from control.

Statistical analysis

Results are expressed as means $\pm$ SE of *n* experiments and were analyzed for statistical significance using Student's *t*-test for paired or unpaired values.

Reagents and antibodies

IL-1 β was obtained from Alexis Biochemicals (ALX-522-056, San Diego, CA). Affinity-purified RGS4 antibody was kindly provided by Dr. Susanne M. Mumby (University of Texas Southwest Medical Center). CPI-17, phosphorylated (Thr³⁸) CPI-17, MLC, phosphorylated (Ser¹⁹) MLC, MYPT1, phosphorylated (Thr⁶⁹⁶) MYPT1, G α_q , and Rho kinase-2 antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). [γ -³²P]ATP was from Amersham Pharmacia Biotech (Piscataway, NJ), and [³H]scopolamine and myo-[³H]inositol were from DuPont NEN (Boston, MA). All other reagents were from Sigma (St. Louis, MO).

RESULTS

IL-1 β inhibits ACh-induced contractions and MLC₂₀ phosphorylation in isolated rabbit colonic circular smooth muscle cells

To identify the effect of IL-1 β on signaling pathways mediating initial and sustained contraction, we examined the effect of IL-1 β on ACh-induced initial and sustained contractions in smooth muscle cells isolated from rabbit colonic circular muscle strips treated with IL-1 β . The dose of IL-1 β (10 ng/ml) was selected according to our preliminary studies and previous reports (43, 45). As shown in Fig. 1A, muscle strips after incubation for 2 days maintained initial and sustained contractile responses of isolated cells to the excitatory stimulator ACh. The contractile response of muscle cells dispersed from muscle strips after 2 days of culture was similar to that observed in muscle cells dispersed from freshly isolated muscle strips (39) (data not shown). Treatment of muscle strips with IL-1 β (10 ng/ml) for 2 days significantly suppressed both initial and sustained contraction. IL-1 β treatment for a short period (3 h) in muscle strips produced a similar inhibition of ACh-induced initial and sustained contraction in isolated muscle cells (data not shown).

Phosphorylation of MLC₂₀ at Ser¹⁹ is a prerequisite for both initial and sustained contraction in smooth muscle and is regulated by the balance between MLCK activity and MLCP activity. Western blot analysis with specific anti-phosphorylated (Ser¹⁹) MLC₂₀ antibody demonstrated that IL-1 β treatment for 2 days caused a significant decrease in MLC₂₀ phosphorylation during both the initial and sustained phases of contraction measured, respectively, at 0.5 and 5 min after ACh stimulation in smooth muscle cells isolated from rabbit colonic circular muscle strips (Fig. 1B).

IL-1 β inhibits ACh-stimulated PLC- β activation

As shown in our previous studies (20, 38, 39), the initial transient phase of muscle contraction (~1 min) induced by G protein-coupled receptor agonists in circular smooth muscle cells is Ca²⁺ dependent and involves stimulation of PLC- β activity (PI hydrolysis). As shown in Fig. 2, smooth muscle cells isolated from rabbit colonic circular muscle strips after culture for 2 days maintained a similar response of PLC- β activation to ACh stimulation. Treatment of colonic circular muscle strips for 2 days with IL-1 β caused a significant decrease in ACh-induced PI hydrolysis measured at 0.5 min after stimulation in isolated colonic muscle cells.

IL-1 β upregulates RGS4 expression but has no effect on muscarinic M₃ receptor binding and G α_q expression

ACh-induced PI hydrolysis is mediated by G α_q -dependent activation of PLC- β and attenuated by inactivation of G α_q via RGS4 (20, 39). To address which target(s) of the signaling pathway mediates the IL-1 β -induced inhibitory effect on initial MLC₂₀ phosphorylation and muscle contraction, we examined the expression and/or activities of important components upstream to PLC- β activation. Western blot analysis of the cell lysate from isolated smooth muscle cells showed that treatment of colonic circular muscle strips for 2 days with IL-1 β increased RGS4 protein expression but had no effect on G α_q protein expression (Fig. 3A).

To determine whether treatment with IL-1 β affected receptor binding, [3 H]scopolamine binding was measured in dispersed muscle cells. Treatment with IL-1 β for 2 days had no effect on [3 H]scopolamine binding, suggesting that the decrease in PLC- β activity was not due to the decrease in receptor binding (Fig. 3B).

Smooth muscle strips contain nonmuscle cells such as neurons, glia cells, and resident macrophages. To determine whether IL-1 β directly affects smooth muscle cells leading to upregulation of RGS4 expression, we performed Western blot and real-time RT-PCR analyses using cultured pure smooth muscle cells. As shown in Fig. 3, C and D, IL-1 β treatment induced time-dependent upregulation of RGS4 expression at both protein and mRNA levels in cultured rabbit colonic circular smooth muscle cells at full confluence (*passage 1*) after 24 h of serum starvation. The specificity of the RGS4 antibody was validated by Western blot analysis of HA-tagged RGS4 overexpressed in cultured smooth muscle cells (data not shown).

Increase in RGS4 expression mediates IL-1 β -induced inhibition of PLC- β activation and muscle contraction

G α_q activated by M $_3$ receptors mediates ACh-induced initial contraction, and the strength and duration of G α_q signaling is regulated by RGS4 (20, 39). We hypothesized that upregulation of RGS4 by IL-1 β mediates the inhibitory effect of IL-1 β on initial MLC $_{20}$ phosphorylation and muscle contraction. To test this in cultured smooth muscle cells, we measured PLC- β activity during the initial phase of contraction after ACh stimulation. Due to the difficulty in measuring contraction in cultured smooth muscle cells, ACh-induced PLC- β activation has been used as a marker to reflect the initial contraction (20, 36, 38). HA-tagged RGS4 plasmid or siRNA RGS4 expression vectors were transfected into cultured muscle cells for 3 days, and the expression level of RGS4 was determined by Western blot analysis. As predicted, RGS4 overexpression alone inhibited ACh-induced PLC- β activation in rabbit colonic circular smooth muscle cells (Fig. 4A), which is consistent with our previous demonstration that overexpression of RGS4 inhibited motilin-induced PLC- β activation in gastric smooth muscle cells (20). One of the vector-based RGS4 siRNA constructs (RGS4A) was most effective in silencing the expression of endogenous RGS4 at both mRNA and protein levels (Fig. 4B). When the expression of endogenous RGS4 was suppressed by RGS4A siRNA, the ACh-induced PLC- β activity was increased compared with the control siRNA vector. In addition, no inhibition of PLC β activity by IL-1 β was observed in cells transfected with RGS4A siRNA expression vector (Fig. 4C). Rho kinase activation has been well known to mediate the sustained but not initial contraction stimulated by various agonists in smooth muscle cells. Therefore, the activity of Rho kinase at 5 min after ACh stimulation in cultured smooth muscle cells transfected with RGS4A or control siRNA vector was measured. As predicted, knockdown of RGS4 expression did not affect ACh-induced Rho kinase activation. The activity of Rho kinase was increased from $1,007 \pm 100$ to $5,090 \pm 174$ ($n = 3$) cpm/mg protein after ACh stimulation in cells with control siRNA vector and from 900 ± 139 to $4,782 \pm 254$ ($n = 3$) cpm/mg protein in cells with RGS4A siRNA vector. These data suggest that upregulation of RGS4 expression mediates the inhibitory effect of IL-1 β on ACh-stimulated PLC- β activation during the initial phase of contraction.

To further confirm that upregulated RGS4 expression is responsible for mediating the inhibitory effect of IL-1 β on initial smooth muscle contraction, we transfected muscle strips with synthesized RGS4A siRNA and isolated single smooth muscle cells from the strips for contraction measurements. As shown in Fig. 5A, delivery of synthesized RGS4A siRNA into muscle strips significantly (>90%) suppressed the expression of RGS4 mRNA and protein, whereas scrambled siRNA had no effect. The addition of IL-1 β to muscle strips transfected with RGS4A siRNA restored the ACh-induced initial contraction of dispersed smooth muscle cells to near control levels, whereas RGS4A siRNA had no effect on the IL-1 β -induced inhibition of sustained contraction (Fig. 5B). These data confirm that RGS4 specifically regulates ACh-induced initial contraction in colonic smooth muscle cells and further demonstrate that IL-1 β -induced upregulation of endogenous RGS4 expression contributes to the inhibitory effect of IL-1 β on the initial contraction.

IL-1 β has no effect on RhoA expression or ACh-induced Rho kinase activity

The sustained phase of smooth muscle contraction is Ca²⁺ independent and involves the activation of RhoA leading to inhibition of MLCP and stimulation of MLC₂₀ phosphorylation. Muscarinic M₃ receptors have been shown to stimulate RhoA activation, resulting in inhibition of MLCP via Rho kinase-mediated phosphorylation of MYPT1, the regulatory subunit of MLCP, and PKC-mediated phosphorylation of CPI-17, an endogenous inhibitor of MLCP (35). To identify which signaling target mediates the IL-1 β -induced inhibitory effect on sustained contraction, we first examined the expression of RhoA by Western blot analysis and the activity of Rho kinase by immunocomplex kinase assay. As shown in Fig. 6, there were no differences in the expression of RhoA protein as well as Rho kinase activities in smooth muscle cells isolated from rabbit colonic circular muscle strips between control and IL-1 β treatment. These results are consistent with a previous report in rat ileal muscle strip (45), implying that the inhibitory effect of IL-1 β on sustained contraction may target downstream of RhoA or Rho kinase.

IL-1 β downregulates expression and phosphorylation of CPI-17

We then examined the expression and phosphorylation of MYPT1 and CPI-17 in smooth muscle cells isolated from muscle strips treated with IL-1 β . Western blot analysis showed that protein expression and phosphorylation of CPI-17 were significantly inhibited in colonic smooth muscle cells isolated from muscle strips exposed to IL-1 β for 2 days (Fig. 7, A and B). However, MYPT1 expression and phosphorylation were not affected (data not shown). Treatment of colonic circular muscle strips with IL-1 β for a short period (3 h) caused a decrease in CPI-17 mRNA and protein expression (Fig. 8A). To determine the direct effect of IL-1 β at the cellular level and confirm the inhibitory effect of IL-1 β on CPI-17 expression in muscle strips, cultured smooth muscle cells at full confluence were used for RT-PCR analysis. As shown in Fig. 8B, IL-1 β exposure caused a significant decrease in CPI-17 mRNA expression within 3 h that was maintained for up to 3 days. These data demonstrate that IL-1 β downregulates CPI-17 expression in rabbit colonic circular smooth muscle cells.

DISCUSSION

Numerous receptor agonists induce both initial and sustained contraction in gastrointestinal smooth muscle cells (15, 35). The present study focused on the effect of the proinflammatory cytokine IL-1 β on both initial and sustained contraction induced by muscarinic M₃ receptors (37, 39). We measured initial and sustained contraction in dispersed single smooth muscle cells isolated from rabbit colonic circular muscle strips treated with IL-1 β . We demonstrated that IL-1 β significantly inhibits both initial and sustained contraction and MLC₂₀ phosphorylation stimulated by ACh in the presence of M₂ receptor antagonist (39). By screening the signaling targets mediating ACh-stimulated initial and sustained contraction, we identified RGS4 as a major target responsible for the inhibitory effect of IL-1 β on smooth muscle initial contraction and CPI-17 as a major target for IL-1 β -induced inhibition of smooth muscle sustained contraction. Using cultured colonic circular smooth muscle cells, we also demonstrated that IL-1 β treatment upregulates the expression of RGS4 and downregulates the expression of CPI-17 directly at the muscular cellular level. Based on our previous studies (20, 35, 39), we propose a model for the effect of IL-1 β on the signaling pathways mediating initial and sustained contraction of gut smooth muscle cells (Fig. 9). IL-1 β -induced upregulation of RGS4 expression causes rapid inactivation of G α_q and inhibition of ACh-induced PI hydrolysis (PLC- β activation) and inositol 1,4,5-trisphosphate (IP₃)-dependent Ca²⁺ release (Ca²⁺ mobilization), resulting in decreases in MLCK activity and initial MLC₂₀ phosphorylation and muscle contraction. The sustained contraction is mediated by Rho-dependent inhibition of MLCP, leading to Ca²⁺ sensitization. Muscarinic M₃ receptors engage both Rho kinase-mediated phosphorylation of MYPT-1, a regulatory subunit of MLCP, and PKC-dependent phosphorylation of CPI-17, an endogenous inhibitor of MLCP. IL-1 β inhibits the expression of CPI-17 and its subsequent phosphorylation, thereby suppressing the ability of phosphorylated CPI-17 to inhibit MLCP, leading to the inhibition of sustained MLC₂₀ phosphorylation and muscle contraction.

Although the mechanisms for the direct effect of IL-1 β on contractile responses of gut smooth muscle cells have not been well understood, changes in ion channels and contractile signaling components have been demonstrated (44). Carbacol-induced Ca²⁺ sensitization was significantly reduced in IL-1 β -treated muscle strips (45). H₂O₂, formed in colonic and esophageal sphincter smooth muscle in response to IL-1 β (10, 11), inhibits neurokinin A-induced contraction by interfering with the Ca²⁺ balance. Suppression in L-type Ca²⁺ channel activity in different models of intestinal inflammation has been reported to contribute to the inhibition of smooth muscle contraction. This suppression in Ca²⁺ channel activity may result from the decreased expression of Cav1.2 channels in colonic smooth muscle cells (50, 56, 59) and/or from posttranslational modification such as nitrotyrosylation of Ca²⁺ channels (53). Changes in the transcriptional regulation of ATP-sensitive K⁺ channels has also been shown to be associated with reduced motility during colonic inflammation (17). However, the effect of IL-1 β on the expression and activity of Cav1.2 and other ion channels has not been reported. By screening the signaling targets mediating the sustained contraction in smooth muscle cells, we demonstrated in the present study that CPI-17 protein expression and phosphorylation in dispersed muscle cells are reduced after

IL-1 β treatment in rabbit colonic smooth muscle strips, whereas expressions or activities of other regulatory proteins, such as RhoA, Rho kinase, and MYPT-1, are not changed. These results are consistent with a previous report (45) in rat ileal circular muscle strips. Reports on the phosphorylation of MYPT-1 are inconsistent. IL-1 β -induced reduction in MYPT-1 phosphorylation has been shown in rat ileal smooth muscle strips (45) and cultured human astrocytes (23). In contrast, phosphorylation of MYPT-1 is increased after TNF- α incubation in airway smooth muscle cells (21, 47).

CPI-17 is an endogenous inhibitor of serine/threonine protein phosphatase and activated by several kinases including PKC, Rho kinase, zipper-interacting protein kinase, and integrin-linked kinase (18). Activation of CPI-17 has been widely shown to mediate the Ca²⁺ sensitization and sustained contraction induced by various agonists in smooth muscle cells (18, 35, 44). Ohama et al. (45) were the first to demonstrate the downregulation of CPI-17 after long-term treatment of rat ileal muscle strips with IL-1 β . Downregulation of CPI-17 has been reported in colitis animals (43, 55) and IBD patients (44). These data suggest that CPI-17 downregulation during gut inflammation is associated with the decreased contractile response of inflamed smooth muscle cells. Studies using CPI-17 antibody (43) or siRNA may address the importance of CPI-17 in mediating agonist-induced sustained contraction but not in mediating the inhibitory effect of IL-1 β on contraction due to CPI-17 downregulation by IL-1 β . Direct evidence for the involvement of CPI-17 in mediating IL-1 β -induced inhibition of smooth muscle contraction may derive from studies using a CPI-17 knockout strategy.

The mechanisms underlying the downregulation of CPI-17 by IL-1 β remain elusive. Recent studies using TNF- α conventional knockout mice have suggested that IL-1 β -induced downregulation of CPI-17 in smooth muscle tissues is mediated by TNF- α (sources of cell types unknown) (43). However, TNF- α has been shown to directly activate the RhoA/Rho kinase pathway, leading to calcium sensitization and airway hyperresponsiveness (21, 22). The direct effect of TNF- α on the RhoA pathway and calcium sensitization in gut smooth muscle cells remains unknown. The direct effect of TNF- α on CPI-17 expression in smooth muscle cells needs to be determined.

During initial contraction, the pathway of G protein signaling is different from that during sustained contraction of gut smooth muscle (Fig. 9). ACh-stimulated initial contraction involves G α_q -mediated PLC- β activation and Ca²⁺-dependent rapid phosphorylation of MLC₂₀ (35). RGS4 has been shown to inactivate G α_q signaling by binding to G α_q protein and accelerating its GTPase activity (52). The expression and function of RGS4 have been well studied in cardiomyocytes (29, 33) and nerve tissue (27), whereas little is known in smooth muscle cells. In cardiomyocyte, RGS4 expression is induced by endotoxin and IL-1 β (48, 49) and may contribute to the loss of G α_q -mediated PLC activation by endothelin-1 (34). In human aortic smooth muscle cells, RGS4 is highly expressed at the mRNA level and inhibits sphingosine-1-phosphate receptor-mediated signaling (12). In a neuronal cell line, expression of RGS4 mRNA and protein is reduced after nerve growth factor treatment (27). In the present study, we demonstrated, for the first time, that RGS4 expression is increased in both dispersed and cultured rabbit smooth muscle cells after IL-1 β treatment. The biological relevance of the increased RGS4 expression to IL-1 β -induced inhibition of

smooth muscle contraction is validated through RGS4 overexpression and siRNA silencing in cultured smooth muscle cells using PLC- β activity as a marker to reflect initial contraction (20, 36, 38). Direct evidence was obtained from experiments using muscle strips transfected with synthesized RGS4 siRNA and measurements of cell contraction. Therefore, RGS4 is an important mediator for IL-1 β -induced inhibition on initial contraction in smooth muscle cells.

The regulatory mechanisms of RGS4 expression are unknown. The fact that cAMP reduces the level of RGS4 mRNA suggests that the transcription factor cAMP response element-binding protein may regulate RGS4 expression (51). The existence of several splice variants of RGS4 implies a complex mechanism for RGS4 regulation (13). Cloning and characterization of rabbit full-length RGS4 and its promoter will provide a better understanding of RGS4 gene regulation. IL-1 β is well known to activate NF- κ B and various protein kinases. We are currently investigating the transcriptional and posttranscriptional mechanisms for IL-1 β -induced upregulation of RGS4 expression in colonic smooth muscle cells.

In conclusion, the present study shows that IL-1 β down-regulates CPI-17 expression in colonic circular smooth muscle cells and demonstrates, for the first time, that IL-1 β upregulates RGS4 expression, resulting in the inhibition of initial contraction in gut smooth muscle cells. These data provide insights into molecular mechanisms for the impaired motility after intestinal inflammation.

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Fig. 1.

IL-1 β inhibits initial and sustained muscle contraction and myosin light chain (MLC₂₀) phosphorylation in response to acetylcholine (ACh). Smooth muscle cells isolated from rabbit colonic circular muscle strips treated for 2 days without (control) or with IL-1 β (10 ng/ml) were stimulated with ACh (0.1 μ M) plus methoctramine (0.1 μ M) for the indicated time periods. Contraction was measured by scanning microscopy and expressed as the percent decrease in cell length from before ACh treatment (A). MLC₂₀ phosphorylation was determined by Western blot (WB) analysis using phosphorylated (Ser¹⁹) MLC₂₀-specific antibody (B). β -Actin was used as an internal control. Values are means $\pm$ SE of 3–4 experiments. * P < 0.05 and ** P < 0.01 indicate significant decrease in IL-1 β treatment compared with the corresponding control.

Fig. 2.

IL-1 β inhibits ACh-induced phosphoinositide (PI) hydrolysis in colonic muscle cells.

Smooth muscle cells isolated from muscle strips treated for 2 days without (control) or with IL-1 β (10 ng/ml) were labeled with myo-[3 H]inositol and then stimulated with ACh (0.1 μ M) plus methoctramine (0.1 μ M) for 0.5 min. [3 H]inositol phosphate was determined as described in MATERIALS AND METHODS and expressed as counts per minute (cpm).

Values are means $\pm$ SE of 3 experiments. ** P < 0.01 indicates significant decrease in ACh-induced PI hydrolysis by IL-1 β compared with the control.

Fig. 3.

IL-1 β upregulates regulator of G protein signaling 4 (RGS4) expression but has no effect on muscarinic receptor binding or G α_q expression. **A**: cell lysates were prepared from muscle cells isolated from muscle strips treated with or without IL-1 β (10 ng/ml) for 2 days and analyzed by WB analysis with anti-RGS4 or anti-G α_q antibodies. **B**: muscle cells isolated from muscle strips treated with or without IL-1 β (10 ng/ml) for 2 days were used for muscarinic receptor binding using [³H]scopolamine. **C** and **D**: cultured smooth muscle cells at confluence with 24 h of serum starvation were treated without (control) or with IL-1 β (10 ng/ml) for 3 h and 3 days, and the expression of RGS4 protein (**C**) and mRNA (**D**) were determined by WB analysis and real-time RT-PCR. β -Actin and GAPDH were used as internal controls. ** $P < 0.01$ indicates significant increases in RGS4 mRNA expression compared with the control.

Fig. 4.

RGS4 mediates IL-1 β -induced inhibition of ACh-stimulated PLC- β activation in cultured rabbit colonic circular smooth muscle cells. **A:** overexpression of RGS4 alone inhibited ACh-induced PI hydrolysis. Cultured cells transiently transfected with hemagglutinin-tagged RGS4 expression vector were stimulated with ACh (0.1 μ M) plus methoctramine (0.1 μ M) for 0.5 min, and PI hydrolysis was determined as described in MATERIALS AND METHODS. **B:** vector-based RGS4 short interfering (si)RNA efficiently knocked down the expression of endogenous RGS4 mRNA and protein. Cultured cells were transfected with empty or RGS4 siRNA vector for 3 days, and expression levels of RGS4 protein and mRNA were determined by WB analysis and RT-PCR. β -Actin and GAPDH were used as internal controls. **C:** silencing of RGS4 increased ACh-stimulated PLC- β activation and blocked IL-1 β -induced inhibition of PLC- β activation. Cultured cells were transfected with empty or efficient RGS4 siRNA vector for 2 days, labeled with myo-[3 H]inositol for 1 day, pretreated with IL-1 β (10 ng/ml) for 3 h, and then stimulated with ACh (0.1 μ M) plus methoctramine (0.1 μ M) for 0.5 min. [3 H]inositol phosphate was determined as described in MATERIALS AND METHODS and expressed in cpm. Values are means $\pm$ SE of 3 experiments. * P < 0.05 and ** P < 0.01 indicate significant decreases in ACh-induced PI hydrolysis compared with the control; # P < 0.05 indicates significant increases in ACh-induced PI hydrolysis compared with the corresponding empty siRNA vector.

Fig. 5. RGS4 mediates IL-1 β -induced inhibition of ACh-stimulated initial contraction in dispersed colonic circular smooth muscle cells. Rabbit colonic circular muscle strips were transfected with synthesized scrambled or RGS4 siRNA for 1 day and treated without (control) or with IL-1 β (10 ng/ml) for 2 days. Expression levels of RGS4 protein and mRNA were determined by WB analysis and real-time RT-PCR, respectively (*A*). Contractile responses of freshly dispersed muscle cells to ACh (0.1 μ M) plus methoctramine (0.1 μ M) were measured by scanning microscopy (*B*). Contraction was expressed as the percent decrease in cell length from before ACh treatment. β -Actin and GAPDH were used as internal controls. Values are means $\pm$ SE of 3–4 experiments. ** P < 0.01 indicates significant differences from the corresponding control.

Fig. 6.

IL-1 β has no effect on RhoA expression and ACh-induced Rho kinase activity. Smooth muscle cells dispersed from muscle strips treated for 2 days without (control) or with IL-1 β (10 ng/ml) were stimulated with ACh (0.1 μ M) plus methoctramine (0.1 μ M) for 5 min, and Rho kinase activity was determined by immunocomplex kinase assay, as described in MATERIALS AND METHODS. *Inset*: WB analysis of RhoA expression. Cont, control.

Fig. 7.

IL-1 β decreases CPI-17 expression and ACh-induced CPI-17 phosphorylation. Cell lysates of smooth muscle cells dispersed from muscle strips treated for 2 days without (control) or with IL-1 β (10 ng/ml) were analyzed for CPI-17 expression using anti-CPI-17 antibody (A). Dispersed smooth muscle cells were stimulated with ACh (0.1 μ M) plus methoctramine (0.1 μ M) for 5 min, and phosphorylation of CPI-17 was determined by WB analysis using phosphorylated (Thr³⁸) CPI-17-specific antibody (B). Relative density is shown as means $\pm$ SE of 3 experiments. β -Actin was used as an internal control. ** $P < 0.01$ indicates significant decreases in CPI-17 expression and ACh-induced CPI-17 phosphorylation by IL-1 β .

Fig. 8.

IL-1 β causes a time-dependent decrease in CPI-17 expression in colonic muscle strips and cultured cells. Rabbit colonic circular muscle strips (A) or cultured muscle cells at confluence with 24 h of serum starvation (B) were treated without (control) or with IL-1 β (10 ng/ml) for 1–3 h and 3 days. Tissue lysates were analyzed for CPI-17 protein expression using CPI-17 antibody (A). Total RNAs were analyzed for CPI-17 mRNA expression by conventional and real-time RT-PCR (A and B). β -Actin and GAPDH were used as internal controls. ** $P < 0.01$ indicates significant decreases in CPI-17 mRNA expression compared with the corresponding control.

Fig. 9.

Diagram for the effects of IL-1 β on signaling pathways mediating initial and sustained contraction in colonic smooth muscle cells. ACh-induced smooth muscle contraction consists of a transient Ca^{2+} -dependent phase and a sustained Ca^{2+} -independent phase. Initial contraction is mediated by Ca^{2+} /calmodulin (CaM)-dependent activation of MLC kinase (MLCK) and phosphorylation of MLC_{20} via $G\alpha_q$ -dependent activation of $PLC-\beta$ and inositol 1,4,5-trisphosphate (IP_3) generation. Sustained contraction is mediated by inhibition of MLC phosphatase (MLCP) via RhoA-dependent pathways involving phosphorylation of the regulatory protein of MLC phosphatase (MYPT1) by Rho kinase and the endogenous inhibitor of MLCP CPI-17. IL-1 β inhibits both initial and sustained contraction induced by ACh. Inhibition of initial contraction is mediated by increasing the expression of RGS4, leading to rapid inactivation of $G\alpha_q$ and inhibition of $PLC-\beta$ activity. Inhibition of sustained contraction is mediated by decreasing the expression of CPI-17, leading to inhibition of CPI-17 phosphorylation and activation of MLCP.