Acceleration of Myosin Light Chain Dephosphorylation and Relaxation of Smooth Muscle by Telokin

SYNERGISM WITH CYCLIC NUCLEOTIDE-ACTIVATED KINASE*

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Incorporation of 32P into telokin, a smooth muscle-specific, 17–18-kDa, acidic (pI 4.2–4.4) protein, was increased by forskolin (20 μM) in intact rabbit ileum smooth muscle (ileum) and by 8-bromo-cyclic GMP (100 μM) in α-toxin-permeabilized ileum. Native telokin (5–20 μM), purified from turkey gizzard, and recombinant rabbit telokin, expressed in Escherichia coli and purified to >90% purity, induced dose-dependent relaxation, associated with a significant decrease in regulatory myosin light chain phosphorylation, without affecting the rate of thiophosphorylation of regulatory myosin light chain of ileum permeabilized with 0.1% Triton X-100. Endogenous telokin was lost from ileum during prolonged permeabilization (>20 min) with 0.1% Triton X-100, and the time course of loss was correlated with the loss of 8-bromo-cyclic GMP-induced calcium desensitization. Recombinant and native gizzard telokins were phosphorylated, in vitro, by the catalytic subunit of cAMP-dependent protein kinase, cGMP-dependent protein kinase, and p42/44 mitogen-activated protein kinase; the recombinant protein was also phosphorylated by calmodulin-dependent protein kinase II. Exogenous cGMP-dependent protein kinase (0.5 μM) activated by 8-bromo-cyclic GMP (50 μM) phosphorylated recombinant telokin (10 μM) when added concurrently to ileum depleted of its endogenous telokin, and their relaxant effects were mutually potentiated. Forskolin (20 μM) also increased phosphorylation of telokin in intact ileum. We conclude that telokin induces calcium desensitization in smooth muscle by enhancing myosin light chain phosphatase activity, and cGMP- and/or cAMP-dependent phosphatase activity, and cAMP-dependent protein kinase (PKA) can also relax permeabilized smooth muscle at constant [Ca2+]i (11). We have reported that 8-Br-cGMP reverses G-protein-coupled Ca2+-sensitization and accelerates relaxation and dephosphorylation of MLC20 at constant [Ca2+], (12). The mechanism of the Ca2+-desensitizing effect of cAMP and cGMP is not known, and, in search of it, we identified telokin as the major cytosolic protein phosphorylated under the influence of 8-Br-cGMP.

Telokin was first discovered by Hartshorne and colleagues (13) and found to be identical with the COOH terminus (155–156 amino acids) of smooth muscle MLCK (13), and it is independently expressed in certain smooth muscles (14) through the activities of serum response factor (15) and a smooth muscle-specific promoter located in an intron of the MLCK gene (16). The crystal structure of telokin solved at 2.6-Å resolution showed a characteristic immunoglobulin fold, but the NH2 terminus containing the phosphorylation sites could not be visualized (17). Telokin, also known as kinase-related protein (18), binds to the S1/S2 region of unphosphorylated smooth muscle myosin (19), modulates, in vitro, the oligomerization of MLCK (20), and, also in vitro, inhibits the phosphorylation of myosin by MLCK (19, 20) through competitive inhibition for the MLCK site (21). Telokin also prevents the folding of the 6 S myosin into 10 S conformation (22) and stabilizes filamentous myosin in solution (19); therefore, it has been suggested that stabilization of myosin filaments is a physiological function of telokin in smooth muscle (19).

Our results suggest that the major in situ effect of telokin is desensitization to [Ca2+]i, through a mechanism that does not significantly affect thiophosphorylation but accelerates the dephosphorylation of MLC20 and acts synergistically with the Ca2+-desensitizing effect of an 8-Br-cGMP-activated kinase. Preliminary results of some of these findings have been published (23).
Experimental Procedures

Labeling of Intact and Permeabilized Smooth Muscle—Longitudinal muscle of the ileum was removed from rabbits anesthetized by halothane and exsanguinated according to approved animal protocols. Two groups of intact rabbit ileum longitudinal smooth muscle strips were incubated in 5 mM D-glucose, 250 mM sucrose, 2 mM EDTA, 1 mM diethiothreitol, 1 mM 4-(2-aminoethyl)-benzenesulfonil fluoride (Calbiochem), and 0.1 μM okadaic acid. Aliquots of cell lysates were then mixed with Laemmli sample buffer (24) for SDS-PAGE (15% acrylamide) and electrophoresed. After 2 h, cells were harvested and broken open by a French press (25) followed by autoradiography and Western blot analysis. In another series of experiments, rabbit ileum muscle strips were permeabilized with a generous gift of Drs. David Hartshorne and Masaaki Ito), and two-dimensional gel electrophoresis together with an

Identification of Small, Soluble, Acidic Protein(s) in Rabbit Ileum Smooth Muscle—In order to identify the proteins with increased 32P incorporation in telokin-58B-GMP treatment, we consider the supernatant of ~1 g of rabbit ileum longitudinal smooth muscle (with or without treatment with 20 μM forskolin) homogenized in buffer A and centrifuged at 100,000 g for 30 min. After 2 h, cells were harvested and broken open by a French press, and peptides eluted from the gel matrix were sequenced by LC mass spectrometry (Biomolecular Research Facilities, University of Virginia).

Purification of Native and Recombinant Telokins—Native telokin was purified from frozen turkey gizzards by chromatography on DEAE-AE2 (23) and phenyl-Sepharose (Amersham Pharmacia Biotech) according to the published method (13). Recombinant rabbit telokin was expressed from a pET vector expression plasmid (25) followed by autoradiography and Western blot analysis. Recombinant rabbit telokin antibody at a 1:5000 dilution in PBST (26) containing 10 μM EDTA and 0.5 mM γ-32P[ATP] (NEN Life Science Products, with or without 100 μM S-Brc-GMP. Similar procedures as above were carried out to screen for phosphoproteins showing increased 32P incorporation upon treatment with 100 μM S-Brc-GMP.

Western blot Analyses—Treated or control smooth muscle tissues were frozen with liquid N2 and homogenized with buffer A. Approximately 20 μg of total protein, determined by the Bradford assay (BioRad), from each tissue homogenate was subjected to 15% SDS-PAGE. Proteins were electrophoretically transferred to PVDF (Millipore Corp.) membranes. Post-transfer gels were stained with Coomassie Blue. PVDF membranes were incubated with 0.2% glutaraldehyde in phosphate-buffered saline containing 0.05% Tween 20 (PBST) for 30 min and then blocked with 5% nonfat dry milk in PBST for 1 h. After PBST washes (10 min), the PVDF membranes were incubated with anti-telokin antibody at a 1:5000 dilution in PBST (1 μg/ml) for 3 h at room temperature or overnight at 4 °C. Following PBST washes (20 min), incubation with a secondary anti-rabbit IgG conjugated with horseradish peroxidase (Amersham Pharmacia Biotech) at 1:8000 dilution in PBST, blots were developed with ECL (Amersham Pharmacia Biotech). ECL detections were carried out according to the manufacturer's directions, and the normal exposure time of the x-ray film (DuPont) to PVDF blots was 30 s. Ready gels (4–20%, Bio-Rad) were used for immunoblotting with anti-PKG and anti-PKA antibodies. Two anti-PKG antibodies were used: a goat polyclonal anti-bovine PKG as a generous gift from Dr. Thomas Lincoln (University of Alabama at Birmingham) and a rabbit polyclonal anti-human PKG type Ia and Iβ purchased from Upstate Biotechnology, Inc. A rabbit polyclonal anti-PKA (NH2-terminal residues 7–21 of the human a-isofrom of the catalytic subunit of PKA) was also purchased from Upstate Biotechnology, Inc. Immunoblotting procedures were according to the protocol provided by Dr. Padmini Komalavilas, which uses nitrocellulose instead of PVDF, for the goat anti-bovine PKG and the manufacturer's directions for the rabbit polyclonal anti-PKG and anti-PKA antibodies. Secondary anti-bodies were donkey anti-goat IgG (Jackson Laboratories) and goat anti-rabbit IgG (Amersham Pharmacia Biotech) conjugated with horse-radish peroxidase.

Measurements of MLC20 Phosphorylation and Thio phosphorylation—The smooth muscle strips used for recording tension were frozen at selected time points by quickly transferring the specimen to Freon 22 precooled with liquid N2 and cutting the frozen strips away from the hooks on the tension recording apparatus. To identify phosphorylated and nonphosphorylated MLC20, tissue homogenates were separated by two-dimensional gel electrophoresis with amyllole pH 4.5–5.4, transferred to nitrocellulose membranes, and stained with AuroDye (Amersham Pharmacia Biotech) as described previously (25). Protein spots were analyzed by densitometry (Molecular Analyst, Bio-Rad). The percentage of MLC20 phosphorylation or thio phosphorylation was expressed as (P1 + P2)/(U + P1 + P2) (where U represents unphosphorylated, P1 represents singly phosphorylated, and P2 represents doubly phosphorylated). To measure telokin-induced phosphorylation of MLC20, muscle strips were contracted with pCa 6.2 or pCa 6.0 solutions, and without or with telokin treatment at 10 min, and frozen at 15 min after exposure to Ca2+. For thio phosphorylation studies, stored rabbit ileum smooth muscle strips were incubated in Ca2+-free, ATP-free solution at room temperature for 9 min, followed by ATP-free pCa 6.2 solution with 0.5 μM calmodulin in the presence or absence of telokin (20 μM). This

zyme required to transfer 1 pmol of phosphate to a peptide substrate, GTGRRSSNI, in 1 min at 30 °C, p42/44 mitogen-activated protein (MAP) kinase (27), or calmodulin-dependent protein kinase II (Neuroglioblasts). Assays with the commercially available kinases were carried out exactly according to protocols provided by the manufacturer.

In Vitro Kinase Assays—Phosphorylation of native and recombinant telokin was determined in kinase assay systems using the following reagents: catalytic subunit of CAMP-dependent protein kinase (5–10 units/reaction, New England Biolabs; 1 unit is the amount of enzyme required to transfer 1 nmol of phosphate to Leu-Arg-Arg-Ala-Ser-Leu-Gly (Kemptide) in 1 min at 30 °C), GMP-dependent protein kinase (5000–10,000 units/reaction, Calbiochem; 1 unit is the amount of en-
phosphorylation was initiated by the addition of 1 mM ATP-S (Boehringer Mannheim), followed by incubation for 1 or 5 min as indicated. Strips were then washed with Ca2+-free, ATP-free solution, and subsequently force was measured upon exposure to G1 solution containing 4.5 mM ATP.

Statistics—Student’s t test was performed as indicated under “Results” (Microsoft Excel). All values are means ± S.D. unless otherwise indicated, and n is the number of observations.

Materials—All reagents were purchased from Sigma unless otherwise specified.

RESULTS

Protein Phosphorylation in Smooth Muscle Induced by 8-Br-cGMP or Forskolin—Both 8-Br-cGMP (100 μM) and forskolin (20 μM) induced protein phosphorylation in, respectively, a-toxin-permeabilized and intact rabbit ileum smooth muscle (Fig. 1) and markedly increased 32P incorporation into a group of small (22–24 kDa upon SDS-PAGE), soluble, acidic (pI 4.2–4.4) proteins (Fig. 2, A and B). There was also a significant, but lesser, amount 32P incorporation into these same proteins in control intact muscle, indicating a basal level of phosphorylation. Analysis of the partially purified rabbit ileum smooth muscle (with or without 20 μM forskolin treatment) cytosolic proteins “spiked” with the 32P-labeled tissue lysates (see “Experimental Procedures”) showed that the two more acidic spots on two-dimensional gels detected by silver staining (vertically smeared) exactly matched 32P-containing spots shown by autoradiography (data not shown). An aliquot (60 μl) of the concentrated partially purified intact rabbit ileum smooth muscle cytosol, enriched with the small, acidic proteins, was then subjected to another two-dimensional gel electrophoresis, and the second dimensional gel was stained with Coomassie Blue. At least six faintly stained, distinct, small spots were detected, four of which were subjected to in-gel trypsin digestion. Six eluted peptides sequenced by LC mass spectrometry unambiguously matched the sequence of telokin (Table I). Western blotting of the two-dimensional gel of the 32P-labeled muscle lysate, using a rabbit polyclonal anti-telokin antibody, further confirmed that each of the group of small, acidic, phosphoproteins consisted of telokin (Fig. 2C).

We also found significantly enhanced 32P incorporation into several unidentified proteins in intact ileum smooth muscle upon forskolin (20 μM) stimulation (Fig. 1A), as well as another small (24–kd) acidic (pI ~4.9) protein in α-toxin-permeabilized ileum muscle upon stimulation with 8-Br-cGMP (100 μM) (data not shown).

Isolation of Turkey Gizzard Telokin—Telokin was isolated from frozen turkey gizzards by the method published (13), and purified telokin migrated as a group of at least three protein bands of 24–26 kDa on a 15% SDS-PAGE gel (Fig. 3A); each band was an acidic protein as shown on the two-dimensional gel (pI ~4.4), stained blue with Stains-all, and was recognized by the anti-telokin antibody (data not shown); the heterogeneity of avian telokin was recently characterized (30) and probably accounts for the appearance of multiple bands upon SDS-PAGE.

Expression and Purification of Recombinant Telokin—We also expressed a rabbit clone of telokin using a pET vector in BL21 (DE3) pLysS E. coli cells and purified the protein based on the method used for isolation of turkey gizzard telokin with some modifications (see “Experimental Procedures”). A yield of 10 mg of telokin/liter of culture was achieved, and the purified.
recombinant telokin was also consistently detected as multiple bands (22–24 kDa) on one-dimensional 15% SDS-PAGE gels (Fig. 3A) and as multiple spots (pI ~4.4) on two-dimensional gels by silver staining and/or Western blotting. The difference between the apparent molecular weights of native (avian) and recombinant (rabbit) telokins has been reported (14) and arises from small differences in amino acid sequences including three different translational initiation sites as well as COOH-terminal glutamate residues in the avian protein (30). Following storage at 4 °C, native gizzard telokin migrated as one band upon SDS-PAGE (Fig. 3B) without an apparent decrease in molecular mass (~26 kDa), whereas recombinant telokin migrated as one major band around 17 kDa (Fig. 3B). Sequencing the 17-kDa recombinant telokin by Edman degradation (Protein Sequencer; Applied Biosystems, Inc.) showed that the NH2-terminal 10 residues were FLEAVAAEKP, indicating that the 34 NH2-terminal amino acids were missing. The 17-kDa recombinant telokin (amino acids 35–155) was also an acidic protein (pI 4.2–4.4) and stained blue by Stains-all (data not shown), suggesting that it retained the COOH-terminal glutamate residues.

**In Vitro Phosphorylation of Telokin by PKA, PKG, MAP Kinase, and Calmodulin-dependent Protein Kinase II**—Both turkey gizzard and recombinant telokin were good substrates for the catalytic subunit of PKA in *vitro*, but the time course of recombinant telokin phosphorylation was much faster (reaching a stoichiometry of ~7 mol of phosphate/mol of protein in 8 min) than that of native telokin (reaching a stoichiometry of ~7 mol of phosphate/mol of protein in 32 min) (Fig. 4). This difference in phosphorylation may be due to a basal level of phosphorylation in native gizzard telokin. The holoenzyme of cGMP-dependent protein kinase (activated by 0.5 μM 8-Br-cGMP) phosphorylated turkey gizzard and recombinant telokin slowly to a stoichiometry of 0.4 mol of phosphate/mol of protein in 64 min (Fig. 4). Mass spectrometry of rabbit ileum smooth muscle telokin peptides eluted from two-dimensional gels showed a possibly phosphorylated serine residue (with an additional mass of ~80Da) on one of the six peptides sequenced (Table I, underlined S on peptide 4), which was a proline-directed consensus site and suggested phosphorylation of telokin by MAP kinase in *vitro*. Indeed, an activated p42/44 MAP kinase phosphorylated both turkey gizzard telokin and recombinant telokin in *vitro* (Fig. 4). Within the region of the primary structure of rabbit telokin that differs from avian telokin, there is a threonine residue in the context of RLET (absent from avian telokin) that may be a consensus phosphorylation site for cAMP-dependent protein kinase II. A catalytic subunit of cAMP-dependent protein kinase II phosphorylated, much more effectively, the purified recombinant than turkey gizzard telokin, in *vitro* (Fig. 4). None of the kinases could phosphorylate the 17-kDa recombinant telokin (residues 35–155), produced by storage at 4 °C (data not shown), indicating that all of the phosphorylation sites discussed above are within the NH2-terminal 34 amino acids.

**Telokin-induced Calcium Desensitization Associated with MLC20 Dephosphorylation in Triton X-100-permeabilized or Stored Smooth Muscle**—Both isolated turkey gizzard telokin and recombinant telokin (5–15 μM) induced dose-dependent relaxation of submaximal tension induced by Ca2+ in stored rabbit ileum smooth muscle strips (see “Experimental Procedures”) (Fig. 5). Following permeabilization with 0.1% Triton X-100 for 30 min or after storage, the recombinant telokin (20 μM) also induced significant relaxation (30 ± 7.5% (n = 3) of the respective pCa 6.2 tension response) in femoral artery, a smooth muscle that, like aortic smooth muscle (14, 16), contains only trace amounts of endogenous telokin (Fig. 6). Of the tissues examined by Western blot analysis using the rabbit polyclonal antibody raised against purified turkey gizzard telokin, rabbit bladder, intact ileum, vas deferens, portal vein, and chicken amnion contained similar high levels of telokin (Fig. 6); rabbit mesenteric artery, femoral artery, cerebral artery, skeletal muscle, and brain tissue showed no detectable level of telokin (Fig. 6); and a marked decrease in telokin content was found in Triton X-100-permeabilized ileum (Fig. 6). Telokin (20 μM) did not relax the submaximal tension generated by ~40% thiophosphorylated MLC20 in stored rabbit ileum smooth muscle strips (data not shown), suggesting that telokin-induced relaxation is associated with dephosphorylation of MLC20.

Isolated turkey gizzard telokin (20 μM) significantly relaxed tension induced by pCa 6.0 in rabbit ileum smooth muscle permeabilized with 0.1% Triton X-100 for 40 min and induced a decrease in MLC20 phosphorylation, from 44 ± 3.6% in control to 25 ± 6.3% (n = 10 each, p < 0.001) in relaxed muscle. Recombinant telokin (20 μM) also significantly relaxed the force developed at pCa 6.2 by stored rabbit ileum smooth muscle and induced a decrease in MLC20 phosphorylation, from 34 ± 3.1% (S.E.) in control to 24 ± 3.7% (S.E.) (n = 8 each, p < 0.05) in relaxed muscle.

**Lack of Effect of Telokin on the Rate of Thiophosphorylation of the Regulatory Myosin Light Chain**—To further verify that the reduction of MLC20 phosphorylation by telokin was due to activation of SMPP-1M rather than inhibition of MLCK, we used ATPγS as substrate, because MLC20 thiophosphorylation resists dephosphorylation (31). The rate of MLC20 thiophosphorylation was not affected by telokin: at 1 min, 35 ± 7.0% in control and 36 ± 6.5% in telokin-treated tissue MLC20; at 5 min, 47 ± 0.1% in control and 49 ± 4.5% in telokin-treated tissue MLC20 (n = 3 each, p > 0.05; values are means ± S.E.). These results suggest that telokin has no major effect on MLCK activity, *in vivo*, in thiophosphorylating MLC20.

**Loss of Endogenous Telokin and 8-Br-GMP-dependent Relaxation by Heavy Permeabilization**—Although 8-Br-cGMP induced significant calcium desensitization of force and MLC20 dephosphorylation in α-toxin-permeabilized rabbit ileum and femoral artery smooth muscle treated with 10 μM A23187 to eliminate Ca2+ transport by intracellular organelles (12), the calcium-desensitizing effect in both tissues was greatly diminished when they were permeabilized with 0.1% Triton X-100 in G1 solution for 10 min or longer. In α-toxin-permeabilized rabbit ileum smooth muscle strips where the amount of endogenous telokin was 97 ± 9.8% (n = 10) (Fig. 7) of that in intact...
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Fig. 4. Autoradiographs showing the time courses of in vitro phosphorylation of both recombinant and native telokin by the kinases indicated. See “Experimental Procedures.”

Fig. 5. Relaxation induced by exogenous native gizzard telokin in stored rabbit ileum smooth muscle strip. Rabbit ileum smooth muscle strips permeabilized stored at −20 °C (up to 4 weeks) were contracted by exposing them to pCa6.3 solution with 1 μM calmodulin. Native telokin (up to 15 μM, concentrated by centrifuge filters; Microcon-3K, Amicon) or filtrate as controls was added as indicated. Results are representative of more than three experiments.

Fig. 6. Western blot showing tissue distribution of telokin. Different tissues were dissected, frozen in liquid N2, and then subjected to Western blot analyses as described under “Experimental Procedures.” For each lane, −20 μg of total protein was loaded. RMA, rabbit mesenteric artery; RFA, rabbit femoral artery; RB, rabbit bladder; RI, rabbit ileum; RI (Triton), rabbit ileum after 0.1% Triton permeabilization (30 min); RPV, rabbit portal vein; Am, chicken amnion; 1, purified turkey gizzard telokin (~100 ng); 2, purified recombinant rabbit telokin (~200 ng); CA, cerebral artery; SKA, skeletal muscle; VD, vas deferens; BR1, brain tissue; BR2, brain tissue.

Fig. 7. Loss of telokin correlated with significantly diminished and slower relaxant effect by 8-Br-cGMP in rabbit ileum smooth muscle upon Triton X-100 permeabilization and storage. Rabbit ileum smooth muscle strips were permeabilized with α-toxin (αTX) as reported previously (12, 28). Permeabilization with 0.1% Triton X-100 was carried out in G1 solution in the presence of 10 μM A23187 for 10 min (T10) or 20 (T20) min at room temperature. For storage (STR), after 20-min permeabilization with 0.1% Triton X-100, muscle strips were washed with G1 solution and then immersed in relaxing/glycerol (1:1, v/v) solution (29) at −20 °C prior to use. Muscle strips can be stored up to a month without significant loss of tension response to calcium. For Western blotting, intact or permeabilized or stored muscle strips were washed with G1 solution, blotted briefly to remove excess solution, frozen with liquid nitrogen, and stored at −80 °C prior to homogenization and subsequent SDS-PAGE analyses. For tension measurements, permeabilized or stored muscle strips were mounted onto a force transducer (A9S01; AME, Horten, Norway), and immersed in solution bubbles on a “bubble plate.” Tension responses were obtained by exposing the strips to pCa 6.0 solution (for Triton X-100-permeabilized or stored strips, 0.5 μM calmodulin was added); 50 μM 8-Br-cGMP was added at the plateau of pCa tension to induce relaxation. Summarized data showing loss of telokin detected by Western blots paralleled the decrease and slowing (increased t½) of the relaxant effect of 8-Br-cGMP (50 μM) in Triton X-100-permeabilized and stored muscle strips. The OD of the telokin band on Western blot was obtained by Molecular Analyst (Bio-Rad) and normalized against the OD of the respective actin band. The ratio of telokin/actin from each sample was normalized against that from intact tissue; the percentage of relaxation was calculated as the ratio of amplitude of 8-Br-cGMP-induced relaxation to the respective tension response (Fig. 7). Furthermore, the rate of 8-Br-cGMP-induced relaxation became significantly slower in the muscles (Fig. 7). After 40 min of 0.1% Triton X-100 permeabilization (data not shown) or overnight storage in 50% relaxing/glycerol solution at −20 °C, there was a further loss of endogenous telokin (Fig. 7), and 8-Br-cGMP-dependent relaxation was also significantly decreased further to 15 ± 3.2% of the respective tension response (Fig. 7). Furthermore, the rate of 8-Br-cGMP-induced relaxation became significantly slower (Fig. 7) along with the loss of endogenous telokin. The increase
in $^{32}$P incorporation into small, soluble proteins stimulated by 100 μM 8-Br-cGMP in α-toxin-permeabilized smooth muscle (Figs. 1 and 2A) was also abolished in stored muscles or in muscles permeabilized with 0.1% Triton X-100 for 40 min (Fig. 8A, inset). Purified telokin (up to 20 μM) did not affect Ca$^{2+}$-induced tension in α-toxin-permeabilized smooth muscle, indicating that its effect required intracellular penetration ($n > 3$, data not shown).
Mutual Enhancement of Relaxation by Activated PKG and Telokin—To determine whether the significant reduction of the relaxant effect of 50 μM 8-Br-cGMP on stored rabbit ileum smooth muscle was due to inhibition or loss of endogenous PKG during permeabilization and storage, we added 0.5 μM PKG activated by 50 μM 8-Br-cGMP to stored muscle strips. However, this activated exogenous PKG did not affect the pCa 6.0-induced tension (Fig. 8A, right part), suggesting that loss of PKG substrate(s) rather than PKG was responsible for the markedly diminished relaxant effect of 8-Br-cGMP in the stored fibers. Furthermore, Western blotting analyses using both the goat polyclonal antibody raised against bovine lung PKG and the rabbit polyclonal antibody raised against human PKG type Iα and Iβ indicated no significant loss of endogenous PKG during permeabilization and storage of rabbit ileum smooth muscle and significantly less abundance of PKG in intact ileum, bladder, and vas deferens (not shown) than that in femoral artery, mesenteric artery, and portal vein (Fig. 8D). PKA content was very similar in the different smooth muscles tested (Fig. 8D), but a significant loss of PKA was observed in stored rabbit ileum smooth muscle. Recombinant telokin (10 μM) relaxed pCa 6.0-induced tension of stored muscle strips by 31 ± 6.1%, and the subsequent addition of PKG (0.5 μM) activated by 8-Br-cGMP (50 μM) induced a further 41 ± 7.7% relaxation. However, without previous addition of telokin, PKG (0.5 μM) activated by 8-Br-cGMP (50 μM) relaxed the stored muscle strips by only 22 ± 7.3%, whereas the subsequent addition of telokin (10 μM) induced further relaxation of 52 ± 5.0% (Fig. 8, A and C). Therefore, telokin and activated PKG enhance mutually their relaxant effects. Activated PKG (0.5 μM) phosphorylated exogenous telokin added to the stored muscle strips (Fig. 8A, inset). The shortened 17-kDa telokin (amino acids 35–155) (Fig. 3D) could not be phosphorylated by PKA/PKG, and when we added to stored muscle strips this short telokin (10 μM) in the presence of activated PKG (0.5 μM) by 8-Br-cGMP (50 μM), no significant mutual enhancement of relaxant effects was observed (Fig. 8C).

DISCUSSION

The major new findings of this study are that telokin accelerates relaxation and dephosphorylation of the MLC20 in smooth muscle, that the loss of endogenous telokin parallels the loss of the relaxant effect of 8-Br-cGMP and PKG, and that the relaxant effects of 8-Br-cGMP and telokin are synergistic. Furthermore, phosphorylation of telokin, measured by 32P incorporation, was very significantly enhanced by forskolin in intact smooth muscle and by 8-Br-cGMP in permeabilized smooth muscle. In conjunction with earlier studies (12), these findings lead to the hypothesis that phosphorylation of endogenous telokin contributes to Ca2+-desensitization by PKG and/or PKA, which is mediated by activation of smooth muscle myosin phosphatase.

In vitro phosphorylation of telokin by PKA has been previously reported (13), and after our studies were completed, we were informed of a preliminary report also indicating such phosphorylation in situ (32). The serine 15 phosphorylated by an 8-Br-cGMP-dependent kinase corresponds to site B of the cyclic nucleotide kinase (PKG and PKA) phosphorylation site of the COOH terminus of MLCK and is, therefore, responsible for the functional effects observed in our studies. An additional serine-phosphorylated peptide within a proline-directed consensus site detected in situ was consistent with MAP kinase phosphorylation, and both MAP kinase and calmodulin-dependent protein kinase II phosphorylated telokin in vitro; the functional effects, if any, of phosphorylation of these latter sites, remain to be determined. None of the above kinases phosphorylated the NH2-terminally truncated telokin (see "Results"), indicating that the amino acids phosphorylated under our experimental conditions were consistent with the consensus phosphorylation sites identified within the first 35 NH2-terminal amino acids of the MLCK/telokin sequence (33).

Several cytosolic and membrane-associated proteins are phosphorylated by cyclic nucleotide-activated kinase(s), and some of these may be involved in regulating cytosolic Ca2+ (for a review, see Ref. 9). There is also considerable evidence showing that agents that increase cyclic nucleotide levels can reduce cytosolic [Ca2+]i (for a review, see Refs. 8 and 9). Therefore, it is likely that both mechanisms, reduction in [Ca2+]i, and Ca2+-desensitization, make variable contributions to relaxation of smooth muscle induced by agonists that increase cyclic nucleotide levels. However, cyclic nucleotides and their regulated kinases can also relax smooth muscle independently of [Ca2+]i (11, 12, 34–36) (Ca2+-desensitization), and we had suggested that increased activity of smooth muscle myosin phosphatase is at least one of the mechanisms of desensitization (1, 12, 37). Our attention was drawn to telokin as a possible mediator of Ca2+-desensitization, because we found it to be the most abundant protein showing increased 32P incorporation when intact or permeabilized smooth muscles were stimulated, respectively, forskolin or 8-Br-cGMP.

Desensitization of smooth muscle contraction to Ca2+- can be mediated, however, by inhibition of MLCK (2) and/or activation of SMPPP-1M (12, 37). Telokin inhibits MLC20 phosphorylation by MLCK in vitro (19, 38) through competition with MLCK for a common binding site in the S1/S2 region of myosin (21). Should telokin also act in situ primarily through this mechanism, we would expect it to reduce the rate of thophosphorylation of MLC20. Because we did not detect such an effect, we examined in greater detail the hypothesis that Ca2+-desensitization induced by telokin is mediated by activation of myosin dephosphorylation.

Telokin (5–20 μM) (both the wild type, isolated from turkey gizzard, and the recombinant rabbit protein) relaxed submaximally contracted, permeabilized smooth muscle and accelerated dephosphorylation of MLC20 at fixed [Ca2+]i. The concentration of endogenous telokin in phasic smooth muscles is 70–90 μM (19), and the relaxant effect of telokin became greater in parallel with the loss of endogenous telokin, suggesting that, at least in permeabilized smooth muscle, endogenous telokin may have a Ca2+-desensitizing activity even in the absence of exogenous cyclic nucleotide.

That the effect of telokin is enhanced by its phosphorylation by a cGMP- or cAMP-dependent protein kinase is supported by three observations: 1) the significant increase in telokin phosphorylation during relaxation induced by forskolin or 8-Br-cGMP; 2) the concomitant reduction of the effect of 8-Br-cGMP and PKG with the loss of endogenous telokin from permeabilized smooth muscle; and 3) the greater than merely additive relaxant effects of combined PKG and telokin (Fig. 8). The exact mechanism of these interactions is not clear. A "short telokin" that lacks the phosphorylation sites had a significant relaxant effect, but 8-Br-cGMP and PKG did not potentiate its activity, and this suggests that phosphorylation enhances, but is not required for, the Ca2+-desensitizing action of telokin. Similarly, neither phosphorylation with PKA nor removal of the phosphorylatable amino terminus affects the binding of telokin to myosin, and that binding depends largely on the acidic COOH terminus (21). The rapid loss of telokin from permeabilized smooth muscle (Fig. 7) indicates that, unlike calmodulin, an acidic protein of similar molecular weight (39, 40), telokin is not strongly bound to nondiffusible proteins. Depletion of endogenous telokin had no detectable effect on
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contractility,\(^2\) suggesting that telokin, although facilitating myosin assembly in vitro (19), is not required for the maintenance of myosin filaments in situ.

Telokin is a high abundance protein in phasic, but not in tonic, smooth muscles (Refs. 14 and 16; this study). Therefore, it remains to be determined whether Ca\(^{2+}\) desensitization is a function of telokin specific to phasic smooth muscle or whether another acidic protein, perhaps a yet to be detected isoform, plays a similar role in tonic smooth muscles. The higher phosphatase activity of phasic than tonic smooth muscle (41) may reflect its higher telokin content, and the decrease in endogenous phosphatase activity observed following storage of chicken gizzard muscle fibers (42, 43) may also have been in fact due to loss of telokin.

A single, identical cyclic nucleotide kinase-directed consensus site on MLCK phosphorylated in the presence of calmodulin by both PKA and PKG (13, 44). This is the only such consensus site on telokin, and both PKA and PKG phosphorylated telokin (Fig. 4). Forskolin, an agent that increases cAMP, but not cGMP, also increased telokin phosphorylation in intact intestinal, as contrasted to vascular, smooth muscle (2–4), suggesting that telokin, although facilitating contractility,\(^2\) suggesting that telokin, although facilitating myosin assembly in vitro (19), is not required for the maintenance of myosin filaments in situ.

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