Site-specific Phosphorylation and Point Mutations of Telokin Modulate Its Ca\textsuperscript{2+}-desensitizing Effect in Smooth Muscle*  

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Forskolin and 8-bromoguanosine 3’-5’-cyclic monophosphate (8-Br-cGMP) induce phosphorylation of Ser-13 of telokin and relaxation of smooth muscle at constant calcium. Comparison with the effect of wild type with aspartate (D; to mimic phosphorylation) and alanine (A; non-phosphorylatable) mutants of telokin showed that the S13D mutant was more effective than wild type in relaxing smooth muscle at constant calcium. The efficacy of the Ser-13A, Ser-12A, and Ser-12D mutants was not significantly different from that of wild-type telokin. The effect of neither S13D nor Ser-13A was affected by 8-Br-cGMP, whereas the effect of wild type, S12A, and S12D was enhanced by 8-Br-cGMP, indicating the specificity of Ser-13 charge modification. Mutation of Ser-19 (a mitogen-activated protein kinase site) showed the S19A to be more effective than, and S19D to be not different from, wild-type telokin. The effect of both mutants was slightly enhanced by 8-Br-cGMP. A truncated (residues 1–142) form lacking the acidic C terminus had the same relaxant effect as wild-type telokin, whereas the C-terminal peptide (residues 142–155) had no effect. We conclude that site-specific modification of the N terminus modulates the Ca\textsuperscript{2+}-desensitizing effect of telokin on force.

Contraction and relaxation of smooth muscle are dependent mainly upon the intracellular free Ca\textsuperscript{2+} concentration ([Ca\textsuperscript{2+}]). The magnitude of contractile force developed depends on the level of myosin regulatory light chain (MLC\textsubscript{20}) phosphorylation that, in turn, is determined by the relative activities of myosin light chain kinase (MLCK) and smooth muscle myosin light chain phosphatase (SMPP-1M). The Ca\textsuperscript{2+} sensitivity of contraction can be affected by any change in kinase and/or phosphatase activities that alters this ratio. At a fixed [Ca\textsuperscript{2+]i}, both MLCK and SMPP-1M can be modulated by second messenger-mediated signaling pathways, resulting in a change in MLC\textsubscript{20} phosphorylation and, consequently, in an increase or decrease in force developed (reviewed in Refs. 1 and 2). The Ca\textsuperscript{2+} sensitivity of the contractile apparatus can be regulated by a number of agents such as RhoA (3–5), cyclic nucleotides (6, 7), arachidonic acid (8), and diacylglycerol (8). Dephosphorylation of the regulatory myosin light chains by SMPP-1M can be inhibited by a G-protein-coupled pathway that can increase the level of MLC\textsubscript{20} phosphorylation, with no change in cytosolic calcium (Ca\textsuperscript{2+} sensitization) (1, 9, 10), and we suggested that the reverse mechanism, activation of SMPP-1M, could lead to Ca\textsuperscript{2+} desensitization. Stimulation of permeabilized smooth muscle with 8-Br-cGMP relaxes submaximal Ca\textsuperscript{2+}-induced contractions and can also reverse G-protein-coupled inhibition of SMPP-1M (6), suggestive of a mechanism mediated by activation of SMPP-1M.

Telokin, also known as kinase-related protein, is a low molecular mass (17 kDa) protein whose sequence is identical to the C terminus of MLCK (11) and is independently expressed at high concentrations in some smooth muscles (12) through a promoter located within an intron of the MLCK gene (13). Telokin binds to the S1/S2 region of unphosphorylated smooth muscle myosin (14, 15) and prevents the folding of the 6 S myosin into 10 S conformation, thus stabilizing filamentous myosin in solution (14). Stabilization of unphosphorylated myosin filaments has been suggested to be a physiological function of telokin in smooth muscle (14), and its acidic C terminus was believed necessary for high affinity binding to myosin and filament stabilization. Furthermore, it has been shown (16) that there are at least 6 C-terminal variants of telokin, differing from each other in the number of C-terminal glutamates (from 0–5), and it was suggested that the heterogeneity of the C terminus may play an important role in the regulation of macromolecular protein complexes at the structural level (16). A linearly related loss of 8-Br-cGMP-induced relaxation and an enhanced responsiveness to exogenously added native and recombinant telokin that can relax permeabilized smooth muscle and reduce MLC\textsubscript{20} phosphorylation (17). The relaxant effect of telokin is potentiated by 8-Br-cGMP and cyclic GMP-dependent kinase (PKG), and we suggested that phosphorylation of telokin by PKG (or cAMP-dependent protein kinase) modulates desensitization of smooth muscle to calcium, as telokin is the major cytosolic protein phosphorylated during cGMP-induced relaxation and dephosphorylation of MLC\textsubscript{20} (17). Most recently, we have mapped the in vivo site of phosphorylation of mammalian telokin to Ser-13 in intact, forskolin-stimulated and permeabilized, 8-Br-cGMP-stimulated rabbit ileum (18).
Therefore, to determine whether Ser-13 is also the functionally relevant site of telokin phosphorylation, we have produced mutant telokin with replacement of Ser-13, Ser-12, and Ser-19 with either an aspartate or alanine to determine their respective contributions to calcium-independent effects on force and MLC20 phosphorylation. Additionally, we have expressed a telokin mutant lacking the acidic C terminus to examine the other proposed functions (14, 19, 20) of telokin.

EXPERIMENTAL PROCEDURES

Tissue Preparation, Force Measurement, and Electron Microscopy—Longitudinal ileal smooth muscle was removed from rabbits anesthetized with halothane and killed by exsanguination according to approved animal protocols. Small strips (250 μm × 2 mm) were dissected and permeabilized with Triton X-100 (0.1%) in a Ca2+-free solution containing 1 mM EGTA (G1) for 30 min. For storage, muscle strips were washed with relaxing solution containing 10 mM EGTA and 40 mM reduced glutathione, placed in the same relaxing solution containing 50% glycerol, and stored at −20 °C. Details of the solutions used for studies of permeabilized smooth muscle strips, storage of muscles, and the time course of depletion of endogenous telokin have been published (17, 21). For force measurement, strips were removed from the freezer, washed briefly in G1 solution, and tied with silk monofilaments to the tips of two fine wires. One wire was fixed, and the other was connected to a force transducer (SensNor, AES01). The strip was mounted in a well on a bubble plate to allow rapid solution exchange and freezing. Strips were stretched to 1.5× resting length. All experiments were carried out at room temperature. The telokin content of paired strips was determined by gel electrophoresis and Western blotting.

For in situ labeling of exogenously added telokin, permeabilized, telokin-depleted strips were mounted in a bubble chamber, contracted with submaximal calcium, washed briefly in calcium-free solution containing 0.5 mM ATP, and then loaded for 10 min in the same solution with ϕ-32P]ATP (5 μCi/ml). After loading, strips were relaxed by the addition of either wild-type or mutant (10 μM) telokin in the presence or absence of PKG and PKA. After 10 min, strips were rapidly frozen and homogenized in Laemmli sample buffer and separated by 15% SDS-PAGE. Separated proteins were transferred to polyvinylidene fluoride membranes, air-dried, and placed on film for autoradiography. After autoradiography, membranes were re-wetted and immunoblotted for telokin.

For electron microscopy, strips of ileal smooth muscle were prepared, prior to fixation, as described above, fixed overnight in 2% glutaraldehyde plus 0.1% tannic acid followed by osmium, stained with uranyl acetate en bloc, and dehydrated and embedded in Spurr’s resin. Non-permeabilized strips served as controls.

Construction of Recombinant Telokin Mutants—Rabbit telokin cDNA was obtained as a generous gift from Dr. Paul Herring of Indiana University. A gel purified reading frame amplified by polymerase chain reaction (22) using the 5′ primer, GACTccatggTCTCAGGGCT-, and the 3′ primer, −3CATGAAATTTTTGCTGTCG, which introduces a NcoI site (lowercase letter), was cloned into pGEX Universal (23) from Calbiochem. Unless noted, all other reagents were from Sigma.

RESULTS

Relaxation of Ca2+-induced Tension by Wild-type (WT) and Ser-13 Mutant Recombinant Telokins—As previously shown (17), recombinant WT telokin relaxed telokin-depleted rabbit ileal smooth muscle in a dose-dependent manner. In the absence of 8-Br-cGMP, 10 μM WT telokin caused 35% ± 5.6% relaxation of a submaximal calcium (pCa 6.5)-induced contraction (Fig. 1), and 20 μM WT telokin relaxed by 49% ± 6.9% (not shown). Preincubation with 8-Br-cGMP for 5 min potentiated the effect of WT telokin and increased the relaxation evoked by 10 μM WT telokin to 59% ± 5.5% (Fig. 1) and 20 μM to 80% ± 2.3% that of the contractile response to submaximal calcium. 10 μM S13D mutant telokin (which mimics the negative charge on phosphorylated telokin) relaxed telokin-depleted ileum to 55% ± 5.9% (Fig. 1) and 20 μM S13D telokin by 76% ± 8.7% (not shown); 8-Br-cGMP did not significantly change the relaxation evoked by S13D telokin at any concentration (Fig. 1). 10 μM Ser-13A telokin (to block phosphorylation of Ser-13) relaxed telokin-depleted ileum to 28% ± 2.4% (Fig. 1) and was not affected by preincubation with 8-Br-cGMP.
Relaxation by Ser-12 Mutants—To determine whether phosphorylation of the residue adjacent to Ser-13, Ser-12 (also revealed by in situ phosphorylation of the S13D/S19A double mutant, see below) influenced the relaxant activity of telokin, we constructed both the S12A and S12D mutants. In the absence of 8-Br-cGMP, the S12A mutant relaxed submaximally contracted permeabilized ileum by 26.3 ± 1.8%, and its effect was potentiated to 48.7 ± 1.8% by 8-Br-cGMP (Fig. 2). Similarly, the S12D mutant, in the absence of 8-Br-cGMP, relaxed calcium-stimulated smooth muscle by 28.8 ± 1.2% and was potentiated to 45.2 ± 2.2% (Fig. 2). These results are not significantly different from those of WT, suggesting that Ser-12 is not a physiologically relevant phosphorylation site in the absence of other (i.e. Ser-13) phosphorylation.

The Effect of the Ser-19 Mutation and in Situ Phosphorylation of S13D/S19A Telokin—A telokin mutant with a mutation at the presumptive mitogen-activated protein kinase site, S19A, resulted in a 53 ± 5.6% relaxation of telokin-depleted strips at 10 μM (Fig. 1), and the S19D mutant induced in only 20.9 ± 9.2% relaxation, suggesting that phosphorylation of Ser-19 may indeed exert a physiological (inhibition of relaxation) effect. Therefore, we constructed a double mutant with an Asp at position 13 and an Ala at position 19 (S13D/S19A). 10 μM S13D/S19A relaxed submaximally contracted ileum by 37 ± 5.4%, not significantly different from WT. Surprisingly, 8-Br-cGMP and PKG catalytic subunit increased the relaxation evoked by this double mutant (10 μM) to 71.2 ± 2% (n = 4) (Fig. 1), suggesting either a separate additional site of phosphorylation of the S13D/S19A telokin or the contribution of another phosphoprotein that, most likely, can only interact with telokin that is not phosphorylated at Ser-19.

To determine whether potentiation of the relaxant effect of S13D/S19A telokin by 8-Br-cGMP was related to phosphorylation of this mutant at a site other than Ser-13 or 19, we examined the in situ phosphorylation of the S13D/S19A mutant. In the absence of exogenously added telokin, phosphorylation of the small amount of endogenous telokin that remains in these strips even after storage (<15%), was detectable both in the presence and absence of 8-Br-cGMP. However, the lack of an increase in phosphate incorporation into endogenous telokin in the presence of 8-Br-cGMP (Fig. 3) suggests that this remaining telokin is not a good substrate for PKG or is already fully phosphorylated. Exogenously added WT, S19A, S19D, and S13D/S19A telokin were further phosphorylated in the presence of 8-Br-cGMP (Fig. 3), suggesting that indeed, the S13D/S19A double mutant could be phosphorylated at yet another site.

To identify the site of phosphorylation of the S13D/S19A telokin, the recombinant double mutant was phosphorylated in

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**Fig. 1.** Summary of relaxation induced by wild-type and Ser-13 and Ser-19 mutant telokins in the absence and presence of 8-Br-cGMP. Stored, telokin-depleted rabbit ileum strips were contracted with pCa 6.3 and then 10 μM telokin, either WT or mutant was added, and strips were allowed to relax for 10 min. Alternatively, calcium-contracted muscle was incubated with 50 μM 8-Br-cGMP plus 2500 units of PKG for 5 min before relaxation with telokin. Relaxation by 8-Br-cGMP by itself (no added telokin) was less than 5%. Percent relaxation is calculated from the plateau of the pCa 6.3 contraction. n = at least four for each mutant.

**Fig. 2.** Summary of relaxation induced by Ser-12 telokin mutants in the absence and presence of 8-Br-cGMP. Stored, telokin-depleted rabbit ileum strips were contracted with pCa 6.3, and then 10 μM S12A, S12D, S12A/S13D/S19A, or S12D/S13D/S19A was added, and strips were allowed to relax for 10 min. Alternatively, calcium-contracted muscles were incubated with 50 μM 8-Br-cGMP plus 2500 units of PKG for 5 min before relaxation with telokin. Relaxation by 8-Br-cGMP by itself (no added telokin) was less than 5%. Percent relaxation is calculated from the plateau of the pCa 6.3 contraction. n = at least four for each mutant.

**Fig. 3.** Autoradiograms showing in situ labeling of exogenously added telokin mutants in the presence and absence of 8-Br-cGMP. Telokin-depleted strips were contracted with pCa 6.3 for 10 min, washed briefly in pCa 6.3 containing 0.5 mM ATP, and incubated for 10 min in the same solution containing [γ-32P]ATP (5 mCi/ml). After loading, strips were relaxed by the addition of either wild-type or mutant (10 μM) telokin in the presence or absence of 8-Br-cGMP and PKG. After homogenization, proteins were separated by 15% SDS-polyacrylamide gel electrophoresis and transferred to polyvinylidene fluoride membranes, air-dried, and placed on film for autoradiography. Bottom panel, lanes 9 and 10 show phosphorylation of the endogenous telokin (<10%) that remains after skinning and storage of smooth muscle.
interfere with subsequent relaxation induced by 10 μM Ca\(^{2+}\) in Triton-skinned smooth muscles (28) and/or the presence of 50 μM 8-Br-cGMP and the mutant protein (Fig. 3). However, we were able to phosphorylate, in vitro, both mutants (on serine 28) with cAMP-dependent protein kinase or PKG catalytic subunit.

**MLC\(_{20}\) Phosphorylation Decreases with Telokin-induced, Ca\(^{2+}\)-independent Relaxation**—As shown in Fig. 1, WT and S13D telokin (10 μM) induced significant relaxation of stored, telokin-depleted ileum smooth muscles. Therefore, we examined the phosphorylation of the regulatory light chains of myosin and found telokin-induced relaxation to be associated with a proportional decrease in MLC\(_{20}\) phosphorylation. WT-telokin induced a decrease in phosphorylation from 60% in control tissues (at the plateau of submaximal calcium contraction) to 42%, and S13D telokin induced a decrease in MLC\(_{20}\) phosphorylation to 26% after 10 min. Resting phosphorylation in the skinned, stored ileum muscles in the absence of calcium was ~22%. This high level of phosphorylation in the absence of calcium may be due to a decrease in endogenous phosphatase activity in Triton-skinned smooth muscles (28) and/or the presence of a Ca\(^{2+}\)-independent kinase activity.

The Acidic Polyglutamate Tail of Telokin Is Neither Sufficient Nor Necessary for Telokin-induced Relaxation of Smooth Muscle—Polyanionic as well as polybasic peptides can modulate PP-1C activity in vitro on certain substrates (29, 30), and it has been suggested (20) that the acid-rich C-terminal region, corresponding to residues Gly-138–Glu-150, is the primary site of interaction between telokin and myosin. To test whether the polyglutamate C-terminal tail of telokin had similar relaxant properties as the protein itself (i.e. through either a direct effect on the phosphatase or through competitive binding to myosin), we synthesized a 14-amino acid peptide corresponding to the C terminus (residues 142–155) of rabbit telokin (MEEGEGEEEEE). The addition of this acidic C-terminal peptide (20 μM) of telokin did not relax submaximal calcium-induced contractions of rabbit ileum (n = 3, data not shown) nor did it interfere with subsequent relaxation induced by 10 μM telokin (n = 3, data not shown).

In contrast, a truncated telokin without the acidic C terminus relaxed telokin-depleted ileum at constant calcium in a dose-dependent manner, with no significant difference from wild type; 10 μM truncated telokin relaxed a submaximal calcium contraction by 32.0% and was potentiated to 52.1% in the presence of 50 μM 8-Br-cGMP, and 20 μM C-terminally truncated-telokin relaxed 48.2 ± 6.2% in the absence of 8-Br-cGMP (Fig. 4) and was potentiated to 72.0 ± 3.0%.

**Microcystin-induced Contraction Is Unchanged by Telokin**—Since MLC\(_{20}\) phosphorylation is regulated by both SMPP-1 and MLCK, we tested the remote possibility that telokin was inhibiting MLCK (31), although it does not inhibit the rate of MLC\(_{20}\) thio-phosphorylation (17). Stored telokin-depleted rabbit ileum strips were incubated in G1 solution ±10 μM WT telokin for 10 min. Microcystin (5 μM) was added to irreversibly inhibit SMPP-1M. After 5 min, the muscle was stimulated with submaximal calcium. Preincubation of stored rabbit ileum with 10 μM telokin did not slow the rate nor change the amplitude of calcium-induced contraction in the presence of microcystin used to irreversibly inhibit SMPP-1 (Fig. 5). Had telokin inhibited MLCK, it would be expected to affect the rate and/or magnitude of calcium-evoked contraction, even in the presence of a phosphatase inhibitor.

**Triton-skinned, Telokin-depleted Ileum Smooth Muscle Retains Normal Filament Arrays**—It has been suggested that one major role of telokin is stabilization of myosin filaments in vivo (14); therefore, we examined the filament arrays in control and telokin-depleted, Triton-skinned smooth muscles. Rabbit ileum longitudinal smooth muscle was permeabilized with 0.1% Triton X-100 as described under “Experimental Procedures.” Western blots for telokin showed that more than 90% of the telokin was removed within 20 min in Triton X-100. Electron microscopy of thin cross-sections of skinned, telokin-depleted ileum strips shows a normal number and array of thick myosin filaments surrounded by thin actin filaments and of dense bodies (Fig. 6) when compared with paired, nonpermeabilized preparations. Discontinuity in the plasma membrane and organelle damage were consistent with the permeabilizing effects of the detergent.

**DISCUSSION**

Our major findings show that 1) mutation of the in vivo cAMP and/or cGMP kinase site of phosphorylation of telokin (Ser-13) to an Asp enhances its ability to relax smooth muscles, whereas 2) mutation of Ser-13 to Ala does not affect its relaxant effect; 3) cGMP does not potentiate the relaxant activity of either (S13D or S13A) mutant; 4) mutation of the Ser-12 to either an Asp or an Ala does not change the activity of telokin;
and 5) the acidic C-terminal peptide of telokin alone does not mimic the relaxant effect of telokin, whereas the C-terminally truncated mutant is as effective as WT telokin in evoking relaxation of smooth muscle. We also show that depletion of endogenous telokin does not lead to the loss or disorganization of myosin filaments. Taken together, these data suggest that Ser-13 is indeed a relevant in vivo phosphorylation site associated with cyclic nucleotide-induced, Ca\(^{2+}\)-independent relaxation and that the acidic C terminus of telokin is neither necessary nor sufficient for its relaxant effect.

A double mutation of Ser-13 to Asp to mimic phosphorylation and Ser-19, a putative mitogen-activated protein kinase site, to a (non-phosphorylatable) Ala, revealed an alternative in situ phosphorylation site, but the physiological role of phosphorylation of Ser-19, if any, is less clear. The present results are consistent with our previous suggestion (17) that cyclic nucleotide-induced relaxation is mediated at least in part by phosphorylation of telokin and increased activity of myosin light chain phosphatase (17). The Ser residue at position 19 (Ser-19) that is a potential in vivo site of phosphorylation by mitogen-activated protein kinase (17) as expected did not show an increase in phosphorylation when intact rabbit ileal smooth muscle was stimulated with either forskolin or when permeabilized ileal muscle was stimulated with 8-Br-cGMP (18). Nevertheless, mutations of Ser-19 to either an Asp or an Ala are consistent with phosphorylation of this site being a potent inhibitory site (even though the S19D mutant can be substantially phosphorylated (Fig. 3), presumably on Ser-13 or Ser-12). Furthermore, it has recently been shown (32) that stimulation of either chicken carotid arterial smooth muscle or chicken gizzard with phorbol ester results in an increase in phosphorylation of telokin at an as yet unidentified site but that only carotid arterial smooth muscle (and not gizzard) contracts when stimulated with phorbol ester. These data further suggest differences in the regulation of tonic and phasic smooth muscle. Unlike phosphorylation of Ser-13, it remains to be shown whether Ser-19 phosphorylation is relevant in the presence of physiological concentrations of cGMP in intact muscles or if telokin may be a target for other signaling pathways.

We should consider the functional effects of the various mutations of telokin within the context of the native telokin structure, a compact immunoglobulin fold bordered by an extended N-terminal 32 amino acid and C-terminal 19 amino acid sequence. Neither the N nor the C terminus is resolved in either the original crystal structure at 2.8 Å (33) or a more recent refinement at 1.9-Å resolution (Protein Data Bank code 1FHG), presumably as the result of flexibility of these regions. The fact that the highly acidic C terminus is neither sufficient nor necessary for the Ca\(^{2+}\)-independent relaxant effect of telokin does not exclude the possible importance of electrostatic interactions being involved in such activity, because the truncated structure is also acidic (calculated P\(_i\), 4.37), with a significant solvent-exposed acidic surface (33). Truncation of the N terminus that contains the phosphorylation sites does not eliminate the relaxant effect of telokin (17), although it abolishes its potentiation by 8-Br-cGMP. Mutations involving charge modification of exposed acidic residues of the immunoglobulin fold may be useful in elucidating the main structural requirement for the relaxant activity of telokin. Furthermore, circular dichroism studies on the various telokin mutants revealed changes in the secondary structure, as suggested by a slight increase in the \(\alpha\)-helical content. That is, the WT, S13D, S13A,
and S19D telokin mutants contained no α-helix detectable by circular dichroism,2 but mutation of Ser-19 to an alanine increased the predicted α-helical content to ~25%. Although it is unlikely that a single mutation changes the overall structure so dramatically, it is very likely that mutation of the Ser-19 and further mutations (the double and triple mutants) changed the native structure, allowing further potentiation by 8-Br-cGMP and PKG perhaps via an interaction with the phosphatase, myosin, or another protein. Therefore, we are reluctant to assign a physiological function to the effects of multiple mutations.

In the presence of a phosphatase inhibitor, preincubation of relaxed, telokin-depleted ileal strips with 10 μM wild-type telokin did not change either the rate or amplitude of calcium-stimulated contraction, supporting the conclusion (17) that telokin acts most likely through direct or indirect activation of the phosphatase rather than inhibition of myosin light chain kinase (31). However, we have thus far been unable to demonstrate in vitro a direct effect of telokin on phosphatase activity (18). This may reflect the inability to recapitulate the correct structural relationships between myosin filaments, phosphatase regulatory, and catalytic subunits, and telokin. It is also possible that another protein(s) is involved in the cascade between phosphorylation of telokin and relaxation (through activation of the phosphatase) of smooth muscle.

A variety of proteins in addition to telokin are phosphorylated during cyclic nucleotide-evoked relaxation including but not limited to HSP 20 and HSP 27 (35, 36), SM-223 and calmodulin.3 The possibility that one or more of these proteins are acting in conjunction with telokin to activate the myosin phosphatase is currently under investigation. However, we know of no direct evidence to show that these proteins, acting either alone or with telokin, can activate SMPP-1M. Since exogenous telokin is active in Triton-treated preparations, membrane proteins phosphorylated by cyclic nucleotide-activated kinases (34, 37, 38) are unlikely to be relevant to telokin activity.

The hypothesis that the physiological role of telokin is to stabilize unphosphorylated myosin filaments through binding via its acidic tail to myosin (14, 20) is not consistent with the normal myosin filament arrays (Fig. 6) in tissues skinned with Triton X-100 and depleted of endogenous telokin (17) and with the lack of effect of removal of the acidic C terminus on the relaxant effect of telokin (this study). We cannot, however, rule out the possibility that in vivo telokin contributes to the structural and functional interactions between MLCK, SMPP-1M, myosin, and actin because in our preparations there was a small (<10%) amount of telokin that remained after skinning. It is unlikely, though, in view of the very low stoichiometry of remaining telokin to myosin (perhaps less than 5 μM versus ~52 μM), that the small amount of telokin remaining in permeabilized preparations is required for filament stability. In conclusion, we present evidence (through point mutations) that demonstrates the importance of phosphorylation of the Ser-13 residue of telokin in cyclic nucleotide-induced dephosphorylation of MLC20 and relaxation of smooth muscle.

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