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Novel Phosphospecific Antibodies for Monitoring Phosphorylation of Proteins Encoded by the Myosin Light Chain Kinase Genetic Locus

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Abstract—Myosin light chain kinase (MLCK) and the kinase-related protein (KRP), also known as telokin, are the major independent protein products of the smooth muscle/non-muscle MLCK genetic locus. They share a common C-terminal part and major sites phosphorylated *in vivo*. Whereas MLCK is critically involved in myosin activation and contraction initiation in smooth muscle, KRP is thought to antagonize MLCK and to exert relaxation activity. Phosphorylation controls the MLCK and KRP activities. We generated two phosphorylation and site-specific antibodies to individually monitor levels of MLCK and KRP phosphorylation on critical sites. We quantified the level of KRP phosphorylation in smooth muscle before and after an increase in intracellular free Ca^{2+} and stimulation of adenylate cyclase, protein kinase C, and mitogen-activated protein kinases (MAP-kinases). Forskolin and phorbol-12,13-dibutyrate increased KRP phosphorylation at Ser13 from 25 to 100% but did not produce contraction in rat ileum. The level of Ser13 phosphorylation was not altered during Ca^{2+}-dependent contraction evoked by KCl depolarization or carbachol, but subsequently increased to maximum during forskolin-induced relaxation. These data suggest that several intracellular signaling pathways control phosphorylation of KRP on Ser13 in smooth muscle and thus may contribute to relaxation. In contrast, phosphorylation level of Ser19 of KRP increased only slightly (from 30 to 40-45%) and only in response to MAP-kinase activation, arguing against its regulatory function in smooth muscle.

Key words: smooth muscle, contraction, relaxation, myosin light chain kinase, KRP, telokin, MAP-kinase, protein kinase A, phosphorylation

Calcium ions play a major role in initiation of smooth muscle contraction [1-3]. An increase in cytosolic Ca^{2+} concentration following membrane depolarization or agonist binding to membrane receptors leads to subsequent activation of calmodulin and myosin light chain kinase (MLCK), rapid phosphorylation and activation of myosin enabling its interaction with actin, and contraction onset [4, 5]. The force of contraction is primarily determined by this Ca^{2+}-dependent mechanism through the control of myosin phosphorylation level determined by the balance of intracellular activities of MLCK and specific myosin light chain phosphatase. Ca^{2+}-independent changes in MLCK and myosin phosphatase activities alter the Ca^{2+}-sensitivity of smooth muscle force [1-3].

Several intracellular mechanisms that mediate an increase in Ca^{2+}-sensitivity (Ca^{2+}-sensitization) include either consequent activation of small G-protein Rho and Rho-kinase [6] or activation of protein kinase C (PKC) [7-9]. The signaling cascades triggered by PKC are not

Abbreviations: ECL) enhanced chemiluminescence; GSK3) glycogen synthase kinase 3; MLCK) myosin light chain kinase; KRP) kinase-related protein, telokin (protein related to MLCK); MAP-kinase) mitogen-activated protein kinase; PAK) p21-activated protein kinase; PDBu) phorbol-12,13-dibutyrate; PKA) cyclic AMP-dependent protein kinase A; PKC) Ca^{2+}-phospholipid-dependent protein kinase C; PVDF) polyvinylydenedifluoride.

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completely understood. Earlier we have found that, in intact smooth muscle, the direct activation of PKC by phorbol-12,13-dibutyrate (PDBu) results in phosphorylation of MLCK and tonic contraction. In contrast, in phasic smooth muscles, PDBu stimulated phosphorylation of KRP (telokin), but not that of MLCK, and produced no contraction [10]. We have suggested that PKC-dependent mechanisms, coupled to phosphorylation of KRP and MLCK, function differently in tonic and phasic smooth muscles and bidirectionally alter the Ca^{2+}-sensitivity and contractility [10].

Ca^{2+}-independent activation of MLCK may occur as a result of direct phosphorylation by mitogen-activated protein kinases (MAP-kinases). This conclusion came from observations that direct phosphorylation of MLCK by MAP-kinase *in vitro* stimulated the MLCK activity [11], and the increased phosphorylation of MLCK correlated with increased smooth muscle contraction [12] and non-muscle cell migration [13, 14]. Because the major MAP-kinase phosphorylation site in MLCK is Ser834 [10], it is most likely involved in MLCK activation. However, several closely located sites phosphorylated by other protein kinases preclude individual analysis of Ser834 phosphorylation by standard protein and peptide chemistry methods [10]. The goal of this study was to develop a tool to quantitatively measure MLCK phosphorylation on Ser834 in intact tissue and cells.

Decrease in Ca^{2+}-sensitivity (Ca^{2+}-desensitization), associated with a reduction in myosin phosphorylation level, leads to smooth muscle relaxation. KRP is an independent protein product derived from the MLCK genetic locus, and its primary sequence is identical to the C-terminal myosin-binding domain of MLCK [15-17]. Due to competition with MLCK for myosin binding, KRP inhibits myosin phosphorylation and activation [18, 19]. Thus, KRP can be considered as a smooth muscle relaxation factor [15].

Another mechanism of KRP action has been suggested to involve acceleration of myosin dephosphorylation, presumably through activation of myosin phosphatase [20]. Furthermore, phosphorylation of KRP at Ser13 has been suggested to increase its relaxing activity [20, 21]. These observations led to hypothesis that phosphorylation of Ser13 in KRP by cAMP- (PKA) or cGMP-activated protein kinases is involved in cyclic nucleotide dependent relaxation of smooth muscle induced by nitric oxide donors or forskolin [22]. Based on the site mutagenesis studies, the same authors suggested that phosphorylation of KRP by MAP-kinase on Ser19 (that is equivalent to Ser834 in MLCK) reverses the effect of Ser13 phosphorylation [21]. We found that KRP is the major phosphoprotein in smooth muscle [10] and phosphorylation proceeds mainly on the two sites mentioned above [22, 23]. However, similar to the MLCK case, several closely located phosphorylation sites preclude individual analysis of KRP phosphorylation at Ser13 and Ser19.

Thus, phosphorylation of Ser834 in MLCK and the equivalent Ser19 in KRP by MAP-kinases may result in increased level of myosin phosphorylation due to a shift in the balance of MLCK and myosin phosphatase activities, whereas phosphorylation of Ser13 of KRP by cyclic nucleotide activated protein kinases may be linked to smooth muscle relaxation. We have developed phospho- and site-specific antibodies to analyze the extent of MLCK and KRP phosphorylation at these two sites *in vivo*. In this study, we investigated dynamics of KRP phosphorylation during contraction–relaxation of smooth muscle. Our data favor the hypothesis that phosphorylation of KRP on Ser13 correlates with smooth muscle relaxation, but do not support regulatory role of KRP phosphorylation on Ser19.

MATERIALS AND METHODS

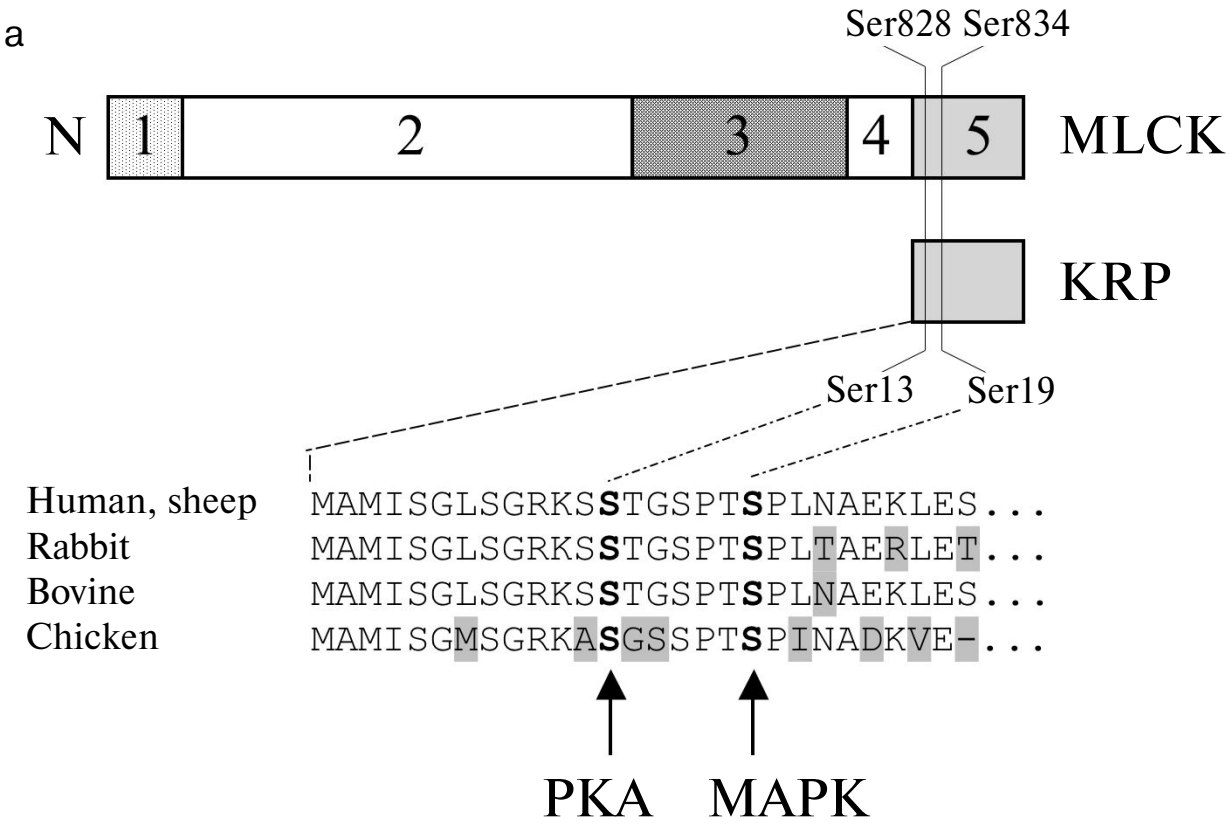
Physiological measurements. Male rats (250-300 g) were anesthetized by Nembutal and decapitated. Smooth muscle strips (3 × 6 mm) were separated from the circular layer of proximal section of the intestine and mounted on isometric force transducers (Kent Scientific, USA) in Tyrode's solution (0.42 mM NaH_2PO_4, 11.9 mM $NaHCO_3$, 136.9 mM NaCl, 2.68 mM KCl, 1.05 mM $MgCl_2$, 1.8 mM $CaCl_2$, 5.55 mM D-glucose) under constant carbogen administration at 37°C. Two identical strips were maintained in twin organ bath chambers for 1 h at initial load of 0.1-0.15 g and their contractile force was registered in parallel with the use of MacLab 2 and PowerLab computer software (ADInstruments Pty Ltd., Australia). The contraction was stimulated either by depolarization upon the addition of KCl to 60 mM, or by carbachol (20 μM), or by PDBu (0.3-1 μM). After reaching the maximum tension, relaxation was stimulated by forskolin addition to 10 μM without exchange of the medium.

For the following biochemical analysis, the strips were removed at different time points, drained of excessive liquid, and immediately frozen in liquid nitrogen, all within 4-6 sec. The tissue was weighed and pulverized while frozen, supplied with 20-fold (v/w) of 2-fold SDS-PAGE sample buffer, and pulverized again. The resultant powder was transferred to microtubes and boiled for 5 min. The extracts were clarified by spinning for 5 min at 12,000*g* and analyzed by SDS-PAGE and immunoblotting.

Immunoblotting. Proteins were separated by SDS-PAGE and transferred onto polyvinylydenedifluoride (PVDF) membranes in 10 mM Caps, pH 11, 10% ethanol, and 5 mM 2-mercaptoethanol as described earlier [23]. The membranes were blocked for 1 h at room temperature in 20 mM Tris-HCl, pH 7.6, 165 mM NaCl, 0.05% Tween-20, and 5% fat-free milk. All the following incubations with antibodies were done at the room tem-

perature for 1 h or at 4°C overnight in this buffer, except that the milk concentration was reduced to 1-2.5%. Protein bands were visualized by enhanced chemiluminescence (ECL) detection using reagents from Amersham-Pharmacia Biotech. (UK). To ensure the linearity of chemiluminescence signal, the membranes were exposed to film for various times, scanned, and quantitatively processed with the use of Scion Image 1.63 software (Scion Corp., USA).

Antibodies. Commercial phosphospecific and polyclonal antibodies against MAP-kinases (New England Biolabs, USA) were used as before [24] to quantify activation of MAP-kinases by the extent of concurrent phosphorylation of Thr and Tyr residues within the activation loop of the enzymes. Affinity purified polyclonal antibodies to KRP have been described [23]. To obtain phosphospecific antisera to KRP, the phosphopeptides shown in Fig. 1b were synthesized in an automated solid-phase synthesizer (Applied Biosystems 431A, USA), using the Fmoc-technique on polystyrene polymer (Wang resin) with hydroxymethylphenoxymethyl group at 0.73 mmol/g substitution. The phospho-Ser residue was introduced into the polypeptide chain as the Fmoc-Ser(PO(Obzl) OH) derivative (Navabiochem, USA) in the presence of 2-(1H-benzotriazol-1-yl)-1,1,3,3-tetra-methyluronium tetrafluoroborate/1-hydroxybenzotriazole and diiso-



b

Fig. 1. Domain organization of MLCK and KRP and sequences of synthetic peptides used for production of phosphospecific antibodies. a) Domain organization of MLCK and KRP. Shown are the relative positions of the N-terminal actin-binding domain (*1*), tandem repeats (*2*), catalytic domain (*3*), regulatory calmodulin-binding segment (*4*), and the myosin-binding domain of MLCK that is separately expressed in smooth muscle as KRP (*5*). The N-terminal sequence of KRP from different species is zoomed underneath. The numbering of common MLCK and KRP sites phosphorylated by protein kinase A (PKA) and MAP-kinase (MAPK) is according to chicken proteins (see [5] for details). b) Sequences of phosphopeptides used for immunization. Asterisks denote position of residues with associated phosphate group.

propylethylamine to yield the amide bond. Resulting polypeptides were deblocked and cleaved from the matrix by trifluoroacetic acid and then purified by reverse-phase HPLC and ion-exchange chromatography. The structure of phosphopeptides was verified by mass-spectrometry, ^{1}H-NMR spectroscopy, and amino acid analysis. The synthetic phosphopeptides were coupled via the sulfhydryl group of the N-terminal cysteine to keyhole limpet hemocyanin using *p*-maleimidobenzoyl-N-hydroxysulfosuccinimide ester (Pierce, USA). Each conjugate was resuspended in complete Freund's adjuvant and injected in equal volumes (50 μg of phosphopeptide) into four lymphatic gland regions of rabbits. Additional injections of 0.1 mg of phosphopeptide dissolved in incomplete Freund's adjuvant per rabbit were made twice at fortnight intervals. The blood (50 ml) was taken from the outer ear artery on the 7th, 9th, and 11th day after the last boost, coagulated at 4°C, and the serum was clarified by centrifugation. On dilution 1 : (10,000-40,000), the resultant antisera revealed satisfactory phospho- and site-specificity on Western blots towards phosphorylated avian KRP, but weakly stained unphosphorylated recombinant human KRP. Therefore, an additional purification of phosphospecific antibodies against human KRP phosphorylated at Ser13 (R5 antibody) and KRP phosphorylated at Ser19 (R8 antibody) was performed by passing the antisera through immobilized human KRP and collecting the flow-through fraction. The affinity-purified antibodies were used at 1 : (1000-5000) dilution in immunoblotting.

cDNA constructs. To obtains recombinant proteins, the plasmids encoding GST-p44^{erk1} MAP-kinase and constitutively active His$_6$-ddMEK1ca [23], constitutively active XPAK1 (kindly donated by Dr. N. Morin, Centre de Recherches de Biochimie Macromoleculaire, CNRS ERS 155, 34293 Montpellier, France), and chicken His$_6$-KRP [19] were used. The plasmid pCR2.1-TOPO, containing cDNA of human KRP, was kindly given by Prof. D. M. Wastterson (Northwestern University, Chicago, IL, USA). The open reading frame was amplified by polymerase chain reaction (PCR) using the forward primer 5′-GTATCATATGGCAATGATCTCAGGG-3′ to introduce the *Nde*I restriction site (underlined), and reverse primer 5′-TATCTCGAG*TCA*CTCTTCTTCCTCTTCCCC-3′ containing *Xho*I restriction site (underlined) and nucleotides complementary to stop-codon (shown in italic), to match the KRP reading frame. The PCR product was cloned into pET-22b(+) vector at the *Nde*I and *Xho*I sites, so that stop-codon was retained at the 3′-end of the insertion and resulting mRNA did not code for the 3′-flanking polyhistidine of the original vector. After verification by sequencing using the universal T7 primers to vector sequences, the construct was used to express protein with primary structure identical to human KRP.

Proteins. Recombinant proteins were expressed in BL21 (DE3) pLys *E. coli* strain. Chicken His$_6$-KRP and His$_6$-ddMEK1ca were purified from bacterial lysates on TALON affinity matrix (Clontech, USA), glutathione-S-transferase (GST)-p44^{erk1}-MAP-kinase was purified on Glutathione-Sepharose (Amersham-Pharmacia Biotech.) [19, 23]. The catalytic domain of PAK1, fused to the maltose-binding protein (MBP-XPAK1) was purified on amylose-Sepharose resin (Clontech). A standard protocol [18, 23] was used to purify the recombinant human KRP that contained no tag sequences. Briefly, KRP was precipitated from bacterial lysate with ammonium sulfate and purified by successive anion-exchange and hydrophobic chromatographies on the Q- and Octyl-Sepharose columns (Amersham-Pharmacia Biotech.), respectively. Recom-binant rabbit MLCK expressed in a baculovirus system was kindly donated by Dr. Z. Grabarek (Boston Biomedical Research Institute, MA, USA).

Protein phosphorylation *in vitro*. Recombinant GST-p44^{erk1}-MAP-kinase was activated by phosphorylation with His$_6$-ddMek1ca in molar ratio 20 : 1, respectively, for 30 min at 30°C in 10 mM MOPS, pH 7.0, 20 mM NaCl, 1 mM dithiothreitol, 0.2 mM EGTA, 0.5-1 mM ATP, and 5 mM $MgCl_2$. KRP (40 μM) was phosphorylated by thus activated GST-p44^{erk1}, or by MBP-XPAK1, or by commercial PKA catalytic subunit (New England Biolabs), or by glycogen synthase kinase 3 (GSK3, New England Biolabs) at 30°C in the buffer stated above. The extent of phosphorylation was determined by radioactive phosphate incorporation using $(0.5\text{-}1.0)\cdot10^6$ cpm/nmol of [γ-^{32}P]ATP [10]. The enzyme/KRP molar ratio was 1 : 100 for PKA, 1 : 20 for GST-p44^{erk1} and GSK3, and 1 : 10 for MBP-XPAK1. MLCK (3 μM) was similarly phosphorylated by GST-p44^{erk1} and PKA for 2-6 h at 30°C.

RESULTS

Preparation and characterization of phosphospecific antibodies against KRP and MLCK. Figure 1 illustrates the domain organization of MLCK and KRP and localization of sites phosphorylated by MAP-kinase (Ser19 in KRP and Ser834 in MLCK) and PKA (Ser13 in KRP and Ser828 in MLCK). To obtain antibodies that specifically recognize these residues while in phosphorylated form, we used the strategy and reagents developed by Pierce (see "Materials and Methods"). Phosphopeptides MG2 and MG3 corresponding to human MLCK and KRP sequences were chemically synthesized and used as the antigens (Fig. 1b). An additional N-terminal Cys residue was incorporated into each phosphopeptide to allow the coupling to carrier protein and affinity matrix should it be required to purify antibodies. The antibody R5 against phospho-Ser13 and antibody R8 against phospho-Ser19 of KRP were generated in rabbits and purified as described in the "Materials and Methods" section.

The antibodies were characterized with respect to their phospho- and site-specificity, independence of their

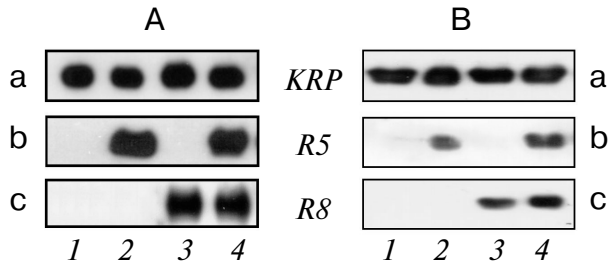


Fig. 2. Phospho- and site-specificity of the R5 and R8 antibodies. Recombinant human (A) and chicken (B) KRP (40 ng in each lane) was fully phosphorylated by PKA (*2*), $p44^{erk1}$-MAP-kinase (*3*), or both kinases (*4*). Lane *1* contains unphosphorylated KRP. The Western blot was probed with polyclonal antibody against KRP (a) and phospho-specific antibody against Ser13 (R5 (b)) or Ser19 (R8 (c)).

binding on phosphorylation of the neighbor sites, and selectivity for KRP and MLCK in tissue extracts.

The phospho-specificity is shown in Fig. 2. On Western blots, the R5 antibody recognized human KRP phosphorylated by either PKA alone, or with both PKA and MAP-kinase (Fig. 2a). This antibody did not react with unphosphorylated KRP and KRP phosphorylated by MAP-kinase alone suggesting it is highly phosphospecific and selective for Ser13 in KRP. Phosphorylation of Ser19 by MAP-kinase did not notably alter the R5 antibody binding to phosphorylated Ser13, since mono- and diphosphorylated KRP were similarly stained. Likewise, the R8 antibody recognized MAP-kinase phosphorylated KRP and this was not affected by the Ser13 phosphorylation. Essentially the same results were obtained when MLCK phosphorylated *in vitro* by PKA and MAP-kinase was analyzed (data not shown, see also Fig. 4).

Thus, antibodies R5 and R8 are phospho- and site-specific towards MLCK and KRP, while their antigen determinants appear to be larger than a single phosphoserine and to include neighbor residues, possibly also depending on the polypeptide chain configuration. In this respect, it was important to assess a specie-specificity of the antibodies, because sequence of human MLCK/KRP involved in their binding is not identical to that in the other species commonly used in experiments (Fig. 1a). Therefore, we repeated the described above analysis using recombinant chicken KRP, whose sequence is farthest away from that of the human protein, and confirmed the phospho- and site-specificity of both antibodies (Fig. 2b). Together with data obtained for recombinant rabbit MLCK and rat tissue extracts shown below, these results argue against possibility that the R5 and R8 antibodies are species-specific.

Earlier we reported that at least three closely located sites are phosphorylated in KRP in chicken smooth muscle, and Ser16 of KRP is a substrate for glycogen synthase kinase GSK3 *in vitro* [23]. In addition, the sequence surrounding Ser12 of KRP resembles that of Ser814 phosphorylated by p21-activated protein kinase PAK2 in MLCK [25], suggesting that PAK may also phosphorylate Ser12 of KRP and affect the R5 antibody binding to KRP phosphorylated by PKA at Ser13. Therefore, we investigated whether recombinant PAK phosphorylates KRP *in vitro* and alters binding of R5 and R8 antibodies.

Figure 3a demonstrates that recombinant PAK1 effectively phosphorylates human, but not chicken KRP, which contains Ala in place of human Ser12, whereas other phosphorylatable residues, except for Thr14, are conserved in the human and chicken KRP sequences (see Fig. 1a). Phospho amino acid analysis revealed that PAK1 phosphorylates exclusively serine (data not shown), indi-

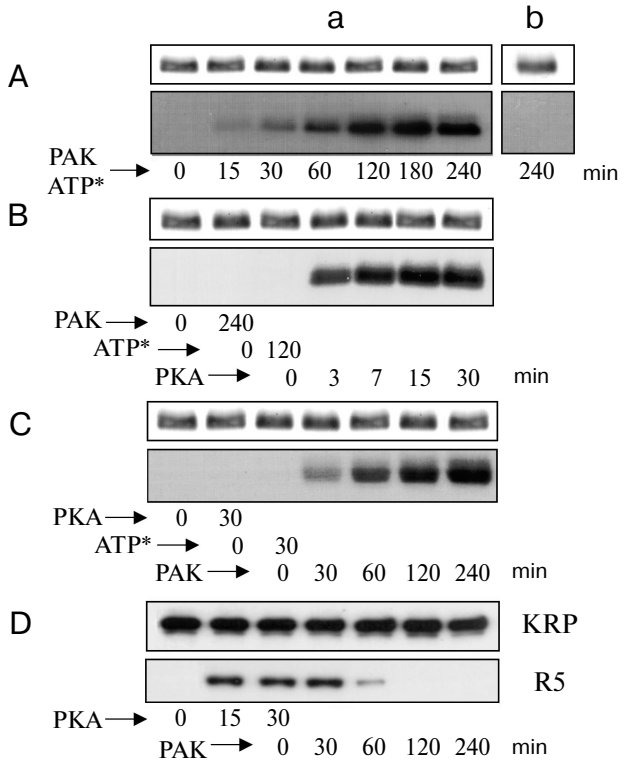


Fig. 3. Phosphorylation of KRP by p21-activated protein kinase PAK1 *in vitro* and its effect on the R5 antibody binding. Shown is the kinetics of KRP phosphorylation after addition of protein kinase A (PKA) and PAK1 (PAK). ATP* indicates time of the [γ-^{32}P]ATP addition. For each experiment, the Coomassie R-250 stained gels and autoradiograms are shown on the upper and lower panels, respectively. A) Phosphorylation of recombinant human (a) and chicken (b) KRP by PAK1; B) sequential phosphorylation of KRP by PAK1 in the absence of [γ-^{32}P]ATP and then by PKA added 2 h after the intermediate incubation with [γ-^{32}P]ATP to ensure completeness of former phosphorylation with PAK1; C) sequential phosphorylation of KRP by PKA and then by PAK1; the details as in (B); D) KRP was sequentially phosphorylated by PKA and PAK1 as in (C), but in the absence of [γ-^{32}P]ATP and analyzed by Western blotting using the R5 antibody against phospho-Ser13 and polyclonal anti-KRP antibody for the load control as indicated.

cating that PAK1 phosphorylates Ser12 in human KRP. Furthermore, phosphorylation of Ser12 by PAK1 did not alter the rate of subsequent Ser13 phosphorylation by PKA (Fig. 3b) and *vice versa* (Fig. 3c). However, the binding of R5 antibody to KRP phosphorylated by PKA gradually decreased with increase in the level of Ser12 phosphorylation (Fig. 3d), suggesting that Ser12 belongs to the antigen recognition sequence of R5 antibody. Reciprocal experiments revealed that phosphorylation of either Ser12 by PAK1 or Ser16 by GSK3 did not affect the R8 antibody reactivity against phosphorylated Ser19 (data not shown).

Finally, we examined the selectivity of the R5 and R8 antibodies to MLCK and KRP in rat ileum extracts. PKA or MAP-kinase were incubated with the extracts in the presence of ATP for 30 min, and reaction mixture aliquots were taken at various intervals and analyzed by Western blots with R5 and R8 antibodies, respectively. The total amount of MLCK and KRP in the extracts was monitored on identical membranes by polyclonal anti-KRP antibody described earlier [23]. R5 antibody cross-reacted only with KRP, MLCK, and a few proteolytic fragments of MLCK in the extracts incubated with PKA, and binding of the antibody increased time-dependently (Fig. 4a). A similar pattern of recognition by the R8 antibody was observed on Western blots of ileum extracts incubated with MAP-kinase; however, this antibody also weakly recognized a 45-50 kD protein band (Fig. 4b), which may represent an additional, still poorly characterized, protein product of the MLCK genetic locus, whose mRNA has been described [16].

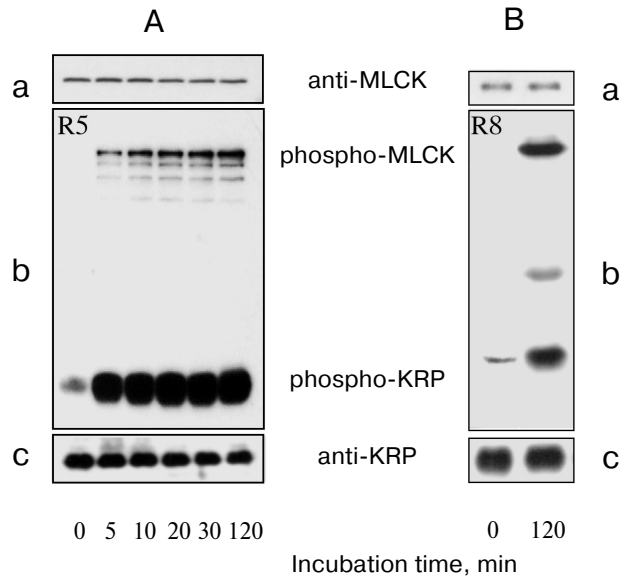


Fig. 4. Selectivity of phosphospecific antibodies against MLCK and KRP in smooth muscle tissue extracts. Rat ileum extracts were incubated at 30°C with 4 mM Mg^{2+}-ATP (pH 7.4) and either PKA (A) or activated GST-$p44^{erk1}$-MAP-kinase (B). Equal aliquots of the reaction mixtures were withdrawn at indicated time intervals and analyzed by immunoblotting. The total amount of MLCK (a) and KRP (c) was determined by the polyclonal anti-KRP antibody staining. Phosphorylated MLCK and KRP were analyzed with phosphospecific R5 (A) or R8 (B) antibodies (b).

We conclude that novel phosphospecific R5 and R8 antibodies can be effectively used to monitor site-specific phosphorylation of MLCK and KRP in vertebrate tissues. The minor restrictions applied to these antibodies are the dependence of R5 reactivity on phosphorylation of KRP and KRP-domain of MLCK by PAK1, should it happen *in vivo*, and an uncertain nature of the 45-50 kD protein recognized by the R8 antibody, although this protein may be also a product of the MLCK genetic locus.

Quantification of KRP phosphorylation in tissue. The basal level of KRP phosphorylation in rat ileum smooth muscle was determined by quantitative immunoblotting. Tissue extracts were prepared as described in the "Materials and Methods" section and total amount of KRP was measured with polyclonal anti-KRP antibody and recombinant human KRP as a standard (Fig. 5a). The fraction of KRP phosphorylated at Ser13 and Ser19 was determined with R5 or R8 antibodies and recombinant KRP fully phosphorylated *in vitro* by PKA or MAP-kinase, respectively. The empirically established increasing volumes of tissue extract and standard KRP were blotted onto the same membrane and developed with corresponding antibodies to obtain overlapping linear relationships between the ECL signal and loaded protein (Fig. 5). The total amount of KRP (Fig. 5a) and that of KRP phosphorylated at either Ser13 (Fig. 5b) or Ser19 (Fig. 5c) were determined from the intercepts, and fraction of phosphorylated KRP was calculated as ratios of phosphorylated to total amount of KRP in equal volumes of extract. The level of KRP phosphorylation was found to be 26 ± 2% at Ser13 and 31 ± 3% at Ser19, as determined from seven independent extractions. The content of KRP was calculated to be 0.5 μg per 1 mg of the wet tissue, which is in a good agreement with the value reported earlier for chicken gizzard smooth muscle [18].

KRP phosphorylation in the smooth muscle contraction–relaxation cycle. Two models were used to monitor level of KRP phosphorylation in stimulated rat ileum smooth muscle. First, Ca^{2+}-dependent contraction was elicited by depolarization with KCl (Fig. 6a) or by carbachol (Fig. 6b) and subsequent relaxation was induced by forskolin. The muscle strips were rapidly frozen at different phases of contraction and changes in KRP phosphorylation were determined by immunoblotting of extracts. The Western blots were developed with phosphospecific or polyclonal antibodies against KRP and relative level of site-specific phosphorylation was calculated as above as the ratio of corresponding ECL signals. We found that phosphorylation of KRP at Ser13 was not altered during contraction but increased 4-5-fold upon relaxation. In the second model, ileum was stimulated by PDBu and a

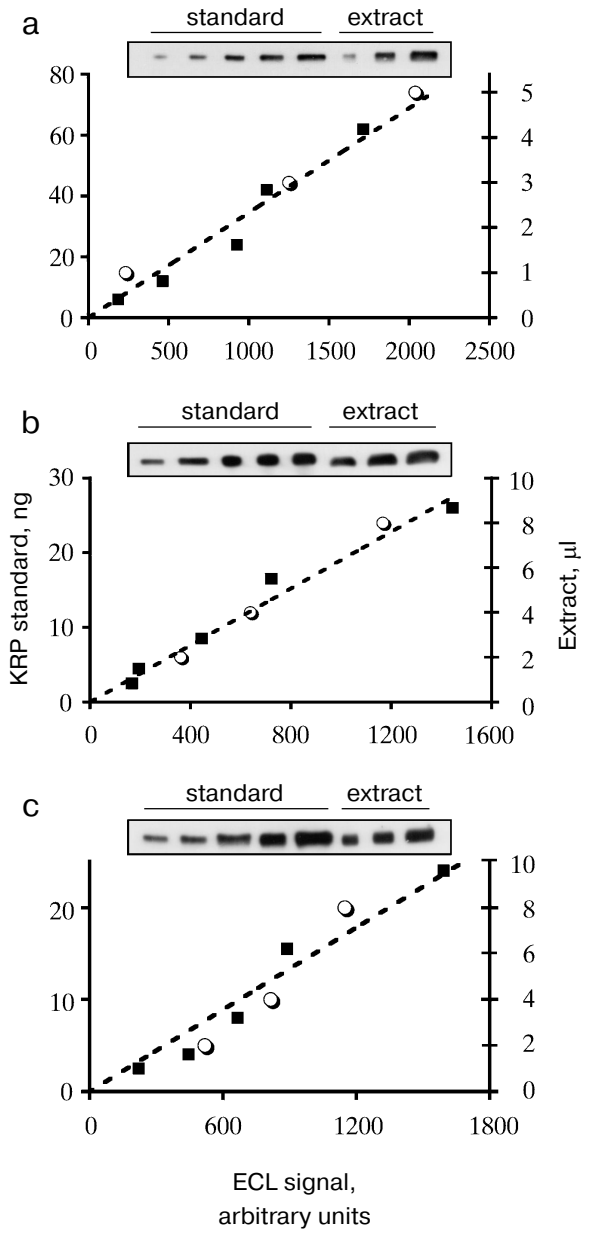


Fig. 5. Determination of basal level of KRP phosphorylation in rat ileum. Increasing amounts of recombinant human KRP (a), KRP phosphorylated *in vitro* by PKA (b) or by MAP-kinase (c) (all designated as standard) were blotted onto PVDF membrane along with the increasing amounts of ileum extracts (designated as extract) prepared as described in the "Materials and Methods" section. The membranes were developed with polyclonal (a) and phosphospecific R5 (b) or R8 (c) antibodies (typical images are shown at the top of each graph). The images were scanned, and the loaded amounts of standard KRP (left ordinates and filled squares) or that of the extract (right ordinates and open squares) were plotted against the peak areas of KRP (abscissa). The plots were linear and amount of KRP in a certain volume of tissue extract was calculated from the intercepts.

4-5-fold increase in the Ser13 phosphorylation was observed in the absence of contractile response (Fig. 6c). Subsequent addition of forskolin had no effect on either muscle tension or KRP phosphorylation. Thus, assuming that basal level of Ser13 phosphorylation in rat ileum is 0.25 mol phosphate per 1 mol of KRP (Fig. 5), the 4-5-fold increase accounts for its complete phosphorylation following PDBu or forskolin stimulation. These findings indicate that, in intact smooth muscle, phosphorylation of Ser13 of KRP is mediated by cyclic nucleotide activated protein kinases and further suggest that PKC, the major PDBu target, is also involved in the control of Ser13 phosphorylation in KRP.

In contrast to Ser13 phosphorylation, the level Ser19 phosphorylation in KRP was increased no more than 1.3-1.5-fold upon stimulation (Fig. 6, a-c). Since we found that PDBu activates both p42/44^{erk1,2} and p38 MAP-kinases in rat ileum (Fig. 6d), the changes in Ser19 phosphorylation were qualitatively expected. However, correction of these values for basal level of Ser19 phosphorylation gives an increase from 0.3 to 0.4-0.45 mol of phosphate per 1 mol of KRP, suggesting it is not significant.

DISCUSSION

Reversible phosphorylation of regulatory proteins plays an essential role in regulation of smooth muscle contraction and cell motility [3]. MLCK is a crucial enzyme to phosphorylate myosin and activate its motor activity. MLCK is in turn controlled by a number of protein kinases that phosphorylate residues located mostly in the C-terminal domain of MLCK [5, 10, 26]. In smooth muscle, this domain is expressed as an independent protein KRP (telokin) that retains myosin-binding activity and major phosphorylation sites of MLCK [10, 23]. KRP is a smooth muscle relaxation factor [15, 20], and phosphorylation modulates the relaxing activity of KRP [20, 21]. Phosphorylation of distinct but closely located sites in KRP and MLCK is suggested to have different regulatory effects (reviewed in [3]). Therefore, a simple and reliable method is required to monitor phosphorylation of individual sites of MLCK and KRP *in vivo*.

In this report, we describe generation and characterization of phospho- and site-specific antibodies against major phosphorylation sites common to MLCK and KRP. These antibodies appear useful for qualitative and quantitative analyses of MLCK and KRP phosphorylation in vertebrate tissues and thus add to the array of already available antibodies aimed to examine signaling mechanisms of smooth muscle and non-muscle cells that are based on phosphorylation of the regulatory proteins of the contractile machinery. Similar antibodies against phosphorylated myosin regulatory light chains [24, 27], myosin phosphatase [8, 28] and its inhibitor CPI-17 [29], caldesmon [30], and calponin [31] are also available on

the market. In addition to the commercial phospho-specific antibodies that recognize active components of various signaling cascades (such as MAP-kinases, Fig. 6d) and the dominant-negative transfection technology, the novel tool could be used to address intracellular pathways that couple the activation of cell surface receptors to a particular cell response.

Specifically, the activation of cyclic nucleotide dependent protein kinases leads to smooth muscle relaxation, but the molecular mechanisms remain unclear. It has been suggested that Ser13 of KRP is one of the targets that accepts this signal to increase the relaxing activity of KRP [20]. We investigated this possibility and determined the level of KRP phosphorylation at Ser13 in intact

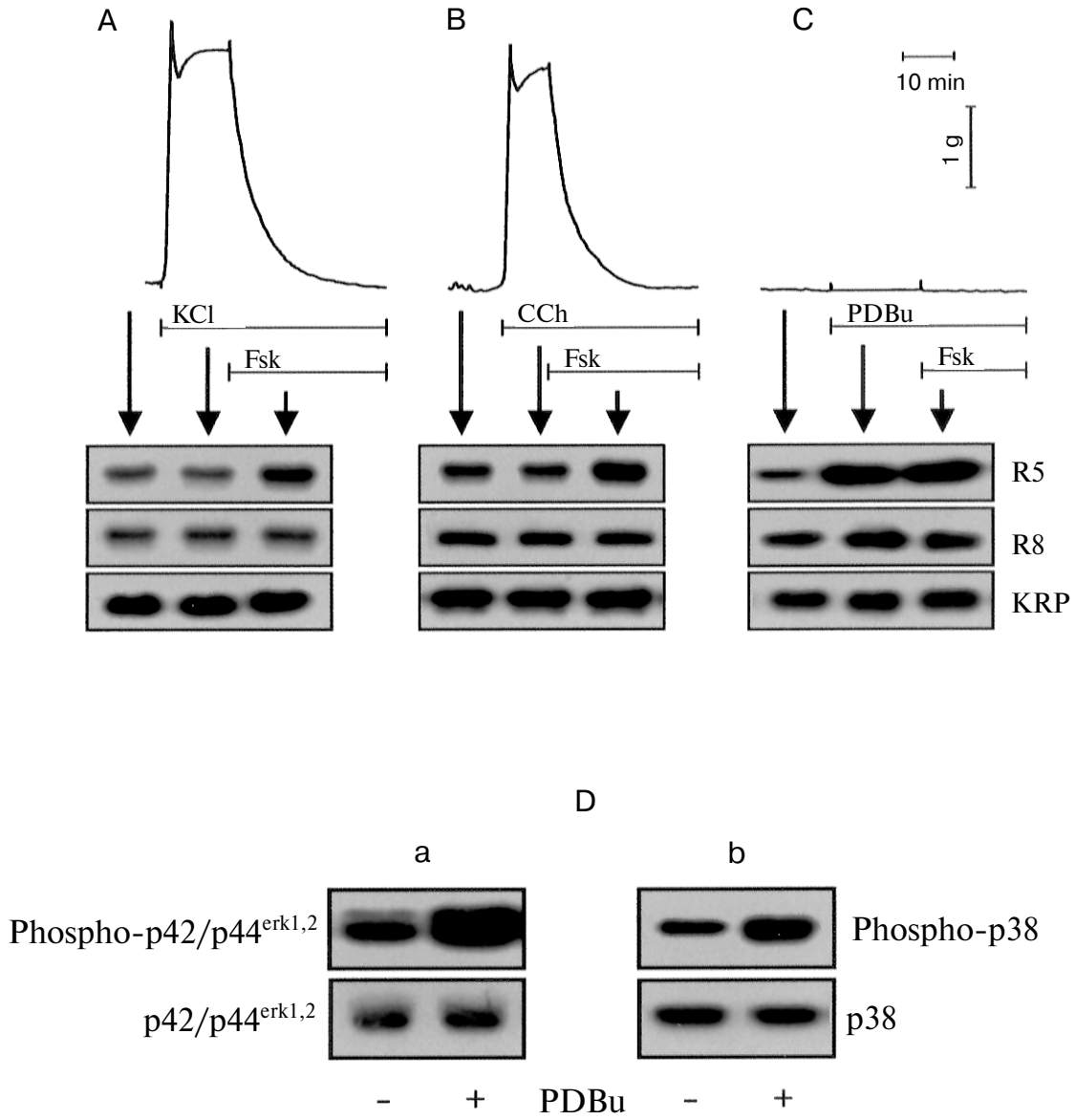


Fig. 6. Phosphorylation of KRP in the smooth muscle contraction–relaxation cycle. The experiments were performed with intact ileum strips as described in the "Materials and Methods" section. The level of KRP phosphorylation was measured in extracts of rapidly frozen tissue using the phospho-specific or polyclonal antibodies. Shown are typical immunoblots from three independent experiments. A) Mechanograms of rat ileum contraction induced by membrane depolarization with 90 mM KCl. After contraction had reached a plateau, the relaxation was stimulated by adding forskolin to 10 μM (Fsk). Phosphorylation of KRP at Ser13 (R5) and Ser19 (R8) and its total amount (KRP) were determined by Western blots at the time points indicated by arrows; B) as in (A), contraction was stimulated by 20 μM carbachol (CCh); C) as in (A), the muscle was stimulated with 1 μM PDBu. Note that PDBu does not evoke contraction in phasic smooth muscle [23]; D) 1 μM PDBu activates both $p42/44^{erk1,2}$ (a) and p38 (b) MAP-kinases in intact ileum. MAP-kinase activation was measured with phospho-specific (upper panels) and polyclonal (lower panels) antibodies against MAP-kinases as described in "Materials and Methods".

smooth muscle using the novel phosphorylation-specific antibody. We found that in unstimulated tissue up to 25% of KRP is phosphorylated at Ser13 as determined by quantitative immunoblotting. Increasing the intracellular cAMP concentration by activation of adenylate cyclase with forskolin led to a 4-fold increase in Ser13 phosphorylation, i.e., to its complete phosphorylation. This indicates that PKA mediates phosphorylation of Ser13 in KRP *in vivo*.

Important observations were made when the extent of KRP phosphorylation was analyzed after smooth muscle stimulation that does not involve activation of cyclic nucleotide activated kinases. It appeared that PDBu, the direct PKC activator, also stimulates maximum phosphorylation of Ser13 in KRP. This suggests a likely function of PKC in intracellular control of KRP phosphorylation at Ser13. Interestingly, phosphorylation of Ser13 was also found increased in the GTPγS treated permeabilized rabbit portal vein [22], further suggesting possible involvement of a G-protein and PKC activation. Although the substrate recognition sequences are similar for PKC and PKA, we earlier found that conventional Ca^{2+}- and PDBu-activated α and β isoforms of PKC fail to phosphorylate KRP *in vitro* [32]. Furthermore, these isoforms become activated upon the increase in intracellular Ca^{2+} concentration after membrane depolarization [33]; however, this does not result in increased phosphorylation of KRP at Ser13 (Fig. 6a). It remains to be established whether the Ca^{2+}-independent, PDBu-activated PKCs such as PKCε or PKCδ that are virtually ubiquitous in smooth muscles [33], can phosphorylate KRP at Ser13 *in vitro*. Thus, although determination of the kinases that phosphorylate Ser13 of KRP *in vivo* requires the further studies, our results demonstrate that Ser13 of KRP is targeted by different signaling cascades.

Addition of KRP caused partial relaxation of skinned rabbit ileum at constant Ca^{2+} concentration [20]. The relaxing effect of KRP was further increased in the presence of 8-Br-cGMP or cGMP-activated protein kinase regardless of their addition order, and this was accompanied by increased phosphorylation of KRP at Ser13 [20, 21]. Mutation of Ser13 into unphosphorylatable Ala did not alter the relaxing effect of KRP, but abolished its potentiation by 8-Br-cGMP. Furthermore, the phosphorylation mimicking mutation of Ser13 into Asp enhanced relaxation effect of KRP in the absence of 8-Br-cGMP [21]. Similar experiments with KRP site-mutated on Ser19 led to suggestion that Ser19 phosphorylation reverses the effect of Ser13 phosphorylation [21].

Although these results suggested a role for KRP phosphorylation in Ca^{2+}-desensitization of smooth muscle contraction, the relevant changes in the level of Ser13 and Ser19 phosphorylation of KRP during a contraction–relaxation cycle of intact smooth muscle have not been established. To reveal them, we used newly developed phospho-specific antibodies in two different experimental protocols. First, we found that the extent of Ser13 phosphorylation in KRP is not altered during Ca^{2+}-dependent contraction, but considerably increases during cAMP-induced relaxation of the rat ileum (Fig. 6, a and b). Second, we took an advantage of the earlier found phenomenon that PDBu evokes strong contraction only in tonic smooth muscle, but does not contract phasic smooth muscle [10, 23]. We observed that, in phasic smooth muscle, PDBu utterly increased phosphorylation of Ser13 in KRP, suggesting this may preclude the contraction development. Notably, this mechanism does not operate in tonic smooth muscles because these barely express KRP [23]. Clearly, phosphorylation of KRP is not the sole mechanism of the phasic smooth muscle relaxation, and other signaling pathways, including those activated by monomeric G-protein Rho and coupled to regulation of myosin phosphatase activity [6], should provide for variety of contractile responses to external stimuli. We suggest that comparative model based on contrasting contractile responses of tonic and phasic smooth muscles [10, 23] can be used in the future to reveal individual contributions of these mechanisms to vascular tonic contraction.

PDBu activates p42/p44^{erk1,2} and p38 MAP-kinases in phasic smooth muscle (Fig. 6d), and Ca^{2+}-dependent activation of p42/p44^{erk1,2} was earlier reported [33]. The active MAP-kinases are shown to translocate into contractile domain [34] where they phosphorylate actin bound caldesmon [3]. *In vitro*, both p42/44^{erk1,2} [22, 23] and p38 MAP-kinases (A. Yu. Khapchaev, unpublished data) phosphorylate KRP at Ser19. However, *in vivo* depolarization of rat ileum smooth muscle or PDBu stimulation result only in partial increase in Ser19 phosphorylation from 0.3 up to 0.4-0.5 mol P_i per 1 mol of the protein. It seems unlikely that Ser19 becomes hindered when KRP binds contractile proteins, because it has a substantial steady-state level of phosphorylation that may be further increased by PDBu or GTPγS stimulation [22]. Perhaps, MAP-kinases phosphorylate only a fraction of KRP that is localized in contractile domain, whereas the rest of the protein is diffusely distributed in cytosol, inaccessible and readily lost upon even mild permeabilization [20].

Thus, the presented results may explain the earlier reported pattern of KRP phosphorylation in ^{32}P-labeled intact smooth muscle preparations [23]. We found that PDBu increased total level of KRP phosphorylation 2-2.5-fold, and it was not further altered by forskolin. The level of Ser19 phosphorylation is now found to change insignificantly, implying that it is the change in Ser13 phosphorylation that mainly contributes to total increase in KRP phosphorylation in intact smooth muscle.

Our data demonstrate that phosphorylation of KRP at Ser13 is associated with relaxation of the contracted intact phasic smooth muscle, or with the absence of initial contractile response to PDBu, which is a powerful

tonic smooth muscle constrictor. In contrast, phosphorylation of Ser19 in KRP is unlikely to have a regulatory role because of the small and rigid changes during contraction–relaxation. Perhaps this phosphorylation may be related to a so far unknown function of KRP, or is redundant in KRP compared to that of equivalent Ser834 in MLCK. In this line, MAP-kinase has been reported to activate MLCK [11, 13, 14], whereas Ser834 appears to be major target in MLCK in intact smooth muscle [10]. Availability of novel phosphorylation specific antibody to Ser834 in MLCK allows a simple and easy analysis of its *in vivo* phosphorylation dynamics in terms of functional proteomics. Similarly, phosphorylation of MLCK at Ser828 that is equivalent of Ser13 in KRP can be now addressed with phosphospecific R5 antibodies to study its role in intracellular signaling.

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