Phosphorylation of telokin by cyclic nucleotide kinases and the identification of in vivo phosphorylation sites in smooth muscle

Justin A. MacDonald[a,1], Lori A. Walker[b,1], Robert K. Nakamoto[b], Isabelle Gorenne[b], Avril V. Somlyo[b], Andrew P. Somlyo[b], Timothy A.J. Haystead[a,*]

[a] *Department of Pharmacology and Cancer Biology, Duke University Medical Center, Durham, NC 27710, USA*
[b] *Department of Molecular Physiology and Biological Physics, University of Virginia, Charlottesville, VA 22908, USA*

Received 28 June 2000; accepted 4 July 2000

Edited by Shmuel Shaltiel

Abstract The Ca^{2+}-independent acceleration of dephosphorylation of the regulatory light chain of smooth muscle myosin and relaxation of smooth muscle by telokin are enhanced by cyclic nucleotide-activated protein kinase(s) [Wu et al. (1998) J. Biol. Chem. 273, 11362–11369]. The purpose of this study was to determine the in vivo site(s) and in vitro rates of telokin phosphorylation and to evaluate the possible effects of sequential phosphorylation by different kinases. The in vivo site(s) of phosphorylation of telokin were determined in rabbit smooth muscles of longitudinal ileum and portal vein. Following stimulation of ileum with forskolin (20 μM) the serine at position 13 was the only amino acid to exhibit increased phosphorylation. Rabbit portal vein telokin was phosphorylated on both Ser-13 and -19 as a result of forskolin and GTPγS stimulation in vivo. Point mutation of Ser-13 (to Ala or Asp) abolished in vitro phosphorylation by cyclic nucleotide-dependent protein kinases.

Key words: Telokin; Smooth muscle; Phosphorylation; Ca^{2+}-desensitization

1. Introduction

The major mechanism of activating smooth muscle contraction is through changes in $[Ca^{2+}]_i$ and the consequent phosphorylation of the regulatory myosin light chain (MLC_{20}) by the Ca^{2+}-calmodulin dependent myosin light chain kinase (MLCK) [1,2]. MLC_{20} phosphorylation and contractile force can also be increased by 'Ca^{2+} sensitization' through a G-protein coupled, Ca^{2+} independent inhibition of smooth muscle myosin phosphatase [3]. Phosphorylation of site A of MLCK by calmodulin-dependent protein kinase II (CaMKII) in vivo can reduce MLCK's affinity for Ca^{2+}-calmodulin and leads to 'Ca^{2+}-desensitization' [4]. Ca^{2+}-desensitization is manifested as a decline in MLC_{20} phosphorylation and force in the absence of proportional, or any, decline in $[Ca^{2+}]$ [5]. Another physiologically significant pathway of Ca^{2+}-desensitization is mediated by the activation of cyclic nucleotide-dependent protein kinases.

Several hormones and clinically administered nitrovasodilators (such as atrial natriuretic peptide, endothelium-derived relaxing factor (nitric oxide), and exogenous vasodilators) exert their actions to widen vessels and inhibit vasoconstriction through activation of smooth muscle guanylate cyclase to elevate cGMP levels (for a review see [6–8]). One of the effects of elevated intracellular cGMP is the reduction of $[Ca^{2+}]_i$. The mechanisms by which cGMP-dependent protein kinase (PKG) and smooth muscle relaxants that increase intracellular cGMP lower intracellular Ca^{2+} are numerous and have been well characterized [8]. However, elevated intracellular [cGMP], presumably through the activation of PKG, or cAMP-dependent protein kinase (PKA), can also cause Ca^{2+} desensitization. This can be induced experimentally by treatment of permeabilized smooth muscle with 8-Br-cGMP at fixed $[Ca^{2+}]$, reversing G-protein coupled Ca^{2+}-sensitization and accelerating relaxation and dephosphorylation of MLC_{20} at constant $[Ca^{2+}]$ [9]. Importantly, the effect is mimicked by the addition of exogenous PKG or PKA to permeabilized smooth muscle [10]. The mechanism of the Ca^{2+}-desensitizing effect of cAMP and cGMP is not known; however, telokin was identified as the major cytosolic protein phosphorylated under the influence of 8-Br-cGMP and was proposed to induce Ca^{2+}-desensitization in smooth muscle by up-regulating the relaxant effects of myosin light chain phosphatase activity [11].

Indeed, the addition of recombinant telokin to permeabilized smooth muscles produces a profound relaxation and decrease in MLC_{20} phosphorylation of Ca^{2+}-induced contractions [11]. Telokin, also known as kinase-related protein (KRP), is an abundant, small acidic protein (17 kDa) that was first identified by Hartshorne and colleagues [12] and found to be identical to the carboxy terminus of MLCK [13]. Telokin and MLCK are expressed independently from two different promoters; telokin expression is through the action of serum response factor [13] and a smooth muscle specific promoter located in an intron of the MLCK gene [14]. The protein is only expressed in certain smooth muscles, with the highest levels of expression found in phasic smooth muscle [15] with little if any expression in tonic muscle [11,13]. Telokin binds to the S1–S2 region of myosin through the acidic carboxyl terminus [15] and prevents myosin from adopting the 10 S conformation [16]. Telokin stabilizes unphosphorylated myosin filaments in vitro against ATP-dependent depolymerization [15], and modulates, in vitro, the oligomerization of MLCK [17] as well as the phosphorylation rate of myosin by the competitive inhibition of MLCK [18] [19]. It has been suggested that telokin's physiological function is mediated through its stabilization of myosin filaments; however, tonic smooth muscles with little or no telokin have normal arrays of myosin filaments [20] indicating that telokin is not necessary for in vivo filament stability.

*Corresponding author.
E-mail: hayst001@mc.duke.edu

[1] These authors contributed equally to this manuscript.

0014-5793/00/$20.00
PII: S0014-5793(00)01884-6

The crystal structure of telokin shows that the protein consists of an immunoglobulin-like β-barrel and a flexible amino terminal 35 amino acids [21]. The unstructured amino terminus is serine and threonine rich and contains substrate recognition sequences for a variety of kinases, including cyclic nucleotide-dependent protein kinases (PKA and PKG), mitogen-activated protein kinase (MAPK), CaMKII, [11,12,22] and MLCK [18]. To gain a better understanding of the role of phosphorylated telokin in smooth muscle, we mapped its in vivo phosphorylation site(s) in stimulated ileum and portal vein and investigated the role of phosphorylation on its relaxant activity. Our results suggest that telokin is phosphorylated on a single residue (Ser-13) in ileum and on two residues (Ser-13 and Ser-19) in portal vein.

2. Materials and methods

2.1. Materials

Recombinant $p42^{MAPK}$ was purified from *Escherichia coli* strain BL21(DE3)[pET-MK][23]. The constitutively active S218D/S222D MEK1 was a gift from Dr. Andrew Cattling (Department of Microbiology, University of Virginia, VA, USA). Rabbit telokin cDNA in a pET expression plasmid was a gift of Dr. Paul Herring (Indiana University, IA, USA). cAMP-dependent protein kinase catalytic subunit (PKAc) was purchased from Calbiochem Inc. (San Diego, CA, USA).

2.2. Preparation of ^{32}P-phosphorylated recombinant telokin

Recombinant rabbit telokin was prepared as previously described [11]. The recombinant protein was phosphorylated for 2 h at 25°C to a stoichiometry of 1 mol/mol in a 5-ml reaction containing 3 mg of recombinant telokin, 25 mM HEPES, pH 7.2, 5 mM $MgCl_2$, 0.1 mM DTT, 0.3 mM [γ-^{32}P]ATP (250 cpm/nmol) and either 3 μg PKAc or 3μg PKG. Excess [γ-^{32}P]ATP was removed by dialysis against 25 mM HEPES, pH 7.2, 0.1 mM EDTA, 1 mM DTT, 0.1 mM phenylmethylsulfonyl fluoride (PMSF), and 1 mM benzamidine.

2.3. Phosphorylation site analysis of ^{32}P-recombinant telokin

^{32}P-Telokin samples (0.5 mg) from in vitro phosphorylation reactions were incubated at 37°C for 15 h with lysyl endopeptidase (20 μg/ml). The digests were acidified by addition of trifluoroacetic acid (TFA) and applied to a reverse phase column (Waters Nova-Pak C18, 3.9×150 mm) that had been equilibrated in 0.1% TFA (Buffer A). The flow rate was maintained at 1 ml/min. The column was washed for 10 min with Buffer A before peptides were eluted with a linear gradient of acetonitrile (0–80% in 80 min) in buffer A. Fractions (1 ml) were collected, and peptides containing ^{32}P were identified by measuring Cerenkov radiation. Those fractions were pooled and evaporated to dryness before the peptides were immobilized to Immobilon membrane (Millipore) following the manufacturer's instructions. The ^{32}P-labeled peptides generated by phosphorylating recombinant telokin with [γ-^{32}P]ATP were identified with a vapor phase amino acid sequencer (Applied Biosystems, Procise 494). Phosphorylated residues within phosphopeptides were located by determining the cycles in which ^{32}P was released when samples were subjected to sequential Edman degradation under conditions that optimize recovery of ^{32}P [24].

As an alternative, solid phase mixed peptide sequencing [25] with ^{32}P release was used to establish the precise ^{32}P-labeled site. Phosphorylated recombinant telokin was run on SDS–PAGE gels and transferred to PVM membrane. The membranes were stained with amido black and then exposed to film. The autoradiograms were overlaid onto the PVM membrane to align the phosphoprotein signal with the distinct amido black staining protein. The protein band was excised from the membrane with a razor, and after treatment with cyanogen bromide, the membrane piece was placed directly into a 494 Procise sequencing cartridge (Perkin-Elmer) for mixed peptide sequencing. Phosphorylated residues were identified by sequential Edman degradation as described above.

2.4. In vivo phosphorylation site determination of telokin

The portal vein and the longitudinal muscle of the ileum were removed from rabbits anesthetized by halothane and exsanguinated according to approved animal protocols. Two groups of intact smooth muscle strips (250 μm×2 mm) were incubated in HEPES-buffered Krebs solution in the presence of [^{32}P]PO_4^{3-} (5 mCi/ml) at room temperature for 1 h, followed by the addition of forskolin (50 μM) to one group. After 15 min, both control and agonist-treated groups were flash frozen in liquid N_2 and then homogenized in lysis buffer (20 mM Tris–HCl, pH 7.5, 250 mM sucrose, 5 mM EDTA, 1 mM DTT, 10 nM microcystin, 2 μg/ml aprotinin, 2 μg/ml leupeptin, and 0.1 mM PMSF). After a 45-min centrifugation at 100 000×g, the soluble component was fractionated by anion exchange chromatography (MiniQ; 2.3×3 cm) on a SMART-System FPLC (Pharmacia). A linear gradient of 0–1 M NaCl in 20 mM Tris–HCl, pH 8.0, 0.1 mM EDTA, 1 mM DTT at a flow rate of 50 μl/min over 30 min was employed, and 50-μl fractions were collected. Aliquots (10 μl) of each fraction were run on SDS–PAGE gels and silver-stained. Telokin containing fractions were identified by autoradiography and immunoblotting. The remaining volume (40 μl) of telokin containing fractions was treated with endolysylpeptidase, and the phosphopeptides generated were purified over reverse phase HPLC. Phosphorylated residues were identified by sequential Edman as described above.

In another series of experiments, ileum muscle and portal vein strips were permeabilized with β-escin based on published protocols [9] and then incubated in a Ca^{2+}-free solution containing 10 mM EGTA and 0.5 mM [γ-^{32}P]ATP (NEN Life Science Products). Strips were incubated in pCa 6.3, ileum, and pCa 6.5, with 10μM microcystin, portal vein, with or without added 50 μM GTPγS. Similar procedures as outlined above were used to isolate ^{32}P-labeled telokin and identify the in vivo site(s) of phosphorylation.

2.5. Preparation and treatment of Triton X-100-permeabilized smooth muscle ileum

Details of methods and solutions have been described elsewhere [11]. For storage, small strips of rabbit ileum were permeabilized for 15 min with 0.1% Triton X-100 in a Ca^{2+}-free solution containing 1 mM EGTA (G1), immersed in a relaxing solution with 50% glycerol, and stored at −20°C for up to 4 weeks. Stored strips were washed three times with G1 solution prior to use. The strips were connected to an isometric force transducer (AM801), and mounted in a well on a bubble plate. After permeabilization, unless noted otherwise, all experiments were carried out in the presence of 1 μM calmodulin at room temperature. The maximal responses evoked by calcium (pCa 4.5) were assessed and then the strips were washed three times in G1. The strips were incubated in pCa 6.3 to obtain a sub-maximal contraction prior to the addition of exogenous telokin. Telokin or phosphorylated telokin (20 μM) in 30 mM PIPES, 5 mM magnesium methane sulfonate, 165 mM potassium methane sulfonate, pH 7.0 was added at the plateau of pCa tension and the magnitude and rate of relaxation measured.

2.6. Telokin phosphorylation by PKA and MAPK

Phosphorylation of telokin and telokin mutants was performed at 23°C in 25 mM HEPES, pH 7.2, 1 mM $MgCl_2$, 0.1 mM DTT with 0.2 mM [γ-^{32}P]ATP and either 3 μg PKA catalytic subunit or 3 μg activated MAPK. MAPK was activated by phosphorylation with recombinant S218D/S222D MEK1 as described previously [23]. The stoichiometry of telokin phosphorylation was measured by terminating the kinase reactions with 25% TCA after pre-determined time intervals. The radioactivity incorporated into the precipitated proteins was assessed by Cerenkov counting.

3. Results

3.1. Telokin phosphorylation

In vitro phosphorylation of recombinant telokin protein by both PKG- and PKA-catalyzed phosphate incorporation exclusively onto serine as determined by phospho-amino acid analysis (data not shown). To determine the precise amino acid residue that was phosphorylated, 1 nmol of the ^{32}P-labeled protein was digested overnight with endolysylpeptidase. Reverse phase phosphopeptide mapping of the digest showed that >95% of the total radioactivity present in the protein

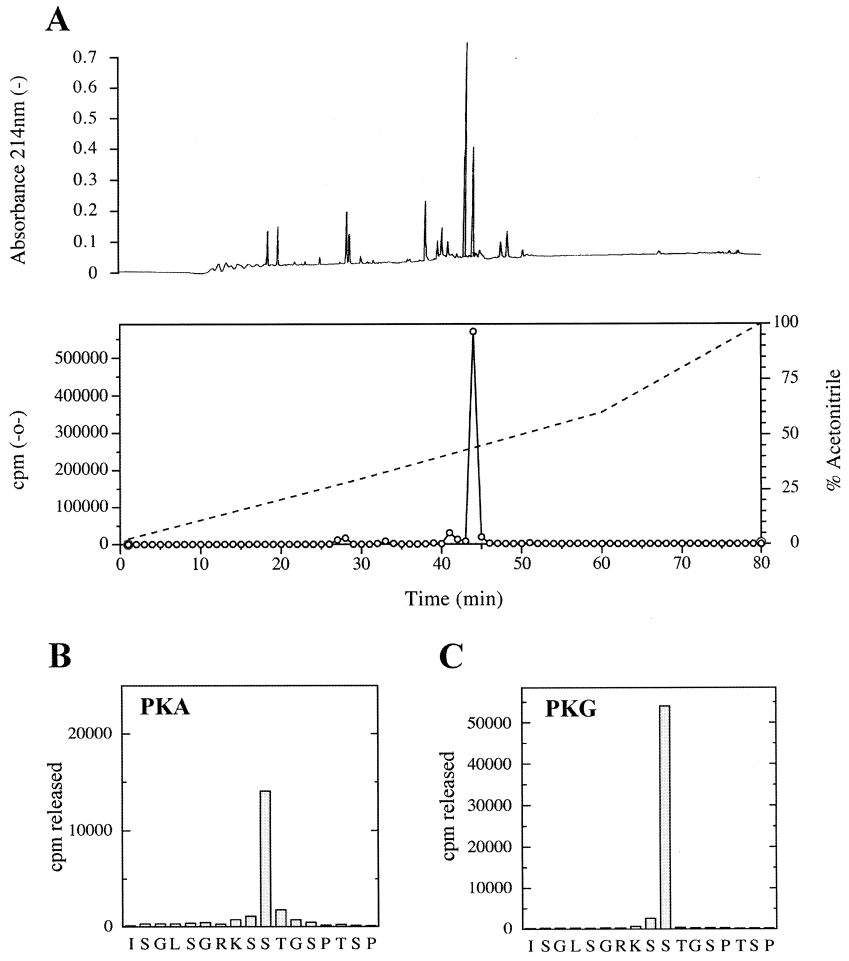

Fig. 1. A: Resolution of ^{32}P-phosphopeptides generated by lysyl endopeptidase digestions of recombinant telokin phosphorylated in vitro by PKA. Telokin was incubated at 25°C for 2 h. The ^{32}P-labeled proteins were then digested with lysyl endopeptidase, and then the resulting peptides were applied to a reverse phase column and eluted with an increasing gradient of acetonitrile (dashed line). The results show the absorbance profile at 214 nm (solid line), and the amount of ^{32}P recovered in fractions (1 ml) (open symbols). B: Identification of the in vitro site(s) of phosphorylation in purified phosphopeptides obtained from endolysylpeptidase-digested telokin phosphorylated by PKA. Peptides were coupled to Sequelon AA disks and phosphorylated residues were identified by sequential Edman degradation. The amount of ^{32}P released in each cycle was quantitated by Cerenkov counting. C: Identification by mixed peptide sequencing of sites in telokin phosphorylated in vitro by PKA. Recombinant telokin was incubated for 2 h with [γ-^{32}P]ATP and either PKA or PKG prior to mixed peptide sequencing with ^{32}P release to establish the ^{32}P-labeled site(s) on the protein. Telokin was excised from a PVM membrane with a razor, and after treatment with cyanogen bromide, the membrane piece was placed into a 494 Procise sequencing cartridge (Perkin-Elmer) for mixed peptide sequencing. The results represent the amount of ^{32}P released in each cycle when the peptides were subjected to sequential Edman degradation.

was incorporated into a single peptide (Fig. 1). After further purification of the phosphopeptide over capillary reverse phase HPLC, the amino acid sequence was determined by Edman sequencing. The purified phosphopeptide contained a single amino acid sequence, SSTGSPTSPLTAE. Solid phase sequencing, in which radioactivity was collected after each cycle, suggested that the incorporated ^{32}P was split between the first and second cycle. Subsequently, solid phase-mixed peptide sequencing [25] with detection of ^{32}P release was used to unambigously establish the in vitro PKA/PKG site as Ser-13 (Fig. 1).

In ileum strips, the in vivo phosphorylation site(s) of telokin were determined by ^{32}P-labeling in HEPES-buffered Krebs solution followed by treatment with either DMSO (control), GTPγS, or forskolin. Forskolin induced protein phosphorylation of Ser-13 (Fig. 2). There was also a significant, but lesser, amount of ^{32}P incorporation onto this same site in control ileum, indicating a basal level of phosphorylation on Ser-13. GTPγS treatment did not generate results different from that induced by the other treatments. We were unable to identify ^{32}P incorporation on either of the two putative MAPK sites, Ser-16 or Ser-19, following stimulation of ileum with GTPγS or forskolin.

The in vivo phosphorylation site(s) of telokin were determined in a similar manner in portal vein. In this case, an increase in phosphorylation of the Ser-13 site was evident following stimulation with either GTPγS or forskolin. Minor phosphorylation of the Ser-19 site was observed with forskolin treatment. Phosphorylation of the Ser-19 site was most pronounced when GTPγS was used to stimulate portal vein in the presence of microcystin (Fig. 3). There also appeared to be a small amount of basal phosphorylation of these two sites.

3.2. *Effects of telokin and phospho-telokin on smooth muscle contraction*

To determine whether in vitro phosphorylation of Ser-13

affects the ability of telokin to relax smooth muscle, we treated Triton X-100-permeabilized smooth muscle with recombinant telokin and phospho-telokin (20 μM). There was no difference in the rate or extent of relaxation obtained from the addition of telokin when compared with the addition of phosphorylated telokin (data not shown).

3.3. *In vitro phosphorylation of telokin mutants by PKA and MAPK*

With evidence showing phosphorylation of both Ser-13 and Ser-19 in vivo, telokin mutants were created in order to assess phosphorylation potential in vitro. Wild-type and the S19A mutant were good substrates for PKAc in vitro with no differences in the overall stoichiometry of phosphorylation (reaching a stoichiometry of ~1.0 mol of phosphate/mol of protein), as shown in Fig. 4A. The rate of phosphorylation of the S19A mutant was faster (reaching a stoichiometry of ~0.8 mol of phosphate/mol of protein in 10 min) than that of the wild-type telokin (reaching a stoichiometry of ~0.8 mol of phosphate/mol of protein in 40 min). The S13A and S13D mutants were not phosphorylated to any significant extent by PKAc; the maximal phosphorylation levels ranged from 0.05 to 0.1 mol/mol.

There were no differences in the rate or overall stoichiometry of phosphorylation among any of the recombinant telokin species when phosphorylated by MAPK (Fig. 4B). MAPK

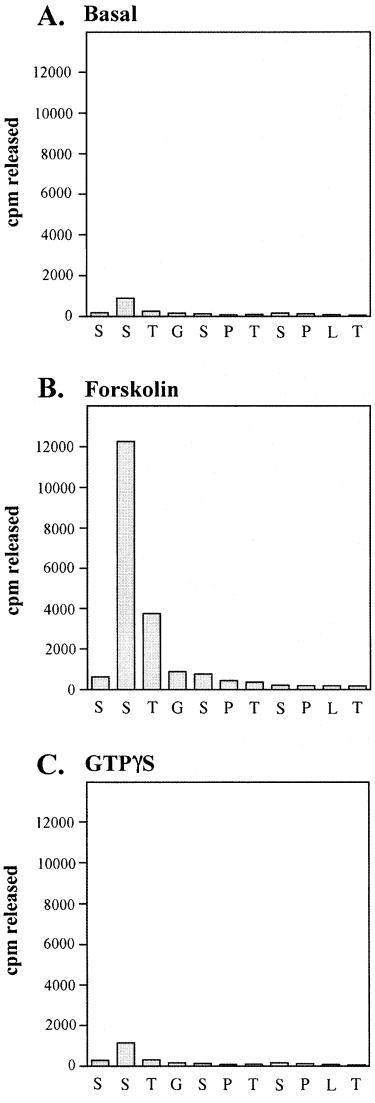

Fig. 2. Identification of sites in rabbit ileum longitudinal smooth muscle telokin phosphorylated after treatment with (A) DMSO; (B) forskolin; or (C) GTPγS. Ileum smooth muscle strips were α-toxin-permeabilized, except those used for the forskolin treatment, prior to contraction in pCa 6.3 solution with 1 μM calmodulin. Samples of telokin from control and stimulated ^{32}P-labeled ileum were digested with endolysylpeptidase. Phosphopeptides were resolved by reverse phase HPLC, coupled to Sequelon AA disks, and subjected to sequential Edman degradation. The results represent the amount of ^{32}P released in each cycle when the peptides were subjected to sequential Edman degradation.

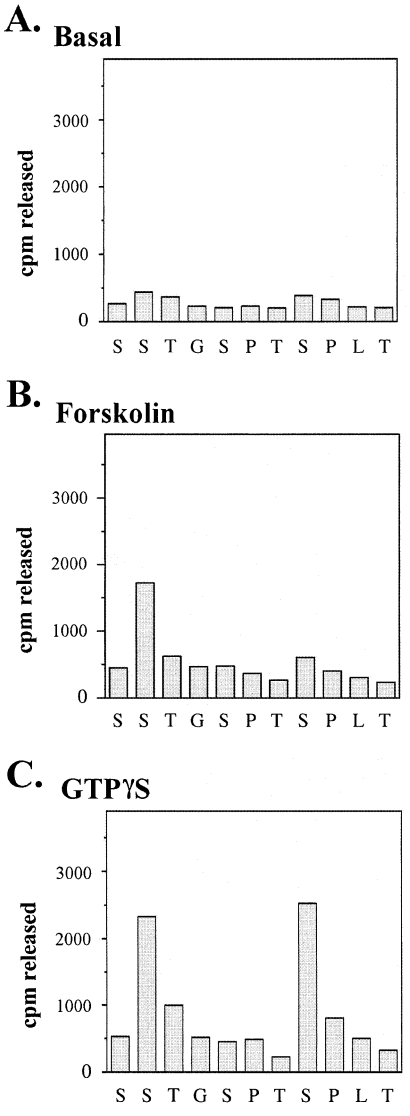

Fig. 3. Identification of sites in rabbit portal vein telokin phosphorylated after treatment with (A) DMSO; (B) forskolin; or (C) GTPγS. Portal vein strips were permeabilized with β-escin (50 μM for 30 min), except those used for the forskolin treatment, prior to contraction in pCa 6.5 solution with 1μM calmodulin and 10 μM microcystin. Forskolin-treated portal vein was contracted in pCa 6.5 solution without the addition of calmodulin or microcystin. Telokin samples were prepared as in Fig. 2. The results represent the amount of ^{32}P released in each cycle when the peptides were subjected to sequential Edman degradation.

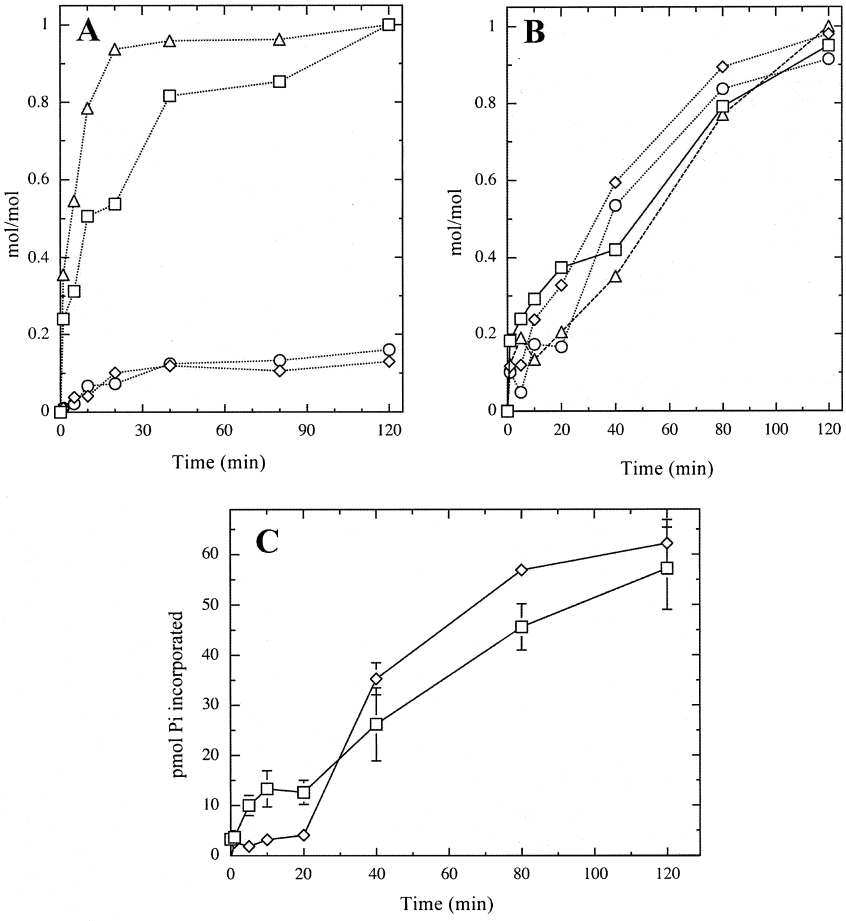

Fig. 4. Time course for the phosphorylation of recombinant telokin mutants, 0.1 mg wild-type (□), 13A (○), 13D (◇), or 19A (△) by (A) PKA catalytic subunit and (B) activated MAPK. A 250-μl reaction mixture containing 25mM HEPES, pH 7.2, 200 μM ATP (250 cpm/nmol), 5 mM $MgCl_2$, and 3 μg of either PKA catalytic subunit or activated MAPK. The stoichiometry of phosphorylation was determined by removing 10-μl aliquots of the reaction mixtures and measuring ^{32}P incorporation into TCA-precipitated protein. C: The time courses of phosphorylation by MAPK for telokin pre-phosphorylated with PKA catalytic subunit (◇) and unphosphorylated telokin (□) are shown. Wild-type telokin was phosphorylated to stoichiometry with PKA catalytic subunit prior to the addition of activated MAPK. Data are means ± S.E.M., $n = 4$.

phosphorylated all telokin mutants to ~1 mol of phosphate/mol of protein by 120 min. Interestingly, the S19A mutant was as good an in vitro substrate for MAPK as the wild-type protein. Using sequential Edman degradation, we found that the in vitro MAPK site present on wild-type telokin was exclusively Ser-19; however, on the S19A mutant, the phosphorylation occurred exclusively on Ser-16 (data not shown). These results suggest that the in vitro phosphorylation of wild-type telokin by MAPK is preferred and exclusive to Ser-19.

We also tested whether prior phosphorylation by PKAc affected the subsequent rate of phosphorylation by MAPK (Fig. 4C). Wild-type telokin was incubated at 25°C with PKAc+unlabeled ATP or PKAc alone. Telokin incubated with PKAc+ATP was phosphorylated to a stoichiometry of approximately 0.8 mol/mol (~2 h), and excess PKA inhibitor peptide (10 μM) was added to both reaction tubes. Activated MAPK and [$^{32}\gamma$P]ATP was added, and the stoichiometry of phosphorylation was determined. Pre-phosphorylation with PKAc did not affect the stoichiometry of phosphorylation by MAPK. Phosphate incorporation onto both PKAc phospho-telokin and non-phosphorylated telokin reached ~1 mol/mol within 120 min.

4. Discussion

The phosphorylation of telokin in situ has been reported previously [11,12,22]. Because telokin contains multiple consensus phosphorylation sites for both cyclic nucleotide-dependent protein kinases and MAPK, it was not clear which of the phosphorylation sites was involved in the regulatory function. Mass spectrometry results of rabbit ileum smooth muscle telokin peptides eluted from two-dimensional gels showed possible phosphorylation of a proline-directed consensus site and suggested phosphorylation of telokin by MAPK in vivo [11]. However, the resolved peptide, RKSSTG**S**P**T**SPLTAER, contained consensus sites for both cyclic nucleotide-dependent protein kinases (underlined) and MAPK (bold). An additional mass of ~80 kDa associated with this peptide could result from the phosphorylation of either MAPK or cyclic nucleotide-dependent protein kinase sites. The goal of this study was to determine the precise phosphorylation sites on telokin that result from stimulation.

In vitro phosphorylation by cyclic nucleotide-dependent protein kinases results in phosphorylation solely at Ser-13, and if this site is replaced by a non-phosphorylatable residue (S → A/D mutant), phosphorylation is inhibited. By using sol-

id phase mixed peptide sequencing with ^{32}P release, we established the in vivo ^{32}P-labeled site on the protein. Telokin is phosphorylated solely at Ser-13 in intact ileum stimulated with forskolin. We were unable to find evidence of in vivo phosphate incorporation on either MAPK site, Ser-16 or Ser-19, in forskolin or GTPγS-stimulated ileum. Interestingly, there was ^{32}P incorporation on Ser-19 in telokin isolated from portal vein; phosphorylation of this site was more pronounced when the muscle had been treated with microcystin, a potent phosphatase inhibitor, plus added agonists. These results suggest that phosphorylation of the Ser-19 site may be strongly regulated by the action of protein phosphatases. The functional significance of the Ser-19 site in the two different muscles remains unclear.

We tested the hypothesis that phosphorylation of Ser-13 may alter the ability of Ser-19 to be phosphorylated by MAPK. Ser-13 was phosphorylated to a 1 mol/mol stoichiometry with PKA and then treated with MAPK. There was no enhancement of phosphorylation at the MAPK sites when the protein had been previously phosphorylated with PKA at Ser-13, or alternatively, no enhancement of phosphorylation at Ser-13 when a 19D telokin mutant was used (data not shown). Furthermore, exogenous, activated MAPK (5 μM) added to permeabilized ileum, had no relaxant effect as seen previously [26]. The importance of telokin phosphorylation by MAPK remains to be elucidated. One possibility is that Ser-19 phosphorylation regulates potentiation of telokin-induced relaxation by cyclic nucleotides. Relaxation in permeabilized ileum is super-potentiated by cyclic nucleotides in the presence of exogenous 13D/19A telokin [27].

Thus, it appears that in vitro phosphorylation at Ser-13 is not required for the Ca^{2+}-desensitizing action of telokin; although, we cannot rule out the possibility that telokin phosphorylated in vitro was dephosphorylated when added to smooth muscle. The thiophosphorylation of telokin was attempted, but it labeled with very low stoichiometry. It is possible that the phosphorylation of additional protein(s) is required for full potentiation of telokin's relaxant properties. The concomitant reduction of the effect of 8-Br-cGMP and PKG with the loss of endogenous telokin from permeabilized smooth muscle reported previously [11] may be a result of the loss of both telokin and additional diffusible proteins. We are currently identifying by mixed peptide sequencing other small molecular weight proteins that are phosphorylated in response to cyclic nucleotide agonists and may have a cooperative role in smooth muscle Ca^{2+}-desensitization.

Acknowledgements: The authors wish to thank Dr. Paul Herring (Indiana University) for providing the telokin cDNA expression plasmid and Amy DeZern and Xiaopu Liu for their technical assistance. Supported in part by NIH Grants HL19242-24 (T.A.J.H.) and HL48807-07 (A.P.S.).

References

[1] Hartshorne, D.J. (1987) in: Physiology of the Gastrointestinal Tract (Johnson, L.R., Ed.), pp. 423–482, Raven Press, New York.
[2] Somlyo, A.P. and Somlyo, A.V. (1994) Nature 372, 231–236.
[3] Kitazawa, T., Masuo, M. and Somlyo, A.P. (1992) Jap. J. Pharmacol. 58, 348.
[4] Gallagher, P.J., Herring, B.P. and Stull, J.T. (1997) J. Muscle Res. Cell Motil. 18, 1–16.
[5] Somlyo, A.P., Wu, X., Walker, L.A. and Somlyo, A.V. (1999) Rev. Physiol. Biochem. Pharmacol. 134, 201–234.
[6] Lincoln, T.M., Komalavilas, P. and Cornwell, T.L. (1994) Hypertension 23, 1141–1147.
[7] McDaniel, N.L., Rembold, C.M. and Murphy, R.A. (1994) Can. J. Physiol. Pharmacol. 72, 1380–1385.
[8] Warner, T.D., Mitchell, J.A., Sheng, H. and Murad, F. (1994) Adv. Pharmacol. 26, 171–174.
[9] Wu, X., Somlyo, A.V. and Somlyo, A.P. (1996) Biochem. Biophys. Res. Commun. 220, 658–663.
[10] Pfitzer, G., Merkel, L., Ruegg, J.C. and Hofmann, F. (1986) J. Pflug. Arch. 407, 87–91.
[11] Wu, X., Haystead, T.A., Nakamoto, R.K., Somlyo, A.V. and Somlyo, A.P. (1998) J. Biol. Chem. 273, 11362–11369.
[12] Ito, M., Dabrowska, R., Guerriero Jr., V. and Hartshorne, D.J. (1989) J. Biol. Chem. 264, 13971–13974.
[13] Gallagher, P.J. and Herring, B.P. (1991) J. Biol. Chem. 266, 23945–23952.
[14] Herring, B.P. and Smith, A.F. (1997) Am. J. Physiol. 272, C1394–C1404.
[15] Shirinsky, V.P., Vorotnikov, A.V., Birukov, K.G., Nanaev, A.K., Collinge, M., Lukas, T.J., Sellers, J.R. and Watterson, D.M. (1993) J. Biol. Chem. 268, 16578–16583.
[16] Olney, J.J., Sellers, J.R. and Cremo, C.R. (1996) J. Biol. Chem. 271, 20374–20384.
[17] Nieznanski, K. and Sobieszek, A. (1997) Biochem. J. 322, 65–71.
[18] Sobieszek, A., Andruchov, O.Y. and Nieznanski, K. (1997) Biochem. J. 328, 425–430.
[19] Silver, D.L., Vorotnikov, A.V., Watterson, D.M., Shirinsky, V.P. and Sellers, J.R. (1997) J. Biol. Chem. 272, 25353–25359.
[20] Somlyo, A.V. (1980) in: The Handbook of Physiology: Vascular Smooth Muscle (Bohr, D.F., Somlyo, A.P. and Sparks, H.V., Eds.), pp. 33–67, Williams and Wilkins, Baltimore, MD.
[21] Holden, H.M., Ito, M., Hartshorne, D.J. and Rayment, I. (1992) J. Mol. Biol. 227, 840–851.
[22] Vorotnikov, A.V., Silver, D.L., Seller, J.R., Watterson, D.M. and Shirinsky, V.P. (1996) J. Muscle Res. Cell Motil. 17, 153.
[23] Scott, A., Haystead, C.M. and Haystead, T.A. (1995) J. Biol. Chem. 270, 24540–24547.
[24] Russo, G.L., Vandenberg, M.T., Yu, I.J., Bae, Y.S., Franza Jr., B.R. and Marshak, D.R. (1992) J. Biol. Chem. 267, 20317–20325.
[25] Damer, C.K., Partridge, J., Pearson, W.R. and Haystead, T.A. (1998) J. Biol. Chem. 273, 24396–24405.
[26] Nixon, G.F., Iizuka, K., Haystead, C.M., Haystead, T.A., Somlyo, A.P. and Somlyo, A.V. (1995) J. Physiol. 487, 283–289.
[27] Walker, L.A., MacDonald, J.A., Nakamoto, R.K., Somlyo, A.V., Haystead, T.A., and Somlyo, A.P. (2000) submitted.