Chronic Treatment with Interleukin-1β Attenuates Contractions by Decreasing the Activities of CPI-17 and MYPT-1 in Intestinal Smooth Muscle*

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Interleukin-1β (IL-1β) is a proinflammatory cytokine that plays a central role in inflammatory bowel disease (IBD). In order to elucidate the mechanism of motility disorders frequently observed in IBD, we investigated the long term effects of IL-1β on rat ileal smooth muscle contractility by using an organ culture system. When ileal smooth muscle strips were cultured with IL-1β (10 ng/ml), contractions elicited by high K+ and carbachol were inhibited in a time-dependent manner. IL-1β more strongly inhibited the carbachol-induced contractions than high K+ with decreasing myosin light chain phosphorylation. In the α-toxin-permeabilized ileal muscle, carbachol with GTP or guanosine 5′-3-O-(thio)triphosphate increased the Ca2+ sensitivity of contractile elements, and this G protein-coupled Ca2+ sensitization was significantly reduced in the IL-1β-treated ileum. Among the functional proteins involved in the smooth muscle Ca2+ sensitization, CPI-17 expression was significantly reduced after the culture with IL-1β, whereas the expressions of RhoA, ROCK-I, ROCK-II, MYPT-1, myosin light chain kinase, and myosin phosphatase (PP1) were unchanged. The phosphorylation level of CPI-17 by carbachol was low in accordance with the decrease in CPI-17 expression due to IL-1β treatment. In contrast, constitutively phosphorylated MYPT-1 was also decreased in the IL-1β-treated muscles. These results suggest that long term treatment with IL-1β decreases either CPI-17 expression or MYPT-1 phosphorylation, which may result in an increase in myosin phosphatase activity to reduce force generation. Based on these findings, we consider IL-1β to be an important mediator of gastrointestinal motility disorders in IBD, and CPI-17 and MYPT-1 are key molecules in the decreased smooth muscle contractility due to IL-1β.

Interleukin-1β (IL-1β)1 is a proinflammatory cytokine that activates many immune and inflammatory cells. It is produced by various cell types, including monocytes/macrophages, neutrophils, endothelial cells, and smooth muscle cells. The biological actions of IL-1β are regulated at a transcriptional and post-transcriptional level. Expression of IL-1β is regulated by activation of mitogen-activating protein kinase and transcriptional repressors. In addition, as post-transcriptional regulation, IL-1 receptor antagonist, which is produced by the same cells as IL-1β itself, competitively counters the actions of IL-1β (1).

Motility disorders of the gastrointestinal tract are extremely important clinically because they can lead to systemic disease. Intestinal inflammation results in a disturbance of motility in human and in animal models, which may reflect an alternation in the function of smooth muscle and/or the enteric nervous system (2). Elevated IL-1β levels are frequently seen in both mucosa and the muscle layer during acute and chronic intestinal inflammation, including inflammatory lesions of patients with forms of inflammatory bowel disease (IBD) such as Crohn’s disease and ulcerative colitis (3, 4). As for the IL-1β-producing cells in the intestinal smooth muscle layer during chronic intestinal inflammation, it is attractive to consider resident macrophages, which are regularly distributed with a network structure in the subserosa, at the level of the myenteric plexus, and inside the muscle layer (5–7).

Regarding the short term action of IL-1β, IL-1β has been reported to inhibit smooth muscle contractility by modulating the release of acetylcholine, norepinephrine, and substance P, all of which are neuromediators located in the rat myenteric plexus. However, these inhibitory effects disappear within a few hours (8–10). In contrast, concerning the chronic effects of IL-1β on intestinal motility, few data are available in published reports. The main reason for this lack of data is the difficulty of keeping the smooth muscle phenotype in cultured cells, i.e. smooth muscle cells in culture rapidly lose their contractile phenotype (11). One explanation for this loss is that the absence of extracellular matrix constituents accelerates a change of phenotype to that of proliferative smooth muscle (12, 13). Therefore, because of the presence of a surrounding matrix, an organ culture system is effective for use in assessing the long term effects of IL-1β on modification of intestinal smooth muscle functions. In vascular diseases such as arteriosclerosis, it is well known that many inflammatory cytokines, including IL-1β and growth factors, affect vascular smooth muscle differentiation (14). Smooth muscle cells change from a contractile to a proliferative phenotype, which in turn leads to a change in the expression levels of contractile proteins such as α-actin and polynvinylidene difluoride; PKC, protein kinase C; iNOS, inducible NO synthase; PIPES, 1,4-piperazinediethanesulfonic acid; DBP, 12-deoxyphorbol 13-isobutyrate; PKN, protein kinase N.

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1 The abbreviations used are: IL, interleukin; IBD, inflammatory bowel disease; MLC, myosin light chain; MLCK, myosin light chain kinase; GTPγS, guanosine 5′-3-O-(thio)triphosphate; PP1, myosin phosphatase; DTT, dithiothreitol; PBS, phosphate-buffered saline; PVDF, polyvinylidene difluoride; PKC, protein kinase C; iNOS, inducible NO synthase; PIPES, 1,4-piperazinediethanesulfonic acid; DBP, 12-deoxyphorbol 13-isobutyrate; PKN, protein kinase N.
myosin heavy chains, resulting in a reduction in force generation (15, 16). These phenomena observed in vascular proliferative disease have been well reproduced in rabbit mesenteric artery using organ culture method in which tissues have been treated with fetal bovine serum, a mixed source of various growth factors (17).

Smooth muscle contractile system is basically regulated by myosin light chain (MLC) phosphorylation, which is driven by the balance between MLCK activity and myosin phosphatase activity (18). During receptor agonist-induced contractions, contractile elements are sensitized to Ca\(^{2+}\) to induce a greater MLC phosphorylation and greater force at a given cytosolic Ca\(^{2+}\) level (19–21). Recent studies have shown that a small GTP-binding protein, RhoA, and RhoA-dependent coiled-coil serine/threonine kinases (ROCKs) play a major role in Ca\(^{2+}\) sensitization. The activated RhoA (RhoA-GTP) activates ROCKs, which in turn phosphorylate a noncatalytic subunit of myosin phosphatase (MYPT-1) inactivating the myosin phosphatase activity. On the other hand, receptor stimulation also activates PKC. The activated PKC phosphorylates PKC-potentiated phosphatase inhibitor protein-17 (CIPI-17) (22, 23), which in turn inactivates myosin phosphatase. As a result, MLCK-induced MLC phosphorylation is augmented to induce a greater contraction at a given cytosolic Ca\(^{2+}\). In addition, it appears that CIPI-17 phosphorylation can also be produced by RhoA/ROCKs or RhoA/PAK after the receptor stimulation (24–26).

In the present study, using an organ culture system, we investigated whether long term treatment with IL-1β can affect the contractile response of ileal smooth muscle, and how it changes the contractile mechanism. We found that long term exposure of IL-1β selectively reduces the force generation due to activation of G protein-coupled receptor, by increasing myosin phosphatase activity resulting from the changes in CIPI-17 and MYPT-1 activities in intestinal smooth muscles.

**EXPERIMENTAL PROCEDURES**

**Tissue Preparation and Organ Culture Procedure**—Animal care and treatment were conducted in conformity with the institutional guidelines of the University of Tokyo and are consistent with the Guide for the Care and Use of Laboratory Animals from the National Institutes of Health. Male Wistar rats (200–250 g) were euthanized by a sharp blow to the neck and exsanguination. A 10–15-cm segment of the ileum was detached from mesentery and placed in sterile Hank’s balanced salt solution. Strips were teased along the natural line of cleavage from the longitudinal smooth muscle. The strips were then transferred to culture dishes with Medium 199 (M199; Invitrogen) supplemented with 1% antibiotic and antimycotic (Invitrogen) and 1-glutamine (200 μM; Invitrogen). The culture dishes were incubated at 37 °C in an atmosphere of 95% air and 5% CO\(_2\). The incubation medium was replaced every day.

**Permeabilized Muscle**—Permeabilized muscle was prepared by treating the intestinal smooth muscle strips with *Staphylococcus aureus* α-toxin (80 μg/ml, 30 min, Sigma), as described previously (27, 28). The relaxing solution containing Mg\(^{2+}\)-dimethanesulfonate 7.1 mM, K+ methanesulphonate 108.0 mM, ATP 5.9 mM, creatine phosphate 2.0 mM, PIPES 20.0 mM, EGTA 2.0 mM, creatine phosphokinase 10 units/ml, carbohydrate cyano p-trifluoromethoxyphenylhydrazine 1 μM, and E-64 1 μM, pH 6.8. Free Ca\(^{2+}\) concentrations were changed by adding an appropriate amount of Ca\(^{2+}\)-dimethanesulfonate. The apparent binding constant of EGTA for Ca\(^{2+}\) was considered to be 1 μM. Contractile force was measured by an isometric transducer under a resting tension of 1 mm at room temperature (22–24 °C). Concentration-response curves were obtained by cumulative application of Ca\(^{2+}\) (0.1–30 μM).

Western blots of rat ileum were homogenized in each suitable homogenizing buffer to extract each protein. Homogenizing buffer to extract α-actin and MLC contained 500 mM KCl, 0.1 mM EGTA, 8 mM urea, and 50 mM Tris-HCl, pH 6.8. Homogenizing buffer to extract MLCK and PP1 contained 500 mM KCl, 0.1 mM EGTA, and 50 mM Tris-HCl, pH 8.0. Homogenizing buffer to extract RhoA and ROCKs contained 50 mM KCl, 1 mM EGTA, and 20 mM Tris-HCl, pH 8.8. Homogenizing solution to extract CIPI-17 and MYPT-1 contained 60 mM β-glycerophosphate, 0.5% Nonidet P-40, 0.2% SDS, 1 mM Na\(_3\)VO\(_4\), 2 mM EGTA, and 50 mM Tris-HCl, pH 8.0. All homogenizing solutions contained 1 mM dithiothreitol (DTT), 1 mM phenylmethylsulfonyl fluoride, 1 mM benzamidine, and 1 μg/ml aprotinin. After centrifugation of homogenized samples for 30 min at 15,000 × g at 4 °C, the supernatant was used for the analysis. 10 μg (α-actin, MLC) 20 μg (MLCK, PP1, RhoA, ROCK-II, MYPT-1, and CIPI-17), or 40 μg (ROCK-I) of total proteins purified from rat ileum were mounted in each lane, and SDS-PAGE of 7.5, 10, or 15% was performed to separate each protein. After electrophoresis was completed, separated proteins were transferred to a PVDF membrane. The PVDF membrane was washed with phosphate-buffered saline (PBS; pH 7.4) and then treated with PBS containing 5% skim milk as a blocking solution for 20 min at room temperature. Anti-α-actin antibody (1:1000 dilution; Dako A/S, Denmark), anti-MLC antibody (1:1000 dilution of monoclonal rabbit anti-bovine tracheal MLC antibody, kindly donated by Dr. J. Stull, University of Texas), anti-MLCK antibody (1:1000 dilution, Sigma), anti-PP1 (PP1 (E-9), 1:1000 dilution; Santa Cruz Biotechnology, Santa Cruz, CA), anti-MYPT-1 antibody (1:1000 dilution; Santa Cruz Biotechnology), anti-phosphorylated MMYPT-1 antibody (p-MYPT-1 (Thr-696), 1:1000 dilution; Santa Cruz Biotechnology), anti-ROCK I antibody (Rock-1 (C-19), 1:1000 dilution; Santa Cruz Biotechnology), anti-ROCK II antibody (1:50 dilution; Santa Cruz Biotechnology), anti-CPI-17 antibody (CPI-17 (N-20), 1:500 dilution; Santa Cruz Biotechnology), anti-phosphorylated CPI-17 antibody (p-CPI-17 (Thr-38), 1:250 dilution; Santa Cruz Biotechnology), and anti-RhoA antibody (Rho A(26C4), 1:1000 dilution; Santa Cruz Biotechnology) were treated with blocking buffer overnight as the first antibody at 4 °C. After washing the membrane with PBS/Tween (four times for 5 min), matched secondary antibodies were treated with blocking buffer for 1 h. Each protein was detected using alkaline phosphatase staining or peroxidase staining. The stained membranes were saved as images using an EPSON GT-9600 color scanner (EPSON, Tokyo, Japan).

**Western Blots**—Western blots were performed as described previously (29) under the isometric conditions described under “Measurement of Muscle Tension.” At the planned times during contractions induced by 72.7 mM high K+ solution and carbachol, the strips were quickly frozen in acetone/dry ice containing 10 mM DTT, 10 mM sodium thiglycolate, and 10% (w/v) trichloroacetic acid. The strips and trichloroacetic acid/acetone/dry ice solution were then gradually warmed to 4 °C. The strips were homogenized in sampling buffer containing 10 mM DTT, 8 mM urea, 20 mM Tris, and 23 mM glycine, pH 8.6. Equal amounts of protein (8–12 μg/lane) were loaded on each lane of glycerol-PAGE. The proteins were separated by electrophoresis and transferred to PVDF membranes. The membranes were incubated for 30 min in PBS containing 5% bovine serum albumin and then incubated with anti-MLC, anti-ROCK, or anti-CPI-17 antibody (1:1000 dilution of monoclonal rabbit anti-bovine tracheal MLC antibody, kindly donated by Dr. J. Stull, University of Texas) overnight as the first antibody at 4 °C. After washing the membrane with PBS/Tween (four times for 5 min), matched secondary antibodies were treated with blocking buffer for 1 h. Each antibody was detected using alkaline phosphatase staining or peroxidase staining. The stained membranes were saved as images using an EPSON GT-9600 color scanner (EPSON, Tokyo, Japan).

**Measurement of Muscle Tension**—The strips were placed in normal physiological salt solution, which contained (mM) NaCl 136.9, KCl 5.4, CaCl\(_2\) 1.5, MgCl\(_2\) 1.0, NaHCO\(_3\) 25.8, and glucose 5.5. 1 μM ETA was also added to remove contaminating heavy metal ions. The high K+ (72.7 mM) solution was prepared by replacing NaCl with equimolar KCl. These solutions were saturated with 95% O\(_2\), 5% CO\(_2\) mixture at 37 °C and pH 7.4. Muscle tension was recorded isometrically with a force-displacement transducer. Each of the muscle strips was attached to a holder under a resting tension of 10 mN. After equilibration for 15 min in a 10-ml 20-ml bath, each strip was repeatedly exposed to high K+ solution until responses became stable. Concentration-response curves were obtained by the cumulative application of agonists. At the end of the tension measurements, the wet weight of each muscle strip was measured. Contractile force is shown in mN/g wet weight of tissue.
FIG. 1. Time-dependent change in contractions induced by high K⁺ (72.7 mM) (A) and carbachol (CCh) (1 μM) (B) after cultivation in Medium 199 with (closed circles) or without IL-1β (10 ng/ml) (open circles). C shows the effects on carbachol-induced contractions of various concentrations of IL-1β (0.1–10 ng/ml) in a 3-day culture. Results are expressed as means ± S.E. of 8–75 experiments. **, significantly different from control, p < 0.01.

Contractions in the Organ-cultured Tissue—We examined the effects of chronic treatment with IL-1β on contractility in response to high K⁺ and carbachol in the organ-cultured rat ileum. The high K⁺- and carbachol-induced contractions were well maintained in the tissue cultured in M199 for 3 days, as reported previously in other intestinal tissues (30, 31). In contrast, the presence of 10 ng/ml IL-1β, contractions elicited by high K⁺ and carbachol were inhibited in a time-dependent manner, and the degree of IL-1β-induced inhibition was much greater in the carbachol-induced contractions than the high K⁺-induced contractions. The concentration dependence of IL-1β indicates that 0.1 and 1 ng/ml IL-1β induce less inhibition of contractile forces in ileal smooth muscles cultured for 3 days (Fig. 1C). We also attempted to determine whether IL-1β affects the IC₅₀ value for carbachol. Cumulative addition of carbachol (1 nM to 100 μM) caused concentration-dependent contractions elicited with or without IL-1β for 3 days and also in freshly isolated tissue. There was no difference between concentration-response curves in freshly isolated, control, and IL-1β-treated tissues (IC₅₀ values: fresh, –6.73 ± 0.07 versus control, –6.81 ± 0.23 versus IL-1β, –6.83 ± 0.24; n = 8).

In vascular smooth muscles, IL-1β has been shown to stimulate iNOS expression that results in a reduction of contractile force (32, 33). Furthermore, iNOS expression is often associated with the expression of COX-2 (34–36). Therefore, we next examined the effects of NO synthase inhibitor, L-NMMA (300 μM), and also COX inhibitor, indomethacin (10 μM), on smooth muscle contractility that had already been modified by IL-1β treatment. To maintain intracellular L-Arg concentrations high enough to support the NO generation, L-Arg (1 mM) was added to the bath solution (37). L-Arg alone had no effect on the carbachol-induced contractions in both control tissues and those treated with IL-1β for 3 days. In addition, L-NMMA (300 μM) did not change the carbachol-induced contractions in tis-

RESULTS

As a preliminary experiment, we performed a morphological examination of the long term effects of IL-1β in rat ileal tissue. In the layer of freshly isolated ileal smooth muscle tissue, flat-shaped smooth muscle cells were arranged in an ordinary fashion. In the layer of either control or IL-1β-treated tissue (10 ng/ml for 3 days), most of the smooth muscle cells were also arranged in the ordinary fashion, indicating that the tissue was intact after the culture in M199 with or without IL-1β for 3 days (data not shown).

Chemicals—The chemicals used are as follows: ATP, creatine phosphate, creatine phosphokinase, E-64, EGTA, carbonyl cyanide p-trifluoromethoxyphenylhydrazone, indomethacin, GTP (Sigma), EDTA, PIPES (Dojindo Laboratories, Japan), DTT, trichloroacetic acid, t-NMMA (Wako Pure Chemical, Japan), sodium thioglycolate (Nakarai Chemicals, LTD, Kyoto, Japan), GTP·SγS (Roche Applied Science), DFB (Funakoshi, Japan), K252a, Y-27632, HA-1077, and bisindolylmaleimide I (Calbiochem).
sues treated with or without IL-1β. Furthermore, indomethacin (10 μM) did not affect the carbachol-induced contractions of smooth muscle tissues cultured with or without IL-1β (p = 0.457). These results are summarized in Table I. Moreover, treatment with both L-NMMA and indomethacin did not restore the reduced high K⁺-induced contractions elicited by IL-1β treatment (data not shown).

**MLC Phosphorylation**—We measured the phosphorylation of 20-kDa MLC, which regulates the contractions of smooth muscle. As shown in Fig. 2, the levels of MLC phosphorylation in IL-1β-treated strips in the resting state did not differ from the control (n = 10 each). When contractions were induced in response to treatment with carbachol (1 μM) for 30 s, the MLC phosphorylation levels significantly increased. In the IL-1β-treated tissues, however, the levels of MLC phosphorylation induced by carbachol were significantly smaller than those in the control tissues (control, 23.5 ± 2.0%, n = 9, versus IL-1β, 13.4 ± 1.6%, n = 9; p < 0.01). Treatment of the tissue with high K⁺ for 30 s also increased MLC phosphorylation, and this was not significantly reduced by the IL-1β treatment (control, 25.7 ± 1.8%, n = 13, versus IL-1β, 20.2 ± 2.6%, n = 13).

**Contractions in Permeabilized Muscle**—In α-toxin-permeabilized muscle, functional proteins in cytosol are retained, and internal Ca²⁺ concentrations can be clamped in order to evaluate Ca²⁺ sensitivity and the contractility of the contractile apparatus (27, 28).

In the permeabilized muscle prepared from the control tissues, cumulative addition of Ca²⁺ (0.1–30 μM) caused concentration-dependent contractions. As shown in Fig. 3A, in control tissues Ca²⁺ (1 μM) induced 30–50% of the maximum contractions induced by 30 μM Ca²⁺. Pretreatment of the muscle with 10 μM carbachol in the presence of 100 μM GTP increased the contractions elicited by 1 μM Ca²⁺ (p < 0.01). The Ca²⁺ sensitization by carbachol with GTP in the presence of 1 μM Ca²⁺ was significantly reduced by the IL-1β treatment (control, 74.2 ± 4.7%, n = 8, versus IL-1β, 53.8 ± 2.8%, n = 6, p < 0.01). Instead of carbachol with GTP, the addition of 100 μM GDPβS also significantly increased the Ca²⁺ sensitivity of the contractile element (Fig. 3A), and the level of contractile force was significantly reduced by IL-1β treatment (control, 122.0 ± 4.1%, n = 9, versus IL-1β, 82.5 ± 8.1%, n = 7, p < 0.01).

We next examined the effects of DBP, an activator of PKC, on Ca²⁺-induced contractions in the muscle permeabilized with α-toxin. In both control and IL-1β-treated tissues, the relative contractile force induced by 1 μM Ca²⁺ was significantly increased by 10 μM DBP (p < 0.01). However, the degree of augmentation of the contractile force was much smaller than that of the GTP-mediated responses. In addition, the relative force induced by 1 μM Ca²⁺ with DBP was slightly but not significantly decreased by the IL-1β treatment.

We finally compared the contractile force induced by 30 μM Ca²⁺ (Fig. 3C). The absolute force induced by 30 μM Ca²⁺ was significantly decreased by the IL-1β treatment (control, 133.6 ± 15.6 mN/mm² versus IL-1β, 72.8 ± 12.3 mN/mm², n = 14–16; p < 0.01).

**Change in Protein Expression of Contractile Elements**—We examined the protein expression of α-actin, 20-kDa MLC, MLCK, and PP1. By comparing the amounts of the freshly isolated tissues, the expression of α-actin remained constant (88.8 ± 5.5%, n = 8) in the cultured tissue after the 3-day culture in M199. When muscle tissue was treated with IL-1β in M199, the expression of α-actin (80.3 ± 4.9%, n = 8) was not significantly different from the control (Fig. 4A). In addition, the expression of MLC (control, 83.3 ± 4.8%, n = 5, versus IL-1β, 81.8 ± 9.2%, n = 5), MLCK (control, 101.1 ± 8.3%, n = 4, versus IL-1β, 98.9 ± 9.3%, n = 4), and PP1 (control, 90.9 ± 4.4%, n = 4, versus IL-1β, 97.3 ± 10.5%, n = 4) did not change after the IL-1β treatment (Fig. 4, B–D).

All these results suggest the possibility that IL-1β treatment alters the mechanism responsible for the G protein-coupled Ca²⁺ sensitization of the contractile element. Therefore, we next examined the expression levels of proteins involved in the Ca²⁺ sensitization of smooth muscle.

By comparing the amounts in the freshly isolated tissues, the expression of RhoA did not change (96.9 ± 7.1%, n = 4) after the 3-day culture in M199 (Fig. 4A). When muscle tissue was treated with IL-1β in M199 for 3 days, the RhoA expression did not change (125.0 ± 21.8, n = 4) (Fig. 5A). The expressions of ROCK I and ROCK II also did not differ between control tissue (ROCK I, 105.4 ± 5.8%, n = 6; ROCK II, 98.3 ± 24.7%, n = 4) and IL-1β-treated tissue (ROCK I, 89.2 ± 8.1%, n = 6; ROCK II, 91.5 ± 8.9%, n = 4) (Fig. 5, B and C).

As shown in Fig. 5D, the expression of MYPT-1 in IL-1β-treated tissue (77.9 ± 3.0%, n = 4) did not differ from that in control tissues (87.2 ± 4.2%, n = 4) (Fig. 5D). However, CPI-17 protein expression in control tissue (95.5 ± 3.2%, n = 5) was significantly reduced in the tissues treated with IL-1β for 3 days (42.0 ± 4.9%, n = 5, p < 0.01).

**MYPT-1 Phosphorylation**—In some types of smooth muscle, the activated RhoA activates ROCKs, which in turn phosphorylates MYPT-1 to inactivate myosin phosphatase activity. Therefore, we examined whether the MYPT-1 phosphorylation pathway participates in carbachol-induced contractions in rat ileum. Fig. 6 shows time-dependent phosphorylation of MYPT-1 at Thr469 in freshly isolated rat ileum upon stimulation with carbachol (1 μM). Phosphorylated MYPT-1 was already detected even in the resting state using anti-phosphorylated MYPT-1 antibody. MYPT-1 was already phosphorylated under the resting conditions, and the level of phosphorylation of MYPT-1 did not significantly change within 1 min after stimulation with carbachol. Approximately 5 min after the stimulation, the MYPT-1 phosphorylation was slightly but significantly increased.

Fig. 7 shows the effects of K252a (1 μM; nonspecific kinase inhibitor), Y-27632 (10 μM; ROCKs inhibitor), and HA-1077 (2 μM; ROCKs and PKN inhibitor) on the phosphorylation of

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**Table I**

*Effects of the substrate of NO synthesis, L-Arg (1 mM), NO synthase inhibitor, L-NMMA (300 μM), and COX-2 inhibitor, indomethacin (10 μM), on carbachol (1 μM)-induced contractions in rat ileal smooth muscle cultured with or without IL-1β (10 ng/ml) for 3 days.*

Results are expressed as means ± S.E. of 6–12 experiments.

| Treatment                  | Control (mN/mg w/w) | IL-1β (mN/mg w/w) |
|----------------------------|--------------------|-------------------|
| Exp. 1                     |                    |                   |
| Control                    | 14.4 ± 2.8 (n = 12) | 6.3 ± 1.9 (n = 6) |
| L-Arg (1 mM)               | 13.9 ± 2.6 (n = 12) | 5.9 ± 2.0 (n = 6) |
| L-Arg (1 mM) + L-NMMA (300 μM) | 13.4 ± 2.5 (n = 12) | 5.7 ± 2.1 (n = 6) |
| Exp. 2                     |                    |                   |
| Control                    | 12.3 ± 3.9 (n = 12) | 6.5 ± 3.3 (n = 6) |
| Indomethacin (10 μM)       | 10.8 ± 3.8 (n = 12) | 5.1 ± 2.7 (n = 6) |
IL-1β and Smooth Muscle Contractility

Fig. 2. Effects of high K+ (72.7 mM) and carbachol (CCh) (1 μM) on MLC phosphorylation at 30 s in ileal smooth muscle cultured with (closed columns) or without (open columns) IL-1β (10 ng/ml) for 3 days. A, typical pattern of MLC phosphorylation. B, summarized MLC phosphorylation levels. Results are expressed as means ± S.E. of 9–16 experiments. **, significantly different from control, p < 0.01.

MYPT-1 phosphorylated with carbachol (1 μM) for 10 min. MYPT-1 phosphorylation in the presence of carbachol (1 μM) (23.8 ± 1.5%, n = 6) was inhibited by K252a to the value of 9.7 ± 2.2% (n = 4), which was significantly smaller than the resting state (18.9 ± 0.9%, n = 6). Y-27632 and HA-1077 also inhibited the carbachol-induced phosphorylation of MYPT-1 to 9.8 ± 2.1% (n = 4) and 11.9 ± 0.6% (n = 4), respectively. In the present study, we also examined the effects of adenylate cyclase activator, forskolin (1 μM), and guanylate cyclase activator, sodium nitroprusside (10 μM), on MYPT-1 phosphorylation in the resting state, but MYPT-1 phosphorylation could not be detected using this anti-phosphorylated MYPT-1 antibody (forskolin, 20.2 ± 1.7%, n = 4; sodium nitroprusside, 19.7 ± 1.5, n = 4).

CPI-17 Phosphorylation—As an alternative pathway to induce Ca2+ sensitization by carbachol, one should next consider the CPI-17 phosphorylation pathway, which is mediated not only by PKC but also by RhoA/ROCKs and RhoA/PKN, which leads to an elimination of myosin phosphatase activity (see Introduction). In the freshly isolated ileum, in contrast to the MYPT-1 phosphorylation, phosphorylated CPI-17 at Thr38 was hardly detected in the resting state. Stimulation of the tissue with 1 μM carbachol increased the levels of CPI-17 phosphorylation at Thr38 within 30 s, with these levels being maintained for over 10 min (Fig. 8).

Fig. 9 shows the effects of K252a, Y-27632, HA-1077, and bisindolylmaleimide I (10 μM) on the phosphorylation of CPI-17 by carbachol. K252a inhibited the carbachol-induced phosphorylation of CPI-17 in the resting state from 9.2 ± 1.2% (n = 10) to 5.3 ± 2.2% (n = 4), indicating that this anti-phospho-CPI-17 antibody can only detect the phosphorylated form of CPI-17. Y-27632 partly (~30%) inhibited the carbachol-induced CPI-17 phosphorylation (carbachol 30 s; 88.9 ± 5.3%, n = 10 versus carbachol 30 s with Y-27632; 58.9 ± 5.7%, n = 4). HA-1077 more potently inhibited the phosphorylation to 20.0 ± 1.0% (n = 4). In addition, bisindolylmaleimide I also significantly inhibited the phosphorylation of CPI-17 to 29.8 ± 3.9% (n = 4).

The Effects of IL-1β on Phosphorylation of MYPT-1 and CPI-17—Fig. 10A shows the effects of IL-1β treatment on the phosphorylation of MYPT-1 in tissues cultured in M199 for 3 days with or without IL-1β. In the control tissue cultured for 3 days (without IL-1β), treatment with 1 μM carbachol for 30 s did not change the phosphorylated MYPT-1 content (resting, 96.0 ± 5.4% versus carbachol, 96.6 ± 10.5%, n = 4 each). In the IL-1β-treated tissues, the MYPT-1 content phosphorylated at the resting state was significantly less than that in control tissues (resting, 62.7 ± 5.3, n = 4; carbachol, 68.4 ± 3.6, n = 4).

We also examined the CPI-17 phosphorylation in the tissue cultured in M199 for 3 days (Fig. 10B). At resting state, CPI-17 was hardly phosphorylated in the cultured tissue. Addition of carbachol (1 μM) immediately increased the phosphorylation level at 30 s. In accordance with the decrease in the total content of CPI-17, the content of the phosphorylated form of CPI-17 in the muscle stimulated with 1 μM carbachol for 30 s was significantly smaller in IL-1β-treated tissue (27.6 ± 7.1%, n = 5) than in control tissue (83.7 ± 7.9%, n = 5).

DISCUSSION

In the present experiments, we examined the chronic effects of IL-1β on ileal smooth muscle contractility in an organ culture system, and we found that treatment with IL-1β for 3 days inhibits smooth muscle contractility. An important finding of this study was that IL-1β more strongly inhibits carbachol-induced contractions than contractions induced by simple membrane depolarization with high K+.

It has been demonstrated that inducible NO synthase (iNOS) is expressed in intestinal or vascular smooth muscle exposed to IL-1β or tumor necrosis factor-α (32, 33). In rat ileal smooth muscle, IL-1β has been shown to mediate lipopolysaccharide-induced increases in iNOS activity and the decreases in muscle contractility (38). Therefore, we examined whether NO re-
leased from ileal tissue is involved in the IL-1β-induced decrease in contractility. However, the inhibition of contractions was not affected by pretreatment of the tissues with i-NMMA, an inhibitor of iNOS, and/or l-Arg, a supplement of the NO precursor (Table I). It has also been reported that lipopolysaccharide and IL-1β drive COX-2 expression in macrophages, resulting in an increase in the release of prostaglandins to induce muscle relaxation (6, 39, 40). However, indomethacin, a nonselective COX inhibitor, did not modify the IL-1β-mediated decrease in contractions. These results suggest the possibility that the IL-1β-induced inhibition of muscle force may be mediated by a change in the myogenic mechanism.

Smooth muscle contractility is regulated by MLC phosphorylation via the Ca2+-calmodulin-MLCK system (18). Therefore, we next examined the effects of IL-1β on the levels of MLC phosphorylation induced by carbachol and high K+. Although the resting levels of MLC phosphorylation were similar in control and IL-1β-treated tissues, the MLC phosphorylation induced by carbachol at 30 s was significantly smaller in the muscle treated with IL-1β for 3 days than in the control muscle (Fig. 2). In agreement with the data that high K+-induced contractions are only slightly inhibited by IL-1β treatment (Fig. 1A), the inhibitory effects of IL-1β on high K+-stimulated MLC phosphorylation were found to be weak. These results suggest that IL-1β may primarily down-regulate smooth muscle contractions at the level of the regulatory process of MLC phosphorylation responsible for the agonist-inducing Ca2+ sensitization of contractile elements.

As described in the Introduction, it is generally accepted that receptor agonist-induced Ca2+ sensitization is mediated by at least two signal transduction pathways, i.e. small GTP-binding protein, the RhoA pathway, and the PKC pathway (41–45). In permeabilized intestinal smooth muscles, it has been reported that carbachol plus GTP or GTPγS alone induces greater force...
generation at constant Ca\(^{2+}\), thereby showing Ca\(^{2+}\) sensitization (46, 47). Phorbol esters, an activator of PKC, also induce Ca\(^{2+}\) sensitization in the permeabilized ileum (48, 49). In the present study, in rat ileum permeabilized with \(\alpha\)-toxin, we confirmed that carbachol with GTP or GTP\(\gamma S\) results in Ca\(^{2+}\) sensitization of contractile elements (Fig. 3). DPB (1 \(\mu\)M) was also found to induce Ca\(^{2+}\) sensitization in the ileum, although the degree of Ca\(^{2+}\) sensitization was much smaller than that induced by carbachol plus GTP or GTP\(\gamma S\). Consistent with the results obtained from intact tissue, our results demonstrate that the Ca\(^{2+}\) sensitization induced by carbachol with GTP or GTP\(\gamma S\) is significantly reduced by IL-1\(\beta\) treatment. In contrast, IL-1\(\beta\) slightly but not significantly inhibited DPB-induced Ca\(^{2+}\) sensitization (Fig. 3B). These results indicate that long term treatment with IL-1\(\beta\) may affect the RhoA-dependent pathway rather than the PKC-dependent pathway.

Activated RhoA-GTP activates ROCKs, which leads to the phosphorylation of MYPT-1, a noncatalytic subunit of myosin phosphatase (20, 21, 50, 51). The phosphorylated MYPT-1 inhibits the catalytic subunit of myosin phosphatase (PP1c), resulting in Ca\(^{2+}\) sensitization. Although ROCKs phosphorylate chicken gizzard MYPT-1 at two sites (at Thr\(^{695}\) and Thr\(^{795}\)), only phosphorylation at Thr\(^{695}\) is considered to be responsible for the inhibition of myosin phosphatase activity (52). The present results indicate that MYPT-1 at Thr\(^{695}\) is phosphorylated to some level in the resting condition (Fig. 7). However, the stimulation with carbachol did not change MYPT-1 phosphorylation in 30 s, during which time the contractile tension reached a maximum. Incubation of the tissue for 5–15 min with carbachol significantly increased MYPT-1 phosphorylation, but its extent was very small (less than 20\%). These results coincide with those of the most recent studies in the rabbit portal vein and vas deferens (24) and the rabbit femoral artery (53). Therefore, we concluded that MYPT-1 phosphorylation does not play an essential role in the Ca\(^{2+}\) sensitization induced by receptor stimulation in rat ileum. Although we did not observe a close correlation between the time course of MYPT-1 phosphorylation and contractions in the carbachol-stimulated ileum, we noticed that the levels of MYPT-1 phosphorylation were significantly reduced by IL-1\(\beta\) not only under the resting condition but also under the carbachol-stimulated condition (Fig. 10). It is therefore possible that decreased levels of the constitutively phosphorylated MYPT-1 change the kinase/phosphatase balance, which may result in a decrement of MLC phosphorylation upon stimulation with the agonist.

It has been reported that MYPT-1 phosphorylation is regul-
lated by endogenous kinases other than ROCKs, including ZIPK (54), ILK (55, 56), and DMPK (57). Kitazawa et al. (24) have reported that MYPT-1 phosphorylation in the resting state is not inhibited by the ROCKs inhibitor, Y-27632, in intact rabbit vas deferens, indicating the presence of ROCK-independent phosphorylation of MYPT-1. However, in the ileal smooth muscle, ROCKs inhibitors, Y-27632 and HA-1077, and nonselective kinase inhibitor, K252a, equally inhibited the MYPT-1 phosphorylation in the resting state (Fig. 7), indicating that MYPT-1 is primarily phosphorylated by the ROCK-dependent cascade in the smooth muscle of rat ileum. Further study is necessary to clarify the down-regulation of MYPT-1 phosphorylation in rat ileum, and this clarification may prove the importance of the MYPT-1-mediated mechanism in the decreased contractions occurring in response to IL-1β.

On the other hand, PKC phosphorylates CPI-17 at Thr38, which directly inhibits PP1c activity to induce Ca^{2+} sensitization (22, 23, 58, 59). However, based on recent in vitro studies, Koyama et al. (25) have reported that CPI-17 is a good substrate for ROCKs. In addition, Kitazawa et al. (24) have recently reported that CPI-17 phosphorylation is partly blocked by a specific ROCKs inhibitor, Y-27632. It has also been reported that RhoA-activated PKN can activate CPI-17 in vitro (26). These results suggest the possibility that the RhoA/ROCKs/CPI-17 and/or RhoA/PKN/CPI-17 pathway may also be involved in carbachol-induced contractions in rat ileum. In the present study, in the freshly isolated rat ileum, CPI-17 was slightly phosphorylated in the resting state (Fig. 8), but the addition of carbachol (within 30 s) immediately phosphorylated CPI-17 at Thr38, and the phosphorylation levels were well maintained for over 10 min during the carbachol stimulation. These results are consistent with previous results (24, 53) obtained in other smooth muscles and suggest that CPI-17 phosphorylation may play a pivotal role in the carbachol-induced Ca^{2+} sensitization in rat ileum. Furthermore, in the ileal smooth muscle, Y-27632 inhibited the carbachol-induced increase in CPI-17 phosphorylation by ~30% (Fig. 9). HA-1077 inhibited the phosphorylation more than Y-27632, indicating that not only ROCKs but also PKN may be activated by RhoA. Moreover, PKC activation may also be involved in the phosphorylation of CPI-17 in rat ileum because bisindolylmaleimide I was found to partially but significantly inhibit the phosphorylation of CPI-17 (Fig. 9). The most important finding of this study is that CPI-17 expression was dramatically decreased by ~60% in ileal tissue treated with IL-1β without changing other components related to Ca^{2+} sensitization of contractile pro-

![Western blot analysis of RhoA (A), ROCK I (B), ROCK II (C), MYPT-1 (D), and CPI-17 (E) in ileal smooth muscle cultured for 3 days with (closed columns) or without (open columns) IL-1β (10 ng/ml). Bands densities are normalized to the respective proteins of freshly isolated tissue analyzed in the same series of experiments. Results are expressed as means ± S.E. of 5–8 experiments. **, significantly different with p < 0.01. n.s., not significant.](http://www.jbc.org/)

**Fig. 5.** Western blot analysis of RhoA (A), ROCK I (B), ROCK II (C), MYPT-1 (D), and CPI-17 (E) in ileal smooth muscle cultured for 3 days with (closed columns) or without (open columns) IL-1β (10 ng/ml). Bands densities are normalized to the respective proteins of freshly isolated tissue analyzed in the same series of experiments. Results are expressed as means ± S.E. of 5–8 experiments. **, significantly different with p < 0.01. n.s., not significant.
proteins such as α-actin, MLCK, RhoA, ROCKs, MYPT-1, and PP1. In accordance with the decrease in CPI-17 content, the phosphorylated CPI-17 content 30 s after carbachol stimulation was markedly reduced. These results strongly suggest that down-regulation of CPI-17 expression plays a crucial role in the decreased Ca\(^{2+}\) desensitization occurring after long term treatment with IL-1β.

As demonstrated in Fig. 1, the chronic effects of IL-1β reduced high K\(^+\)-induced contractions, even though the inhibitory effect was less than that in carbachol-induced contractions. To characterize the effects of IL-1β on high K\(^+\)-induced contractions, we examined the effects on Ca\(^{2+}\)/calmodulin-activated MLCK against phosphatase activity, but this preparation retains other regulatory mechanisms. As already discussed, because MYPT-1 activity in the resting condition was decreased after the IL-1β treatment, constitutively active RhoA-mediated MYPT-1 activity may contribute to the Ca\(^{2+}\)/calmodulin/MLCK system in the permeabilized tissue as an additional mechanism. It is therefore possible that the decrease in the absolute force observed in the permeabilized muscle may at least in part be mediated by the decreased activity of the MYPT-1 pathway. It has been reported recently (60, 61) that not only the Ca\(^{2+}\)/calmodulin/MLCK pathway but also the RhoA/ROCKs pathway is necessary for maintaining the high K\(^+\)-induced contractions. In addition, Sakurada and co-workers (62) have reported the presence of Ca\(^{2+}\)-dependent activation of RhoA/ROCKs in high K\(^+\)-induced contractions. In the rat ileum, ROCKs inhibitor, Y-27632 (1 μM), was found to significantly inhibit high K\(^+\)-induced contractions in freshly isolated ileal tissue by ~35%
These findings suggest the possibility that IL-1β induces an inhibition of high K⁺-induced contractions by changing the RhoA/MYPT-1-mediated cascades. In the present study, we examined the chronic effects of IL-1β on gastrointestinal motility, as assessed in an organ culture system. IL-1β expression is significantly increased in...
both mucosa and the muscle layer during intestinal inflammation in IBD such as Crohn’s disease and ulcerative colitis (3, 4). In these patients, motility disorders of the gastrointestinal tract are frequently observed, and GI dysmotility is thought to be extremely important clinically, as it can lead to systemic disease. In the present study, we first found that long-term exposure to IL-1β induces a myogenic inhibitory action in intestinal muscle motility. This finding raises many interesting issues, such as the role of the IL-1β-producing cell type in the muscle layer, signal transduction through NFκB to down-regulate the RhoA/RoCKs/CPI-17 pathway, and the presence of cross-talk with other inflammatory cytokines. We found that the IL-1β-induced decrease in muscle tension was recovered in response to treatment with an NFκB inhibitor, pyrrolidine dithiocarbamate (PDTC), and we are now investigating the molecular mechanisms of IL-1β-mediated GI dysmotility in IBD.

In conclusion, the present results demonstrate for the first time that long-term treatment with IL-1β reduces contractions in rat ileal longitudinal smooth muscle. This effect is accompanied by a reduction of MLC phosphorylation that is linked to the decreased activity of the regulatory proteins of myosin phosphate such as CPI-17 and MYPT-1. These findings increase our understanding of the mechanisms inducing motility disorders of the gastrointestinal tract in IBD.

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REFERENCES

1. Dinarello, C. A. (1997) Cytokine Growth Factor Rev. 8, 253–265
2. Vermillion, D. L., Huizinga, J. D., Riddell, R. H., and Collins, S. M. (1993) Gastroenterology 104, 1692–1699
3. Rogler, G., and Andus, T. (1998) World J. Surg. 22, 38–39
4. Bauer, A. J., Schwarz, N. T., Moore, B. A., Turler, A., and Kalff, J. C. (2002) Am. J. Physiol. Gastrointest. Liver Physiol. 283, G30–G35
5. Sartore, S., Chiavegato, A., Franch, R., Faggini, E., and Pauletto, P. (1997) Arthritis Rheum. 40, 361–369
6. Seyama, K., Nishimura, J., Kato, M., and Koizumi, H. (1997) J. Immunol. 158, 1527–1533
7. Sakurada, S., Takuwa, N., Sugimoto, N., Wang, Y., Seto, M., Sasaki, Y., and Narumiya, S. (1997) J. Biochem. 121, 91–96
8. Sakamoto, K., Hori, M., Izumi, M., Oka, T., Kohama, K., Ozaki, H., and Karaki, H. (2003) J. Pharmacol. Sci. 93, 556–565
9. Torihashi, S., Ozaki, H., Hori, M., Kita, M., Ohota, S., and Karaki, H. (2000) Cardiovasc. Res. 47, 1–10
10. Hori, M., Sato, K., Miyamoto, S., Ozaki, H., and Karaki, H. (1993) J. Pharmacol. Sci. 69, 1–10
11. Sakurada, S., Takuwa, N., Sugimoto, N., Wang, Y., Seto, M., Sasaki, Y., and Narumiya, S. (1997) Nature 390, 999–994
12. 2 T. Ohama, M. Hori, K. Sato, H. Ozaki, and H. Karaki, unpublished observations.
Chronic Treatment with Interleukin-1β Attenuates Contractions by Decreasing the Activities of CPI-17 and MYPT-1 in Intestinal Smooth Muscle
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