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Modulation of myosin filament activation by telokin in smooth muscle Liberation of myosin kinase and phosphatase from supramolecular complexes

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Abstract

The mechanism of telokin action on reversible phosphorylation of turkey gizzard myosin was investigated using a native-like filamentous myosin. This myosin contained endogenous calmodulin (CaM) and myosin light chain kinase (MLCK) at a molar ratio to myosin of about 1 to 40 or less depending on the initial extractions conditions. These levels were sufficient to fully phosphorylate myosin within 20–40 s or less after addition of [γ-^{32}P]ATP, but when the ATP was depleted, they became dephosphorylated indicating the presence of myosin light chain phosphatase (MLCP). Addition of telokin at the 1 to 1 or higher molar ratio to myosin caused a three- to five-fold inhibition of the initial phosphorylation rates (without reduction of the overall extent of phosphorylation) and produced a similar increase in the rate of dephosphorylation. The inhibition was also observed for myosin filaments free of MLCK and CaM together with constitutively active MLCKs produced by digestion, or by expression of a truncated mammalian kinase as well as for the wild-type enzyme. Thus, neither N- nor C-terminal of MLCK was necessary for interaction of myosin with telokin and the inhibition resulted from telokin-induced change of myosin head configuration within the filament that prevented their ordered, paracrystaline-like, aggregation. Sedimentation of the filamentous myosin in glycerol gradients showed that this change made the filaments less compact and facilitated release of the endogenous MLCK/CaM complex. For a mixture of the filaments with or without the complex, the configuration change resulted in an increase of the phosphorylation rate but not in its inhibition. The increase of the rate resulting from the liberation of the complex was also observed in mixtures of the filamentous myosin with added isolated regulatory light chain (ReLC) or soluble myosin head subfragment. This observation reinforces the above conclusions. The acceleration of the MLCP activity by telokin was shown to result from dissociation of its catalytic subunit from a MLCK/MLCP complex bound to the filamentous myosin. Analogous desensitizing effects of telokin were also demonstrated for the contraction and relaxation cycle of Triton-skinned fibers from guinea pig *Teania coli*. Taken together, our results indicate that telokin acted as an effective modulator or chaperone of the myosin filament and a scheme for its action in smooth muscle was proposed.

Keywords: Smooth muscle; Myosin filament; Regulation of phosphorylation; Endogenous kinase and phosphatase; Telokin

1. Introduction

It is well established that myosin light chain kinase (MLCK) and myosin light chain phosphatase (MLCP) are the two key regulatory enzymes responsible for the contraction and relaxation of smooth muscle [1,2]. At

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actomyosin level, the contraction pathway is initiated by the formation of an active MLCK–calmodulin (CaM) complex that phosphorylates myosin and enables cyclic interaction of myosin heads with thin filaments. The amino acid sequence and domain structure of several MLCK species has been characterized in detail. The kinase is composed of a catalytic core of about 61 kDa with an adjacent regulatory segment that includes CaM binding and autoinhibitory sequences. In addition, there are three immunoglobulin-like and one fibronectin-like domains [3]. The C-terminal immunoglobulin-like domain is abundantly expressed as an independent protein called telokin, which is the subject of the present study. The mechanism of MLCK activation by CaM has also been extensively investigated (see Ref. [4]). The proposed model includes a steric inhibition of the kinase activity by an autoinhibitory segment that has a sequence analogy to the phosphorylation site on the regulatory light chain (ReLC). In the presence of calcium, the affinity of CaM for MLCK increases dramatically and as a result they form an active complex in which the inhibition is removed.

Despite of these intensive studies, some basic questions such as the localization of MLCK and/or CaM within the contractile apparatus are at present controversial. In view of the tight association of MLCK and of CaM with purified myosin, we concluded that, at relaxed state, they are localized on myosin filaments (see Ref. [5]). This does not exclude the possibility of a functional interaction or binding to the actin filament proposed by others [6] but the affinity of such binding is 100 or more folds lower than that for myosin filaments as observed by us [7–10]. The formation of the active CaM/MLCK complex in the presence of calcium results in the reduction of this affinity and thus enables cooperative activation of the myosin filament [11]. In the presence of calcium, the affinity of the complex for myosin may be comparable to that for actin. In addition, there are also some differences in the domain structure between the gizzard and mammalian kinases related to the presence of the DFRXXL motives that have been shown to be responsible for the binding to actin filaments [12]. These motives are absent in gizzard kinase from which our data were obtained.

The relaxation pathway of myosin as well as the regulatory mechanism of the key enzyme involved is even less understood. For a long time, the smooth muscle MLCP could not be identified and it was simply assumed that during relaxation the MLCP activity was amplified by other factors so it overrides the activity of MLCK thus ensuing myosin dephosphorylation and relaxation. More recently, a MLCP has been identified and purified and shown to be composed of three subunits: a catalytic one of 38 kDa responsible for its activity, a myosin targeting subunit of 110 kDa responsible for its association with myosin and another 20-kDa subunit of unknown function (for review, see Ref. [13]). We have purified another MLCP that is associated not only with the myosin but also with the MLCK [14]. It appears that both phosphatases possess the same catalytic subunit but their targeting subunits are different. Our targeting subunit appeared to be responsible for association of the catalytic subunit with the kinase and was smaller in size (67 kDa) and together with CaM they formed a functional multi-enzyme complex [15]. Thus, it is still not clear which of these two phosphatases represents the smooth muscle MLCP and their relationship remains to be established.

In view of the association of both regulatory enzymes with the myosin, it is possible that some protein factors related to the thick filaments are involved in the modulation of the contraction and relaxation pathways of smooth muscle. The modulatory proteins tropomyosin, calponin and caldesmon exhibit such a role for the thin filament and most of the research effort has been aimed at elucidation of their role (for review, see Ref. [16]). This however has not brought about a clear mechanism of their modulatory action [17]. The myosin-related factors have so far been more difficult to discover and the only candidate is telokin discovered earlier [18] or more recently, the 38-kDa protein described by Okagaki et al. [19]. Telokin appears to be associated with MLCK [20] and is also called kinase-related protein because it has the amino acid sequence identical to that of the C-terminal, immunoglobulin-like, domain of the kinase [18,21–23]. However, it is expressed independently because telokin and MLCK mRNAs are induced by two different promoters within the kinase gene [21,24]. The amino acid sequence of telokin exhibits similarity to the proteins associated with myosins such as C-protein, titin and twitchin [25–27] as well as to other proteins not associated with muscle tissue such as immunoglobulin and chaperonin (see Ref. [28]). Although the 3-dimensional structure of telokin has been determined from X-ray diffraction studies [28] and several of its unique properties have been described, the role of telokin in smooth muscle is unclear because the results obtained so far do not give clear clues about its function in vivo.

It has been originally demonstrated that telokin stabilized unphosphorylated myosin mini-filaments in the presence of ATP [29] but there is no need for such stabilization because at the high concentrations, comparable to those in vivo, myosin forms stable filaments [30,31]. The weak binding of telokin to myosin is inconsistent with the rather effective inhibition of the myosin filament phosphorylation by telokin [20]. This, however, cannot be readily reconciled with the observed moderate inhibition of heavy meromyosin (HMM) phosphorylation [29] or even absence of such inhibition for the soluble substrates: the isolated ReLC [22,23] and the myosin head subfragment [20]. Nevertheless, from these observations, it was clear that it is the neck region of the myosin molecule that is necessary for its interaction with telokin. Additional observations pointed to the possibility that telokin may induce a change of myosin conformation from a folded one (10S) to an extended (6S) conformation [31], thus providing explanation for the stabilization of myosin mini-filaments. However, the inhibitory effect of telokin could not be explained in these terms and this

analogous inhibition has not been observed for muscle fibers permeabilized with Triton X-100 [32].

Our studies on the oligomeric suprastructure of MLCK [33] and the effects of telokin on its oligomeric properties [20] as well as a very slow phosphorylation of telokin by this kinase [34] provide explanation for at least some of these controversial observations. The inhibition of myosin phosphorylation was shown to result from dimerization (or monomerization) of the kinase by telokin because only the oligomers appeared to be tightly bound to myosin filaments. This dimerization could also affect the activity of the MLCP, the one that forms a functional complex with the oligomeric MLCK [15]. The present report represents an extension of our previous studies in which we investigated the effect of telokin on MLCK and MLCP endogenous to smooth muscle filamentous myosin and actomyosin preparations and we put forward a possible scheme of telokin action in smooth muscle.

2. Materials and methods

2.1. Protein preparations

Turkey gizzard actomyosin, myosin, MLCK and CaM were used throughout this study. A myofibril-like preparation [35] was used as a starting material for all these protein preparations. First, MLCK was extracted with a buffer containing 25 mM $MgCl_2$ [36] and purified as described previously (see Ref. [11]). Then, the myofibrils were washed to remove the extraction buffer and used for the following extraction of a crude actomyosin [9] from which native-actomyosin was purified by $MgCl_2$ precipitation [36]. Freshly extracted crude actomyosin was also used for purification of myosin in a three-step procedure that included: formation of filaments by addition of $MgCl_2$, ammonium sulfate fractionation and slow dialysis to form long filaments. Details of the myosin purification procedure are given in Sobieszek [5]. MLCK and CaM were removed from myosin by a passage of the purified myosin (dissolved in a buffer containing 0.6 M NaCl and 0.2 mM $CaCl_2$) through CaM-affinity and Phenyl Sepharose 6B-CL columns connected in tandem [10]. Such myosin, in turn, represented a convenient source for isolation of its regulatory ReLC because it was depleted of endogenous CaM. The light chain fraction was obtained as previously described [37] and, after precipitation at its isoelectric point (pH 5.6) and solubilization, required no further purification.

Telokin was purified as described previously [20] and its effect on MLCK and MLCP activities was evaluated by comparison of myosin phosphorylation progresses obtained in the absence and in the presence of purified telokin. The myosin or actomyosin stock suspensions frozen at −70 °C (with or without 25% glycerol) were first rinsed to refresh the buffer and to remove glycerol. Thus, they were resuspended in 4–5 volumes of fresh BW buffer (composition in mM: 40 KCl, 2 $MgCl_2$, 1.0 DTE and 10 imidazole, 10 Bis Tris with pH adjusted to 6.6 at 4 °C) using a small size tight glass–glass homogeniser. After centrifugation in an SS-34 Sorval rotor at 18,000 rpm for 20 min, the pellet was resuspended as above in an appropriate volume of our BW buffer to obtain the required protein concentration. The phosphorylation reaction was initiated by addition of [γ-^{32}P]ATP (0.3–0.6 mM) to the myosin (or actomyosin) suspension containing 0.1 mM $CaCl_2$. Phosphorylation progress points were collected by withdrawing 100 μl aliquots from the reaction mix (using a positive displacement pipette), and pipetting them into tubes containing 100 μl of quenching solution [8.5 M urea and 40 mM of 4-(hydroxymercuril) benzoic acid] while on a vortex. A 175 μl of the quenched mix was then spotted on 2×4 cm pieces of Wathman 3 MM chromatographic paper that were processed for radioactivity counting as described previously [14]. After two-fold dilution with 8.5 M urea, the remaining 20–25 μl of the mix was used, if necessary, for urea–glycerol PAGE analysis of the maximal phosphorylation levels of the regulatory light chain of myosin [38].

2.2. Skinned fibers and tension measurements

Preparations of guinea pig *Teania coli* were skinned with Triton X-100 as described by Albrecht et al. [39]. Strips of 4–7 mm length and 0.2 mm diameter were mounted isometrically for mechanical studies on an AME 801 force transducer. The composition of the relaxing solution was (in mM or other units): 21 KCl, 36 NaCl, 10 $MgCl_2$, 4 EGTA, 7.5 ATP, 1 sodium azide, 10 phosphocreatine, 140 U/ml creatine phosphokinase, 2 μM CaM, 2 DTE, 20 imidazole with pH adjusted to 6.7 at 22–23 °C. The contraction solution was identical except for the partial replacement of EGTA with Ca-EGTA, thereby increasing the free calcium concentration to the desired value (see Ref. [39]). During a calcium-induced contraction, rapid relaxation was induced by using an "EGTA jump" technique to minimize diffusional delays: first the contraction solution was replaced by a low EGTA contraction solution in which 4 mM Ca-EGTA was replaced by 0.2 mM Ca-EGTA. After about 5 min, the fibers were then immersed in standard relaxing solution containing 4 mM EGTA.

2.3. Sedimentation experiments

Glycerol gradients were prepared by a slow casting of the buffer containing required concentration of glycerol directly into the centrifuge tubes. In case of upper layers of glycerol solutions, this was facilitated by use of plastic syringes with fine bore needles. Gradients were made either in 7- or 14-ml tubes, the latter made possible collection of 15–20 fractions with a Densi-Flow Apparatus (Labconco, Kansas City, MO) that were used for measurements of MLCK activity and/or for SDS-PAGE analysis. The myosin suspensions containing telokin and/or the required additions were then carefully

pipetted on top of the gradient and placed in a cold swing rotor (HB-4; Sorvall, USA) equipped with necessary adaptors. The tubes were then centrifuged for 4–20 min at required speeds that were usually between 4000 and 8000 rpm. After centrifugation, the tubes were photographed by a digital camera on a black background to facilitate contrast. The filaments bands in the glycerol gradient often exhibited little contrast but this improved after 10–20 min standing at RT probably because of filament aggregation.

3. Results

3.1. MLCK and MLCP content of native-like myosin and actomyosin

Our preparations of purified myosin and actomyosin may be considered being native-like because they contained endogenous MLCK, CaM and MLCP. Thus, a simple addition of Ca^{2+} and ATP suffices to attain a maximum phosphorylation of myosin. At 35 °C, myosin became fully phosphorylated within the first 10–20 s (Fig. 1A) indicating a relatively high content of MLCK and CaM and this contrasts with standard preparations of this type to which MLCK and CaM has to be added to obtain much slower phosphorylation rates. The presence of MLCP was apparent from the dephosphorylation that took place after depletion of ATP by myosin ATPase. As a result, the phosphorylation progress for this type of preparations was biphasic: after reaching a maximum, the level of myosin phosphorylation did not remain constant but declined to the baseline level. When, however, MLCP activity was inhibited by Microcystin, then the second dephosphorylation phase became extremely slow or absent (Fig. 1, upper curves).

This type of progress curves is analogous to a phasic contraction–relaxation cycle of smooth muscle. Its contraction phase depended mainly on the relative (to myosin) concentration of MLCK, while the relaxation phase was controlled by the MLCP activity present and the rate of ATP hydrolysis by myosin. The cycle can be depicted by the following sequence of reactions:

$$M + A \xrightarrow{k_k} P + ADP; \quad P \xrightarrow{k_p} M + Pi; \quad M + A \xrightarrow{k_h} ADP + Pi;$$

where M and P correspond to concentration of unphosphorylated and phosphorylated myosin, and A represents ATP concentration. The reactions are, in turn, described by a set of three differential equations:

$$dA/dt = -k_k{*}A{*}M - k_h{*}A{*}(M - P) - c{*}k_h{*}A{*}P$$

$$dM/dt = -k_k{*}A{*}M + k_p{*}P$$

$$dP/dt = -k_k{*}A{*}M - k_p{*}P.$$

The three rate constants represent the kinase (k_k), the phosphatase (k_p) and ATP hydrolysis (k_h). A simple constant c is a factor by which myosin ATPase increases after phosphorylation and this was equal approximately 2. The set was integrated to give a single complex equation describing phosphorylation–dephosphorylation progress curve of this type.

$$P = M_o\{1 - \exp\{-k_p t - \frac{A_o k_k}{M_o k_h}[1 - \exp(-M_o k_h t)]\} \times \{1 + k_p \int_0^t \exp\left\{+k_p t - \frac{A_o k_k}{M_o k_h}[1 - \exp(-M_o k_h t)]\right\} dt\}$$

The experimental phosphorylation progress curves for myosin were satisfactorily approximated by this mathemat-

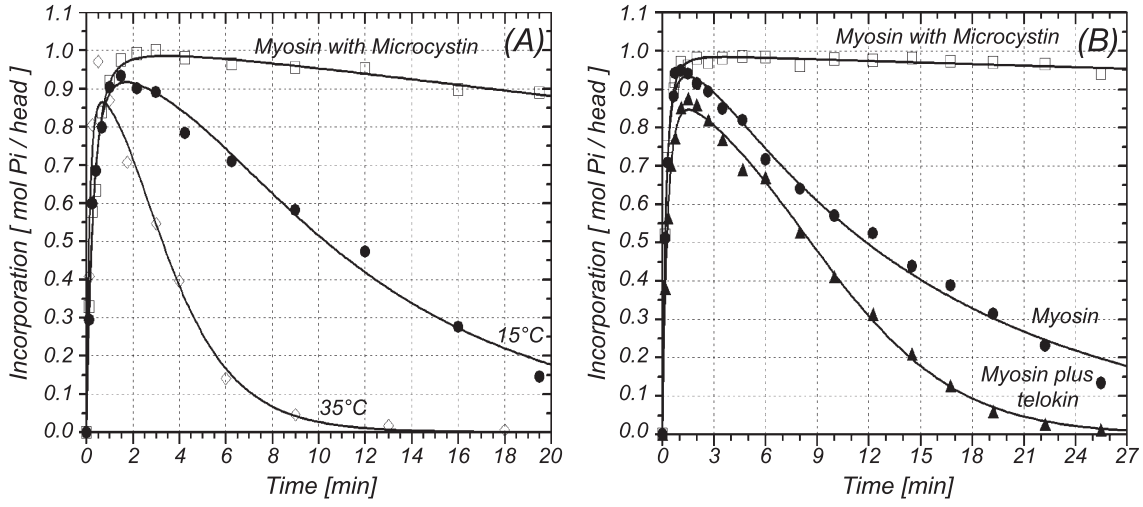

Fig. 1. Reversible phosphorylation of native-like filamentous myosin. (A) Endogenous MLCK rapidly phosphorylated the myosin (e.g. within the first 10–20 s at 35 °C) and this became dephosphorylated some 4–6 min later by the endogenous MLCP when ATP was depleted by myosin ATPase. Note that the dephosphorylation phase could be extended by reduction of the myosin ATP hydrolysis rate (15 °C) or by inhibition of the MLCP by Microcystin (50 nM). (B) The effect of telokin on myosin dephosphorylation in the presence of 250 μM MgATP and at 25 °C. The phosphorylation levels are expressed as a relative concentration of phosphorylated myosin heads.

ical approach, which can be used for evaluation of the values of the rate constants. In view of the limited number of experimental points, more reliable values are obtained when one of the constants (e.g. k_h) is determined independently. We have used this approach for computer estimation of the relative concentrations of MLCK and MLCP together with the Scientists software from MicroMath (Salt Lake City, UT). The rate constants k_k and k_p were optimized under initial boundary conditions A_o and M_o, corresponding to initial concentrations of ATP and myosin heads and the k_h constant given below.

For myosin preparations purified from myofibrils pre-extracted for MLCK (see legend to Table 1), the molar ratio of the kinase to myosin was about 1 to 150 with a similar and saturating CaM contents (Table 1). Their presence resulted in full phosphorylation of myosin within 40–60 s at 4 °C with the rate being a little too fast for manual determination of ^{32}P incorporation of the initial phase (Figs. 1 and 2). As expected, the kinase content of myosin purified directly from our myofibrils was higher and in this case the rates doubled by addition of purified CaM. Thus, the latter filamentous myosin contained a 2-fold excess of the kinase apoenzyme relative to CaM indicating the presence of MLCK hetero trimers (complexes of two kinase molecules with one CaM).

The relative MLCP content of filamentous myosin was lower and more variable (Table 1) and could only be determined for fresh preparations. The preparations frozen in liquid nitrogen (with or without 25% glycerol) contained even less of the phosphatase and its content decreased further after rinsing of the filaments (seeSection 2). Nevertheless, filamentous myosin contained sufficient levels of the phosphatase to bring about a full dephosphorylation of myosin to complete the cycle (Figs. 1 and 2) and their approximate ratios to myosin are given in Table 1. An important additional factor was not only the temperature but also ATP concentration and the relative rate of its hydrolysis due to myosin ATPase that we estimated to be 5 min^{-1} (10 nmol/mg·min). This rate approximately doubled when myosin became phosphorylated. At the myosin concentration used (about 30 μM) and 0.3–0.5 mM ATP present, full dephosphorylation was attained within 5–30 min.

Table 1
Molar ratios of the endogenous MLCK, CaM and MLCP present in native-like filamentous myosin preparations

Myosin prepared from	MLCK to MY	CaM to MLCK	MLCP to MY
Pre-extracted MYF[a,b]	1:150±30	1:1.5±0.2	1:1750±250
MYF at folding conditions	1:45±15	1:2.0±0.2	1:700±200
MYF by filament isolating madium (FIM)[c]	1:350±50	1:2.1±0.3	Not evaluated
Myosin free of MLCK and CaM	1:2500±200	Not detectable	Not detectable

[a] Myofibrils (MYF) were prepared as described by Sobieszek and Bremel [35] that included extensive fragmentation of gizzard muscle with the subsequent five homogenization/centrifugation washing cycles. MLCK was then extracted at 25 mM $MgCl_2$ with two additional washing cycles to remove the $MgCl_2$.

[b] Extraction solution contained in mM: 90 KCl, 5 ATP(Na) and 2 EDTA and 10 imidazole at pH 7.2.

[c] FIM composition was in mM: 40 KCl, 2.5 MgATP, 5 $MgCl_2$, 10 imidazole and 10 histidine at pH 6.5.

3.2. Inhibition of myosin phosphorylation by telokin

Fig. 2 shows the effect of telokin on the phosphorylation−dephosphorylation cycle of the myosin and actomyosin preparations. In the case of myosin (Figs. 1B and 2A) telokin not only reduced by three-fold (or more) the phosphorylation rate but it also accelerated the dephosphorylation rate by approximately the same factor. It is clear from the figure that modifications of the rates made easier our manual quenching of the reaction at required experimental points during the phosphorylation phase. The modifications (the reduction and acceleration) were optimal at an approximately 1 to 1 telokin to myosin molar ratio; a ratio close to that estimated to exist in vivo. At the higher ratios there was, as expected, a substantial reduction of the maximal phosphorylation level resulting from acceleration of the MLCP activity (e.g. Figs. 1B and 2A). The reduction approached 50% at telokin to myosin ratio of about 10 to 1 or higher. This made more difficult visual evaluation of the effects of telokin during phosphorylation progress; however, such evaluation was possible with the computer-aided approach used. The inhibitory effect of telokin could more readily be seen when the endogenous MLCP activity was inhibited by addition of 100 nM of Microcystin. Importantly, the progress curves obtained this way showed no significant inhibition of the overall phosphorylation levels independently of ATP concentration present (Fig. 3A, insert). This indicates that the inhibition did not result from a simple competition of telokin and MLCK for the same binding site on myosin filaments. A competition should bring about not only inhibition of the rate but also a reduction of the total phosphorylation sites.

A similar phosphorylation−dephosphorylation cycle was observed for purified actomyosin and this was analogously modified by telokin (Fig. 2B). In this case, however, the progress curves were more complex and could not be analyzed by the computer aided approach. Clearly, the additional presence of thin filament containing tropomyosin and traces of other proteins made the system more complex. The reason for this difference was unclear and therefore we restricted our analysis to the native-like filamentous myosin system.

As indicated above the inhibition of myosin phosphorylation rates by telokin and not of their optimal levels of phosphorylation cannot be explained by a competition between MLCK and telokin for the same binding site on the myosin filament. Fig. 3B shows that MLCK appeared to be bound weaker to the filaments because it was liberated

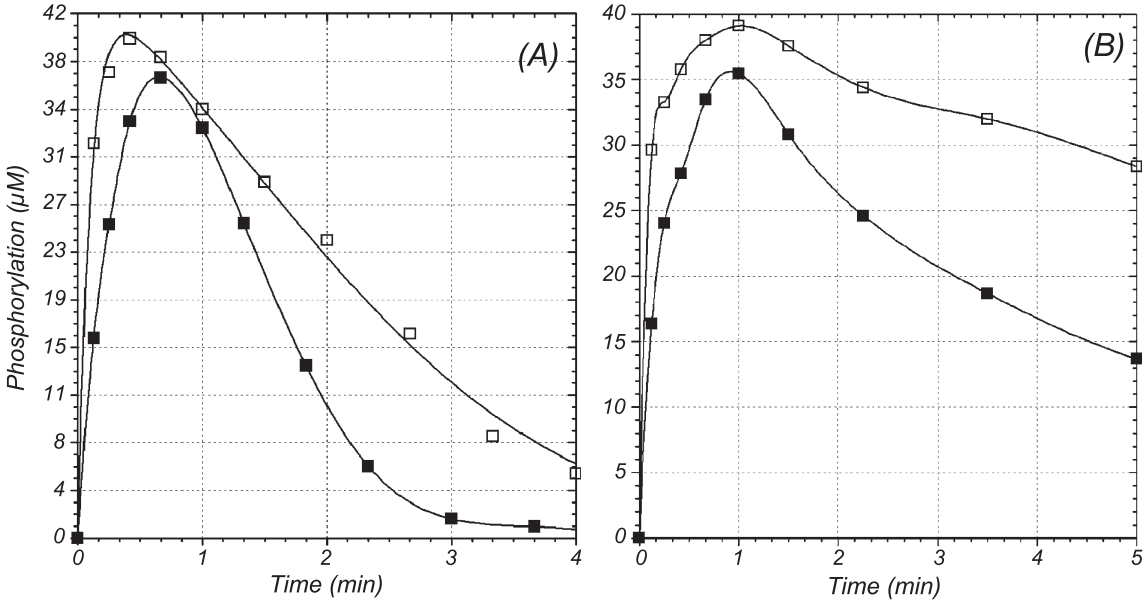

Fig. 2. Modulation of myosin phosphorylation and dephosphorylation phases by telokin for gizzard filamentous myosin (A) and actomyosin (B) systems. The solid squares correspond to the time courses in the presence of telokin, while the open squares are the controls with no telokin added. Concentrations of the myosin heads for both myosin and actomyosin were approximately the same (40 μM) and 60 μM of telokin was added. Note that the initial rapid phosphorylation was inhibited by telokin while the subsequent and slower dephosphorylation rate was accelerated by this modulator. The decrease in the phosphorylation amplitude is also indicative of an increase of the MLCP activity.

from the filaments. In this experiment, increasing concentrations of purified telokin were added to the native-like filaments and, after a short period of incubation, the filaments were removed by high-speed centrifugation. Relative activities of the kinase liberated by telokin were then measured in the supernatants using isolated ReLC of myosin as substrate; the latter substrate is not inhibited by telokin. As expected the amount of MLCK liberated from the filaments was proportional to the telokin concentration for the range up to about 1 to 2 telokin to myosin. At the

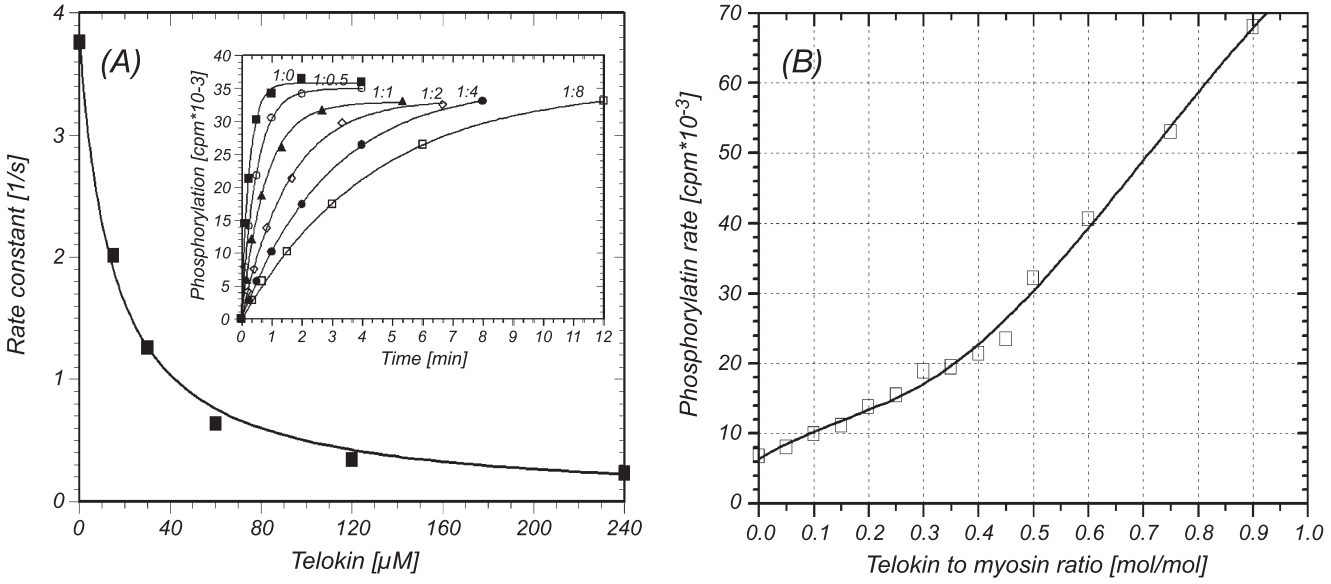

Fig. 3. Effects of telokin on myosin phosphorylation and binding of MLCK to filamentous myosin. (A) Inhibition of the first order rate constant is shown as a function of telokin concentration obtained in the presence of 250 nM of Microcystin that inhibited endogenous MLCP. Insert shows that there was no reduction of the maximal phosphorylation levels as the telokin to myosin molar ratio (given on each curve) increased. (B) The inhibition of the phosphorylation rates seen in (A) resulted from apparent "liberation" of MLCK from myosin filaments induced by telokin. In this experiment, increasing amounts of telokin were added to myosin filaments and, after 15 min incubation at 25 °C, the filaments were removed by centrifugation (30 min at 45,000×g). MLCK activities of the supernatants were then measured using the isolated ReLC and plotted as a function of telokin to myosin ratio. Note that there is no saturation in the "liberating" effect of telokin. At telokin to myosin ratio of about 1 to 1 or higher increasing amounts of non-sedimentable myosin were seen in the SDS-PAGE of the supernatants.

same time and as it is clear from the figure, there was no saturation for the liberating effect of telokin. SDS-PAGE analysis of the supernatant samples indicated that they also contained small amounts of non-sedimentable myosin cross-linked with telokin. These amounts were however high, relative to those of the MLCK present, and increased even more at higher telokin concentrations. This indicated that, under unphysiologically high telokin to myosin ratio, not only there was no simple competition between telokin and the kinase but also its interaction with myosin was more complex.

3.3. Sedimentation of myosin filaments

The interaction of telokin with myosin filaments was more clearly seen in another type of experiments in which telokin–myosin mixtures were sedimented into a step glycerol gradient of 10%, 25% and 50%. After 5 min centrifugation at 2000 rpm, filamentous myosin alone sedimented at the border region between 25% and 50% glycerol while in the presence of telokin the sedimentation was slower and the filaments were seen within the 10–25% border region (Fig. 4). Saturation of the effect was observed already at 2 to 1 myosin to telokin molar ratio and further increase of the telokin concentration resulted in higher amounts of non-sedimentable myosin being present on top of the gradient or in the 10% zone. Our analysis of the gradient fractions by SDS-PAGE showed that all telokin remained in this zone together with the non-sedimentable myosin. MLCK could not be detected by the SDS-PAGE but its activity was spread from the top to the 50% border region. Interestingly, MLCK alone had a similar effect on the sedimentation of myosin filaments and the effects of telokin and of the kinase were additive (Fig. 5a–d). In this

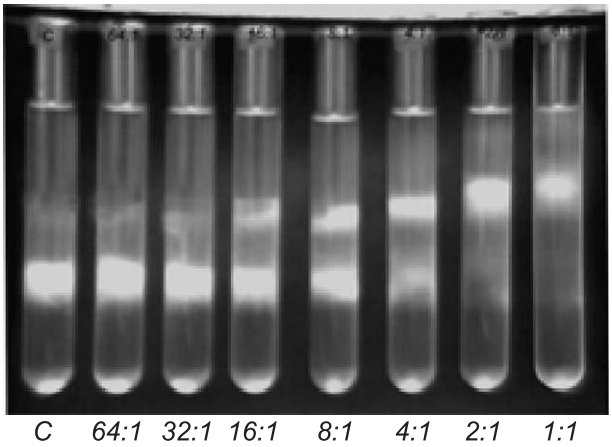

Fig. 4. Sedimentation of filamentous myosin into glycerol step gradient in the presence of increasing concentrations of telokin. Telokin was added to the filament suspensions at molar ratios to myosin indicated below each tube (C; no telokin present) and centrifuged for 5 min at 2000 rpm. Note that, in the absence of telokin, myosin filaments sedimented more rapidly and approached the 50% glycerol region while in its presence the sedimentation was slower and the filaments remained within the 25% region.

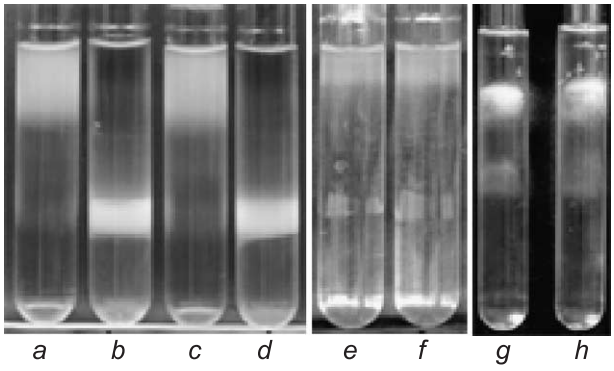

Fig. 5. Sedimentation of different type of filamentous myosin preparations in the presence and absence of telokin. (a–d) MLCK-rich gizzard, (e–f) rabbit skeletal and (g–h) molluscan filamentous myosin. The myosins were applied onto the glycerol gradients as in Fig. 4 with telokin (at 1 to 1 molar ratio) present in (a), (c), (e) and (g) and the tubes were centrifuged for 4 min at 4000 rpm. Note that telokin did not affect sedimentation of the skeletal and molluscan myosin. The effect of telokin was independent of calcium (a, b: 2 mM EGTA; c, d: 0.1 mM $CaCl_2$ present) and was additive to that of MLCK apoenzyme telokin (see Fig. 4 for a comparison). Under the conditions used MLCK and CaM-free myosin filaments sedimented to the bottom of the tube.

case, the kinase-rich filaments sedimented in-between the 25–50% zone, while, in the presence of telokin, they remained within the 10% zone even though a higher centrifuge force was used (4000 rpm, 4 min). Under these conditions, CaM- and MLCK-free myosin sedimented to the bottom of a tube. As it also shown in the figure, telokin effects were Ca-insensitive because exactly the same sedimentation pattern was observed in the presence and absence of this ion. Replacement of telokin for the isolated ReLC showed a similar but weaker effect. This indicates that there are some similarities between telokin and the ReLC, which we also noticed earlier [34].

Similar sedimentation experiments were also carried out using myosin filaments from other types of muscles and they indicated that the effects of telokin were very specific for vertebrate smooth muscle. Correspondingly, telokin did not modify the sedimentation properties of neither molluskan smooth nor rabbit skeletal muscle myosin although these two myosins by themselves exhibited quite different sedimentation properties (Fig. 5e–h).

3.4. Effects of telokin on activity of MLCP

Possibly as a result of protein losses during the myofibril preparations and the subsequent purification, the endogenous MLCP content of our myosin and actomyosin was rather variable and generally low in comparison to that of the MLCK (see Table 1). Nevertheless, the specific activity of MLCP must be very high because this phosphatase could not be detected by SDS-PAGE even in 40-fold concentrated samples obtained from myosin supernatants. Its presence could only be demonstrated by antibody staining. Cross reactivity was seen with our antibodies directed against both the catalytic and targeting subunits and the other antibodies

specific for the catalytic and myosin targeting subunits (Langsetmo, Strickland and Sobieszek, unpublished results). From the activity measurements of such supernatants during aging, it was apparent that the losses resulted from proteolysis degradation of the phosphatase targeting subunit that is responsible for its binding to myosin filaments. Correspondingly, the accelerating effect of telokin for the MLCP was, in general, lower than its inhibitory effect for the MLCK. This effect was more clearly seen after addition of a purified MLCP (see Ref. [14]) to our filamentous myosin which increased the level of MLCP activity present while reducing the myosin phosphorylation level not more than about 50% (Fig. 6A). In these experiments, MLCK activity of the complex was inhibited (by removal of Ca^{2+}) at the end of the phosphorylation phase and the subsequent dephosphorylation rates were used for measuring the effects of telokin on MLCP activity. As can be seen in the figure, the dephosphorylation rates of such MLCP-enriched myosin almost doubled at high concentrations of telokin.

The accelerating effect of telokin on the MLCP activity was also directly demonstrated using purified MLCK/MLCP complex (see Ref. [15]). In this case, much lower MLCP activities were required that could be subsequently accelerated by telokin. Therefore, we used a complex purified on CaM-affinity column which MLCK content was relatively high to that of MLCP. This activity was enhanced proportionally to the concentration of telokin added (Fig. 6B). Significantly, telokin had no effect on the activity of the purified catalytic subunit of this phosphatase (Fig. 6B).

Similar effects of telokin were also observed for actomyosin preparations although from the control experiments it was clear that myosin and actomyosin dephosphorylation phases were somewhat different (Fig. 2B). In the case of actomyosin, however, the dephosphorylation phase never reached the baseline. From the much higher ATP hydrolysis rate, a faster dephosphorylation was expected, and this was only observed at the beginning of the dephosphorylation phase. This was not related to a lower content of the endogenous MLCP because a full dephosphorylation was not attained even after addition of high concentration of the purified phosphatase. Thus, it appeared that a fraction (about 30%) of myosin heads was not accessible to the MLCP when F-actin was present. This problem was not further investigated.

3.5. Contraction and relaxation of skinned fibers in the presence of telokin

The reversible phosphorylation of myosin bears some analogy to the contraction–relaxation cycle of smooth muscle therefore we decided to test the effect of telokin on tension development in skinned smooth muscle. Skinned fibers of guinea pig *T. coli* were first relaxed in ATP-salt solution containing 4 mM EGTA (pCa>8). A graded isometric contraction was elicited by increasing the Ca^{+2} concentration in steps to pCa 6.2 and 4.3, respectively. Fig. 7A shows that at sub-maximal Ca^{2+} concentration telokin (20 μM) reduced the contractile force by about 60% from 50.5±5.2% of the maximally Ca-activated force to 19.5±7.2% (n=5), which remained approximately the same at high calcium levels (pCa>4.2). At the same time, the rate of force development was also reduced in the presence of telokin (Fig. 7B). In both cases, the time course could be fitted to a single asymptotically exponential curve with rate constants of 0.15 and 0.31 (min^{-1}), respectively, in the presence and absence of telokin.

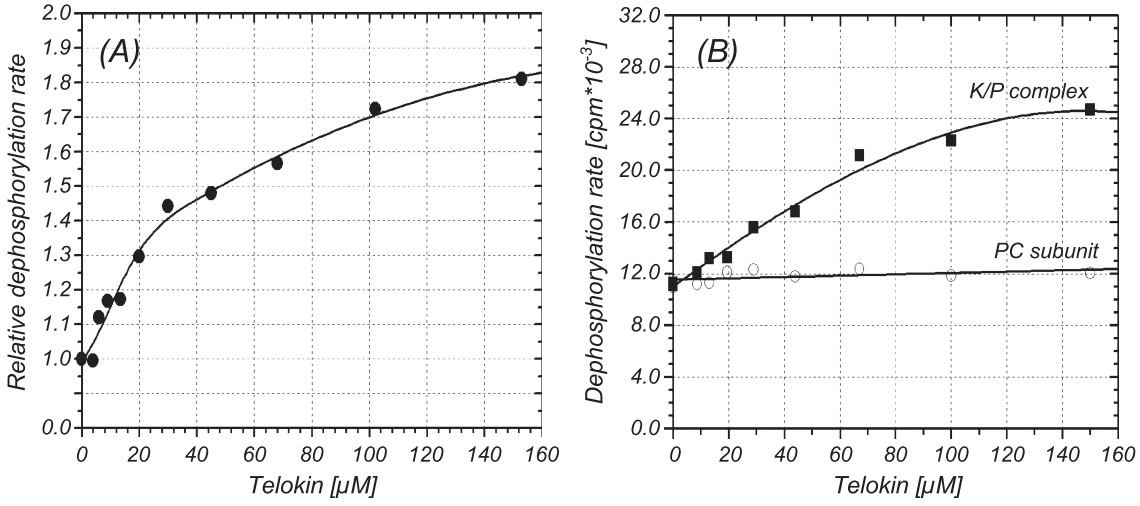

Fig. 6. Telokin induced acceleration of dephosphorylation rate of purified MLCP (A) and that of MLCK/MLCP complex (B). In (A), after addition of radioactive ATP and calcium, the myosin was first fully phosphorylated by the endogenous MLCK (1 min incubation). Subsequently, the kinase was inhibited by 2.5 mM of EGTA added together with the purified MCLP [14] and variable concentrations of telokin. The reaction was terminated 2.5 min later as for normal myosin phosphorylation assays. The myosin phosphorylation levels for the controls (no telokin present) were about 50%. In (B), the phosphatase activity of MLCK/MLCP complex purified by CaM-affinity chromatography [15] was measured as function of the telokin concentration added. Note that telokin accelerated the MLCP activity of the complex although it had no effect on the activity of purified catalytic subunit of this phosphatase.

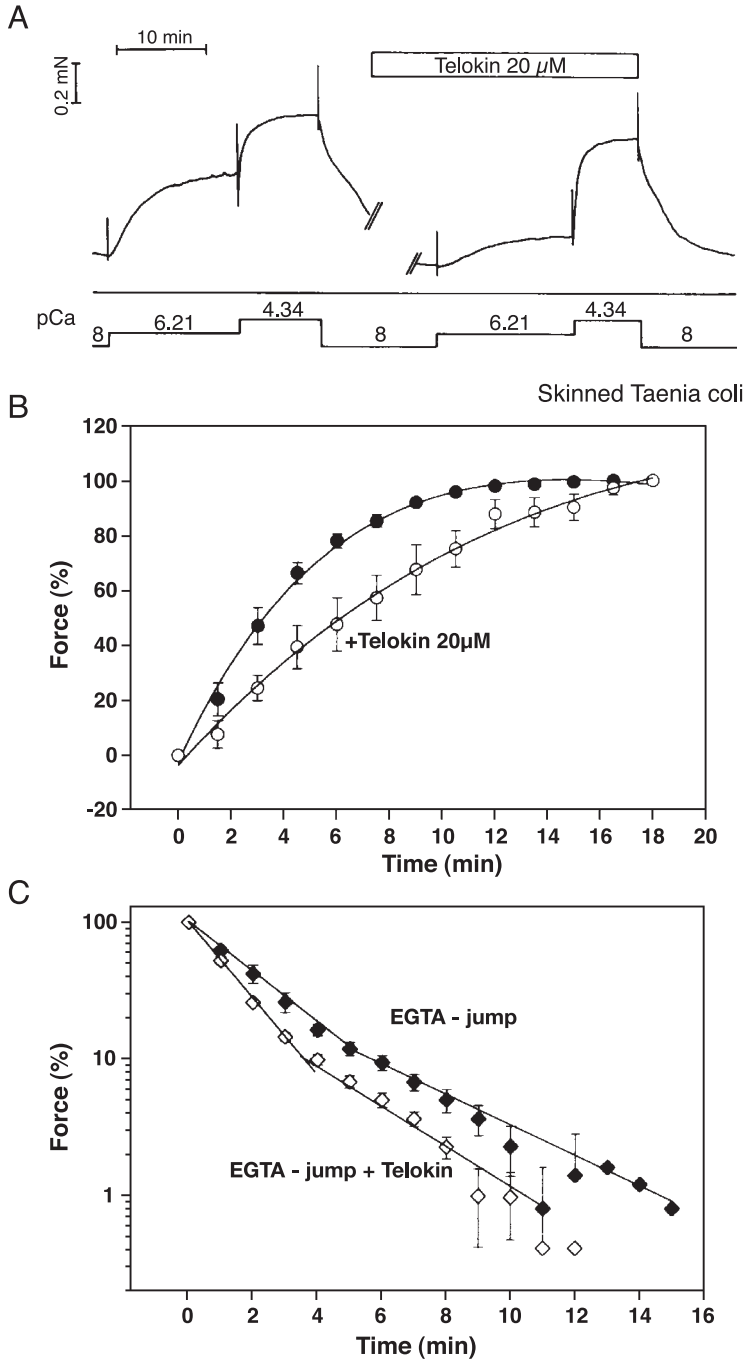

Fig. 7. Effect of telokin on contraction and relaxation of skinned Taenia coli fibers. (A) Telokin inhibited sub-maximal isomeric contraction (at pCa 6.2), but not maximally Ca-activated contraction (pCa 4.3). (B) The rate of force development induced at pCa 6.2 was reduced about two-fold in the presence of telokin. (C) Relaxation of sub-maximally Ca-(pCa 6.2) activated fibers (induced by EGTA jump) was accelerated by the presence of telokin.

Subsequent removal of Ca^{2+} with EGTA caused complete relaxation of the fibers. Prior to relaxation the fibers were, in some experiments, transferred into a contraction solution containing 0.5 mM rather than 4 mM Ca-EGTA (pCa 6.2) in order to reduce their calcium buffering capacity. Relaxation appeared to follow a two exponential process (Fig. 6C). Thus. addition of telokin resulted in an increase in the rate of relaxation of the fibers that were pre-contracted at pCa 6.2 suggesting that their endogenous MLCP became more active. The rate constants of the fast relaxation phase were 0.85 and 0.39 min^{-1} in the presence and absence of telokin, respectively. However, when the fibers were pre-contracted at pCa 4.3 (maximal activation), the fiber bundles relaxed with the same rate constants of about 0.34 min^{-1}. Thus, it is clear that the inhibitory and accelerating effects of telokin on our native-like filamentous myosin system were also observed for skinned muscle fibers exhibiting tonic type contraction.

3.6. Interaction with MLCK catalytic core

In another series of experiments, we attempted to establish which domain of the MLCK was responsible for its interaction with telokin to bring about the observed inhibition of myosin phosphorylation. For this purpose, the initial phosphorylation rates of myosin filaments were measured in the presence of MLCK modified by proteolytic digestion (Fig. 8A). Surprisingly the inhibition was observed for the 61-kDa constitutively active MLCK fragment and was only somewhat lower than that of the intact kinase (Fig. 8A). This indicated again that the inhibition resulted from interaction of telokin with myosin since an interaction with the catalytic domain has to be excluded from the lack of inhibition when isolated ReLC or myosin head subfragment were used as the substrates. In these experiments, MLCK and CaM-free myosin was used together with purified kinases (with or without CaM) at concentrations that gave the same final activities.

Because these above results may disagree with the already published data the experiments were repeated with a genetically truncated mammalian kinase in which either the C-terminal domain or both, the C- and N-terminal domains, were absent. Unexpectedly, the inhibition of myosin phosphorylation by the kinase devoid of the N-terminal domain (77-kDa MLCK) was lower in comparison with that of the wild-type mammalian kinase and the highest inhibition was observed for the 61-kDa kinase containing only catalytic core together with a very short CaM-binding segment (Fig. 8B). This further support our conclusion that the inhibition of myosin phosphorylation by telokin depended on telokin−myosin interaction and was not restricted to the shorter avian MLCK.

3.7. Activation of myosin phosphorylation by telokin

In another series of experiments, we investigated how did the presence of an excess of MLCK apoenzyme on one hand, or an excess of CaM on the other, influence the telokin-induced inhibition. It was expected that in these two situations telokin would behavior differently. This was indeed the case because an excess of the apoenzyme potentiated the inhibition while presence of additional CaM resulted in its reduction (Fig. 9A). At the same time, we noticed that, in the latter case, there was a small but significant acceleration of the rate at low and physiologically relevant myosin to telokin ratios. A comparison of the two curves at this region (Fig. 9A) also indicated that telokin presence results in an increase in the cooperativity of activation by CaM and this is consistent with the previously demonstrated high positively cooperative activation of MLCK by CaM at saturating levels of calcium.

The activation, at the low telokin to myosin ratios, was more clearly seen for the filaments formed from MLCK and CaM-free myosin mixed with small amounts of filamentous myosin. Families of phosphorylation progresses obtained for such mixtures were used in the evaluation of the first order rate constants of phosphorylation. As it is shown in Fig. 9B, telokin accelerated phosphorylation of the MLCK/CaM free myosin three to four folds proportionally to the concentration of the native-like myosin added. It is apparent that this acceleration resulted from telokin-induced liberation of the active MLCK/CaM complex associated with the filamentous myosin so this complex became available for the other MLCK and CaM-free filaments.

It has been established previously that myosin filaments are in equilibrium with their building units (e.g. anti–parallel myosin dimers). Therefore, for a mixture of the filaments

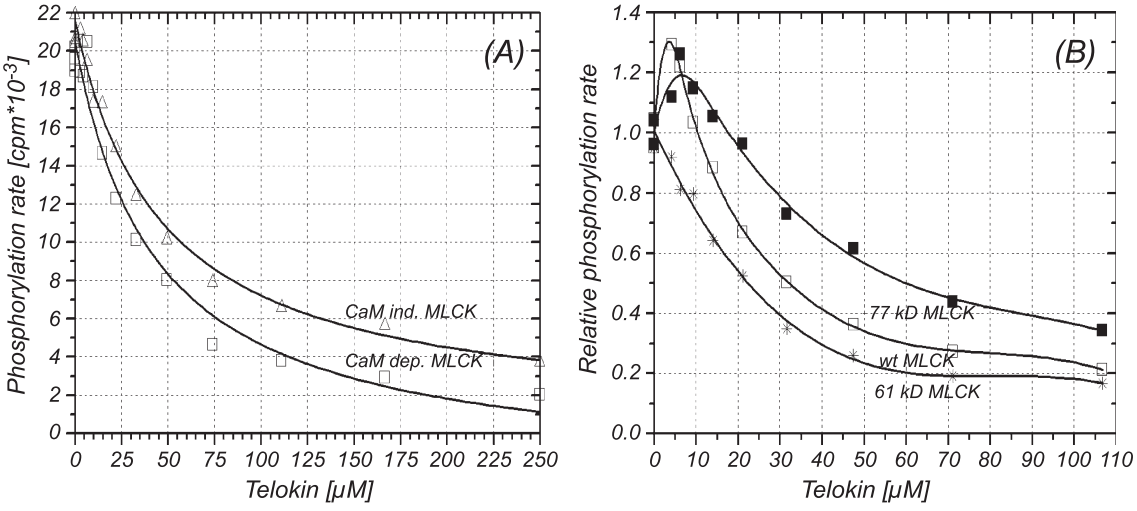

Fig. 8. Inhibitory effect of telokin with constitutively active catalytic core of MLCK. In (A), the phosphorylation rates of MLCK and CaM-free myosin filaments were measured as a function of telokin concentration with added gizzard MLCK catalytic core that was obtained by trypsin digestion (upper curve). The lower curve shows the control that was obtained for the same myosin with CaM-dependent kinase (160 nM) and CaM (240 nM). In (B), truncated recombinant mammalian kinases were tested for their inhibitory effect. Note that the inhibitory effect for the mammalian MLCK core (61-kDa MLCK) was stronger than that of the wild-type enzyme (wt. MLCK). Inhibition of the mammalian kinase devoided of the N-terminal is also shown (77-kDa MLCK).

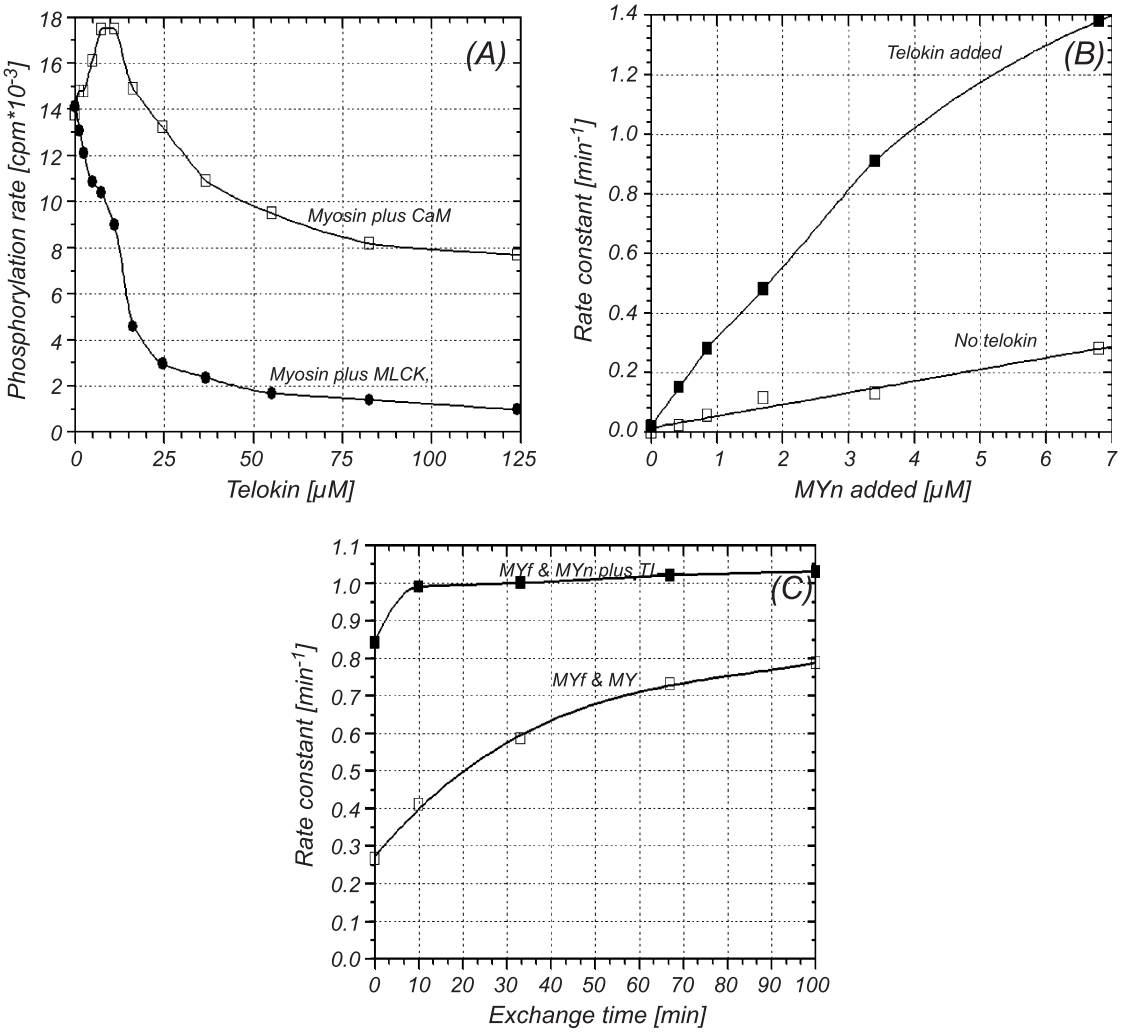

Fig. 9. Liberation of MLCK by telokin in the mixtures of filamentous myosin and the filaments free of MLCK and CaM. The inhibition of the phosphorylation rates was higher for the mixtures that contained MLCK apoenzyme not saturated with CaM (A: lower curve), while addition of an excess of CaM resulted in partial removal of the inhibition (A: upper curve). In the latter case, there was some increase of the rate at low telokin concentrations. This increase became significant when, in the presence of telokin, small amounts of filamentous myosin were added to myosin filaments free of MLCK and CaM (B) and resulted from telokin-induced liberation of the active MLCK/CaM complex. As shown in (C), the liberation was not a result of an exchange of myosin building units with the filaments. In this case, the two myosins (with and without telokin) were added together and their phosphorylation rates measured at different times after formation of the mixture. Note that the activating effect was reduced in time due to the myosin exchange. For more details, see text.

such as those used above, there will be a continuous liberation of the MLCK/CaM complexes resulting from a slow exchange of myosin between the filaments and their building units. The filaments are constantly reassembled and the time scale of this process is about 1 h or more. Therefore, measurements of the phosphorylation rates for mixtures of these two myosins gave us a possibility to test whether the complex was simply liberated by telokin or it remained bond and underwent exchange between the filaments. Fig. 9C shows how the first order rate constant changed with time after combining and mixing of these two myosins together in the presence and absence of telokin. From comparison of the curve obtained in the presence of telokin (upper curve) with the one obtained in its absence (lower curve), it is clear that the increase in the phosphorylation rate was mainly due to liberation of CaM/MLCK complex although, as expected, there was also a slow contribution of the myosin exchange in this process.

3.8. Phosphorylation in the presence of soluble substrates

Liberation of telokin could also be demonstrated in experiments in which isolated ReLC or myosin head subfragment (SF1) was added to filamentous myosin (Fig. 10A). In this case, the liberated MLCK/CaM complex was made available for the phosphorylation of the purified ReLC or that of the SF1. As expected from the proposed mechanism, as the telokin concentration increased, the inhibitory effect on myosin was removed because the rates for the mixtures remained constant. In the case of isolated ReLC, the activation was observed not only at lower range of telokin concentrations but also the rates were not reduced

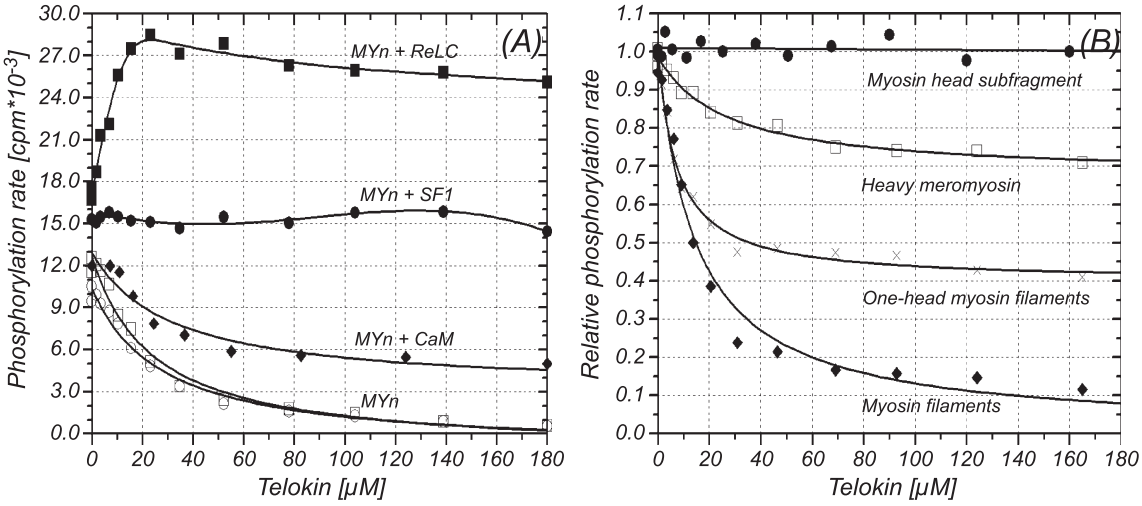

Fig. 10. (A) Telokin induced liberation of the CaM/MLCK complex in the presence of soluble substrates. The complex present on filamentous myosin readily phosphorylated isolated ReLC (upper curve) and myosin SF1 subfragment (middle curve). Myosin concentrations were the same for all the curves including the controls (the lower most curves). Note that, as the concentration of telokin increased, more kinase became available for phosphorylation of the ReLC and of SF1 because no significant reduction of the maximal phosphorylation was observed. Addition of CaM resulted in partial removal of the inhibition (the curve labeled MYn+CaM). (B) A comparison of the inhibitory effect of telokin for filamentous myosin with that of SF1 subfragment and heavy meromyosin as well as the filaments formed from one-head myosin. Note that the inhibition for the filaments formed from one-head myosin was about half as strong as compared to the intact myosin indicating that both heads were required for the inhibition. In all cases MLCK (100 nM) and CaM (150 nM) were added to the preparations.

at the higher range indicating that inhibition of the myosin component was removed (Fig. 10A).

In similar experiments, we evaluated the inhibitory effect of telokin on three myosin fragments and compared this effect with the inhibition of the filamentous myosin. In accordance to previous results, there was no inhibition of the soluble SF1 subfragment and inhibition of the soluble HMM was only moderate (Fig. 10B). Interestingly, the inhibition of the filaments formed from one-head myosin was only about 50% of that exhibited by the native-like myosin. Taken together, these experiments indicated that for effective interaction of telokin with myosin two myosin heads were required with myosin assembled into filaments and that the neck region of myosin was involved in this interaction.

4. Discussion

Myosin preparations used in the present study was extracted from a myofibril-like preparation obtained by extensive fragmentation and washing of the tissue as originally described by Sobieszek and Bremel [35]. These types of preparations are widely used in the smooth muscle field. The subsequent procedures used for purification of myosin vary significantly and, in our case, this amounted to ammonium sulfate fractionation of dissociated filamentous actomyosin and a semi-crystallization step that included formation of long myosin filaments (see Ref. [5]). Although the purified myosin appeared homogenous after SDS-PAGE, it contained relatively high levels of endogenous MLCK, CaM and MLCP, which were tightly bound to the myosin filaments [9,10]. Their tight association with purified myosin must reflect localization of these regulatory enzymes in vivo. This view is not generally shared by others in the field; most consider that MLCK and CaM, but not necessarily MLCP, are localized on actin filaments (for review, see Ref. [4]). The present observation indicated that the filamentous myosin became fully phosphorylated after 5–10 s at 35 °C and 1–2 min were required for its dephosphorylation after removal of Ca^{2+} by EGTA. Because these time courses are comparable with those associated with contraction and relaxation of intact smooth muscle, they fulfill an important criterion for their relevance in vivo [40]. Thus, the conclusions drown from the present study are therefore likely to be relevant to the regulation of smooth muscle.

Relevant to the present discussion is the question of whether or not other endogenous “contaminates”, especially protein kinases, were also present in our purified myosin. Their presence in amounts comparable to those of MLCK is unlikely because we could not detect additional bands in the SDS-PAGE of the various elutes from our affinity chromatography columns. The phosphorylation rates of our myosin and actomyosin preparations were practically zero in the absence of Ca^{+2}; therefore, contamination by kinase such as ZIP kinase, or other Ca-independent kinases, can be excluded. Nonetheless, it is possible that additional regulatory or modulatory proteins in amounts undetectable by SDS-PAGE could be present. In addition, low molecular weight proteins or subunits in the range of 10–20 kDa could be overlooked because they could overlap with the myosin light chains or CaM or they are simple too small to be detected by our SDS-PAGE. It would be particularly interesting to establish whether or not the 20-kDa MLCP subunits of unknown function [13] and the 17-kDa

phosphatase inhibitor (CPI-17 protein) (for references, see Ref. [40]) are present, even if in trace amounts. A possible presence of the first subunit could shed more light on the relationship between the two MLCP complexes discussed below while the level of the MLCP activity could be understated if contamination by the CPI-17 inhibitor was present. Nevertheless, from a comparison of our preparative methodology with that of Eto et al. [41], it is unlikely that this inhibitor was present in our filamentous myosin preparations unless it is a part of the phosphatase complex.

The structural details of MLCK and MLCP association with myosin filament remain unclear. We have recently demonstrated that these two enzymes form a functional complex, which also includes CaM and a novel MLCK targeting subunit [15]. The conclusion that a similar complex was copurified with myosin filaments was not undoubtedly supported by these studies and requires further investigations. An important question is the relationship between our MLCK-targeting subunit and the myosin targeting subunit (MBS) purified by others [40]. From antibody cross-reactivity (Sobieszek and Langsetmo, unpublished data), it is clear that the catalytic subunits of both complexes are the same and this agrees with a general view that there is only one PP1c catalytic subunit associated with the smooth muscle MLCP.

The time course curve of phosphorylation/dephosphorylation of our myosin and actomyosin preparations mimicked the contraction–relaxation cycle of phasic smooth muscle. After addition of ATP and Ca^{2+}, these preparations were first phosphorylated by MLCK and, after depletion of ATP or removal of Ca^{2+}, they were dephosphorylated by the MLCP. As a result, the phosphorylation progression could be separated into "contraction" and "relaxation" phases, respectively. This reversible phosphorylation was found to be convenient for testing the role of regulatory factors that affect myosin phosphorylation (i.e. contraction) and dephosphorylation (i.e. relaxation) of smooth muscle at the actomyosin level. One of such protein factors is telokin, the role of which in smooth muscle is still not fully understood at present. In view of its abundance (the molar content being comparable to that of myosin) and the sequence identity to the C-terminal immunoglobulin-like domain of MLCK [4], it was speculated early on that telokin must act at the level of myosin [29]. In order to understand better the role of telokin in the modulation of actin–myosin interaction, it was necessary to test its effects on phosphorylation progress of our native-like myosin and actomyosin systems.

After successful determination of telokin structure [28], subsequent studies on the role of telokin in smooth muscle have amounted to two main approaches. In the first approach effects of telokin (or its various recombinant forms) on contraction and/or relaxation of intact or skinned smooth muscle fibers have been investigated. In parallel, attempts have been made to find out what is the role of telokin phosphorylation in these processes since it has been known earlier on that telokin can be phosphorylated by cAP kinase in vitro [18]. Correspondingly, Somylo's group [32,40] reported that telokin induced relaxation of the intact fibers via acceleration of myosin dephosphorylation without affecting the rate of myosin phosphorylation. At the same time, it was noted that during permeabilization of muscle fibers most of the telokin was lost and this loss correlated with a decrease of the rate of cGMP dependent relaxation. This desensitization of muscle fibers agrees with our observations of acceleration, in the presence of added telokin, of the endogenous myosin MLCP activity and the relatively high increase in the relaxation rate of the skinned *T. coli* fibers. However, the pronounced telokin-dependent inhibition of the myosin phosphorylation rates that we observed in these and earlier studies does not agree with their conclusion about the lack of telokin effect on myosin phosphorylation. The reason for this discrepancy is not clear at present and could be related to the use of $\gamma S \cdot ATP$ by these authors for myosin phosphorylation in the permeabilized muscle strips. Significantly, we were able to demonstrate a desensitizing effect of telokin on tension development, i.e. contraction in the skinned fibers that must be related to the inhibition of myosin phosphorylation by telokin. It is a first confirmation of this kind and it is consistent with all data published so far on the effect of telokin on myosin phosphorylation in vitro [20,29,42]. From the presence of approximately equimolar amounts of telokin and myosin in smooth muscle [43], it is apparent that telokin should act on the substrate of MLCK, i.e. myosin filament rather than on the enzyme per se. Our sedimentation and kinetics results demonstrate clearly that this was indeed the case. This conclusion agrees with the observed absence of the inhibition for the soluble substrates of MLCK, i.e. isolated ReLC and the SF1 subfragment.

Less clear is telokin desensitization via acceleration of the MLCP activity. Significant in this respect is the recently demonstrated formation of a multi-enzyme complex by these two regulatory enzymes [15] and its possible association with the myosin filament. For such a complex system, MLCP and MLCK activities were, respectively, accelerated and inhibited by telokin while no effects were observed for the purified components, i.e. catalytic subunit of the phosphatase or on the phosphorylation of SF1 subfragment by MLCK. We interpret these observations in terms of dimerization of the MLCK by telokin that results in the dissociation of the complex with a reduction in binding of its subunits to myosin. In agreement with the idea of Johnson and Snyder [1], we also suggest that MLCP should be inhibited when it is complexed with MLCK. Therefore, the acceleration of MLCP activity observed in the present study is a logical consequence of dissociation of the catalytic subunit from the complex. Our preliminary observations on acceleration of the activity of MLCP endogenous to MLCK further support this conclusion (Sobieszek, unpublished results).

The inhibitory effect of telokin on myosin phosphorylation was first demonstrated by Shirinsky et al. [29]. They

have shown the inhibition of HMM phosphorylation, and the absence of such an inhibition for the isolated ReLC has been shown even earlier [22,23]. We confirmed these observations not only for filamentous myosin containing endogenous MLCK and CaM but also for the system reconstituted from the purified components. In contrast, their conclusion about the telokin-like domain of the kinase being responsible for the interaction with the myosin molecule (see also Ref. [43]) does not fit with the observed inhibition of the phosphorylation rates of the constitutively active MLCK core indicating indirect mode of telokin action. Absence of inhibition for the soluble system reconstituted from purified MLCK and CaM together with the myosin SF1 subfragment or ReLC and a moderate inhibition of HMM phosphorylation rate [29] indicates that the neck region of the myosin molecule (not present in the SF1) is involved in modification(s) of the interaction between myosin and telokin. MLCK is not involved in this interaction thus telokin acted exclusively at the level of myosin molecule confirming earlier conclusion of Sellers and his associates [42]. Our observations demonstrated in addition that at physiological concentrations, telokin induced a conformational change of myosin heads when the molecules were assembled into filaments. In this configuration the heads were extended from the filament surface disrupting or preventing lateral aggregation of the filaments. In view of the rather low affinity of telokin for myosin (see binding data in Ref. [42]), this must be a cooperative transition requiring very few telokin molecules to be bound to a filament. This kind of "Christmas tree-like" conformation was accompanied by reduction in the filament density (slower sedimentation) and lower affinity for MLCK and/or CaM (liberation of the MLCK activity). Thus, in the presence of telokin, the active MLCK/CaM could be more readily exchanged between the filaments by a relatively slow diffusional process resulting in the observed inhibition of myosin phosphorylation. The relatively high phosphorylation rates observed in the absence of telokin indicate that, under our experimental conditions, filament aggregation did not represent a diffusional barrier for ATP or the active CaM/MLCK complex. These denser filament aggregates must correspond to the semi-crystalline lateral aggregates of the filaments exhibiting 1.4 nm cross-bridge repeat frequently seen by us (Sobieszek, unpublished results) and by

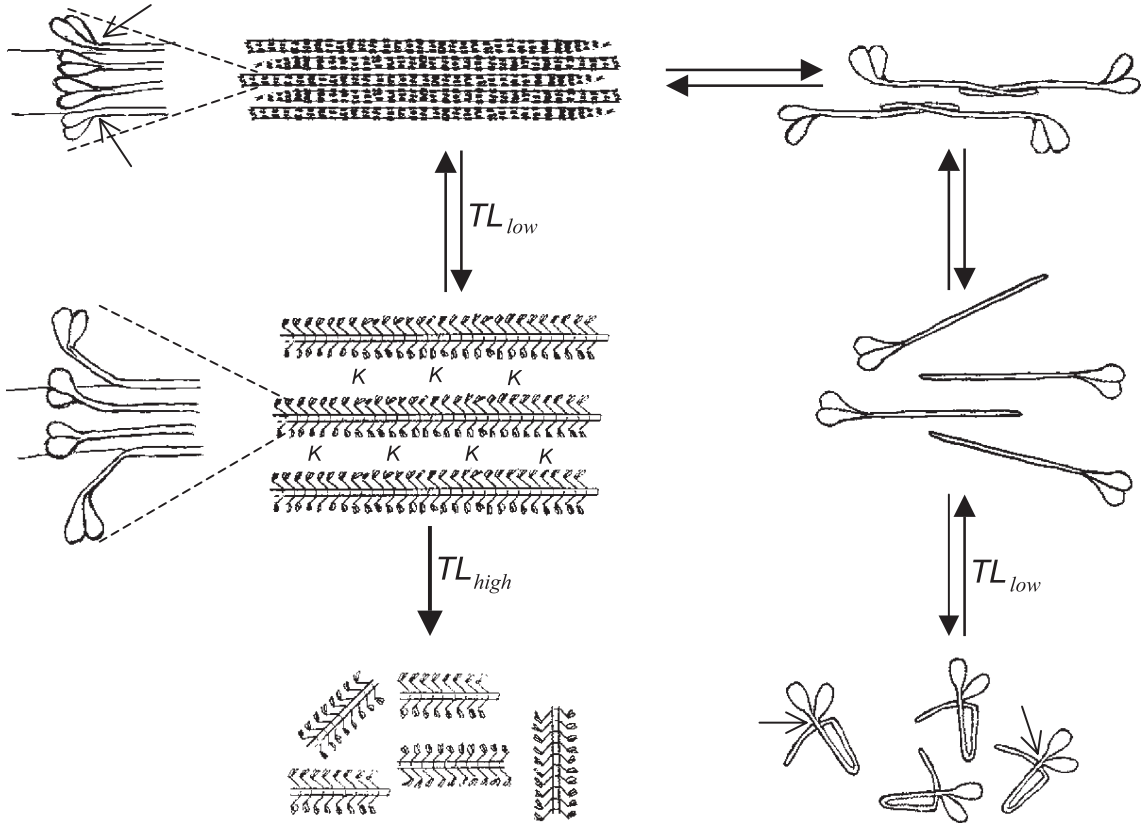

Fig. 11. Schematic representation of the role of telokin in smooth muscle. Previous observations [29,31] demonstrated that telokin induced unfolding of the folded (10S) myosin into its open 6S configuration. We suggest that the telokin-binding site is formed after folding within the "sticky" overlap region of a molecule (small arrows) and after binding of telokin, myosin unfolds eliminating the binding site. This increases concentration of the straight molecules available for formation of the filament. Similar binding site was formed when myosin was assembled into filaments, now between the myosin "sticky" regions and the rod portions of the neighbor molecules on the filament surface. Binding of telokin induced a similar conformation change (as for the folded myosin) with the heads extending from the filament surface and preventing their lateral aggregation. This facilitated liberation of MLCK/CaM complex thus making it available for neighbor filaments. At very high telokin levels the filaments appeared to partly disintegrate into shorter species because more and more non-sedimentable myosin was detected in the supernatants. After SDS-PAGE, the supernatants also contained myosin cross-linked with telokin.

others in electron micrographs of smooth muscle myosin filament preparations. The high phosphorylation rates for these structures and their reduction in the presence of telokin are also consistent with the recently suggested vectorial hypothesis [44] of the myosin filament phosphorylation.

At high concentration of telokin, we observed some disintegration of myosin filaments. This kind of disintegration is also apparent in the recent report of Vorotnikov and his group [45]. From their sedimentation data, it is clear that above 1 to 1 telokin to myosin ratio, significant amounts of myosin remained in the supernatants. Accordingly, in the original report, Sellers and his colleagues concluded that telokin stabilizes only "unphosphorylated mini filaments and in the presence of ATP" [29]. Subsequent observations [31,42] demonstrated that addition of telokin produces a shift from the folded (10S) conformation to the extended (6S) form, which can readily assemble into building units. These results together with our data were incorporated in the scheme of telokin action on the smooth muscle myosin (Fig. 11). In this scheme, we suggest that the binding site for telokin exists only for the folded 10S myosin conformation and that telokin binding induced unfolding, which in turn, abolished the binding site. Most likely the binding site could be located in the area of two oppositely charged sticky patch regions involved in the folding. An analogous binding site was also created within the filament, in this case not for the same molecule but for the two neighbor molecules on the filament surface. Thus, the conformational change or extension of the heads from the filament surface induced by telokin would be analogous to the unfolding of the individual myosin molecule.

Kendrick-Jones [46] originally demonstrated the induction of the 10S to 6S conformational change of myosin after its phosphorylation. From our own observations (Sobieszek, unpublished results), we know that the 6S extended conformation necessary for filaments formation requires only one of the two ReLC being phosphorylated. This together with the previously noted similarities between telokin and this light chain [34] indicates to us that telokin could in fact play a role similar to that of the phosphorylated ReLC. Very slow phosphorylation of telokin by MLCK at a threonine residue [34] is further indicative of this analogy. These properties together with telokin effect on the filament aggregation suggest that telokin may be playing a role of chaperon for the smooth muscle myosin. Therefore, it is perhaps not surprising that there are some similarities in its amino acid sequence to that of another chaperone protein from *Escherichia coli* [28]. Future studies will show whether or not this suggestion is justified.

Acknowledgements

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