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## Kinase Related Protein / telokin inhibits $\text{Ca}^{2+}$ -independent contraction in triton skinned guinea pig *taenia coli*

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## Synopsis

The kinase-related protein, KRP, also known as telokin, has been proposed to inhibit smooth muscle contractility by inhibiting the phosphorylation of the regulatory light chains of myosin (rMLC) by the  $\text{Ca}^{2+}$ -activated myosin light chain kinase (MLCK). Using the phosphatase inhibitor, microcystin, we now show that KRP also inhibits  $\text{Ca}^{2+}$ -independent rMLC phosphorylation and smooth muscle contraction mediated by novel  $\text{Ca}^{2+}$ -independent rMLC kinases. Incubating KRP depleted triton skinned *taenia coli* with microcystin at  $\text{pCa} > 8$  induced a slow contraction reaching 90% of maximal force ( $F_{\text{max}}$ ) at  $\text{pCa} 4.5$  after ~25 min. Loading the fibres with KRP significantly slowed down the force development, i.e. the time to reach 50% of  $F_{\text{max}}$  was increased from 8 min to 35 min. KRP similarly inhibited rMLC phosphorylation of heavy meromyosin (HMM) *in vitro* by MLCK or by the constitutively active MLCK fragment (61K-MLCK) lacking the myosin docking KRP-domain). A C-terminally truncated KRP defective in myosin binding inhibited neither force nor HMM phosphorylation. Phosphorylated KRP inhibited the rMLC phosphorylation of HMM *in vitro* and  $\text{Ca}^{2+}$ -insensitive contractions in fibres similar to unphosphorylated KRP, whereby the phosphorylation state of KRP was not altered in the fibres. We conclude that (i) KRP inhibits not only MLCK induced contractions, but also those elicited by  $\text{Ca}^{2+}$ -independent rMLC kinases; (ii) phosphorylation of KRP does not modulate this effect; (iii) binding of KRP to myosin is essential for this inhibition; and (iv) KRP inhibition of rMLC phosphorylation is most likely due to the shielding of the phosphorylation site on the rMLC.

Short title:

Kinase related protein inhibits  $\text{Ca}^{2+}$ -independent smooth muscle contraction

Keywords:

KRP, telokin, MLCK, MLCP, phosphorylation, smooth muscle contraction

List of abbreviations:

Kinase related protein, KRP; regulatory light chains of myosin, rMLC; myosin light chain kinase, MLCK; myosin light chain phosphatase, MLCP; heavy meromyosin, HMM; zipper-interacting protein kinase, ZIP-kinase; integrin linked kinase, ILK; mitogen-activated protein kinase, MAPK; extracellular-signal-regulated kinase, ERK; MAPK/ERK kinase 1, MEK1, myosin heavy chain, MHC; trichloroacetic acid, TCA, Polyvinylidene Fluoride, PVDF, enhanced chemiluminescence, ECL.

## INTRODUCTION

Phosphorylation of Ser<sup>19</sup> of the regulatory myosin light chain (rMLC) catalyzed by the Ca<sup>2+</sup>-calmodulin activated myosin light chain kinase (MLCK) is a prerequisite for the activation of smooth muscle actomyosin MgATPase and contraction [1]. Dephosphorylation of Ser<sup>19</sup> by rMLC phosphatase (MLCP) results in myosin inactivation and relaxation [2]. The level of rMLC phosphorylation and, hence, of force is determined by the ratio of MLCK to MLCP activity. A rise in the cytosolic free Ca<sup>2+</sup>-concentration ([Ca<sup>2+</sup>]<sub>i</sub>) in response to contractile stimuli is the major determinant of rMLC phosphorylation and, hence, of smooth muscle contraction. However, many contractile agonists increase force and rMLC phosphorylation further at a given [Ca<sup>2+</sup>]<sub>i</sub> leading to a left-ward shift in the force-[Ca<sup>2+</sup>]<sub>i</sub> relationship, a phenomenon now known as Ca<sup>2+</sup>-sensitization [3, 4]. Conversely, relaxant agonists, which act through a rise in the intracellular cyclic nucleotide levels, relax smooth muscle not only by a decrease in [Ca<sup>2+</sup>]<sub>i</sub> but often also by decreasing the Ca<sup>2+</sup>-sensitivity of rMLC phosphorylation.

Ca<sup>2+</sup>-sensitization, has been mainly attributed to G protein mediated inhibition of MLCP which shifts the balance in favour of the rMLC phosphorylation reaction [3]. It is generally held that the kinase responsible for phosphorylation of rMLC under these conditions is MLCK, which is partially activated at the prevailing [Ca<sup>2+</sup>]<sub>i</sub>. However, evidence has been accumulating recently that rMLC can also be phosphorylated in a Ca<sup>2+</sup>- and MLCK-independent manner by distinct protein kinases which are activated in response to certain agonists [5-10]. Thus, the leftward shift in the force-[Ca<sup>2+</sup>]<sub>i</sub> relationship may reflect a concerted action of partially activated MLCK and non-canonical Ca<sup>2+</sup>-independent rMLC kinases. Out of several putative Ca<sup>2+</sup>-independent rMLC kinases (reviewed in [11]), the MYPT1 associated zipper interacting (ZIP) kinase [12], and the myofilament associated integrin linked kinase (ILK), which is spatially distinct from membrane bound ILK [10], emerged as bona fide candidates. In contrast to MLCK, which specifically phosphorylates Ser<sup>19</sup>, these non-canonical rMLC kinases phosphorylate Ser<sup>19</sup> and Thr<sup>18</sup> with equal efficiency [10, 12].

Ca<sup>2+</sup>-desensitization, on the other hand may involve the disinhibition of MLCP by reversing the G protein-coupled Ca<sup>2+</sup>-sensitization [13], or the direct activation of MLCP by interaction between the leucine zipper motifs of protein kinase G and MYPT1 [14], as well as inhibitory phosphorylation of MLCK [15]. In addition, it was proposed that the kinase related protein (KRP), also known as telokin, decreases Ca<sup>2+</sup>-sensitivity, thereby contributing to ~50% of cGMP-induced, Ca<sup>2+</sup>-independent relaxation in ileal smooth muscle [16]. However, the mechanism by which KRP decreases Ca<sup>2+</sup>-sensitivity is not understood. In particular, it is not known whether it can antagonize Ca<sup>2+</sup>-sensitization induced by the non-canonical, Ca<sup>2+</sup>-independent rMLC kinases.

KRP, a small acidic protein, was discovered by Hartshorne and colleagues [17] and was later shown to be a smooth muscle-specific protein whose sequence is identical to the C-terminal domain of sm-MLCK [18-20]. It is independently transcribed through a promoter located in an intron of the *mylk1* gene, and is expressed in very high levels in intestinal, urinary and reproductive tracts, and at lower levels in vascular smooth muscle [18, 19, 21, 22]. KRP is devoid of the calmodulin and nucleotide binding sites of MLCK, as well as its catalytic domain. The crystal structure showed an immunoglobulin-like fold bordered by a C-terminal 19 amino acid and a N-terminal 35 amino acid sequence which were not resolved in the crystal structure, suggesting that these segments are highly flexible and may be of relevance for the function of KRP [23]. KRP binds with the acidic C-terminus to the neck region of unphosphorylated myosin, but not to phosphorylated myosin [24], and thereby promotes the extended conformation of myosin and filament formation [25, 26]. The flexible N-terminus harbours several phosphorylation sites, including a PKA/PKG (Ser<sup>13</sup>) and a MAP kinase phosphorylation site (Ser<sup>19</sup>) [22, 27-30]. In intact ileum, forskolin leads to a significant

phosphorylation of Ser<sup>13</sup>, which correlates with relaxation, whereas phosphorylation of Ser<sup>19</sup> increases only a little in response to PKC mediated activation of MAP kinase [27].

In permeabilized smooth muscle, KRP inhibited submaximal Ca<sup>2+</sup>-activated force, i.e. decreased Ca<sup>2+</sup>-sensitivity [31] and accelerated relaxation [32]. The relaxing effect was potentiated by cGMP mediated phosphorylation of KRP, or by a Ser13Asp phosphorylation mimicking mutation in KRP [21, 30, 32]. KRP's relaxing effect could be due to either inhibition of MLCK or increasing the activity of MLCP. To determine the MLCK activity in the skinned fibres, ATPγS was used as substrate because thiophosphorylated rMLCs are not dephosphorylated by MLCP [16]. As a measure of MLCP activity, the rate of dephosphorylation of prephosphorylated rMCL was used [32]. KRP did not alter the rate of thiophosphorylation but increased the rate of dephosphorylation of rMLC. Therefore, the authors [16] proposed that KRP relaxes smooth muscle by increasing the activity of MLCP by an as yet not defined mechanism. Consistent with these experiments, the rate of dephosphorylation of a so called native myosin preparation which contains MLCK, calmodulin, and MLCP was increased by KRP under cell free conditions [31].

On the other hand, a substantial body of biochemical evidence showed that KRP attenuates the rate of MLCK catalyzed rMLC phosphorylation of intact myosin [24, 26, 31, 33], and the soluble fragment of myosin, HMM [24], but not of isolated rMLC [18, 33]. From this it was concluded that (i) formation of myosin filaments is not required for the inhibition of phosphorylation, and (ii) KRP does not interact with the catalytic core of MLCK in a functionally relevant manner. The C-termini of KRP and MLCK share a common docking site on the myosin S1-S2 junction which is independent from the catalytic interaction of MLCK with rMLC [24]. Because KRP binding affects only the  $K_m$  but not the  $V_{max}$  value of the phosphorylation reaction, it was proposed that KRP inhibits the phosphate transfer onto rMLC by competing with MLCK for docking binding to myosin [34]. Indeed, KRP displaces MLCK from smooth muscle myosin [25, 35], and the binding of KRP to myosin is critical for the inhibition of rMLC phosphorylation [29]. This mechanism, i.e. competition between KRP and MLCK for docking to myosin, is therefore specific to the dedicated MLCK and would not apply to rMLC kinases that do not use the KRP domain mediated docking onto the substrate.

An attractive hypothesis is that KRP, because of binding in the vicinity of rMLC, limits the accessibility of Ser<sup>19</sup> and thereby inhibits rMLC phosphorylation and contraction induced by non-canonical rMLC kinases. The aim of this study was, therefore, to determine whether KRP inhibits (i) rMLC phosphorylation of HMM induced by the constitutively active, proteolytic fragment of MLCK, which lacks the KRP domain and, hence, does not bind to myosin, and (ii) Ca<sup>2+</sup>- and MLCK-independent contractions in triton skinned fibres. To elicit Ca<sup>2+</sup>- and MLCK-independent contractions, the type 1 and type 2A phosphatase inhibitor, microcystin, was employed as it has been reported by several laboratories that inhibition of MLCP by microcystin unmasks the action of the non-canonical rMLC kinases in permeabilized vascular [5, 6, 12, 36] and ileal smooth muscle [7, 8]. In this report, we show that KRP inhibits the microcystin induced, Ca<sup>2+</sup>-independent sustained contraction and rMLC phosphorylation in skinned fibres. It likewise inhibits rMLC phosphorylation of HMM by the constitutively active fragment of MLCK (61K-MLCK), i.e. by a mechanism different from the displacement of MLCK from myosin. We further show that this effect is not modulated by phosphorylation of KRP with PKA, MAPK, or both kinases, whereas it critically depends on the binding of KRP to myosin. From these experiments, we conclude that KRP is a general inhibitor of the phosphorylation reaction catalyzed by the Ca<sup>2+</sup>-dependent MLCK and the non-canonical rMLC kinases.

## EXPERIMENTAL

### Materials

The polyclonal antibodies to KRP [22] and KRP phosphorylated at Ser<sup>13</sup> and Ser<sup>19</sup> [27] have been described. The anti-rMLC antibody (clone Y-21) and microcystin-LR were from Sigma, USA. The polyclonal anti-rMLC antibody (MRLC3) was from Proteintech Group, USA. The protein kinase A catalytic subunit, wortmannin, Y-27632, okadaic acid, and staurosporine were purchased from Calbiochem, USA. Alexa Fluor<sup>®</sup> 488 C<sub>5</sub> maleimide, Alexa Fluor<sup>®</sup> 488 succinimidyl ester, and Alexa Fluor<sup>®</sup> 555 phalloidin were from Invitrogen, Carlsbad, CA, USA; DAPI (4',6-Diamino-2-phenylindole dihydrochloride) was purchased from SIGMA, St Louis, MI, USA. Restriction and modifying enzymes were from New England Biolabs (USA) and Fermentas (Lithuania). The [ $\gamma$ -<sup>32</sup>P]-ATP was from the Institute of Physics and Energetics, Obninsk (Russia) and from Hartmann Analytic (Braunschweig, Germany). Biological buffers and general chemicals were from Sigma (USA) or AppliChem (Germany).

### Plasmids and molecular cloning

GST-tagged mitogen-activated protein kinase with a molecular weight of 44 kDa (p44<sup>erk1</sup>-MAP kinase) expression and activation by phosphorylation with the constitutively active His<sub>6</sub>-tagged MAPK/ERK (extracellular-signal-regulated kinase) kinase 1 (MEK1) were performed as described [27]. The plasmid pET22-b+/DD-hKRP, expressing the phosphorylation-mimicking KRP mutant, S13D,S19D, was generated using the pET22-b+/hKRP construct encoding the full-length human KRP [27] as a template. Plasmids pET21-d(+)/chiKRP-His, pET21-d(+)/ $\Delta$ C-chiKRP-His and pET21-d(+)/ $\Delta$ N-chiKRP-His encoding, respectively, the full-length chicken KRP, its C-terminally truncated ( $\Delta$ C-KRP, aa 1-138) and N-terminally truncated mutants ( $\Delta$ N-KRP, aa 35-157), all containing the His<sub>6</sub>-tag at the C-termini, were described [24]. The  $\Delta$ C-hKRP cDNA (aa 1-136 of human KRP) was amplified by PCR using 5'-TATAGATCTCCATGGCAATGATCTCAGGGC-3' as a forward primer and 5'-AAACTCGAGCTAAAGCTCTGCTGTGCAGGTG-3' as a reverse primer, and subcloned into pET28-a+ vector (Novagen, USA) at NcoI and XhoI restriction sites. The constructs were confirmed by DNA sequencing.

### Proteins

HMM [37, 38] and native 108 kDa MLCK were purified from chicken gizzards. The constitutively active fragment of MLCK (61K-MLCK, molecular weight ~61 kDa) was obtained by tryptic digestion of 108 kDa MLCK, according to [39]. We note that the truncated MLCK lacks the C-terminal KRP domain and possesses somewhat lower catalytic activity than the parent MLCK. Calmodulin was purified from bovine testis [40]. The other recombinant proteins were expressed in BL21 (DE3) *E. coli* strain. His<sub>6</sub>-MEK1 and chicken His<sub>6</sub>-KRP were purified from bacterial lysates on TALON affinity matrices (Clontech, USA). (GST)-tagged p44<sup>erk1</sup> MAP-kinase was purified on Glutathione-Sepharose (Amersham Pharmacia Biotech.). Human recombinant non-tagged KRP proteins were purified by the method of Ito and coworkers [17]. KRP concentrations were determined using specific absorption coefficient  $A_{1\text{cm}}^{0.1\%}_{280}=0.77$  [24]. Human recombinant KRP was used in all fibre

experiments, whereas in the biochemical experiments both chicken and human KRP were used as specified in the figure legends.

### Phosphorylation of KRP in vitro

The full-size human KRP was completely phosphorylated by protein kinase A and/or p44<sup>erk1</sup>-MAP kinase as in [27]. The extent of phosphorylation was confirmed by the shift of PKA and MAPK phosphorylated KRP in urea-glycerol gels [41] or SDS-gels (SDS-PAGE) [42], respectively. Phosphorylated KRP was purified from PKA using ion-exchange chromatography on cellulose phosphate (Sigma, USA) in 15 mM MES (pH 6.5), 0.1 mM PMSF, and 1 mM DTT. Under these conditions, PKA bound to the column and KRP was eluted in the flow through. Glutathione Sepharose and TALON affinity matrices, respectively, were used to remove the GST-tagged p44<sup>erk1</sup>-MAP kinase and His<sub>6</sub>-MEK1. The efficacy of these procedures was checked by adding unphosphorylated human  $\Delta$ C-KRP as a substrate for any remaining kinase to the column purified phospho-KRP. The KRP proteins, i.e. the full length KRP and  $\Delta$ C-KRP which have different electrophoretic mobilities, were separated by SDS-PAGE followed by immunoblotting with phospho-specific antibodies against phospho-Ser<sup>13</sup> and phospho-Ser<sup>19</sup> KRP as described below. No immunoreactivity was associated with the  $\Delta$ C-KRP-band, indicating that no kinase activities remained in the phosphorylated KRP preparations.

### HMM Phosphorylation by MLCK

4  $\mu$ M HMM was phosphorylated by 10 nM 108 kDa MLCK or 40 nM of 61K-MLCK at 30°C in the buffer containing 10 mM MOPS (pH 7.0), 1 mM MgCl<sub>2</sub>, 0.1 mM EGTA, 20 mM or 150 mM NaCl, 1 mM DTT, 0.1 mM PMSF, 0.1-0.5 mM ATP, in the presence or absence of 20  $\mu$ M or 50  $\mu$ M of full-size unphosphorylated KRP or KRP phosphorylated by PKA and/or MAP-kinase. When 108 kDa MLCK was used, 300 nM calmodulin and 0.5 mM Ca<sup>2+</sup> were also added. Equal aliquots of the reaction mixture were withdrawn at different time intervals and their composition was analyzed by either SDS-PAGE or urea-glycerol gels. Anti-total rMLC (MRLC3) antibody was used to determine the level of rMLC phosphorylation after the separation of phospho- and unphospho-rMLC by urea-glycerol gel electrophoresis. The antibody binding was visualized by diaminobenzidine staining. Alternatively, phosphorylation of rMLC was conducted in the presence of 1.5-2.5 kBq/ml [ $\gamma$ -<sup>32</sup>P]-ATP and visualized by autoradiography. The resultant images were scanned and quantified using the BioRad GS800 calibrated densitometer and Quantity One 4.6 software. Phosphorylation levels were calculated as ratios of the phospho-rMLC signal to that of the total rMLC, and in the case of radioactive experiments, they were further referenced to that of completely phosphorylated rMLC in order to obtain the phosphorylation stoichiometry. The completeness of rMLC phosphorylation was controlled by the urea gels.

### Tissue preparation and force measurements

Male or female guinea pigs were anaesthetized with isofluoran and killed by exsanguination following the procedures approved by the Institutional Animal Care and Use Committee of the University of Cologne. The *taenia coli* was removed and permeabilized by chemical

skinning with 1% Triton X-100 for 4 hours on ice, as described in [43]. The skinned fibres were stored at  $-20^{\circ}\text{C}$  in relaxing solution containing 50% glycerol and used within 2 weeks. The relaxing solution contained 20 mM imidazole, 7.5 mM  $\text{Na}_2\text{ATP}$ , 10 mM Mg-acetate, 4 mM EGTA, 1 mM  $\text{NaN}_3$ , 140 U/ml creatine kinase, 10 mM creatine phosphate, 1  $\mu\text{M}$  leupeptin, 2 mM DTT, 0.5 or 1  $\mu\text{M}$  calmodulin (pH 6.7) at room temperature. The contracting solution additionally contained 4 mM  $\text{CaCl}_2$ . The desired  $[\text{Ca}^{2+}]$  was obtained by mixing contracting and relaxing solution in the appropriate ratio,  $[\text{Ca}^{2+}]$  was calculated as in [44]. KRP or the respective volume of KRP dialysis buffer was added to the incubation solutions as specified below.

For force measurements, the strips ( $\sim 0.2$  mm in diameter and 5 mm in length) were mounted between a fixed post and a KG7 force transducer (Scientific Instruments, Germany) while in relaxing solution, stretched by 5% of slack length, and allowed to equilibrate for 15–20 min. After an initial control contraction (pCa 4.5), the fibres were relaxed and incubated with the appropriate KRP species (final concentration 10  $\mu\text{M}$ ) or dialysis buffer (control) for 40 min. The  $\text{Ca}^{2+}$ -independent contraction was elicited by the addition of 10  $\mu\text{M}$  microcystin-LR (final concentration) in the continued presence of KRP or dialysis buffer (control). After recording the  $\text{Ca}^{2+}$ -independent contraction, the fibres were immersed in  $\text{Ca}^{2+}$ -containing contraction solution (pCa 4.5) to determine the maximum force ( $F_{\text{max}}$ ) which was used to normalize the microcystin-induced force values.

### Determination of the KRP content in *taenia coli*

Immediately after isolation and determination of the wet weight, the *taenia coli* was frozen in liquid nitrogen and stored at  $-80^{\circ}\text{C}$ . The *taenia coli* was thawed in 10 volumes of extraction buffer (12.5% glycerol, 2% SDS, 67.5 mM Tris (pH 6.8), and 5%  $\beta$ -mercaptoethanol), homogenised, and incubated on ice for 1 hour. Following centrifugation at 20,800 g for 10 min at  $+4^{\circ}\text{C}$ , the total protein content of the supernatant, referred to in the following as “whole muscle extract, WME”, was determined with the Bradford assay using BSA as a standard. The proteins of the WME were separated by SDS-PAGE, transferred to PVDF membranes, and probed with a polyclonal anti KRP antibody [22]. Three different concentrations of the WME were run for each *taenia coli* sample. In order to construct a calibration curve for KRP, on each gel three different concentrations of recombinant wild type KRP were run in addition. The amount of KRP in the tissue was interpolated from the linear range of the calibration curve. The molar content of the tissue was calculated assuming an extracellular space of 32% as reported for *taenia coli* [45]. Densitometric scans of the myosin heavy chain (MHC) band indicated that the MHC content in intact and triton skinned *taenia coli* is similar.

### Confocal microscopy of KRP-loaded fibres

To assess the loading of *taenia coli* fibres with confocal microscopy, recombinant human KRP was labelled in independent reactions either at the lysine residues or at the cysteine residues. Briefly, for labeling the  $\epsilon$ -amino group of the lysine residues with Alexa Fluor<sup>®</sup> 488 succinimidyl ester, CA), the pH of KRP that was previously dialyzed against phosphate buffered saline (140 mM NaCl, 10 mM  $\text{PO}_4$ , 3 mM KCl) was increased by adding sodium bicarbonate (pH 9.0) to a final concentration of 0.1 M. Labelling efficiency was assessed by adding Alexa Fluor<sup>®</sup> 488 succinimidyl ester dissolved in dimethylfluoride to KRP in either a 8.8, or 17.7 fold excess, assuming a 100% activity of the label. The reactions were stopped by adding hydroxylamine (pH 8.5) to a final concentration of 0.15 M, followed by a 60 minute

incubation at room temperature. KRP was dialysed against 10 mM KPO<sub>4</sub> (pH 6.5), 100 mM KCl, 5 mM EDTA, and 1 mM DTT for labelling the cystein residues with Alexa Fluor<sup>®</sup> 488 C<sub>5</sub> maleimide, CA). After dialysis, the phosphate concentration was adjusted to 50 mM using a 500 mM potassium phosphate stock solution (pH 6.0). In order to reduce the thiol groups, the DTT concentration was raised to 10 mM and the protein was incubated for 30 min at 37°C. In order to prevent an interference of the excess DTT in the following reaction, the protein was subsequently dialyzed with 2 buffer changes against 50 mM Tris (pH 7.5), 100 mM KCl, 1 mM EDTA, and 0.1 mM DTT. A threefold excess of Alexa Fluor<sup>®</sup> 488 C<sub>5</sub> maleimide was added and the mixture was incubated for four hours at 18°C. The reaction was stopped by adding 1 mM DTT. Unincorporated fluorescent dyes were removed by extensive dialysis against 10 mM MOPS (pH 7.0), 20 mM NaCl, 1 mM MgCl<sub>2</sub>, 0.1 mM EGTA, and 1 mM DTT.

Triton skinned *taenia coli* fibres held isometrically were incubated for 40 min at room temperature with 10 µM labelled KRP in relaxing solution. Excess label was removed by washing the fibres twice for 2 min each in relaxing solution containing 10 µM non-fluorescent KRP. The specimens were fixed in 4% paraformaldehyde for 30 min, washed twice with PBS and embedded in Mowiol 4-88. The staining of F-actin with Alexa Fluor<sup>®</sup> 555 phalloidin was performed with 5 U phalloidin per reaction. DAPI (4',6-Diamidino-2-phenylindole dihydrochloride) was used for the staining of nucleic acid. Fluorescent signals were detected by confocal microscopy on a Leica DMI 6000B microscope, using excitation wavelengths of 405 nm for DAPI, 488 nm for Alexa Fluor<sup>®</sup> 488, and 561 nm for Alexa Fluor<sup>®</sup> 555. The laser was operated with 16% intensity at 405 nm, 22% intensity at 488 nm, and 18% intensity at 561 nm.

### Determination of rMLC phosphorylation in fibres

The extent of rMLC phosphorylation was determined using glycerol-urea electrophoresis [41]. In brief, the isometrically contracted fibres were immersed into the ice-cold 15% trichloroacetic acid containing 5% Na-pyrophosphate for 10 min at the desired time intervals, and subsequently homogenized in the urea sample buffer containing 20 mM Tris/HCl (pH 6.8) 9 M urea, and 10 mM DTT. The lysates were clarified from any residual material and subjected to glycerol-urea polyacrylamide gel electrophoresis, followed by Western blotting using the anti-20 kDa myosin light chain primary antibody (dilution 1:200, Sigma), and the alkaline phosphatase conjugated secondary antibody (dilution 1:5000). The immunoreactivity of separated phosphorylated and unphosphorylated rMLC bands was detected with bromochloroindolyl phosphate and nitroblue tetrazolium as chromagen reagents and evaluated by scanning densitometry and Phoretix software. The fraction of phosphorylated rMLC was expressed as a percent of the total rMLC.

### Measurement of KRP phosphorylation in fibres during microcystin induced contraction

The fibres were mounted isometrically onto stainless steel U-tubes with Histoacryl and subjected to the same experimental protocol as described above for the force measurement experiments. The fibres were preloaded with 10 µM of the wild-type (wt) human KRP for 40 min, and then incubated for another 60 min with 10 µM microcystin in relaxing solution containing 3.3 MBq  $\gamma$ -<sup>32</sup>P-ATP / µmol ATP in the continuous presence of the wt-KRP. Fibres incubated with KRP vehicle were used as a control. *In vitro* phosphorylated KRP was used as a standard to estimate the amount of phosphorylated KRP in fibres. *In vitro* phosphorylation of KRP by MAP kinase was performed with the same specific activity of  $\gamma$ -<sup>32</sup>P-ATP as in the fibre experiments.

## Measurement of KRP dephosphorylation

Dephosphorylation of phosphorylated KRP in *taenia coli* was monitored by two approaches, namely directly in fibres, or in the homogenates of *taenia coli* to which phospho-KRP was added as a substrate. To determine whether phosphorylated KRP becomes dephosphorylated in the fibres during the loading period, the fibres were mounted isometrically onto the stainless steel U-tubes with Histoacryl. Following a maximal contraction and relaxation cycle, the fibres were incubated for 60 min with 10  $\mu$ M human KRP prephosphorylated by PKA or MAPK in relaxing solution and then fixed by immersion into trichloroacetic acid (TCA). As a control for the initial level of KRP phosphorylation, the fibres were incubated for 60 min with phospho-KRP and 10  $\mu$ M microcystin in rigor solution (20 mM imidazol pH 6.7, 2.5 mM Mg-acetate, 3 mM EGTA, 1 mM NaN<sub>3</sub>, 50 mM KCl, 1  $\mu$ M leupeptine, and 2 mM DTT). The level of KRP phosphorylation by MAP kinase or PKA was determined by SDS-PAGE and glycerol-urea gels, respectively, with subsequent immunoblotting with antibodies against total KRP.

To measure whether phosphorylated KRP can be dephosphorylated by tissue homogenates, the skinned fibres were homogenized in 5 vol. (w/v) of ice-cold rigor solution. 10  $\mu$ M phospho-KRP was incubated for 40 min at 30°C with the homogenate that was diluted with rigor solution to a final dilution of 1/10 (w/v). The initial phosphorylation level of phospho-KRP was determined in the presence of 10  $\mu$ M microcystin as above. Reactions were stopped by the addition of SDS-sample buffer, and the proteins were resolved by SDS-PAGE. The level of KRP phosphorylation was determined by Western blotting using phosphospecific antibodies as described below.

## SDS PAGE and Western blotting

Fibres were fixed for 10 min with 15% ice-cold TCA and washed with acetone. The removal of TCA was controlled by bromophenol blue in 20 mM imidazole buffer (pH 6.7). The fibres were homogenized on ice in SDS-PAGE sample buffer and heated for 5 min. Thereafter, urea was added to a final concentration of 5 M. The total protein concentration in the extracts was determined by the Bradford Protein Assay Kit (BioRad, USA). 10-15% slab gels were used for SDS-PAGE according to [42] and Western blotting against KRP species were performed as described [22]. The upper part of the gel was stained with Coomassie R-250 as a loading control, the proteins in the lower part were transferred to PVDF or nitrocellulose membranes which were blocked with 5% non-fat milk in TBS (Tris-buffered saline, pH 8.0), supplemented with 0.1% Tween-20, and incubated with primary antibodies in 1% non-fat milk in TBS containing 0.1% Tween-20. As secondary antibodies peroxidase-conjugated IgG or IR Dye<sup>®</sup> 800CW (LI-COR, Lincoln, NE) antibodies were used in dilutions recommended by the vendors. The protein bands were visualized and quantified by enhanced chemiluminescence (ECL, Pierce, USA) and densitometric scanning using Phoretix software or the Odyssey Infrared Imaging System, Li-COR, Lincoln respectively.

## Statistics

The data were analyzed using Student's *t* test and presented as a mean  $\pm$  SEM from *n* independent experiments.  $P < 0.05$  was considered statistically significant.

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## RESULTS AND DISCUSSION

### KRP inhibits the rMLC phosphorylation of heavy meromyosin, HMM.

We have suggested previously that KRP competitively inhibits rMLC phosphorylation by averting the docking binding of MLCK to its substrate, myosin [24, 26]. Additionally, KRP may shelter Ser<sup>19</sup> from phosphorylation by binding to myosin close to the rMLC binding region, and thereby inhibit other rMLC kinases that do not possess the KRP-domain or otherwise employ this non-catalytic docking interaction. This hypothesis was tested using the constitutively active, 61K tryptic fragment of MLCK (61K-MLCK), which lacks the C-terminal regulatory segment including the KRP domain and, hence, unlike full length MLCK, does not bind to myosin [39].

The recombinant avian His<sub>6</sub>-tagged KRP inhibited rMLC phosphorylation of HMM by both the full length MLCK and the 61K-MLCK in a time and concentration dependent manner (Fig. 1). KRP interestingly appeared to more effectively inhibit rMLC phosphorylation by the 61K-MLCK than by full length MLCK. The reason for this difference is presently unclear. A likely explanation is that the 61K-MLCK, unlike full length MLCK, does not compete with KRP for myosin binding. These results suggest that inhibition of rMLC phosphorylation is not confined to competition with MLCK docking to myosin. It is, therefore, conceivable that KRP also inhibits phosphorylation and tension development induced by the non-canonical, Ca<sup>2+</sup>-independent rMLC kinases.

### KRP inhibits Ca<sup>2+</sup>-independent contractions in triton skinned *taenia coli* fibres

Triton skinned *taenia coli* fibres, which have been widely employed to study the effect of exogenously added proteins on the regulation of the contractile mechanism (e.g. [31], [43]), were used to investigate the effect of KRP on contraction because of the ease with which homogenous thin fibre bundles (~100-200 µm in diameter) can be dissected. They are, therefore, well suited to allow the diffusion of proteins into the fibres and the vicinity of the myofilaments. However, the majority of research into the mechanism of microcystin induced, Ca<sup>2+</sup>-insensitive contractions has used vascular smooth muscle as model system. Therefore, we first determined, whether microcystin elicits a Ca<sup>2+</sup>- independent contraction in triton skinned *taenia coli* that cannot be ascribed to activation of MLCK.

As shown in Fig. 2A, addition of 10 µM microcystin-LR to the relaxing solution (pCa >8) induced a sustained Ca<sup>2+</sup>-independent rise in force, which after 40 min amounted to 91±1% of maximum force (F<sub>max</sub>) elicited at pCa 4.5. The tension rise was much slower than that induced by pCa 4.5, and was similar to earlier reports for triton skinned vascular [5] and β-escin permeabilized ileal smooth muscle [8]. The contraction was associated with an increase in rMLC monophosphorylation from its basal value of 2.8 ± 2.8 % to 38 ± 6.4% (n=4) and 43 ± 2.2% (n=9) at 10 and 25 min after microcystin application. Diphosphorylation of rMLC which is considered to be a characteristic feature of the action of non-canonical rMLC kinases ([36], reviewed in [11]) was not regularly observed in our experiments. The reason for this variability could be that rMLC phosphorylation was assessed during the rising phase of the contraction rather than at the plateau like in the previous investigations (e.g. [5, 8, 36]). In the experiments in which diphosphorylation occurred, it ranged between 7 and 14 % of the total rMLC. A similar level of diphosphorylation was observed when myosin was phosphorylated by ILK *in vitro* [10], whereas in triton skinned ileum diphosphorylation amounted to 1.5% of the total rMLC [8]. For comparison, in triton skinned rat caudal artery microcystin induced mono- and diphosphorylation, determined at steady state force, was ~30% and ~50% of the total rMLC, respectively [36]).

In keeping with previous investigations [5, 6, 8, 36], the MLCK inhibitor, wortmannin (10  $\mu$ M) and the Rho kinase inhibitor, Y27632 (10  $\mu$ M), failed to inhibit the microcystin-induced,  $\text{Ca}^{2+}$ -independent contractions, whereas they were inhibited by the general protein kinase inhibitor, staurosporine (2  $\mu$ M; Fig. 2A, cf. [6]). Wortmannin, at the concentration employed here, not only inhibits full length MLCK, which could be activated by autophosphorylation [46], but also its constitutively active,  $\text{Ca}^{2+}$ -independent fragment, which might be generated by proteolytic degradation in the triton skinned fibres [39, 46]. These results indicate that the  $\text{Ca}^{2+}$ -independent contractions are not mediated by MLCK or ROK kinase. Staurosporine has been shown to inhibit ZIP kinase [12], one of the putative non-canonical rMLC kinases [11]. In smooth muscle, two forms of ZIP kinase were detected: a long 54 kD and a shorter 32 kDa form (reviewed in [11]). Western blot analysis of *taenia coli* using commercial antibodies revealed that the short ZIP kinase is expressed in this tissue and that it remains associated with triton skinned fibres (Fig. 2B, cf. also [12]). We also investigated whether the other putative non-canonical rMLC kinase, ILK, is expressed in *taenia coli*. ILK exists in two, spatially distinct pools, a triton soluble, membrane associated and a triton insoluble pool [10]. The triton insoluble fraction associated with the myofilaments amounts to about 75% of the total ILK in chicken gizzard smooth muscle [10]. Western blot analysis revealed that ILK is expressed in *taenia coli* and a significant amount is retained in the triton skinned fibres (Fig. 2C). The lack of specific inhibitors has so far precluded the unequivocal identification of the kinase responsible for  $\text{Ca}^{2+}$ -independent contractions [8, 10-12]. Nevertheless, as our results are in keeping with the previous reports, we propose that the  $\text{Ca}^{2+}$ -insensitive contractions are due to either ZIP kinase, ILK, or both.

Intact *taenia coli*, like other phasic smooth muscles [19], contains a considerable amount of KRP amounting to  $0.15 \pm 0.02$  mg/g tissue wet weight which converts to  $12.978 \pm 1.45$   $\mu$ M (n=8) assuming an extracellular space of 32% [45]. Consistent with a previous report [32], KRP is almost completely lost upon triton skinning (Fig. 3A), suggesting that KRP binds weakly to the myofilaments and therefore its relaxing effect is readily reversible. MLCK, in contrast, is retained in the fibres, due likely to its binding to actin via the N-terminal domain, rather than due to docking of the KRP-domain on myosin [35].

Incubating triton skinned fibres with exogenous recombinant KRP restores the KRP content, as shown by SDS-PAGE. In order to distinguish between endogenous and exogenous KRP, human His<sub>6</sub>-tagged KRP, which has a lower electrophoretic mobility than the endogenous protein, was used (Fig. 3A). Confocal microscopy of triton skinned fibres loaded with Alexa Fluor<sup>®</sup> 488-labelled KRP shows the uniform distribution in the skinned fibres (Fig. 3B, supplementary Fig. 1). We note that the distribution of KRP cannot be detected by indirect immunohistochemistry in intact fibres, because anti-KRP antibodies will obviously cross-react with MLCK in smooth muscle preparations. In conclusion, triton skinned *taenia coli* fibres can be used as an experimental model system allowing the analysis of the effect of exogenously added KRP on  $\text{Ca}^{2+}$ -insensitive rMLC phosphorylation and contraction without the confounding effects of endogenous KRP.

In the KRP loaded fibres, the microcystin induced,  $\text{Ca}^{2+}$ -independent contraction was significantly ( $p < 0.05$ ) slowed down (Fig. 3C). KRP, thus, increased the lag phase from 2-3 min in the control fibres to ~10 min, and the time to reach 50% of  $F_{\text{max}}$  from  $8.4 \pm 0.9$  min (control) to  $35.1 \pm 2.5$  min. The plateau of the contraction was not reached even after 40 min incubation in microcystin. Hence, force at 40 min was  $56 \pm 4\%$  of  $F_{\text{max}}$  (n=7) in KRP loaded fibres, which compares to  $91 \pm 1\%$  (n=7) in the control fibres. The fibres were not contracted for longer times because a run-down of contraction was observed after incubation in microcystin for more than 40 min. KRP also reduced the microcystin induced increase in rMLC phosphorylation from  $43.1 \pm 2.2\%$  (n=9) to  $33.5 \pm 3.7\%$  (n=8,  $p < 0.05$ ) at 25 min after microcystin application (Fig. 3D) suggesting that KRP inhibits  $\text{Ca}^{2+}$ -independent force by inhibiting rMLC phosphorylation. The results shown here extend the observation of

Sobieszek and co-workers that KRP inhibits  $\text{Ca}^{2+}$ -induced contractions [31]. We note that our results are at variance with a previous report [30], but the reason for the diverging results is not clear at present.

### Phosphorylated KRP inhibits microcystin-induced contraction with similar efficiency as the unphosphorylated KRP

As detailed in the *Introduction*, PKG mediated phosphorylation of KRP at Ser<sup>13</sup> was suggested to potentiate its relaxing effect, whereas the functional significance of the phosphorylation of the MAP kinase site Ser<sup>19</sup> is not yet clear. It was therefore of interest to determine whether the inhibition of  $\text{Ca}^{2+}$ -insensitive contractions is modulated by the phosphorylation of KRP. We tested this with different experimental approaches.

Firstly, we determined whether unphosphorylated KRP becomes phosphorylated in the fibres during incubation with microcystin. This was important because inhibition of phosphatases by microcystin may not only unmask kinase activities directed towards rMLC, but also residual kinase activities directed towards KRP. These kinases are retained in triton skinned fibres. Cyclic nucleotides, for instance, have been shown to decrease  $\text{Ca}^{2+}$ -sensitivity by phosphorylating endogenous substrates in triton skinned smooth muscle (e.g. [47]). To test for this possibility, KRP loaded and control fibres were incubated with microcystin in the presence of  $\gamma\text{-}^{32}\text{P}\text{-ATP}$  for 60 min. These experiments revealed that  $^{32}\text{P}$  was incorporated into protein bands with a relative mobility of ~20 and ~35 kDa, but not into the protein band corresponding to the recombinant KRP (supplementary Fig. 2). Furthermore, a KRP mutant in which the N-terminal residues Ser<sup>13</sup> and Ser<sup>19</sup> were changed to alanines (AA-KRP) and, therefore, could not be phosphorylated in the skinned fibres, was as effective in inhibiting microcystin induced contractions as wild type KRP.

In the next series of experiments, we determined whether phosphorylation of KRP modulates its inhibitory effect on HMM phosphorylation by 61K-MLCK and on  $\text{Ca}^{2+}$ -independent contraction. KRP was prephosphorylated *in vitro* with PKA (at Ser<sup>13</sup>), MAP kinase (at Ser<sup>19</sup>), or both kinases [27]. Each site was completely phosphorylated, as was evident from the mobility shift of phosphorylated KRP species on the urea and SDS gels (Fig. 4A). Because these kinases may phosphorylate other substrates in smooth muscle [47], they were removed from the KRP preparation to avoid confounding effects. HMM was fully phosphorylated within 3 min by 61K-MLCK. Recombinant KRP inhibited this reaction consistent with the data shown in Fig. 1. Different from the experiments shown in Fig. 1, which were carried out at low ionic strength (20 mM) and with His<sub>6</sub>-tagged avian KRP, the experiments shown in Fig. 4 were carried out with non-tagged, human recombinant KRP at high ionic strength (150 mM) in order to match the conditions more closely to those in the fibre experiments. The observation that KRP inhibits HMM phosphorylation at high ionic strength is in keeping with our previous report that KRP binding to myosin exhibits little salt dependence (26). As shown in Fig. 4B and 4C, the different phosphorylated KRP species were equally effective in delaying rMLC phosphorylation as unphosphorylated KRP, indicating that phosphorylation of these N-terminal residues did not affect the ability of KRP to inhibit rMLC phosphorylation *in vitro*. Moreover, deletion of the N-terminal residues harbouring all the phosphorylation sites had no effect on the inhibitory effect of KRP (cf. Fig. 5).

In line with these *in vitro* data, phosphorylation of KRP did not modulate its inhibitory effect on  $\text{Ca}^{2+}$ -independent contraction of the triton skinned fibres (Fig. 4D). As phosphorylated KRP is loaded into the fibres in the absence of a phosphatase inhibitor, the possibility exists that it becomes dephosphorylated during the loading period. Control experiments showed that this was not the case. Firstly, loading the fibres with phosphorylated

KRP showed that the phosphorylation level of KRP was maintained in the skinned fibre (Fig. 4 E) and, secondly, incubation of phosphorylated KRP with lysates of triton skinned fibres did not lead to dephosphorylation of KRP (supplementary Fig. 3). In addition, a phosphorylation mimicking mutant in which both serine residues were changed to aspartates inhibited contraction and rMLC phosphorylation with similar efficiency as the wild type protein (supplementary Fig. 4). The important conclusions emerging from these results are that neither phosphorylation of the PKA/PKG site, nor phosphorylation of the MAP kinase site altered the inhibitory effect of KRP on  $\text{Ca}^{2+}$ -insensitive contractions.

Our findings differ from the previous observations that the relaxing effect of KRP was potentiated in triton skinned ileum in the presence of PKG, or its activator 8-bromo-cGMP or when the N-terminal Ser<sup>13</sup> of KRP was mutated to mimic phosphorylation [30, 32]. If, however, as we did, KRP was directly phosphorylated at Ser<sup>13</sup>, inhibition by KRP remained unchanged [28]. This raises the interesting possibility that other PKG substrates in the fibres act in synergy with phosphorylated KRP. In this context it is worth reminding that ablation of KRP in the mouse reduces cGMP-mediated  $\text{Ca}^{2+}$ -desensitization by only 50% [16], in other words these other substrates contribute to ~50% of  $\text{Ca}^{2+}$ -desensitization. Another level of complexity is added by the possibility that KRP or phosphorylated KRP may accelerate relaxation, and perhaps augment inhibition of contraction by interacting with these other substrates. Further studies are needed to identify KRP binding partners and putative synergistic effects with other PKA/PKG substrates.

### **The C-terminus of KRP is required for the inhibition of rMLC phosphorylation and microcystin-induced contraction**

Next we tested whether KRP has to bind to myosin in order to inhibit HMM phosphorylation by 61K-MLCK as well  $\text{Ca}^{2+}$ -insensitive contractions. As shown in Fig. 5, the truncated KRP ( $\Delta\text{C\_KRP}$ ) lacking the acidic C-terminus known to confer strong binding to myosin [24, 34], neither inhibited HMM phosphorylation by 61K-MLCK (Fig. 5A), nor the microcystin induced contraction in skinned fibres (Fig. 5B). The lag phase was comparable to that in the control fibres, and force after 40 min of incubation with microcystin was  $92 \pm 2\%$  of  $F_{\text{max}}$  in  $\Delta\text{C\_KRP}$  loaded fibres compared to  $93 \pm 3\%$  in control fibres ( $n=5$ ;  $P>0.05$ ). Furthermore, there was no significant decrease in the rMLC phosphorylation level in the presence of  $\Delta\text{C\_KRP}$  determined at 25 min after microcystin addition (Fig. 5B, inset). These results indicate that the C-terminal region of KRP is critical for the inhibition of rMLC phosphorylation and  $\text{Ca}^{2+}$ -independent force development.

### **Putative mechanism of KRP action and physiological relevance**

We have previously shown KRP inhibits MLCK mediated phosphorylation of myosin and HMM by competing with MLCK for the non-catalytic docking on myosin [24, 26]. However, this mechanism cannot explain the findings reported here, namely that KRP inhibits the phosphorylation reaction mediated by the 61K-MLCK which lacks the docking KRP-domain. It also cannot explain the inhibition of  $\text{Ca}^{2+}$ -independent contractions because the non-canonical rMLC kinases most likely do not compete with KRP for binding to myosin, an assumption that awaits biochemical confirmation. On the other hand, myosin binding of KRP appears to be pivotal for this inhibition (cf. Fig. 5). Therefore, we propose that KRP interferes with the phosphorylation reaction by binding to the S1-S2 junction adjacent to rMLC thereby restricting the accessibility of Ser<sup>19</sup>. While this novel mechanism is more general than that described above, it incorporates the competitive mechanism. It satisfactorily accounts for the

inhibition of contraction and rMLC phosphorylation in skinned fibres elicited by novel non-canonical rMLC kinases.

In order to operate through this myosin based mechanism, KRP needs to be expressed at a high molar ratio of KRP to myosin. *In vitro* KRP binds with a 1:1 stoichiometry to myosin [24]. The cellular concentration of myosin for non-arterial smooth muscle was reported to be 19.5 mg/g cell wet weight [48] converting to a cellular concentration of ~40  $\mu\text{M}$  based on a molecular weight of 480,000, whereas in portal vein, the myosin head concentration was reported to be 52  $\mu\text{M}$  converting to 26  $\mu\text{M}$  myosin [21]. Based on these values and our determination of the cellular KRP content of ~13  $\mu\text{M}$  a molar ratio of KRP to myosin ranging from 1:3 to 1:2 can be calculated. In solution KRP binds to unphosphorylated myosin with a  $K_d$  of 5.5  $\mu\text{M}$  converting to a KRP myosin complex of 10-11  $\mu\text{M}$ . In other words ~27% to 37% of myosin in the fibres could be occupied by KRP in intact *taenia coli*. It is reasonable to suggest that our experimental conditions mimic the molar ratio of KRP to myosin because the myosin heavy chain content in skinned *taenia coli* was similar to that in intact *taenia coli* (cf. Fig. 2). In other types of phasic smooth muscle (ileum, portal vein) the molar ratio of KRP to myosin likely is higher because their reported KRP content was ~2-fold higher [21]. Hence, the KRP content in phasic smooth muscle is sufficient to inhibit contraction by this novel, myosin based mechanism.

Our results in conjunction with those of other laboratories [16, 21, 30-32] suggest that KRP functions dually in smooth muscle, i.e. it inhibits contraction and accelerates relaxation. The latter effect has been ascribed to the activation of MLCP activity, whereby the mechanism remains elusive [16, 32]. This dual action is supported by biochemical experiments using so-called native myosin and actomyosin, which both contain endogenous MLCK, calmodulin and MLCP activities [31]. In this preparation, the addition of  $\text{Ca}^{2+}$  and ATP resulted in a biphasic phosphorylation progress curve, where a rapid increase in rMLC phosphorylation was followed by a decline to baseline levels once ATP was depleted by myosin ATPase. KRP decelerated the rate of phosphorylation, whereas it accelerated the rate of dephosphorylation. The authors propose that MLCK and rMLC phosphatase form a complex on myosin filaments, and KRP alters its composition by binding to myosin and liberating MLCK. This shifts the balance in favour of the remaining phosphatase. While this hypothesis may provide a mechanistic explanation for the increase in the apparent MLCP activity, our findings suggest that disruption of this complex is not necessary for the inhibition of rMLC phosphorylation. This is because KRP inhibition of rMLC phosphorylation appears to be non-selective for MLCK and occurs with soluble HMM, i.e. it does not require the filamentous state of myosin.

In these two functions, the C-terminal and N-terminal segments of KRP, which flank a large central seven-sheet barrel of a C2 immunoglobulin-like motif [23], may play different roles as depicted in Fig. 6. The N-terminal segment was not required for inhibition of HMM phosphorylation (cf. Fig. 5), but phosphorylation of certain N-terminal residues may modulate KRP's relaxing effect. In contrast, the C-terminal segment was required for the inhibition of rMLC phosphorylation *in vitro* and in skinned fibres, as well as force development (our study), but not for the acceleration of relaxation [30].

At present, we can only speculate about the physiological relevance of our findings. Moreover, the physiological function of the non-canonical rMLC kinases is still not fully understood but they are thought to play role in  $\text{Ca}^{2+}$ -sensitization, in the regulation of tension maintenance at low levels of  $[\text{Ca}^{2+}]$  [11], and in contraction of certain types of smooth muscle [7]. Based on our findings it is possible that KRP plays a role in regulating basal rMLC phosphorylation and tone through the mechanism described here. It could also prevent accidental rMLC phosphorylation at low spurious levels of rMLC kinase activity, thereby setting the threshold for activation. High levels of rMLC phosphorylation, i.e. when the muscle is fully activated, prevent the binding of KRP to myosin [26]. Under this condition,

KRP may decrease rMLC phosphorylation and force by increasing the apparent activity of MLCP. This dual mechanism may allow KRP to act as a brake irrespective of the degree of smooth muscle activation. Studies using intact smooth muscle from KRP deficient mice [16] may help to unravel the physiological function of KRP.

## Summary

In summary, we report here for the first time that KRP inhibits the  $\text{Ca}^{2+}$ -independent phosphorylation of rMLC and force development in triton skinned smooth muscle which is most likely mediated by non-canonical rMLC kinases, such as ZIP kinase and/or integrin linked kinase (ILK). Binding of KRP to myosin is critical for the inhibitory effect, because a KRP mutant which is deficient in myosin binding failed to inhibit rMLC phosphorylation and contraction. Inhibition of the  $\text{Ca}^{2+}$ -independent contraction is not modulated by phosphorylation of the N-terminal residues Ser<sup>13</sup> (PKA), Ser<sup>19</sup> (MAP-kinase), or both. As we removed the enzymes prior to loading of the fibres with the prephosphorylated KRP species, the results are not confounded by phosphorylation of other PKA or MAP-kinase substrates in the fibres. Control experiments showed that the phosphorylation status of KRP was not altered in the fibres. In keeping with these fibre experiments, full length but not the C-terminally truncated KRP inhibited phosphorylation of HMM by the 61K-MLCK. Inhibition of HMM phosphorylation by full length KRP was independent of the N-terminal phosphorylation status. The results presented here extend previous observations that KRP inhibits  $\text{Ca}^{2+}$ -induced force in triton skinned fibres and as well as HMM phosphorylation *in vitro* by the conventional MLCK and provide strong evidence that KRP is a more general inhibitor of smooth muscle contractility. As the 61K-MLCK lacks the C-terminal domain which conveys docking binding to myosin, displacement of MLCK from the non-catalytic binding to myosin is not a prerequisite for inhibition of rMLC phosphorylation. On the other hand, binding of KRP to myosin is critical for inhibition. We, therefore, propose that KRP inhibits rMLC phosphorylation by shielding rMLC from being phosphorylated by the dedicated MLCK as well as non-canonical rMLC kinases.

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## LEGENDS TO FIGURES

### Figure 1. KRP/telokin inhibits in vitro phosphorylation of smooth muscle HMM by full-length MLCK and its 61K proteolytic fragment

KRP inhibits phosphorylation of 4  $\mu\text{M}$  chicken gizzard HMM catalyzed by full length MLCK purified from chicken gizzard (MLCK-108, 10 nM), as well as by its 61K proteolytic fragment obtained by tryptic digestion, according to Ikebe et al. [39] (61K-MLCK, 40 nM). The phosphorylation reactions were carried out at 20 mM NaCl for the time intervals indicated, and in the presence of two concentrations of recombinant chicken His<sub>6</sub>-tagged KRP. Autoradiograms (<sup>32</sup>P) of representative Coomassie R-250 stained gels are shown to confirm the equal loading of rMLC. The bars represent the mean  $\pm$  S.E.M (n=3-6). \*: p < 0.05 with respect to control.

### Figure 2. Incubation of triton skinned fibres with microcystin elicits a Ca<sup>2+</sup>-independent contraction

(A) Original force tracings of microcystin-induced contraction in the presence of vehicle (1), 10  $\mu\text{M}$  wortmannin (2), 10  $\mu\text{M}$  Y-27632 (3), and 2  $\mu\text{M}$  staurosporine (4). The force tracings shown are representative for 3-6 independent experiments. (B) Western blotting of intact and triton skinned *taenia coli* with anti-ZIP kinase antibodies; and (C) with anti-ILK. In (C), the results of three independent intact and skinned fibres, respectively, are shown. Coomassie staining of the actin-containing part of the gel indicates the equal loading. The confirmation of ZIP kinase and ILK immunoreactivity in triton skinned fibers has been obtained in 3 – 6 independent experiments.

### Figure 3: Inhibition of microcystin induced, Ca<sup>2+</sup>-independent contractions by KRP

(A) Western blot analysis with anti-KRP antibodies. Lanes 1-3: increasing concentrations (3, 10, and 20 ng respectively) of purified human recombinant His-tagged KRP; lane 4-6 lysates of *taenia coli* (30  $\mu\text{g}$  of total protein per lane), lane 4: intact *taenia coli*; lane 5: triton skinned *taenia coli*; and lane 6: triton skinned *taenia coli* incubated with his-tagged KRP (20  $\mu\text{M}$  for 30 min). The gel shown is representative of three independent experiments. (B) Representative confocal microscopy images of triton skinned fibres with and without loading with 10  $\mu\text{M}$  Alexa Fluor<sup>®</sup> 488-labeled human KRP. Nuclei and F-actin are visualised by DAPI and Alexa Fluor<sup>®</sup> 555 phalloidin staining, respectively. (C) Time course of microcystin-induced (10  $\mu\text{M}$  for 40 min) force development by *taenia coli* skinned fibres in the presence of human wt-KRP, or the respective dialysis buffer (control). Force was expressed in percent of Ca<sup>2+</sup>-activated force at pCa 4.5 (experimental conditions as in Fig. 2); values are mean  $\pm$  SEM (n=7). (D) rMLC phosphorylation determined 25 min after the addition of microcystin. The experimental protocol is as in Fig. 3C. The level of rMLC phosphorylation was determined by quantitative urea-glycerol-PAGE, as described in 'Experimental' section. Values are mean  $\pm$  SEM (n=9), \*p < 0.05.

### Figure 4. Phosphorylation of KRP did not alter its inhibitory effect on HMM phosphorylation and Ca<sup>2+</sup>-independent contractions.

Recombinant human wt-KRP was phosphorylated *in vitro* at Ser<sup>13</sup> by PKA, at Ser<sup>19</sup> by p44<sup>erk1</sup> MAP-kinase (MAPK), or at both sites as described in the 'Experimental' section. The completeness of phosphorylation at each site was verified by the in-gel mobility shift assay.

Panel (A) shows that PKA phosphorylated KRP migrates slower on urea-glycerol-gels, whereas the MAP-kinase phosphorylated protein has lower mobility on SDS-gels. The shifted position of phosphorylated vs. unphosphorylated protein is confirmed by the simultaneous presence of two bands after 20 min of incubation when only half protein was phosphorylated, as well as by the position of the unphosphorylated control on the gel. (B) shows representative gels and (C) the statistical evaluation of the time course of phosphate incorporation into rMLC of HMM (4  $\mu$ M) catalyzed by 40 nM 61K-MLCK in the absence (control) or presence of 20  $\mu$ M prephosphorylated or nonphosphorylated human recombinant KRP at 150 mM NaCl for the time intervals indicated. Phosphorylation of HMM was determined by the rMLC mobility shift assay using urea-glycerol gels. Note that KRP prephosphorylation does not modulate inhibition of phosphorylation of HMM. Values shown are mean  $\pm$ SEM (n=2-7), \*:  $p < 0.05$  with respect to control. (D) KRP prephosphorylation does not modulate inhibition of the  $\text{Ca}^{2+}$ -independent contraction elicited by microcystin; values shown are mean  $\pm$ SEM (n=5-7). (E) phosphorylated KRP does not get dephosphorylated in the skinned fibres. Skinned fibres were incubated either without added KRP (lane: "taenia") or with phosphorylated KRP (lanes: "taenia+P-KRP") for 60 min. Lysates were analyzed by SDS-PAGE or urea-glycerol-PAGE. The positions of KRP and phosphorylated KRP were identified by Western blotting using corresponding standards loaded onto lanes 1-3 as indicated. No mobility shift of phosphorylated KRP was observed after 60 min of incubation (taenia + P-KRP) indicating it was not dephosphorylated. The electrophoretic mobility of this band remained exactly the same as that of phosphorylated KRP incubated with skinned fibres in the presence of 10 nM okadaic acid (taenia + P-KRP + OA) or 10  $\mu$ M microcystin (taenia + P-KRP + MC) which were added in separate experiments to inhibit any potential dephosphorylation of KRP. For reference, the right lane shows the migration of unphosphorylated KRP that was incubated with separate fibres. Equal loading was confirmed by staining the upper part of the gels with Coomassie R-250. Representative experiments of two independent experiments are shown.

Figure 5. C-terminus of KRP was essential for the inhibition of rMLC phosphorylation *in vitro* and of microcystin-induced skinned fibre contraction.

(A) The full length KRP and N-terminally truncated KRP ( $\Delta$ N-KRP), but not the C-terminally truncated KRP ( $\Delta$ C-KRP), inhibit phosphorylation of 4  $\mu$ M HMM by 40 nM 61K-MLCK *in vitro*. Recombinant chicken His<sub>6</sub>-tagged KRP proteins at a final concentration of 20  $\mu$ M were assayed at 20 mM NaCl, essentially as in Fig. 1. The autoradiogram shown (top) of the SDS-gel fragment that contains rMLC (bottom). Bars represent mean  $\pm$ SEM (n=3-6), \* $p < 0.05$  with respect to control, n.s.: not significant. (B) recombinant human  $\Delta$ C-KRP (10  $\mu$ M) does not affect the time-course of microcystin-induced force development of skinned fibres. Experimental conditions as in Fig. 3. The force values were normalized to  $\text{Ca}^{2+}$ -dependent force elicited at pCa 4.5; values represent mean  $\pm$  SEM (n=5).

Figure 6. Proposed model for the regulatory mechanism of KRP/telokin.

The ribbon diagram shows the core of a KRP molecule that adopts a 7-sheet beta-barrel conformation [23]. The thread-like terminal sequences denoted by N and C are introduced in free style, as they were not resolved in the crystal structure. The N-terminal sequence is suggested to mediate, in a phosphorylation-dependent manner, the acceleration of smooth muscle relaxation and rMLC dephosphorylation, possibly via activation of rMLC phosphatase [16, 26]. The C-terminal sequence, which mediates a myosin binding of KRP [34], is critical

for the inhibition of rMLC phosphorylation and the development of  $\text{Ca}^{2+}$ -independent force as shown in this work. See text for further details.

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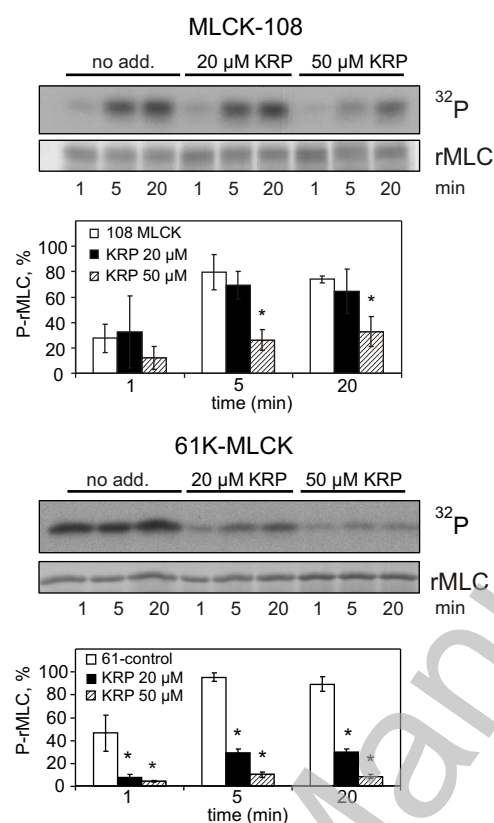


Fig 1

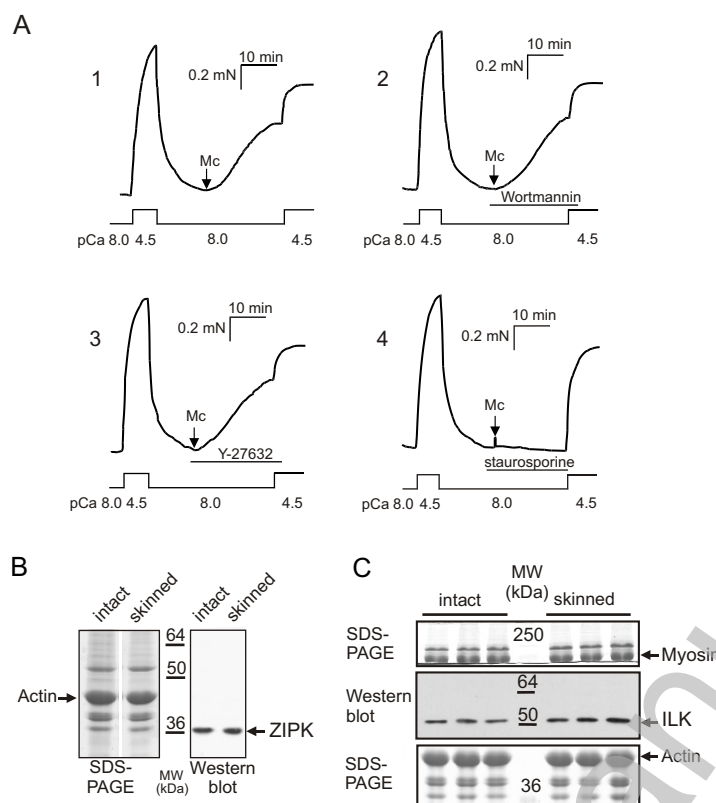


Fig 2

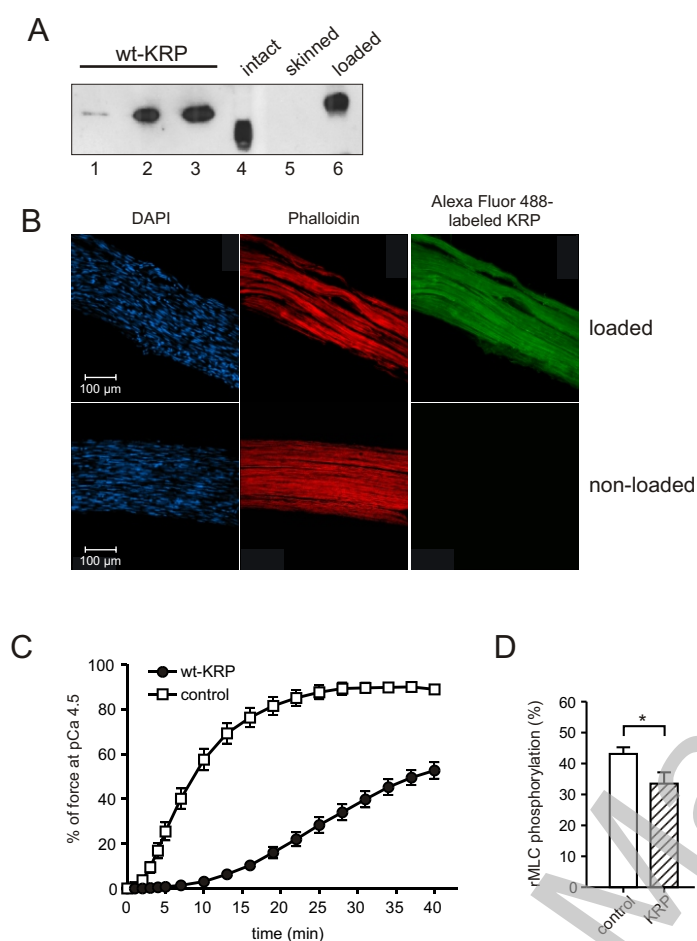


Fig 3

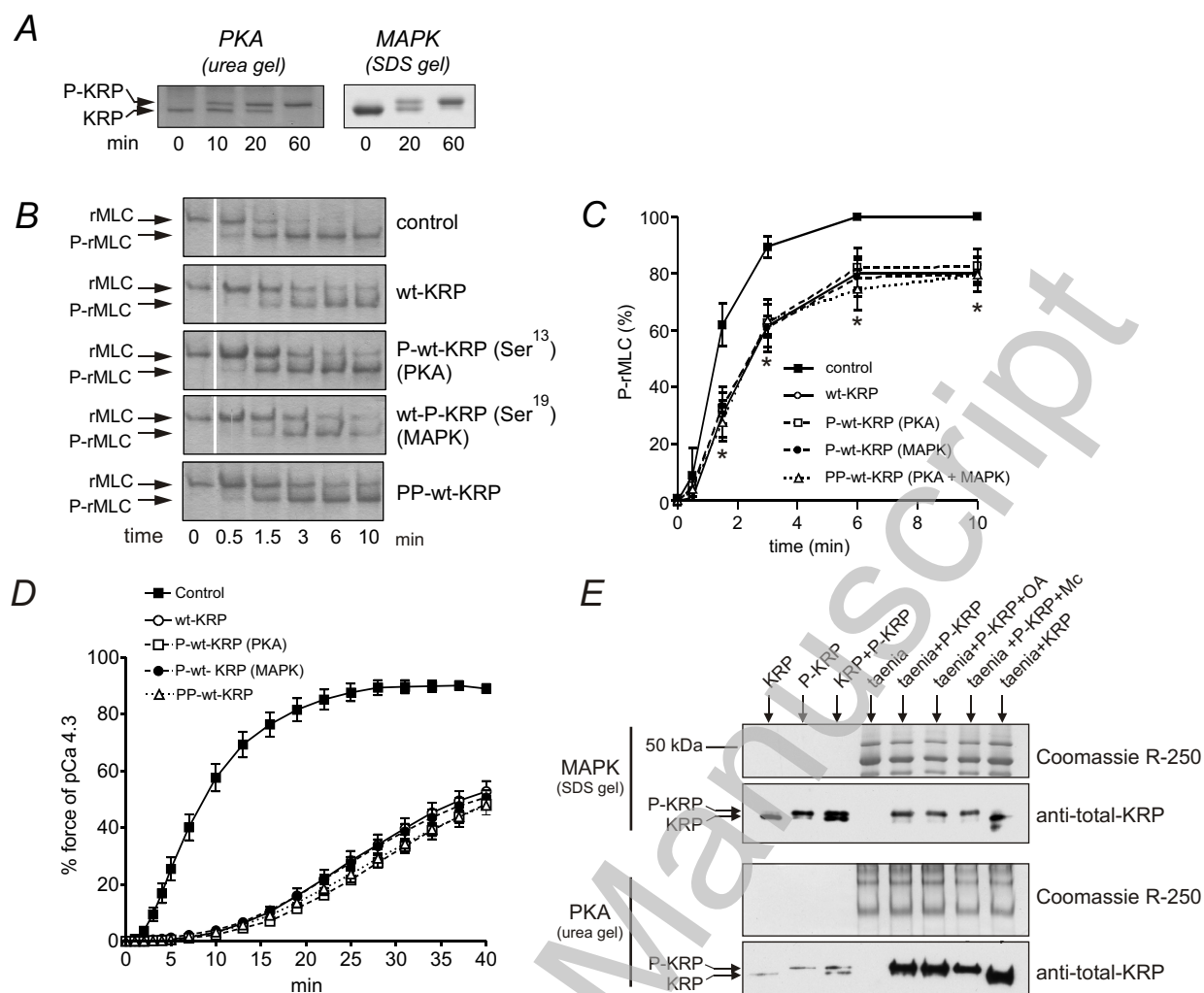


Fig 4

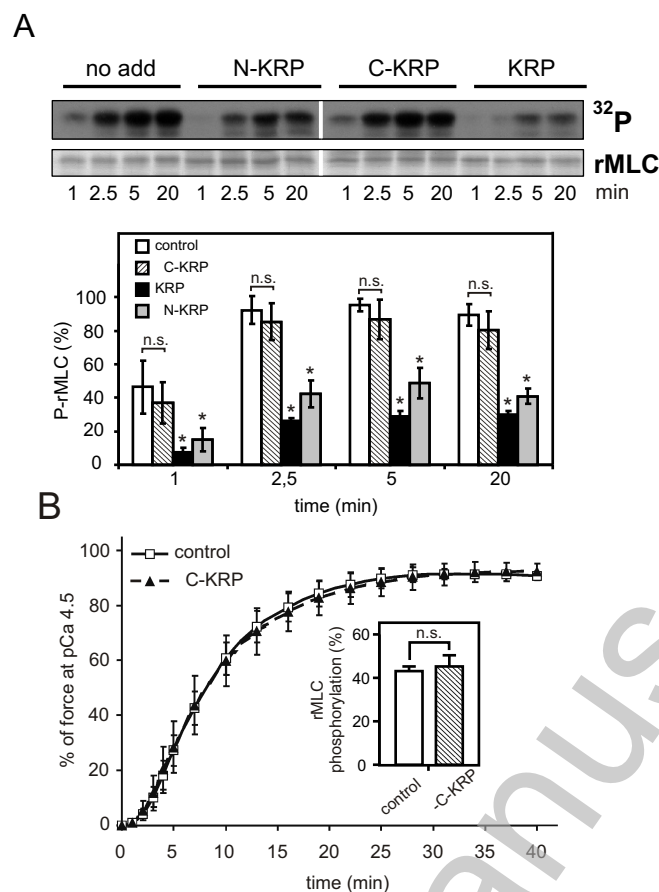


Fig 5

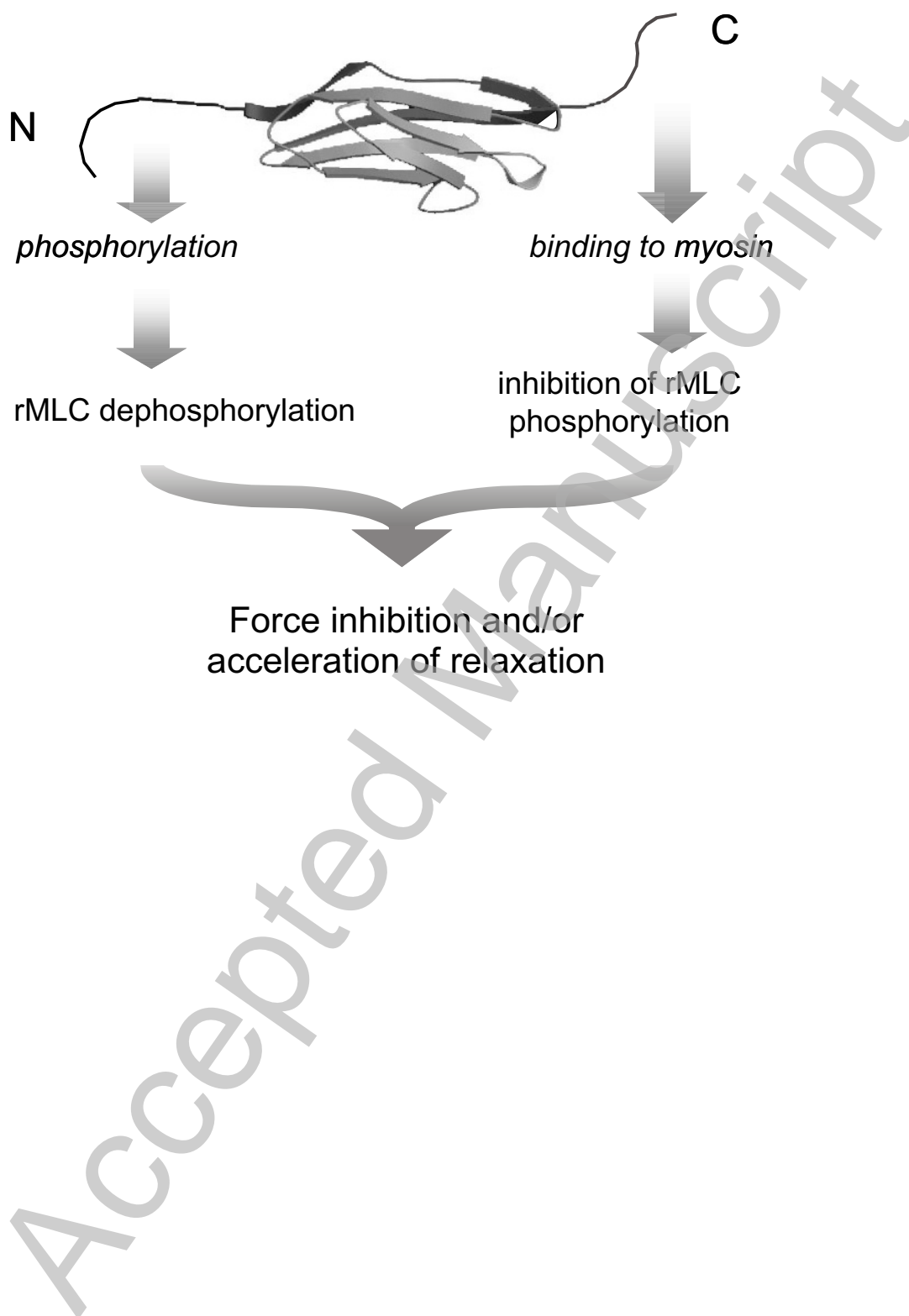


Fig 6