Characterization of protein kinase pathways responsible for Ca\(^{2+}\) sensitization in rat ileal longitudinal smooth muscle

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Submitted 10 May 2007; accepted in final form 24 July 2007

Ihara E, Moffat L, Ostrander J, Walsh MP, MacDonald JA. Characterization of protein kinase pathways responsible for Ca\(^{2+}\) sensitization in rat ileal longitudinal smooth muscle. Am J Physiol Gastrointest Liver Physiol 293: G699–G710, 2007. First published July 26, 2007; doi:10.1152/ajpgi.00214.2007.—We investigated the protein kinases responsible for myosin regulatory light chain (LC\(_{20}\)) phosphorylation and regulation of myosin light chain phosphatase (MLCP) activity during microcystin (phosphatase inhibitor)-induced contraction at low Ca\(^{2+}\) concentrations of rat ileal smooth muscle in the longitudinal axis. Application of 1 \(\mu\)M microcystin inhibited LC\(_{20}\) diphosphorylation and contraction of \(\beta\)-escin-permeabilized rat ileal smooth muscle at pCa 9. The PKC inhibitor GF-109203x, the MEK inhibitor PD-98059, and the p38 MAPK inhibitor SB-203580 significantly reduced this contraction. These inhibitory effects were abolished when the microcystin concentration was increased to 10 \(\mu\)M, indicating that application of these kinase inhibitors generated an increase in MLCP activity. GF-109203x and PD-98059, but not SB-203580, significantly decreased the phosphorylation level of the myosin-targeting subunit of MLCP, MYPT1, at Thr-697 (rat sequence) during microcystin-induced contraction at pCa 9. On the other hand, SB-203580, but not GF-109203x or PD-98059, significantly reduced the phosphorylation level of the PKC-potentiating phosphatase inhibitor of 17 kDa (CPI-17). A zipper-interacting protein kinase (ZIPK) inhibitor (SM1 peptide) and a Rho-associated kinase inhibitor (Y-27632) had little effect on microcystin-induced contraction at pCa 9. In conclusion, PKC, ERK1/2, and p38 MAPK pathways facilitate microcystin-induced contraction at low Ca\(^{2+}\) concentrations by contributing to the inhibition of MLCP activity either through phosphorylation of MYPT1 or CPI-17 [probably mediated by integrin-linked kinase (ILK)]. ILK and not ZIPK is likely to be the protein kinase responsible for LC\(_{20}\) diphosphorylation during microcystin-induced contraction of rat ileal smooth muscle at pCa 9, similar to its recently described role in vascular smooth muscle. The negative regulation of MLCP by PKC and MAPKs during microcystin-induced contraction at pCa 9, which is not observed in vascular smooth muscle, may be unique to phasic smooth muscle.

ileum; protein kinase C; myosin phosphatase; mitogen-activated protein kinase; protein kinase C-potentiated phosphatase inhibitor protein of 17 kDa; myosin-targeting subunit of myosin light chain phosphatase

SMOOTH MUSCLE CONTRACTION is a dynamic and highly regulated process. The contractile state of smooth muscle is mainly regulated by phosphorylation of the 20-kDa myosin regulatory light chain (LC\(_{20}\)) (45), which is controlled by the opposing activities of myosin light chain kinase (MLCK) (48) and myosin light chain phosphatase (MLCP) (20). MLCK activity is dependent on Ca\(^{2+}\)-calmodulin; hence, the intracellular Ca\(^{2+}\) signal (increased intracellular Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_i\))) is the primary determinant of smooth muscle contraction. Force can be further increased through signaling pathways that modulate MLCK and/or MLCP activities. The Ca\(^{2+}\) sensitivity of contraction can be affected by any change in the ratio of MLCK:MLCP activity. A decrease in MLCP activity will shift the balance in favor of MLCK, resulting in a greater degree of LC\(_{20}\) phosphorylation and contraction. The phenomenon of Ca\(^{2+}\) sensitization (45), which is associated with inhibition of MLCP activity, is mediated by an agonist-induced, G protein-coupled process. Inhibition of MLCP can occur either directly by phosphorylation of the myosin-targeting subunit of MLCP (MYPT1) (19, 50) or indirectly via phosphorylation of a protein kinase C (PKC)-potentiating phosphatase inhibitor protein of 17 kDa (CPI-17) (33). The Thr-697 site of MYPT1 (rat sequence) has been identified as a key inhibitory phosphorylation site for the regulation of MLCP activity. Rho-associated kinase (ROK) (25), zipper-interacting protein kinase (ZIPK) (3, 24, 34, 40), and integrin-linked kinase (ILK) (28, 39) have been demonstrated to phosphorylate MYPT1 at this inhibitory site. In addition, ROK (32), ZIPK (35), and ILK (9) contribute indirectly to MLCP inhibition by playing a role in CPI-17 phosphorylation. CPI-17, when phosphorylated at the regulatory Thr-38 site, becomes a potent inhibitor of MLCP.

When microcystin, a type 1 and 2A protein phosphatase (PP) inhibitor, is applied to permeabilized smooth muscle in low-Ca\(^{2+}\) medium, a sustained contraction is observed that cannot be attributed to MLCK (16, 40, 53). Microcystin unmasks Ca\(^{2+}\)-independent protein kinase activity and induces diphosphorylation of LC\(_{20}\) at Ser-19 and Thr-18 to activate crossbridge cycling and force development (53). In vascular smooth muscle, the microcystin-induced contraction at pCa 9 was inhibited by staurosporine (a general protein kinase inhibitor) but was unaffected by several other protein kinase inhibitors, including the ROK inhibitor Y-27632, the PKC inhibitor GF-109203x, the MLCK inhibitor ML-7, and the MLCK and phosophatidylinositol 3-kinase inhibitor wortmannin. To date, only ILK and/or ZIPK have emerged as bona fide candidates for the Ca\(^{2+}\)-independent diphosphorylation of LC\(_{20}\) at Ser-19 and Thr-18 in smooth muscle (55). A synthetic peptide inhibitor, derived from the autoinhibitory domain of MLCK, was used to distinguish between ZIPK and ILK effects in smooth muscle (23). It was concluded that ILK, and not ZIPK, is responsible for Ca\(^{2+}\)-independent diphosphorylation of LC\(_{20}\) and contraction of vascular smooth muscle (55).
Ca\textsuperscript{2+} SENSITIZATION IN INTESTINAL SMOOTH MUSCLE

Gastrointestinal motility is regulated by activation and coupling of muscarinic receptors in numerous cell types, including enteric neurons, interstitial cells of Cajal, and smooth muscle cells. Gastrointestinal smooth muscle expresses both M\textsubscript{2} (major isoform) and M\textsubscript{3} (minor isoform) muscarinic receptors (58), which are known to be indispensable for gastrointestinal smooth muscle contraction (5). The M\textsubscript{3} receptor contributes to the contraction of intestinal smooth muscle in a similar manner to that observed in vascular tissue (15). The stimulation of M\textsubscript{3} receptors, which are coupled to G\textsubscript{q}, activates phospholipase C and produces inositol 1,4,5-trisphosphate and diacylglycerol. These second messengers elicit the activation of PKC and trigger an increase in [Ca\textsuperscript{2+}]\textsubscript{i} (15). On the other hand, M\textsubscript{2} receptors act through G\textsubscript{i}, to regulate adenyl cyclase. Although the inhibition of adenyl cyclase is a classical and established effect of M\textsubscript{2} receptor activation in smooth muscle, other possible downstream signaling pathways that may be coupled to M\textsubscript{2} receptors have been proposed, including Src-family tyrosine kinases (44), ERK1/2 (7), and p38 MAPK (7).

Recently, both ERK1/2 and p38 MAPK have been implicated in the Ca\textsuperscript{2+} sensitization of contraction of esophageal smooth muscle (18). Acetylcholine-induced contraction was reported to be dependent on PKC in the cat esophageal smooth muscle, and this PKC-dependent contraction was mediated by ERK1/2 and p38 MAPK (6). In spite of recent evidence that ERK1/2 and/or p38 MAPK are involved in gastrointestinal smooth muscle contraction, the precise mechanisms by which these protein kinases contribute to smooth muscle contractility have yet to be determined. The phosphorylation of caldesmon and/or calponin is thought to be one mechanism by which ERK1/2 contributes to smooth muscle contraction (26, 31). Alternatively, p38 MAPK has been shown to phosphorylate and activate MAPK-activated protein kinase 2 (MAPAPK2), which in turn can phosphorylate heat shock protein (HSP) 27 (8, 42) and contribute to smooth muscle contraction (18).

Although numerous studies have investigated Ca\textsuperscript{2+} sensitization of smooth muscle contractility, the majority of this research has used vascular smooth muscle as a model system. Thus our understanding of the fundamental mechanisms that regulate Ca\textsuperscript{2+} sensitization in intestinal smooth muscle is limited. The objective of the present study was to explore the signaling pathway(s) responsible for regulation of MLCP activity and LC\textsubscript{20} phosphorylation during contraction of intestinal smooth muscle at low [Ca\textsuperscript{2+}]\textsubscript{i}.

MATERIALS AND METHODS

Materials. All chemicals were reagent grade unless otherwise indicated. Triton X-100, β-escin, PD-98059, SB-203580, and GF-109203x were obtained from Sigma (St. Louis, MO). Gö-6976 and A-23187 were from Calbiochem (San Diego, CA). Microcystin LR was obtained from Alexis Biochemical (San Diego, CA). Anti-MAP kinase 1/2 (which recognizes both ERK1 and ERK2) and anti-phospho-MAP kinase (which recognizes the dual-phosphorylation pT\textsuperscript{180}pY\textsuperscript{182} motif of ERK1 and ERK2) were from Upstate (Charlottesville, VA). Anti-p38 MAPK (which recognizes various SAPK2 isoforms) and anti-phospho p38 MAPK (which recognizes dual phosphorylation of SAPK2 isoforms at Thr-180 and Tyr-182) were from Stressgen Bioreagents (Ann Arbor, MI). Monoclonal antibody specific for MYPT1 phosphorylated at Thr-697 (anti-[phospho-Thr-697]-MYPT1; rat numbering) was purified as previously described (54). Polyclonal MYPT1 antibody generated against the NH\textsubscript{2}-terminal fragment of rat MYPT1 was a gift from Dr. Timothy Haystead (Duke University, Durham, NC) and was used to quantify total MYPT1 levels. Polyclonal antibodies specific for total CPI-17 and phosphorylated CPI-17 (anti-[phospho-Thr-38/-CPI-17] were purchased from Upstate. SM1 peptide (sequence: AKKLSDRMKKYMARQKWKQTG) was produced by the University of Calgary Peptide Synthesis Facility (Calgary, AB), confirmed by amino acid analysis, and shown to be >95% pure by analytical HPLC. The SM1 peptide corresponds to the autoinhibitory domain of smooth muscle MLCK (residues 783–804 of chicken gizzard MLCK) and has recently been shown to be a novel inhibitor of ZIPK (22). This peptide, which has no effect on ILK, can be used to distinguish between ZIPK and ILK effects in smooth muscle tissues.

Tissue preparation and force measurement of longitudinal smooth muscle strips. Ileum was removed from rats anesthetized and euthanized according to protocols approved by the University of Calgary Animal Care and Use Committee. Ideal smooth muscle sheets were dissected and cut into longitudinal smooth muscle strips (250 μm × 2 mm). For force measurement, muscle strips were tied with silk monofilaments to the tips of two fine wires. One wire was fixed, and the other was connected to a force transducer (Sensel, San Diego, CA). The strip was mounted in a well on a stir plate to allow rapid solution exchange. Strips were stretched in the longitudinal axis to 1.3× resting length and were equilibrated for 30 min in normal extracellular solution (NES) containing (in mM) 150 NaCl, 4 KCl, 2 Ca\textsuperscript{2+}, 1 Mg\textsuperscript{2+}, 5.5 glucose, and 1 HEPES, pH 7.3. After obtaining a good contractile response with high-K\textsuperscript{+} extracellular solution [KES; replacement of NaCl in NES solution with equimolar KMS], muscle strips were permeabilized by incubation with 50 μM β-escin for 40 min in an intracellular solution (G1), with 10 μM A-23187 added for the final 10 min to deplete intracellular Ca\textsuperscript{2+} stores. The composition of G1 solution was (in mM) 30 piperazine-1,4-bis(2-ethanesulfonic acid) (K\textsubscript{2}PIPES), 10 creatine phosphate (Na\textsubscript{2}CP), 5.16 Na\textsubscript{2}ATP, 7.92 Mg\textsubscript{2}MS, 46.6 KMS, and 10 K\textsubscript{2}EGTA. Different free Ca\textsuperscript{2+} concentrations (expressed as pCa) were obtained by mixing the CaG and G10 solutions to achieve the desired Ca\textsubscript{EGTA}/EGTA ratio. The composition of CaG solution was (in mM) 30 K\textsubscript{2}PIPES, 10 Na\textsubscript{2}CP, 5.14 Na\textsubscript{2}ATP, 7.25 Mg\textsubscript{2}MS, 47.1 KMS, and 10 K\textsubscript{2}CaEGTA; G10 solution was (in mM) 30 K\textsubscript{2}PIPES, 10 Na\textsubscript{2}CP, 5.14 Na\textsubscript{2}ATP, 7.92 Mg\textsubscript{2}MS, and 10 K\textsubscript{2}CaEGTA; G1 solution was (in mM) 30 K\textsubscript{2}PIPES, 10 Na\textsubscript{2}CP, 5.14 Na\textsubscript{2}ATP, 7.91 Mg\textsubscript{2}MS, 46.6 KMS, and 9.96 K\textsubscript{2}CaEGTA, with 36.1 μM K\textsubscript{2}CaEGTA; and pCa 4.5 solution was (in mM) 30 K\textsubscript{2}PIPES, 10 Na\textsubscript{2}CP, 5.14 Na\textsubscript{2}ATP, 7.25 Mg\textsubscript{2}MS, 47.1 KMS, and 9.99 K\textsubscript{2}CaEGTA, with 6.22 μM K\textsubscript{2}CaEGTA. The force levels obtained with relaxing solution (pCa 9 or G10) and pCa 4.5 were designated as 0 and 100%, respectively. Maximal contraction was obtained with maximal [Ca\textsuperscript{2+}] (pCa 4.5) before and after the protocol in all experiments. All contractile measurements were carried out at room temperature (23°C).

Western blot analysis of smooth muscle tissue. Rat ileum was collected, and squares (5 × 5 mm) were mounted in a silicone-bottom dish. The muscles were permeabilized and washed as described in the same section. To test the phosphorylation of ERK1/2, p38 MAPK, and MYPT1, ileum was treated with vehicle or the protein kinase inhibitors GF-109203x, PD-98059, or SB-203580 (Table 1) before the application of microcystin. Tissue was flash frozen in liquid N\textsubscript{2} and was homogenized in 10 volumes of sample buffer containing 1% SDS, 30 mM Tris, pH 8.8, 12.5% (vol/vol) glycerol, and (p-amidomethyl)phenylmethanesulfonic fluoride (APMSF) by using a glass-class, hand-operated homogenizer. Homogenates were resolved on 12% SDS-polyacrylamide gels and were transferred to polyvinylidene difluoride (PVDF) membranes in 25 mM Tris, 192 mM glycerine, and 20% (vol/vol) methanol at 110 V for 60 min at 4°C. Nonspecific binding sites were blocked with 5% (wt/vol) nonfat dry milk in TBST [25 mM Tris, pH 7.4, 150 mM NaCl, 0.05% (vol/vol) Tween-20]. The blots were washed and incubated for 1 h with primary antibody (1:1,000 dilution of ERK1/2, phospho-ERK1/2, p38 MAPK, phospho-
Table 1. IC\textsubscript{50}/K\textsubscript{i} values for inhibitory compounds of selected protein kinases

<table>
<thead>
<tr>
<th>Compound</th>
<th>Target Protein Kinase</th>
<th>IC\textsubscript{50} or K\textsubscript{i}, Value</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Y-27632</td>
<td>ROK-I</td>
<td>140 nM (K\textsubscript{i})</td>
<td>51</td>
</tr>
<tr>
<td>Y-27632</td>
<td>ROK-II</td>
<td>140 nM (K\textsubscript{i})</td>
<td>51</td>
</tr>
<tr>
<td>GF-109203x</td>
<td>PKC (α, β1, γ, ε, η, θ)</td>
<td>8-20 nM (K\textsubscript{i})</td>
<td>49</td>
</tr>
<tr>
<td>Chelerythrine</td>
<td>PKC (α, β1, γ, ε)</td>
<td>0.66 μM (K\textsubscript{i})</td>
<td>21</td>
</tr>
<tr>
<td>Go-6976</td>
<td>PKC-α</td>
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<td>36</td>
</tr>
<tr>
<td></td>
<td>PKC-β</td>
<td>6.2 μM</td>
<td>36</td>
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<tr>
<td>PD-98059</td>
<td>MAPK1 (MEK1)</td>
<td>4 μM</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>MAPK2 (MEK2)</td>
<td>50 μM</td>
<td>1</td>
</tr>
<tr>
<td>SB-203580</td>
<td>p38 MAPK</td>
<td>0.6 μM</td>
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</tr>
<tr>
<td>SM1</td>
<td>ZIPK</td>
<td>3.8 μM</td>
<td>23</td>
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ROK, Rho-associated kinase; ZIPK, zipper-interacting protein kinase.


d造成了Ca\textsuperscript{2+} sensitization in intestinal smooth muscle. Measurement of LC\textsubscript{20} phosphorylation. Urea/glycerol gel electrophoresis and Western blotting to separate and quantify un-, mono-, and diphosphorylated forms of LC\textsubscript{20} were performed as previously described (23, 53). Contractile responses were halted by immersion of ileal muscle strips in a dry-ice/acetone slurry containing 10% (vol/vol) trichloroacetic acid (TCA) and 10 mM dithiothreitol (DTT). Strips were washed in 10 mM DTT in acetic acid and were lyophilized overnight. Muscle tissues were homogenized in sample buffer in a glass–glass, hand-operated homogenizer. Aliquots of the homogenate were resolved on 18% SDS-polyacrylamide gels and were transferred to PVDF membrane in 10 mM sodium cyclohexylaminopropane sulfonic acid (pH 11) and 10% (vol/vol) methanol at 50 mA for 4 h at 4°C. Nonspecific binding sites were blocked with 1-block solution (Applied Biosystems, Foster City, CA) in TBST overnight at 4°C. Blots were incubated with primary antibody for total or phospho-CPI-17 (1:2,500 dilution) in 1% (wt/vol) nonfat dry milk in TBST for 1 h. The strips were then washed and incubated with horseradish peroxidase-conjugated secondary antibody (1:50,000 dilution) in 1% (wt/vol) nonfat dry milk in TBST for 1 h at 20°C. The membrane was developed with Supersignal West Femto enhanced chemiluminescence (Pierce Chemical, Rockford, IL). Bands were quantified by densitometry, and the relative phosphorylation levels of ERK1/2, p38 MAPK, and MYPT1 were expressed as a function of the density of total protein species.

Western blot analysis of CPI-17. To examine the phosphorylation of CPI-17, ileal smooth muscle strips were frozen by immersion in a dry-ice/acetone slurry containing 10% (vol/vol) trichloroacetic acid (TCA) and 10 mM dithiothreitol (DTT). Strips were washed in 10 mM DTT in acetic acid and were lyophilized overnight. Muscle tissues were homogenized in sample buffer in a glass–glass, hand-operated homogenizer. Aliquots of the homogenate were resolved on 18% SDS-polyacrylamide gels and were transferred to PVDF membrane in 10 mM sodium cyclohexylaminopropane sulfonic acid (pH 11) and 10% (vol/vol) methanol at 50 mA for 4 h at 4°C. Nonspecific binding sites were blocked with 1-block solution (Applied Biosystems, Foster City, CA) in TBST overnight at 4°C. Blots were incubated with primary antibody for total or phospho-CPI-17 (1:2,500 dilution) in 1% (wt/vol) nonfat dry milk in TBST for 1 h. The strips were then washed and incubated with horseradish peroxidase-conjugated secondary antibody (1:50,000 dilution) in 1% (wt/vol) nonfat dry milk in TBST for 1 h at 20°C. The membrane was developed with Supersignal West Femto enhanced chemiluminescence (Pierce Chemical, Rockford, IL). Bands were quantified by densitometry, and the relative phosphorylation levels of CPI-17 were calculated as a function of the density of total CPI-17.

Measurement of LC\textsubscript{20} phosphorylation. Urea/glycerol gel electrophoresis and Western blotting to separate and quantify un-, mono-, and diphosphorylated forms of LC\textsubscript{20} were performed as previously described (23, 53). Contractile responses were halted by immersion of ileal muscle strips in a dry-ice/acetone slurry containing 10% (vol/vol) trichloroacetic acid. The muscle strips were washed with a 10 mM DTT-acetone solution and were lyophilized overnight. Muscle proteins were extracted in a buffer containing 8 M urea, 1 M thiourea, and 10 mM DTT before separation by urea/glycerol gel electrophoresis. Western blotting was carried out with a polyclonal antibody that detects both phosphorylated and unphosphorylated LC\textsubscript{20}. The stoichiometry of LC\textsubscript{20} phosphorylation was calculated from the following equation: mol of P/mol LC\textsubscript{20} = (y + 2z)/(x + y + z), where x, y, and z are the signal intensities of un-, mono-, and diphosphorylated LC\textsubscript{20} bands, respectively.

Data analysis. All smooth muscle contractile data were collected by using a computerized data-acquisition system (PowerLab/SP data-recording unit and Chart software; ADInstruments, Colorado Springs, CO). All data are expressed as means ± SE. Student’s t-test was used to determine statistical significance, with P < 0.05 considered to be significant.

RESULTS

Microcystin-induced contraction of ileal smooth muscle at pCa 9. Because the activity of MLCP (a type 1 protein serine/threonine phosphatase, PP1) is known to be much greater in intestinal smooth muscle than in vascular smooth muscle (16), we hypothesized that the protein kinase networks activated in intestinal smooth muscle, which contribute to contraction and regulation of MLCP, would be different from those in vascular smooth muscle. We set out, therefore, to examine the ability of the phosphatase inhibitor microcystin to induce contractile responses in intestinal smooth muscle. The administration of microcystin (1 μM) to β-escin-permeabilized smooth muscle strips in relaxing solution (pCa 9) elicited a gradual increase in force, reaching a plateau 45 min after application (Fig. 1A). Because microcystin inhibits both PP1 and PP2A phosphatases with similar potency, control experiments with more selective inhibitors of PP2A (i.e., 1 nM okadaic acid and 0.1 μM fostriecin) were conducted to ascertain whether PP2A contributes to Ca\textsuperscript{2+} sensitization. There was no development of contractile force when either okadaic acid or fostriecin was applied to permeabilized ileal muscle strips at concentrations that are selective for PP2A (data not shown). Furthermore, pretreatment with these PP2A inhibitors did not affect the rate of contraction or steady-state force elicited by microcystin at pCa 9. We conclude, therefore, that the contractile response to microcystin is due to inhibition of PP1, not PP2A. The fact that microcystin induces LC\textsubscript{20} phosphorylation supports the conclusion that the targeted PP1 in this context is indeed MLCP.

We further examined the concentration-dependent contractile response of rat ileal smooth muscle to microcystin. Typical contractile responses to microcystin (10 and 30 μM) are depicted in Fig. 1, B and C, respectively. The force obtained with 1 μM microcystin at 25 and 45 min after application was 31.5 ± 2.7 and 59.2 ± 3.3% of pCa 4.5-induced contraction, respectively. A maximal contractile response was obtained at 10 μM microcystin; the force levels at 25 min (42.5 ± 2.4%; n = 5) and at 45 min (69.3 ± 1.4%; n = 5) were significantly higher than those induced by 1 μM microcystin. However, an increase in the microcystin concentration to 30 μM did not elicit further increases in muscle force. The force induced by 30 μM microcystin after 25 min (37.5 ± 2.2%; n = 5) and 45 min (64.4 ± 0.79%; n = 5) was not significantly different from the corresponding force induced by 1 μM microcystin. However, the time required to reach a plateau of muscle tension at pCa 9 was shortened with the application of higher microcystin concentrations. Interestingly, the steady-state tension developed at any concentration of microcystin did not reach 100% of F\textsubscript{max} (i.e., maximal force obtained at pCa 4.5; Fig. 1D), whereas the steady-state tension induced by 1 μM microcystin in the rat caudal artery reached nearly 100% of F\textsubscript{max} (55).

The contraction induced by 1 μM microcystin at pCa 9 was accompanied by diphosphorylation of LC\textsubscript{20} (Fig. 1E). The extent of LC\textsubscript{20} mono- and diphosphorylation was determined to be 20.8 ± 1.3 and 1.5 ± 0.4% of total LC\textsubscript{20}, respectively. This corresponds to an overall phosphorylation stoichiometry of 0.24 mol P/mol LC\textsubscript{20}. In accordance with our force mea-
Fig. 1. A–C: microcystin (MC)-induced diphosphorylation of 20-kDa myosin regulatory light chain (LC20) and contraction of rat ileal smooth muscle at pCa 9. MC [1 μM (A), 10 μM (B), and 30 μM (C)] elicited contraction of β-escin-permeabilized rat ileal smooth muscle strips at pCa 9. Reference responses to pCa 4.5 were obtained before and after MC treatment to define maximal contraction (F_{max}). D: concentration-dependent contractile responses of rat ileal smooth muscle to 1 μM (open bars), 10 μM (hatched bars), and 30 μM (closed bars) MC were measured 25 and 45 min after addition of MC. Forces observed at pCa 9 and pCa 4.5 were designated as 0 and 100%, respectively. Error bars indicate standard error (n = 5). *, Significantly different (Student’s t-test, P < 0.05); n.s., not significantly different. E: following treatment of ileal strips with MC (1 μM) for 45 min, phosphorylated and unphosphorylated LC20 were separated by urea/glycerol gel electrophoresis, detected by Western blotting with anti-LC20, and quantified by scanning densitometry. Different exposure times were used for quantification of these data to ensure that signals lay within linear range of relationship between protein amount and signal intensity in each case. Data are expressed as percentages of total LC20 for unphosphorylated (P0-LC20; open bar), mono-phosphorylated (P1-LC20; hatched bar), and diphosphorylated (P2-LC20; dotted bar) bands. Error bars indicate standard error (n = 5).

PKC involvement in microcystin-induced contraction of ileal smooth muscle at pCa 9. PKC has been linked to microcystin-induced contraction of vascular smooth muscle (40, 55). Therefore, we examined whether PKC was involved in microcystin-induced contraction of ileal smooth muscle. Figure 3, A and B shows representative recordings of force induced by 1 μM microcystin in the presence of either 100 nM GF-109203x or 10 μM chelerythrine. GF-109203x, a broad-specificity PKC inhibitor, reduced contraction induced by 1 μM microcystin. The force levels at 25 min (6.0 ± 1.7%; n = 5) and 45 min (18.1 ± 3.8%; n = 5) following microcystin administration in the presence of GF-109203x were significantly lower than those at 25 min (31.5 ± 2.7%; n = 5) and 45 min (59.2 ± 3.3%; n = 5) in the absence of the inhibitor. Similar results were obtained with chelerythrine, another broad-specificity PKC inhibitor. The forces observed 25 and 45 min after the application of microcystin in the presence of chelerythrine were reduced to 15.2 ± 3.3 and 39.1 ± 4.7% of F_{max}, respectively. To elucidate whether conventional PKCs were involved in microcystin-induced contraction at pCa 9, we examined the effects of Gö-6976, a conventional PKC inhibitor, on ileal smooth muscle contraction. Interestingly, Gö-6976 significantly inhibited muscle tension induced by 1 μM microcystin in a similar manner to that observed with GF-109203x and chelerythrine (Fig. 3C). The force levels at 25 and 45 min in the presence of Gö-6976 were 8.4 ± 2.4 and 32.6 ± 3.6% (n = 5), respectively. These data clearly show that conventional PKCs contribute to microcystin-induced contraction of rat ileal smooth muscle at pCa 9.
involved in the microcystin-induced contraction under resting conditions (pCa 9). We conducted further experiments to confirm that pCa 9 was sufficient for the activation of Ca\textsuperscript{2+}-dependent PKCs in ileal smooth muscle. The contractile response to 1 \textmu M microcystin in G10 solution (Ca\textsuperscript{2+}-free solution) was examined. As expected, the force induced by 1 \textmu M microcystin was much lower in G10 solution than in pCa 9 (Fig. 3F). Together, these data suggest that conventional PKC isoforms contribute to microcystin-induced contraction of ileal smooth muscle even at nanomolar levels of Ca\textsuperscript{2+}.

**Involvement of MAPKs in microcystin-induced contraction of ileal smooth muscle at pCa 9.** MAPKs have been reported to play a role in the regulation of smooth muscle contractility in esophageal phasic smooth muscle (18), so an examination of MAPK involvement in microcystin-induced contraction of ileal smooth muscle at pCa 9 was undertaken. Contraction induced by 1 \textmu M microcystin was recorded in the presence of the MEK antagonist PD-98059 (Fig. 4A) or the p38 MAPK inhibitor SB-203580 (Fig. 4B). PD-98059 reduced 1 \textmu M microcystin-induced force (Fig. 4C). The force developed at 25 min (3.4 \pm 2.1\%; n = 5) and 45 min (19.4 \pm 7.0\%; n = 5) in the presence of PD-98059 was significantly lower than at 25 min (31.5 \pm 2.7\%; n = 5) and 45 min (59.2 \pm 3.3\%; n = 5) in the absence of the inhibitor. However, the inhibition of microcystin-induced contraction by PD-98059 was completely abolished when the microcystin concentration was increased to 10 \textmu M (Fig. 4C). Similar results were obtained with SB-203580 when contraction was stimulated with 1 \textmu M microcystin. The force generated at 25 and 45 min in the presence of SB-203580 was 4.5 \pm 1.9 and 21.4 \pm 5.3\% (n = 5) of F\textsubscript{max}. This inhibition by SB-203580 was completely abolished when the microcystin concentration was increased to 10 \textmu M (Fig. 4C). From these data, we conclude that both ERK1/2 and p38 MAPK play a role in the negative regulation of MLCP during microcystin-induced contraction of ileal smooth muscle at pCa 9.

**MYPT1 phosphorylation at Thr-697 by PKC and MAPK pathways in ileal smooth muscle.** The best-characterized mechanism of MLCP inhibition involves the phosphorylation of MYPT1 that is accompanied by a decrease in phosphatase activity. PKC may play a role not only in the direct phosphorylation of LC\textsubscript{20} but also in the regulation of MLCP activity during microcystin-induced contraction at pCa 9. Thus we examined whether PKC contributes to the phosphorylation of MYPT1. In addition, we investigated whether the negative regulation of MLCP by ERK1/2 and p38 MAPK is mediated by MYPT1 phosphorylation. We examined the effects of GF-109203x, PD-98059, and SB-203580 on the phosphorylation level of MYPT1 at Thr-697. This is the major site of negative regulation of MLCP activity, although phosphorylation at other sites may also have inhibitory effects (38, 54). Ileal smooth muscle tissue was flash frozen 45 min after application of microcystin in the absence and presence of protein kinase inhibitors. Thr-697 phosphorylation of MYPT1 was significantly reduced by administration of either GF-109203x or PD-98059 (Fig. 5). MYPT1 phosphorylation was reduced in the presence of GF-109203x and PD-98059 to 53.3 \pm 2.1\% (n = 3) and 61.4 \pm 10.7\% (n = 3) of the control level (absence of protein kinase inhibitors), respectively. The addition of SB-203580 did not have a significant effect on MYPT1 phosphorylation. These data suggest that PKC and
ERK1/2 pathways, but not the p38 MAPK pathway, contribute to microcystin-induced contraction at pCa 9 via the inhibition of MLCP activity mediated through MYPT1 phosphorylation at Thr-697.

Contribution of CPI-17 to MLCP inhibition by PKC and MAPK pathways during microcystin-induced contraction of ileal smooth muscle at pCa 9. Another important mechanism of MLCP inhibition involves the phosphorylation of CPI-17 (14). We examined the possibility that microcystin-induced contraction of the ileum could be a reflection of CPI-17 phosphorylation status. Lower amounts of CPI-17 were observed in intestinal smooth muscle (e.g., colon and ileum) than in vascular smooth muscle beds (Fig. 6A). However, the small amount of CPI-17 that was contained in the ileal smooth muscle cell was tightly associated with the contractile apparatus. Permeabilization with β-escin or Triton X-100 had no effect on the amount of CPI-17 detected in ileal smooth muscle (Fig. 6B). This is in contrast to our data for caudal artery and previous reports (29) that show the majority of CPI-17 protein diffuses from vascular smooth muscle on treatment with these permeabilization agents. As shown in Fig. 6, C and D, significant amounts of CPI-17 phosphorylation were observed under resting conditions (pCa 9) in β-escin-permeabilized ileal smooth muscle. Furthermore, CPI-17 phosphorylation was not affected by microcystin treatment alone. To assess the importance of PKC and MAPK signaling on CPI-17 phosphorylation status, ileal muscle strips were incubated with protein kinase inhibitors (GF-109203x, PD-98059, or SB-203580) before microcystin administration and were then subjected to Western blot analysis. CPI-17 phosphorylation was reduced following pretreatment with SB-203580 but was unaffected by GF-109203x or PD-98059 (Fig. 6, C and D). The amount of phosphorylated CPI-17 detected in the presence of SB-203580 was significantly lower than that detected in the control. We conclude that the p38 MAPK pathway, but not the PKC and ERK1/2 pathways, participates in the phosphorylation of CPI-17 in ileal smooth muscle.

Complementation of PKC, ERK1/2, and p38 MAPK pathways in the regulation of MLCP activity during microcystin-induced contraction of ileal smooth muscle at pCa 9. The blockade of either PKC or MAPK (ERK1/2 and p38 MAPK) pathways in ileal muscle with pharmacological agents had
similar effects on microcystin-induced contraction at pCa 9. A previous study with esophageal smooth muscle suggested that ERK1/2 and p38 MAPK are likely to participate in PKC-ε-dependent contractile pathways (18). Next, we examined whether microcystin-induced force generation in ileal smooth muscle depends on activation of ERK1/2 or p38 MAPK by PKC pathways. Western blot analyses of activated ERK1/2 and p38 MAPK were performed on ileal tissues following the application of microcystin in the presence of GF-109203x, PD-98059, or SB-203580 (Fig. 7, A and C). The phosphorylation level of ERK1 was reduced by PD-98059 but not by GF-109203x (Fig. 7B). ERK1 phosphorylation in the presence of PD-98059 or GF-109203x was 60.2 ± 5.9% (n = 3) and 92.1 ± 17.8% (n = 3), respectively, of the phosphorylation detected in the absence of protein kinase inhibitor. Also, the phosphorylation level of p38 MAPK was not reduced by GF-109203x (Fig. 7D). p38 MAPK phosphorylation in the presence of GF-109203x was 107.1 ± 15.8% (n = 3). These results are consistent with ERK1 and p38 MAPK activation occurring independently of PKC.

To further assess the integration of PKC and MAPK signaling, we applied the PKC, ERK1/2, and p38 MAPK inhibitors in combination to rat ileal smooth muscle (Fig. 7, E and F). First, we confirmed that 100 nM GF-109203x was sufficient to completely inhibit PKC-dependent contractile pathways. Increasing the GF-109203x concentration to 1 and 10 μM had no further inhibitory effect on the microcystin-induced contraction, demonstrating that 100 nM GF-109203x was sufficient to inhibit PKC contractile signaling in ileal smooth muscle. Addition of either 10 μM PD-98059 or 10 μM SB-203580 in combination with 100 nM GF-109203x did not augment the inhibitory effect of GF-109203x. However, application of all three kinase inhibitors (GF-109203x, PD-98059, and SB-203580) abolished the contractile response to 1 μM microcystin. These data suggest that microcystin-induced contraction of ileal smooth muscle is equally dependent on PKC, ERK1/2, and p38 MAPK pathways. Furthermore, the ERK1/2 and p38 MAPK pathways appear to signal in parallel to PKC and not downstream of this kinase during microcystin-induced contraction at pCa 9.

**ZIPK contribution to microcystin-induced contraction of ileal smooth muscle at pCa 9.** To determine the contribution of ZIPK to microcystin-induced contraction of rat ileal smooth...
muscle at pCa 9, we used a recently described inhibitor of ZIPK, the SM1 peptide. This peptide, which is derived from the autoinhibitory domain of MLCK, exhibits inhibitory potency toward ZIPK in vitro and ex vivo (23) but does not have any effect on ILK or ROK (55). SM-1 peptide significantly inhibited the muscle-tension development induced by the addition of exogenous ZIPK to Triton-skinned rat ileal smooth muscle strips (23). We determined that ZIPK was not responsible for LC20 diphosphorylation and microcystin-induced contraction of rat ileal smooth muscle at pCa 9, consistent with results obtained with vascular smooth muscle (55). As shown in Fig. 8, the application of SM1 peptide (50 μM) had no effect on microcystin-induced force generation, implying that this contraction is mediated by ILK.

**DISCUSSION**

It is widely accepted that pathological alterations in the sensitivity of the smooth muscle contractile response to \([Ca^{2+}]\) (i.e., \(Ca^{2+}\) sensitization) underlie various disorders associated with smooth muscle dysfunction such as hypertension, vasospasm, bronchial asthma, and inflammatory bowel disease. Although ILK was recently suggested to be involved in microcystin-induced contraction of vascular smooth muscle at low \([Ca^{2+}]\) (55), to our knowledge this is the first study undertaken to define the contribution of protein kinases to \(Ca^{2+}\) sensitization of ileal smooth muscle contraction.

Contraction at low \([Ca^{2+}]\) has been reported in a variety of vascular smooth muscle tissues following agonist stimulation (11, 17, 52) or application of microcystin (53). \(Ca^{2+}\) sensitization not only occurs in vascular smooth muscle but also in intestinal smooth muscle. As shown in the present study, the contractile force elicited by microcystin reached 60% of \(F_{\max}\) in rat ileal smooth muscle even when the microcystin concentration was increased to 30 μM, whereas application of 1 μM microcystin was shown to generate essentially 100% of \(F_{\max}\) in a vascular smooth muscle tissue (rat caudal artery) (55). Consistent with this finding, the extent of LC20 phosphorylation during microcystin-induced contraction at pCa 9 was much smaller in the ileum than in the caudal artery. Furthermore, MLCP activity is much greater in intestinal smooth muscle than in vascular smooth muscle (16, 56). Although it is thought that microcystin elicits a contractile response by inhibiting MLCP activity, and this is supported by the observed increase in LC20 phosphorylation, it should be noted that...
microcystin also inhibits other type 1 as well as type 2A protein serine/threonine phosphatases. Using selective inhibitors of PP2A, we confirmed that this class of phosphatase is not involved in microcystin-induced contraction of ileal smooth muscle at pCa 9, lending further support to the conclusion that it is inhibition of the type 1 phosphatase MLCP that is responsible for this contractile response. The relative expression levels of MYPT1 (the myosin-targeting subunit of MLCP) correlate with the rate of LC20 dephosphorylation, with higher levels of MYPT1 (the myosin-targeting subunit of MLCP) contributing to phasic compared with tonic smooth muscle. The expression levels of MLCP and the phosphatase inhibitory protein, CPI-17, both of which are regulated by phosphorylation, dictate the contractile phenotype of the particular smooth muscle bed. We hypothesized, therefore, that the protein kinase networks contributing to the regulation of MLCP and contraction of intestinal smooth muscle at low [Ca$^{2+}$]$_{i}$ would differ from those in vascular smooth muscle.

The PKC, ERK1/2, and p38 MAPK pathways were shown, through the use of pharmacological agents, to be involved in microcystin-induced contraction of rat ileal smooth muscle at pCa 9. ERK1/2 and p38 MAPK have been previously reported to play a role in contraction of both vascular (37, 41) and intestinal smooth muscle (6). The phosphorylation of caldesmon and/or calponin is thought to be one mechanism whereby ERK1/2 contributes to smooth muscle contraction (26, 31). Alternatively, p38 MAPK has been shown to phosphorylate and activate MAPKAPK-2, which in turn can phosphorylate HSP 27 (8, 42) and contribute to smooth muscle contraction (18). However, the precise mechanism by which these MAPKs contribute to ileal smooth muscle contractility remains to be determined. This study demonstrates that both ERK1/2 and p38 MAPK participate in the regulation of MLCP activity but not in the direct phosphorylation of LC20 at pCa 9. These findings are also consistent with a previous report showing that stimulation of $\alpha_1$-adrenoceptors with phenylephrine induced ERK1/2 activation and resulted in MLCP inhibition via the phosphorylation of MYPT1 in uterine artery. This MYPT1 phosphorylation and MLCP inhibition were significantly reduced by PD-98059 (57).

Although it appears that both PKC and ERK1/2 modulate contraction of the ileum at low [Ca$^{2+}$]$_{i}$ by contributing to the phosphorylation of MYPT1 at Thr-697 and hence the activity of MLCP, neither kinase has been shown to phosphorylate MYPT1 directly. Several kinases are known to regulate MLCP activity by phosphorylation of the Thr-697 inhibitory site, including ROK (27), ZIPK (34, 40), myotonic dystrophy protein kinase (39), ILK (28, 39), Raf-1 (4), and p21-activated protein kinase (47). It is possible that one or more of these protein kinases are located downstream of PKC and ERK1/2 and are directly responsible for the observed MYPT1 phosphorylation. Harnett and colleagues (18) have previously suggested that ILK is located downstream of the ERK1/2 pathway and that ZIPK is a downstream target of the p38 MAPK pathway. Because the SM1 peptide (a ZIPK inhibitor) did not affect microcystin-induced contraction at pCa 9 (Fig. 8), it is
degree of constitutive phosphorylation, and furthermore, p38 MAPK but not PKC appears to contribute to its phosphorylation. Because there is no evidence that p38 MAPK can phosphorylate CPI-17 directly, it is likely that some other kinase operates downstream of p38 MAPK to phosphorylate and activate CPI-17. A recent study by Hersch and colleagues (22) has reported CPI-17 phosphorylation by PKC in isolated rabbit intestinal smooth muscle cells. This phosphorylation was potentiated by low concentrations of okadaic acid and the p38 MAPK inhibitor SB-203580, leading the investigators to conclude that PP2A-mediated CPI-17 dephosphorylation in these cells was activated by p38 MAPK. Our findings differ in two key ways: 1) CPI-17 phosphorylation in rat ileum was not induced by inhibitors of PKC, and 2) basal CPI-17 phosphorylation was high following permeabilization with β-escin, and PP inhibitors were unable to elicit further CPI-17 activation. It is possible that the participation of PKC and PP2A in the activation of CPI-17 is restricted to rabbit intestinal tissue. Alternatively, isolated tissue may exhibit differences in active signaling pathways from dispersed smooth muscle cells.

Following stimulation of M₃ muscarinic receptors with acetylcholine, force is generated in cat esophageal smooth muscle by PKC-ε and the subsequent downstream activation of ERK1/2 and p38 MAPK pathways (18). Therefore, we investigated whether PKC could be an upstream modulator of MAPK pathways as previously shown. Treatment of ileum with GF-109203x, however, did not reduce the activating phosphorylation of ERK1 or p38 MAPK. Furthermore, simultaneous inhibition of all three kinase pathways by application of GF-109203x, PD-98059, and SB-203580 reduced microcystin-induced contraction to a greater extent than application of GF-109203x alone. These findings indicate that the ERK1/2 and p38 MAPK pathways operate in parallel with and not upstream of PKC in the regulation of MLCP in rat ileal smooth muscle. We propose that the three protein kinase pathways have different roles in microcystin-induced contraction at pCa 9 (Fig. 9). PKC is associated not only with the direct diphosphorylation of LC₂₀ but also with MLCP inhibition through MYPT1 phosphorylation at the inhibitory Thr-697 site. ERK1/2 is involved in MLCP inhibition through MYPT1 phosphorylation but does not contribute to the direct diphosphorylation of LC₂₀. Finally, p38 MAPK is involved only in

![Fig. 8. Zipper-interacting protein kinase (ZIPK) contribution to MC-induced contraction of rat ileal smooth muscle at pCa 9. Contraction induced by 1 µM MC in presence of 50 µM SM1 peptide, an inhibitor of ZIPK, is shown. SM 1 was applied to muscle in pCa 9 solution 20 min before administration of MC. Cumulative results (B) are presented for force measurement 25 and 45 min after application of MC. Forces observed at pCa 9 and pCa 4.5 were designated as 0 and 100%, respectively. Error bars indicate standard error (n = 5). n.s., Not significantly different from force generated by MC in absence of SM1 (Student’s t-test, P > 0.05).](image)

![Fig. 9. Schematic representation of signaling pathways involved in MC-induced Ca²⁺ sensitization of rat ileal smooth muscle contraction. ILK, integrin-linked kinase; MLCK, myosin light chain kinase; PP, protein phosphatase.](image)
MLCP activity; however, its actions on MLCP are mediated via CPI-17 phosphorylation and not MYPT1 phosphorylation. Ca2+-independent protein kinases can directly contribute to LC20 mono- and diphosphorylation and are thought to play a role in Ca2+ sensitization. Previous reports have implicated several Ca2+-independent MLCKs, including ROK (2), PKC (25), MAPKAPK-2 (30), MAPKAPK-1b (RSK-2) (46), ILK (10), and ZIPK (40). ROK, MAPKAPK-2, and RSK-2 phosphorylation of LC20 is restricted to Ser-19, however, and PKC does not phosphorylate either Ser-19 or Thr-18. Furthermore, among these kinases, only ROK (2), ILK (10), and ZIPK (40) have been shown to induce LC20 phosphorylation and contraction of smooth muscle. Because microcystin induced diphosphorylation of LC20 in low-Ca2+ medium and both Y-27632 and SM11 had no effect on contraction induced by 10 μM microcystin at pCa 9, ILK emerges as the most likely candidate for the Ca2+-independent MLCK in ileal smooth muscle. This conclusion is supported by a recent study (43) that suggested increased [Ca2+]i is required for activation of RhoA and ROK in both KCl- and agonist-induced contractions of vascular smooth muscle. It is possible, therefore, that ILK acts downstream of PKC to contribute to diphosphorylation of LC20 and downstream of MAPKs to regulate MLCP activity.

In conclusion, we describe the mechanism of Ca2+-sensitization in ileal smooth muscle as consisting of several parallel pathways. It appears that PKC, ERK1/2, and p38 MAPK (along with a minor role for ROK) are involved in microcystin-induced contraction through the negative regulation of MLCP activity. The contribution of PKC, ERK1/2, and p38 MAPK to the regulation of LC20 diphosphorylation is thought to be unique to phasic smooth muscle. PKC and ERK1/2 pathways inhibited MLCP activity by modulation of MYPT1 phosphorylation at Thr-697, whereas the p38 MAPK pathway inhibited MLCP activity by modulation of CPI-17 phosphorylation. It is possible that ILK lies downstream of these pathways and PKC, not MAPKs, and may modulate ILK-mediated LC20 diphosphorylation. There are significant differences between the protein kinase networks that contribute to Ca2+-sensitization of vascular (tonic) and intestinal (phasic) smooth muscle contraction. Because these smooth muscle beds have highly specialized functions, it is not unexpected that unique and intricate signaling pathways have developed in each.

GRANTS
This work was supported by grants to aid in research from the Crohn’s and Colitis Foundation of Canada (J. A. MacDonald) and the Canadian Institutes of Health Research (MGP-13101 to M. P. Walsh). E. Ibara is a recipient of a Uehara Memorial Foundation Fellowship (Japan) and Canadian Association of Gastroenterology/Canadian Institutes of Health Research/AstraZeneca Fellowship. L. Mofat is a recipient of a Studentship from the Canadian Institutes of Health Research. J. A. MacDonald is a recipient of a Canada Research Chair (Tier II) in Smooth Muscle Pathophysiology and a Scholarship from the Heart and Stroke Foundation of Canada. M. P. Walsh is an Alberta Heritage Foundation for Medical Research Scientist and recipient of a Canada Research Chair (Tier I) in Vascular Smooth Muscle Research.

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