Myosin Regulatory Light Chain Diphosphorylation Slows Relaxation of Arterial Smooth Muscle

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Background: The regulatory light chains of smooth muscle myosin are phosphorylated at Ser\(^{19}\) and Thr\(^{18}\). Phosphorylation at Thr\(^{18}\) does not increase force elicited by Ser\(^{19}\) phosphorylation, but reduces the rate of relaxation. Phosphorylation of Ser\(^{19}\) or both Ser\(^{19}\) and Thr\(^{18}\) to comparable stoichiometries (0.5 mol of Pi/mol of LC\(_{20}\)) and similar levels of isometric force induced by Ser\(^{19}\) phosphorylation. However, phosphorylation of Ser\(^{19}\) or both Ser\(^{19}\) and Thr\(^{18}\) to comparable stoichiometries (0.5 mol of P/i/mol of LC\(_{20}\)) and similar levels of isometric force increased the rates of dephosphorylation and smooth muscle relaxation following removal of the stimulus: \(t_{1/2}\) values for dephosphorylation were 83.3 and 560 s, and for relaxation were 560 and 1293 s, for monophosphorylated (Ser\(^{19}\)) and diphosphorylated (Thr\(^{18}\), respectively). We conclude that phosphorylation at Thr\(^{18}\) had no effect on steady-state force induced by Ser\(^{19}\) phosphorylation. However, phosphorylation of Ser\(^{19}\) or both Ser\(^{19}\) and Thr\(^{18}\) to comparable stoichiometries (0.5 mol of P/i/mol of LC\(_{20}\)) and similar levels of isometric force increased the rates of dephosphorylation and smooth muscle relaxation following removal of the stimulus: \(t_{1/2}\) values for dephosphorylation were 83.3 and 560 s, and for relaxation were 560 and 1293 s, for monophosphorylated (Ser\(^{19}\)) and diphosphorylated (Thr\(^{18}\), respectively). We conclude that phosphorylation at Thr\(^{18}\) decreases the rates of LC\(_{20}\) dephosphorylation and smooth muscle relaxation compared with LC\(_{20}\) phosphorylated exclusively at Ser\(^{19}\). These effects of LC\(_{20}\) diphosphorylation, combined with increased Ser\(^{19}\) phosphorylation (Ca\(^{2+}\)-independent), may underlie the hypercontractility that is observed in response to certain physiological contractile stimuli, and under pathological conditions such as cerebral and coronary arterial vasospasm, intimal hyperplasia, and hypertension.

Smooth muscle contraction is activated by an increase in cytosolic free Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_{i}\)), whereupon Ca\(^{2+}\) saturates the four Ca\(^{2+}\)-binding sites of calmodulin (1). Ca\(^{2+}\)-calmodulin activates myosin light chain kinase (MLCK), which catalyzes phosphorylation of the motor protein myosin II at Ser\(^{19}\) of its two 20-kDa regulatory light chain subunits (LC\(_{20}\)) (2). This simple phosphorylation reaction markedly increases the actin-activated MgATPase activity of myosin, which provides the energy for cross-bridge cycling and the development of force or shortening of the muscle (3). MLCK is also capable of phosphorylating LC\(_{20}\) at Thr\(^{18}\) in vitro, but this requires very high (unphysiological) concentrations of the kinase (4, 5). Relaxation follows the removal of Ca\(^{2+}\) from the cytosol, which inactivates MLCK, and myosin is diphosphorylated by myosin light chain phosphatase (MLCP), a type 1 Ser/Thr phosphatase (6).

We and others have demonstrated that smooth muscle contraction can be elicited in the absence of Ca\(^{2+}\) by treatment with inhibitors of type 1 protein phosphatases (7–19). For example, treatment of Triton-skinned rat caudal arterial smooth muscle strips with the membrane-impermeant phosphatase inhibitor microcystin in the absence of Ca\(^{2+}\) (presence of EGTA) elicited a slow, sustained contractile response that correlated with LC\(_{20}\) phosphorylation (16). Further investigation revealed that this Ca\(^{2+}\)-independent phosphorylation occurred at both Ser\(^{19}\) and Thr\(^{18}\), referred to as diphosphorylation (16). The kinase responsible was shown not to be MLCK on the basis of the following observations: (i) purified MLCK is inactive in the absence of Ca\(^{2+}\) (20–22); (ii) LC\(_{20}\) diphosphorylation requires unphysiologically high MLCK concentrations (5); (iii) MLCK inhibitors have no effect on Ca\(^{2+}\)-independent, microcystin-induced LC\(_{20}\) diphosphorylation and contraction of Triton-skinned tissue (16, 19); (iv) removal of endogenous calmodulin by treatment of Triton-skinned smooth muscle strips with the calmodulin antagonist trifluoperazine in the presence of Ca\(^{2+}\) does not affect Ca\(^{2+}\)-independent, microcystin-induced LC\(_{20}\) diphosphorylation and contraction (23); (v) the abbreviations used are: MLCK, myosin light chain kinase; ATP, adenosine 5’-O-(3-thiotriphosphate); CaM, calmodulin; ILK, integrin-linked kinase; LC\(_{20}\), the 20-kDa regulatory light chains of smooth muscle myosin II; MLCP, myosin light chain phosphatase; MYP\(_{1}\), myosin targeting subunit of MLCP; ZIPK, zipper-interacting protein kinase; TES, 2-[(2-hydroxy-1,1-bis(hydroxymethyl)ethyl)amino]ethanesulfonic acid.
endogenous LC20 in smooth muscle myofilaments is phosphorylated in the absence of Ca\(^{2+}\) at Ser\(^{19}\) or Thr\(^{18}\) alone, as well as at both sites (16), whereas purified MLCK (at high concentration) only phosphorylates Thr\(^{18}\) after Ser\(^{19}\) has been phosphorylated (4); (vi) stimuli that induce maximal activation of MLCK in smooth muscle tissues (e.g. membrane depolarization of intact vascular smooth muscle strips with an optimal KCl concentration, or addition of a maximal concentration of Ca\(^{2+}\) to permeabilized strips) induce LC20 phosphorylation exclusively at Ser\(^{19}\) (23, 24); (vii) Ca\(^{2+}\)-independent LC20 kinase activity can be separated from MLCK chromatographically (16); and (viii) the Ca\(^{2+}\)-independent LC20 kinase, unlike MLCK, does not use ATP\(_{\gamma}\)S as a substrate (this study). We purified this Ca\(^{2+}\)-independent LC20 kinase activity from chicken gizzard myofilaments and identified it as integrin-linked kinase (ILK) (17). Bacterially expressed ILK phosphorylated LC20 in intact myosin in a Ca\(^{2+}\)-independent manner (17). Approximately 50% of cellular ILK was retained in Triton-skinned smooth muscle and may be associated with MLCP because purified phosphatase preparations contain co-purifying ILK (19). It should be noted that ILK has often been described as a pseudokinase (25), but the evidence for its bona fide kinase activity is substantial (26, 27). Zipper-interacting protein kinase (ZIPK) has also been implicated in the diphosphorylation of LC20 (18, 28), although inhibition of ZIPK activity in Triton-skinned rat caudal arterial smooth muscle did not affect microcinin-induced LC20 diphosphorylation or contraction (19), suggesting that ILK is likely the responsible kinase in these conditions.

The diphosphorylation site in LC20 is highly evolutionarily conserved: the sequence around Thr\(^{18}\)–Ser\(^{19}\) (Arg-Ala-Thr-Ser-Asn-Val-Phe-Ala-Met-Phe; residues 16–25), is identical throughout the animal kingdom and is also found in a homolog of LC20 (29) in the genome of the unicellular choanoflagellate *Monosiga brevicollis* (30); choanoflagellates appear to be the closest living relatives of metazoa (30, 31). LC20 isoforms are also found in non-muscle myosin II, and contain phosphorylation sites corresponding to Thr\(^{18}\) and Ser\(^{19}\) of smooth muscle LC20 that play an important role in regulation of motility (32).

The functional effects of phosphorylation of LC20 at Ser\(^{19}\) and Thr\(^{18}\) have been investigated in vitro using purified LC20 or intact myosin as substrates at high concentrations of MLCK. Ikebe and Hartshorne (4) showed that the actin-activated MgATPase activity of diphosphorylated myosin was 2–3-fold greater than that of myosin phosphorylated exclusively at Ser\(^{19}\). This increase in actomyosin MgATPase activity can be attributed to a doubling of the *V*\(_{\text{max}}\) when both sites are phosphorylated (33–35). In the in vitro motility assay, however, myosin phosphorylated at both Ser\(^{19}\) and Thr\(^{18}\) moved actin filaments at a rate similar to myosin phosphorylated at Ser\(^{19}\) alone (35, 36).

LC20 diphosphorylation has been observed in various smooth muscle tissues treated with a variety of contractile stimuli (37–41), and several instances of diphosphorylation of LC20 have been reported in pathological conditions associated with hypercontractility (42–46). This prompted us to further investigate the functional effects of LC20 diphosphorylation in vascular smooth muscle.

**EXPERIMENTAL PROCEDURES**

**Materials**—All chemicals were analytical grade unless otherwise indicated and purchased from EMD Chemicals (Gibbstown, NJ). Triton X-100 and ATP\(_{\gamma}\)S were purchased from Sigma, microcystin-LR from Alexis Biochemicals (San Diego, CA), calyculin-A and okadaic acid from Calbiochem, and dithiothreitol (DTT) from ICN Biochemicals (Aurora, OH). Calmodulin (47) and MLCK (48) were purified from chicken gizzard as previously described. Antibodies to LC20 (polyclonal anti-pan LC20) were from Santa Cruz Biotechnology (Santa Cruz, CA) and used at 1:500 dilution; phosphospecific antibodies to LC20 phosphorylated at Ser\(^{19}\) (monoclonal anti-pS19-LC20) were from Cell Signaling (Danvers, MA) and used at 1:1,000 dilution; phosphospecific antibodies to LC20 phosphorylated at Thr\(^{18}\) (polyclonal anti-pT18-LC20) were from 21st Century Biochemicals (Marlboro, MA) and used at 1:2,000 dilution; phosphospecific antibodies to LC20 phosphorylated at both Thr\(^{18}\) and Ser\(^{19}\) (polyclonal anti-pT18,pS19-LC20) were from Cell Signaling and used at 1:500 dilution. Polyclonal phosphospecific antibodies to MYPT1 phosphorylated at Thr\(^{697}\) or Thr\(^{555}\) were purchased from Upstate USA (Charlotteville, VA) and used at 1:1,000 dilution. Polyclonal anti-actin was from Cytoskeleton Inc. (Denver, CO) and used at 1:1,000 dilution. Secondary antibodies coupled to horseradish peroxidase were purchased from Chemicon (Temecula, CA).

**Buffer Compositions**—HEPES-Tyrode (H-T) buffer contained 137 mM NaCl, 2.7 mM KCl, 1 mM MgCl\(_2\), 1.8 mM CaCl\(_2\), 5.6 mM glucose, 10 mM HEPES, pH 7.4. Ca\(^{2+}\)-free H-T buffer contained 140.6 mM NaCl, 2.7 mM KCl, 1 mM MgCl\(_2\), 5.6 mM glucose, 10 mM HEPES, pH 7.4. Buffer A contained 30 mM TES, 0.5 mM DTT, 50 mM KCl, 5 mM K\(_2\)EGTA, 150 mM sucrose, pH 7.4. pCa 9 solution contained 4 mM K\(_2\)EGTA, 5.83 mM MgCl\(_2\), 0.5 mM dithioerythritol, 20 mM TES, pH 6.9, and an ATP regenerating system composed of 3.9 mM Na\(_2\)ATP, 7.56 mM potassium propionate, 16.2 mM phosphocreatine, and 30 units/ml of creatine kinase. The free [Ca\(^{2+}\)] of this pCa 9 solution was determined to be 6 nM using fura-2. pCa 4.5 solution contained 4 mM CaEGTA, 5.66 mM MgCl\(_2\), 0.5 mM dithioerythritol, 20 mM TES, pH 6.9, and the ATP regenerating system.

**Tissue Preparation and Force Measurements**—Caudal arteries were removed from male Sprague–Dawley rats (300–350 g) that had been anesthetized with halothane and euthanized according to protocols consistent with the standards of the Canadian Council on Animal Care and approved by the University of Calgary Animal Care and Use Committee. The arteries were cleaned of excess adventitia and adipose tissue in Ca\(^{2+}\)-free H-T buffer. Segments were placed over a 0.31-mm needle and moved back and forth 40 times to remove the endothelium, cut into helical strips (1.5 × 6 mm), mounted on a Grass isometric force transducer (model FT03C) connected to a PowerLab (ADInstruments) 8-channel recording device with a resting tension of 0.45 g and incubated for 20 min in H-T buffer (bath volume = 0.8 ml). Tissues were stimulated at least twice with H-T buffer containing 87 mM KCl (the increase in [KCl] was balanced by a decrease in [NaCl]) with a 20-min interval of relaxation in Ca\(^{2+}\)-free H-T buffer. Muscle strips were then incubated in Ca\(^{2+}\)-free H-T buffer and either used
**RESULTS**

**Ca2+**-independent, Microcystin-induced LC20 Diphosphorylation and Contraction—Fig. 1A show the time course of **Ca2+**-independent contraction of Triton-skinned rat caudal arterial smooth muscle strips in response to the phosphatase inhibitor microcystin (t½ = 451.1 ± 13.4 s (n = 8)). Tissues were immersed in TCA/acetone/DTT at the indicated times during the contractile response, washed with acetone, lyophilized, and tissue proteins were extracted in SDS gel sample buffer. Phosphorylated and unphosphorylated forms of LC20 were separated by Phos-tag SDS-PAGE (49) and detected by Western blotting with anti-pan LC20, which recognizes all forms of the protein (Fig. 1B, panel a). The three separated bands were identified by Western blotting with phosphospecific antibodies to LC20 (Fig. 1B, panels b–d). In resting tissue in the absence of **Ca2+** (lane 1), only unphosphorylated LC20 was detected. Treatment with microcystin in the absence of **Ca2+** induced a time-dependent increase in mono- and diphosphorylated LC20. The monophosphorylated band contained a mixture of LC20 phosphorylated exclusively at Ser19 (Fig. 1B, panel c), identified as containing both Thr(P)18 and Ser(P)19 in Fig. 1B, panel d. The cumulative quantitative data in Fig. 1C show the time-dependent increase in mono- and diphosphorylation, and the corresponding decrease in unphosphorylated LC20 in response to microcystin in the absence of **Ca2+**.

**Ca2+**-independent, Calyculin-A-induced LC20 Diphosphorylation and Contraction—Treatment of intact rat caudal arterial smooth muscle with the membrane-permeant phosphatase inhibitor calyculin-A in **Ca2+**-free solution also induced LC20 mono- and diphosphorylation, which correlated with force development with a t½ of 1326 ± 96 s (n = 6) (Fig. 2). In this case, the amount of monophosphorylated LC20 detected was significantly less (Fig. 2C) than was observed in the Triton-skinned tissue in response to microcystin (Fig. 1C). It is also noteworthy that the steady-state force achieved in response to calyculin-A in the absence of **Ca2+** appeared to be significantly higher than the force induced by a strong depolarizing stimulus (87 mM KCl) (Fig. 2A). This prompted us to address the question: does LC20 diphosphorylation elicit more steady-state isometric force than monophosphorylation?

**KCl-induced LC20 Monophosphorylation and Contraction—**We first demonstrated that an increase in cytosolic free **Ca2+** concentration induced exclusively monophosphorylation of LC20 at Ser19. **Ca2+** entry via voltage-gated **Ca2+** channels was activated by KCl-induced membrane depolarization of intact rat caudal arterial smooth muscle strips, which induced a rapid contractile response (t½ = 10.2 ± 0.2 s (n = 29)) (Fig. 3A). Analysis of the LC20 phosphorylation time course revealed phosphorylation at Ser19 (Fig. 3B, panel b) with no phosphorylation at Thr18 (Fig. 3B, panel c) or diphosphorylation at Thr18 and Ser19 (Fig. 3B, panels a and d). LC20 phosphorylation stoichiometry peaked at ~0.6 mol of P1/mol of LC20 (Fig. 3C).

**Effects on Force and LC20 Phosphorylation of Sequential Treatment with **Ca2+** and Microcystin—**Similarly, addition of **Ca2+** to Triton-skinned rat caudal arterial smooth muscle induced phosphorylation of LC20 exclusively at Ser19 (Fig. 4G, lanes A in panels a–d) with a t½ of 151.7 ± 4.8 s (n = 23) and an LC20 phosphorylation level of ~0.5 mol of P1/mol of LC20 (Table 1). Addition of microcystin at the plateau of a **Ca2+**-induced contraction resulted in a further increase in force of ~25% (Fig. 4B and Table 2), which correlated with LC20 diphosphorylation (Fig. 4G, lanes B in panels a–d, and Table 1). If microcystin and **Ca2+** were added together, a rapid contraction occurred (t½ of 65.3 ± 2.3 s (n = 15) compared with 151.7 ± 4.8 s (n = 23) for **Ca2+** alone and 451.1 ± 13.4 s (n = 8) for microcystin at pCa 9), which was again accompanied by LC20 diphosphorylation (Fig. 4G, lanes C in panels a–d, and Table 1).
No force development or LC20 phosphorylation was observed in the absence of Ca\(^{2+}\)/H11001 and phosphatase inhibitor (Fig. 4, D and G, lanes D in panels a–d, and Table 1). If contraction was evoked by addition of microcystin in the absence of Ca\(^{2+}\)/H11001, subsequent addition of Ca\(^{2+}\)/H11001 elicited further force development (20%; Fig. 4F and Table 2) and LC20 diphosphorylation (Fig. 4G, lanes F in panels a–d, and Table 1) compared with control (Fig. 4E and G, lanes E, and Tables 1 and 2). A more detailed analysis of the (Ca\(^{2+}\)/H11001/microcystin)-induced contraction revealed rapid phosphorylation of LC20 at Ser\(^{19}\) that can be attributed to MLCK activation by Ca\(^{2+}\), and a slower rate of phosphorylation at Thr\(^{18}\), due to ILK activity that is unmasked by the phosphatase inhibitor (Fig. 5).

**Effects on Force and LC20 Phosphorylation of Combined Treatment with KCl and Calyculin-A**—Calyculin-A treatment of intact rat caudal arterial smooth muscle in the presence of extracellular Ca\(^{2+}\)/H11001 elicited a slow, sustained contraction (Fig. 6, green trace) with a \(t_{1/2}\) of 1206 ± 102 s (\(n = 6\)), which was indistinguishable from the calyculin-A-induced contraction in Ca\(^{2+}\)-free solution (\(t_{1/2} = 1326 ± 96\) s (\(n = 6\))) (Fig. 2A). Membrane depolarization in the presence of extracellular Ca\(^{2+}\)/H11001 elicited a rapid increase in force (\(t_{1/2} = 10.2 ± 0.2\) s (\(n = 29\)), which subsequently declined to a steady-state level (Figs. 3A and 6, red trace). The simultaneous application of KCl and calyculin-A in the presence of extracellular Ca\(^{2+}\)/H11001 elicited a contractile response (Fig. 6, black trace) that matched the superimposed contractions due to membrane depolarization (Fig. 6, red trace) and phosphatase inhibition (Fig. 6, green trace): the initial rapid contractile response in the presence of KCl and calyculin-A occurred with a \(t_{1/2}\) of 11.2 ± 0.6 s (\(n = 6\)), i.e. similar to the contraction induced by KCl treatment alone (\(t_{1/2} = 10.2 ± 0.2\) s (\(n = 29\)), whereas the slow, sustained contractile response occurred with a \(t_{1/2}\) of 1110 ± 84 s (\(n = 3\)), i.e. similar to the contraction induced by calyculin-A in Ca\(^{2+}\)-free solution (\(t_{1/2} = 1326 ± 96\) s (\(n = 6\))). We hypothesize that the biphasic contractile response to KCl and calyculin-A involves two distinct mechanisms: the rapid response is attributable to membrane depolarization-mediated Ca\(^{2+}\) entry and MLCK activation, and
the slow response to calyculin-A-mediated inhibition of MLCP with unmasking of Ca\(^{2+}\)-independent LC\(_{20}\) kinase activity. These mechanisms are supported by measurements of site-specific LC\(_{20}\) phosphorylation during the time course of contraction in the presence of extracellular Ca\(^{2+}\) and following addition of both KCl and calyculin-A (Fig. 7). Thus, there was a rapid initial increase in LC\(_{20}\) monophosphorylation (Fig. 7B, panel a), which occurred exclusively at Ser19 (Fig. 7B, panels b and c), followed by a slight dephosphorylation (Fig. 7C) leading to partial relaxation (Fig. 7A). It was only at prolonged incubation times that diphosphorylation of LC\(_{20}\) was observed (Fig. 7B, panels a and d), which correlated with the slow, sustained phase of contraction (Fig. 7A).

**Stoichiometric Phosphorylation of LC\(_{20}\) at Ser\(^{19}\) in Triton-skinned Tissue**—The results described above suggest that phosphorylation of LC\(_{20}\) at Thr\(^{18}\) may increase the level of force that is achieved in intact or Triton-skinned rat caudal arterial smooth muscle as a result of Ser\(^{19}\) phosphorylation. Alternatively, the observed increases in force could be due to an increase in the total level of Ser\(^{19}\) phosphorylation, rather than phosphorylation at Thr\(^{18}\). To distinguish between these possibilities, it would be necessary to achieve stoichiometric phosphorylation exclusively at Ser\(^{19}\) and then observe whether or not phosphorylation at Thr\(^{18}\) has an additional effect on steady-state force. The next step, therefore, was to achieve stoichiometric phosphorylation exclusively at Ser\(^{19}\). Unfortunately, treatment of intact tissue with an optimal KCl concentration to elicit a maximal increase in [Ca\(^{2+}\)], leading to maximal activation of MLCK, does not lead to stoichiometric phosphorylation of LC\(_{20}\) at Ser\(^{19}\) (Fig. 3). This is due to competing dephosphorylation of LC\(_{20}\) by MLCP, which is constitutively active. Likewise, in Triton-skinned tissue, addition of a maximal [Ca\(^{2+}\)] fails to elicit stoichiometric LC\(_{20}\) phosphorylation at Ser\(^{19}\) for the same reason (Fig. 4G, lane A in panel a, and Table 1). We tested the possibility that the stoichiometry of LC\(_{20}\) phosphorylation could be increased by addition of exogenous calmodu-
lin and MLCK to Triton-skinned tissue in the presence of Ca\(^{2+}\), recognizing the caveat that, if the MLCK concentration was too high, it would phosphorylate Thr\(^{18}\) as well. Whereas the addition of calmodulin in the absence or presence of MLCK did increase LC\(_{20}\) phosphorylation slightly, there remained a significant amount of unphosphorylated LC\(_{20}\), and a low level of LC\(_{20}\) diphosphorylation was observed (supplemental Fig. S1 and Table S1). This approach was, therefore, unsuitable for achieving stoichiometric phosphorylation at Ser\(^{19}\) in the absence of Thr\(^{18}\) phosphorylation.

An alternative approach to achieve stoichiometric LC\(_{20}\) phosphorylation was to use ATP\(^{32}\)S to thio-phosphorylate LC\(_{20}\): MLCK uses ATP\(^{32}\)S as a substrate (50), but the thio-phosphorylated protein is not a substrate for MLCP (51). This approach was used successfully with Triton-skinned rat caudal arterial smooth muscle (Fig. 8). Triton-skinned tissues were shown to be viable by contraction at \(pCa\ 4.5\) in the presence of ATP and an ATP regenerating system, and relaxation following removal of Ca\(^{2+}\) (Fig. 8A). Following removal of ATP, incubation with ATP\(^{32}\)S in the presence of Ca\(^{2+}\), but absence of ATP or an ATP regenerating system, resulted in stoichiometric thio-phosphorylation of LC\(_{20}\) at Ser\(^{19}\) (Fig. 8B, lanes 2 and 3) was, therefore, not accompanied by contraction (Fig. 8A). Transfer to \(pCa\ 9\) solution containing ATP and an ATP regenerating system following washout of ATP\(^{32}\)S resulted in a rapid contractile response (\(t_{1/2} = 21.2 \pm 0.2\) s (\(n = 8\))) and steady-state force corresponding to 85.4 \(\pm\) 1.9\% (\(n = 8\)) of the \(pCa\ 4.5\)-induced contraction (Fig. 8A). Once the steady-state force was achieved, microcystin was added at \(pCa\ 9\) in the presence of ATP and an ATP regenerating system. No additional force development was observed (77.3 \(\pm\) 4.2\% (\(n = 5\)) of \(pCa\ 4.5\)-induced contraction), although significant di(thio)phosphorylation of LC\(_{20}\) did occur (Fig. 8B, lanes 6 and 7, and Table 3).

The identities of the thio-phosphorylated LC\(_{20}\) species as depicted in Fig. 8B were verified by the use of phosphospecific antibodies (supplemental Fig. S2). Incubation of Triton-skinned rat caudal arterial smooth muscle strips with ATP\(^{32}\)S and microcystin at \(pCa\ 9\), in the absence of ATP and an ATP regenerating system, failed to elicit thio-phosphorylation of LC\(_{20}\) (supplemental Fig. S3, lanes 3 and 4). This is in contrast to

\[\text{ATP}}\]
incubation with ATPγS at pCa 4.5, in the absence of ATP and an ATP regenerating system, which led to LC20 monothiophosphorylation (supplemental Fig. S3, lane 2) at Ser19 (supplemental Fig. S2, lanes 3–5).

**FIGURE 4.** Effect of microcystin on Ca2⁺/H11545-induced contraction and Ca2⁺/H11545 on microcystin-induced contraction of Triton-skinned rat caudal arterial smooth muscle. A–F, Triton-skinned rat caudal arterial smooth muscle strips mounted on a force transducer in pCa 9 solution were treated as indicated. G, LC20 phosphorylation at the end of the protocols shown in A–F was analyzed by Phos-tag SDS-PAGE and Western blotting with antibodies to LC20 (panel a), Ser(P)19-LC20 (panel b), Thr(P)18-LC20 (panel c), and Thr(P)18,Ser(P)19-LC20 (panel d). Letters below the gel lanes correspond to panels A–F. Results are representative of at least 3 independent experiments.

**Effects of Diphosphorylation of LC20 on the Rates of Dephosphorylation and Relaxation**—Finally, we investigated the possibility that LC20 diphosphorylation may affect relaxation, rather than contraction, by comparing the rates of dephospho-
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rlylation and relaxation of Triton-skinned rat caudal arterial smooth muscle following monophosphorylation of LC20 at pCa 4.5 or diphosphorylation of LC20 at pCa 9 in the presence of okadaic acid. Okadaic acid was chosen as the phosphatase inhibitor for these experiments, rather than microcystin, because its effects are readily reversable (54), whereas microcystin can covalently modify the catalytic subunit of type 1 protein phosphatase, resulting in irreversible inhibition of the phosphatase (55). Indeed, we have observed that microcystin-induced contractions cannot be reversed by washout of the inhibitor (data not shown).

Comparable levels of phosphorylation of LC20 were achieved with pCa 4.5 (0.48 ± 0.02 mol of P/(mol of LC20 (n = 4)) or okadaic acid treatment at pCa 9 (0.49 ± 0.09 mol of P/(mol of LC20 (n = 5)), with monophosphorylation occurring exclusively in response to Ca2+ and both mono- and diphosphorylation being detected in the presence of okadaic acid, as expected (Fig. 9C). The steady-state force generated by okadaic acid at pCa 9 was 83.3 ± 1.4% (n = 9) of that at pCa 4.5 (supplemental Fig. S4). Relaxation was initiated by transfer to pCa 9 solution and the time courses of LC20 dephosphorylation and relaxation were quantified (Fig. 9, A and B, respectively). The rate of dephosphorylation of LC20 was markedly reduced in the tissues in which LC20 had been diphosphorylated as compared with tissues containing exclusively monophosphorylated LC20 (Fig. 9A): t1/2 values were 83.3 s for Ca2+-treated tissue and 560 s for okadaic acid-treated tissue. This correlated with a reduction in the rate of relaxation (Fig. 9B): t1/2 values were 560 s for Ca2+-treated tissue and 1293 s for okadaic acid-treated tissue. The slower rate of dephosphorylation following okadaic acid treatment cannot be explained by slow washout of the inhibitor because MYPT1-Thr697 and -Thr855 (the inhibitory phosphorylation sites in the myosin targeting subunit of MLCP) (56) were maximally dephosphorylated at the first time point analyzed during the relaxation, i.e., when force was at 90% (supplemental Fig. S5).

DISCUSSION

LC20 diphosphorylation has been observed in several smooth muscle tissues treated with various contractile stimuli, including carbachol- (37) and neurally stimulated bovine tracheal smooth muscle (38), prostaglandin-E2-stimulated rabbit thoracic aorta (39, 40), and angiotensin II-stimulated rat renal afferent arterioles (41). LC20 diphosphorylation has also been observed in pathological cases of smooth muscle hypercontractility, for example, coronary artery spasm (44, 45), cerebral vasospasm after subarachnoid hemorrhage (43, 46), and intimal hyperplasia (42). More recently, Cho et al. (57) provided evidence for enhanced Ca2+-independent LC20 diphosphorylation and force generation in β-escin-permeabilized mesenteric arterial smooth muscle. Smooth muscle rings of spontaneously hypertensive rats compared with normotensive Wistar Kyoto controls. Furthermore, phentolamine induced significant LC20 diphosphorylation in the spontaneously hypertensive rat arteries. Evidence was also presented that ZIPK contributes to the Ca2+- independent LC20 diphosphorylation through phosphorylation of MYPT1 at Thr697 and possibly direct phosphorylation of LC20, and the expression level of ZIPK, but not ILK, was greater in spontaneously hypertensive rats than Wistar Kyoto tissues (57). Collectively, these data suggest that LC20 diphosphorylation may account for the hypercontractility observed in smooth muscle tissues in response to certain contractile stimuli and in pathological situations. It was, therefore, important to determine the functional effect of LC20 phosphorylation on smooth muscle contractility. The results of these studies led to the following conclusions.

(i) Treatment of Triton-skinned rat caudal arterial smooth muscle with the phosphatase inhibitor microcystin in the absence of Ca2+ induced a slow, sustained contraction, as previously observed (16), which correlated with LC20 phosphorylation at Ser19 and Thr18 (Fig. 1).

(ii) Similar results were obtained when intact tissues were treated with the membrane-permeant phosphatase inhibitor calyculin-A in the absence of extracellular and stored Ca2+ (Fig. 2). However, an interesting difference between the Triton-skinned and intact tissues was observed: microcystin treatment of skinned tissue induced monophosphorylation at Ser19 and Thr18 at similar rates (Fig. 1B, panels b and c), in addition to diphosphorylation (Fig. 1B, panel d), whereas no monophosphorylation was observed at Thr18 following calyculin-A treatment of intact tissue in the absence of extracellular Ca2+ (Fig. 2B, panel c), but instead Ser19 monophosphorylation was followed by Thr18 phosphorylation to form the diphosphorylated species (Fig. 2B). This suggests that LC20 phosphorylation at the two sites was random in the Triton-skinned tissue experiments but sequential in the intact tissue experiments. A possible explanation would be that distinct kinases are involved in the two situations, the most likely candidates being ILK and ZIPK, and we have provided evidence that ILK is responsible for

| Conditions | Force (%) | n |
|------------|-----------|---|
| pCa 4.5/pCa 4.5 | 105.1 ± 1.4 | 4 |
| pCa 4.5/MC, pCa 4.5 | 124.5 ± 2.2 | 5 |
| MC, pCa 4.5/MC, pCa 4.5 | 106.8 ± 0.3 | 4 |
| MC, pCa 9/MC, pCa 9 | 106.1 ± 1.8 | 4 |
| MC, pCa 9/MC, pCa 4.5 | 1214.3 ± 30 | 5 |
(i) Microcystin-induced Ca\(^{2+}\)/H\(_{11001}\)-independent contraction of Triton-skinned rat caudal arterial smooth muscle (19).

(ii) The level of steady-state force induced by calyculin-A in the absence of Ca\(^{2+}\)/H\(_{11001}\) is significantly greater than that induced by a maximally effective concentration of KCl, i.e. an optimal Ca\(^{2+}\) signal (Fig. 2A). This would be consistent with diphosphorylation of LC\(_{20}\) increasing steady-state force compared with Ser19 monophosphorylation. Indeed, addition of microcystin to Triton-skinned tissue pre-contracted at pCa 4.5 (Fig. 4B), or of Ca\(^{2+}\)/H\(_{11001}\) to tissue pre-contracted with microcystin in the absence of Ca\(^{2+}\)/H\(_{11001}\) (Fig. 4F), evoked a significant increase in steady-state force (Table 2), which correlated with increases in LC\(_{20}\) diphosphorylation (Fig. 4G and Table 1). However, Ser19 phosphorylation stoichiometry also increased under these conditions (from ~0.5 mol of P\(_i\)/mol of LC\(_{20}\) to ~1 mol of P\(_i\)/mol of LC\(_{20}\)) (Table 1), suggesting that the enhanced force responses could be due to increased phosphorylation at Ser19 (whether in the form of monophosphorylated or diphosphorylated LC\(_{20}\)).

(iii) In intact (Fig. 3) and Triton-skinned tissue (Fig. 4A and G), Ca\(^{2+}\) elicited exclusively monophosphorylation of LC\(_{20}\) at Ser19, as expected.

(iv) The fact that the rate of contraction of Triton-skinned rat caudal arterial smooth muscle in response to Ca\(^{2+}\) was signifi-
contraction correlated with Ser\(^{19}\) phosphorylation, and the slow sustained contractile response with the diphosphorylation of LC\(_{20}\) (Fig. 7). The contractile effects of KCl and calycin-A, however, could be explained entirely by Ser\(^{19}\) phosphorylation.

It was necessary, therefore, to devise a way to achieve stoichiometric phosphorylation at Ser\(^{19}\) without Thr\(^{18}\) phosphorylation, and then observe whether subsequent phosphorylation at Thr\(^{18}\) has an effect on steady-state force development. This was achieved by using ATP\(_{S}\) to evoke close-to-stoichiometric phosphorylation at Ser\(^{19}\) with very little dithiophosphorylation (Fig. 8A and Table 3). Subsequent phosphorylation of LC\(_{20}\) at Thr\(^{18}\) (Fig. 8B) failed to elicit an increase in force (Fig. 8A). We conclude, therefore, that phosphorylation at Ser\(^{19}\) of LC\(_{20}\) accounts for maximal force development, and no further force results from additional phosphorylation at Thr\(^{18}\).

We then turned our attention to the possibility that diphosphorylation may affect relaxation rather than contraction by comparing the time courses of diphosphorylation of LC\(_{20}\) and...
relaxation of Triton-skinned muscle strips that had been pre-contracted under conditions that evoked phosphorylation exclusively at Ser19 or at both Ser19 and Thr18 to the same overall phosphorylation stoichiometry. The rates of dephosphorylation and relaxation were significantly slower in the case of diphosphorylated LC20 (Fig. 9). We conclude, therefore, that diphosphorylation of LC20 at Thr18 and Ser19 has a marked effect on relaxation compared with monophosphorylation at Ser19.

The mechanism underlying the reduction in the rate of dephosphorylation of diphosphorylated LC20 compared with Ser19-monophosphorylated LC20 remains to be determined. A possibility is that the $K_m$ of MLCP for diphosphorylated LC20 may be significantly higher than that for LC20 phosphorylated exclusively at Ser19. Although such kinetic comparisons have not been performed to date, in vitro assays indicated that dephosphorylation of diphosphorylated LC20 (whether free or in intact myosin) occurred by a random mechanism, with dephosphorylation at Ser19 and Thr18 occurring at similar rates (5).

The principal conclusions from this study are: (i) the level of steady-state force is dictated by the level of Ser19 phosphorylation and is unaffected by Thr18 phosphorylation; and (ii) Thr18 phosphorylation reduces the rate of LC20 dephosphorylation and relaxation, supporting a sustained contractile response. There is abundant literature indicating that most contractile stimuli elicit phosphorylation exclusively at Ser19 and this can be explained by Ca$^{2+}$-induced activation of MLCK, with or without a modest degree of Ca$^{2+}$ sensitization due to MLCP inhibition (58). Specific stimuli and pathophysiological situations associated with hypercontractility induce LC20 diphosphorylation at Thr18 and Ser19. This can be explained by

### TABLE 3

| Conditions                      | 0P       | 1SP      | 1P       | 1SP1P    | 2P       |
|--------------------------------|----------|----------|----------|----------|----------|
| ATP$_7$, pCa 4.5, no RS         | 6.0 ± 2.5$^+$ | 88.4 ± 4.8$^+$ | 0        | 0        | 5.6 ± 4.3$^-$ |
| Then pCa 9, no RS               | 11.1 ± 3.4$^+$ | 81.6 ± 3.9$^+$ | 0        | 0        | 7.2 ± 2.7$^+$ |
| Then pCa 9, RS                  | 16.6 ± 4.7$^+$ | 80.0 ± 4.4$^+$ | 0        | 0        | 3.4 ± 1.9$^+$ |
| Then MC, pCa 9, RS              | 13.9 ± 3.2  | 54.7 ± 4.0  | 3.4 ± 2.1 | 14.0 ± 9.2 | 13.9 ± 8.6 |

Values represent percentage of total LC$_{20}$ ± S.E. (n = 3). $^+$, $^-$, and $^\wedge$ indicate values are not statistically significantly different from each other; 0P, unphosphorylated LC$_{20}$; 1SP, monophosphorylated LC$_{20}$; 1P, monophosphorylated LC$_{20}$; 1SP1P, LC$_{20}$, thiophosphorylated at one site and phosphorylated at the other; 2P, diphosphorylated LC$_{20}$; RS, ATP regenerating system; MC, microcystin.
increased MLCP inhibition, unmasking constitutive Ca$^{2+}$-independent LC$_{20}$ kinase activity (ILK and/or ZIPK), and potentially an increase in activity of Ca$^{2+}$-independent LC$_{20}$ kinases, leading to an increase in Ser$^{19}$ phosphorylation (force) and Thr$^{18}$ phosphorylation (sustained contraction). ILK and ZIPK are therefore potential therapeutic targets for the treatment of cerebral and coronary vasospasm, intimal hyperplasia, hypertension, and other conditions associated with hypercontractility.

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