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Phosphorylation of kinase-related protein (telokin) in tonic and phasic smooth muscles

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Abstract

KRP (telokin), an independently expressed C-terminal myosin-binding domain of smooth muscle myosin light chain kinase (MLCK), has been reported to have two related functions. First, KRP stabilizes myosin filaments (Shirinsky *et al*., 1993, J. Biol. Chem. 268, 16578–16583) in the presence of ATP. Secondly, KRP can modulate the level of myosin light chain phosphorylation. In this latter role, multiple mechanisms have been suggested. One hypothesis is that light chain phosphorylation is diminished by the direct competition of KRP and MLCK for myosin, resulting in a loss of contraction. Alternatively, KRP, through an unidentified mechanism, accelerates myosin light chain dephosphorylation in a manner possibly enhanced by KRP phosphorylation. Here, we demonstrate that KRP is a major phosphoprotein in smooth muscle, and use a comparative approach to investigate how its phosphorylation correlates with sustained contraction and forskolin-induced relaxation. Forskolin relaxation of precontracted artery strips caused little increase in KRP phosphorylation, while treatment with phorbol ester increased the level of KRP phosphorylation without a subsequent change in contractility. Although phorbol ester does not induce contraction of phasic tissues, the level of KRP phosphorylation is increased. Phosphopeptide maps of KRP from both tissues revealed multiple sites of phosphorylation within the N-terminal region of KRP. Phosphopeptide maps of KRP from gizzard were more complex than those for KRP from artery consistent with heterogeneity at the amino terminus and/or additional sites. We discovered through analysis of KRP phosphorylation *in vitro* that Ser^{12}, Ser^{18} and Ser^{15} are phosphorylated by cAMP-dependent protein kinase, mitogen-activated protein (MAP) kinase and glycogen synthase kinase 3 (GSK3), respectively. Phosphorylation by GSK3 was dependent upon prephosphorylation by MAP kinase. This appears to be the first report of conditional or hierarchical phosphorylation of KRP. Peptides consistent with such multiple phosphorylations were found on the *in vivo* phosphopeptide maps of avian KRP. Collectively, the available data indicate that there is a complex relationship between the *in vivo* phosphorylation states of KRP and its effects on relaxation in smooth muscle.

Introduction

Smooth muscle contraction is primarily initiated by the phosphorylation of Ser^{19} of myosin regulatory light chain by specific Ca^{2+}/calmodulin-dependent myosin light chain kinase (MLCK) (for recent review see Lukas *et al*., 1998). However, isometric stress is not always proportional to the degree of myosin light chain phosphorylation which, in turn, may not be strictly proportional to changes in intracellular Ca^{2+} concentration (Somlyo and Somlyo, 1994; Horowitz *et al*., 1996). Several mechanisms exist to increase Ca^{2+}-sensitivity of smooth muscle contractile response and these employ small G-protein-dependent and independent pathways (reviewed in Somlyo and Somlyo, 1994). Ca^{2+}-sensitization for contractions induced by phorbol esters in independent of G-protein pathways (Fu *et al*., 1998). Therefore, these agents, which target a subset of protein kinase C isoforms, trigger contraction by undefined intracellular cascades (Horowitz *et al*., 1996) that may converge on inhibition of myosin light chain phosphatase (MLC phosphatase). Furthermore, contracted smooth muscle can be relaxed upon elevation of intracellular cyclic nucleotide levels, and an involvement of cyclic GMP-(PKG) and/or cyclic AMP-dependent (PKA) protein kinases has been suggested (Eckly-Michel *et al*., 1997). However, evidence is growing that the mechanisms of cyclic AMP- and cyclic GMP-mediated relaxation are markedly different (Ahn *et al*., 1997; Pfeifer *et al*., 1998). Therefore, a description of protein kinase cascades, relevant regulatory protein phosphorylation and critical sites of their modifications is exceptionally important for identification of events directly related to the generation of Ca^{2+}-sensitized contraction and increased vascular tone. Besides MLCK

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and MLC phosphatase, recent studies have been directed at the actin-associated proteins caldesmon (reviewed in Shirinsky *et al.*, 1999), calponin (Horowitz *et al.*, 1996) and the myosin-binding protein, KRP, also known as telokin (Ito *et al.*, 1989; Vorotnikov, 1997; Somlyo *et al.*, 1998) as potential targets for phosphorylation-dependent regulation of the smooth muscle contractile machinery.

KRP is an independently expressed protein derived from a distinct gene embedded within the MLCK gene. The promoter for KRP is located within an intron of the MLCK gene, but the initiator ATG is an internal Met codon for MLCK. Thus, the KRP coding region uses the same reading frame as that for the independently regulated kinase and its amino acid sequence is identical to the C-terminal domain of MLCK (Collinge *et al.*, 1992; Watterson *et al.*, 1999).

KRP binds *in vitro* to the neck region of unphosphorylated smooth muscle myosin with 1:1 stoichiometry, stabilizes the extended conformation of myosin and thus facilitates filament formation in the presence of ATP (Shirinsky *et al.*, 1993; Masato *et al.*, 1997; Silver *et al.*, 1997). Estimation of the KRP content in chicken gizzard smooth muscle revealed that its concentration approximates that of myosin (Shirinsky *et al.*, 1993) consistent with a potential structural as well as regulatory function of KRP. KRP competes *in vitro* with the KRP domain of MLCK for interaction with myosin, which results in the inhibition of the initial rate of myosin phosphorylation (Shirinsky *et al.*, 1993; Silver *et al.*, 1997). The observation that phosphorylation of Ser^{19} on the regulatory myosin light chain prevents KRP binding to myosin (Shirinsky *et al.*, 1993) led to the hypothesis that *in vivo* KRP might control the rates of the light chain phosphorylation and dephosphorylation via a competition mechanism (Vorotnikov, 1997). It was found, however, that KRP does not affect the rate of myosin thiophosphorylation, but does enhance the rate of relaxation of permeabilized smooth muscle, presumably through activation of MLC phosphatase activity (Sobieszek *et al.*, 1998; Wu *et al.*, 1998).

The potential roles of KRP in regulation of smooth muscle cell contractile elements suggests intracellular signalling mechanisms could modulate its activity. KRP is a phosphoprotein in resting smooth muscle and its phosphorylation *in situ* is enhanced following various treatments including phorbol ester (Vorotnikov *et al.*, 1996), forskolin, and 8-bromo-cGMP in α-toxin permeabilized muscle (Vorotnikov *et al.*, 1996; Wu *et al.*, 1998; MacDonald *et al.*, 2000). The relaxing effect of KRP on permeabilized smooth muscle was potentiated by following addition of active PKG, suggesting that phosphorylation of KRP might have a functional importance (Wu *et al.*, 1998). In a later study, the relaxing effect of exogenously added KRP, or KRP phosphorylated by PKA at a single site, were not distinguishable. Therefore, whether PKA, PKG, or other kinase phosphorylation actually changes the relaxing activity of KRP remains in question and requires additional investigation.

In situ, KRP is apparently phosphorylated at several sites because multiple phosphorylated protein bands were found by two-dimensional (2D) isoelectrofocusing and SDS–PAGE (Wu *et al.*, 1998). KRP is phosphorylated in unstimulated muscle and okadaic acid treatments leads to a further several-fold increase in associated phosphate (Vorotnikov *et al.*, 1996). There are several potential and known phosphorylation sites within the N-terminal sequence of chicken KRP: AMISGMSGRKAS^{12}GSSPTS^{18}PINADKVENE, and at least two of these sites (Ser^{12} and Ser^{18}) are also conserved in the same region of human KRP: AMSGLSGRKSS^{12}TGSPTS^{18}PLNAEKLESE. Ser^{12} of KRP was identified as a site phosphorylated by PKA both *in vitro* (Ito *et al.*, 1989) and *in vivo* (MacDonald *et al.*, 2000), while Ser^{18}, a putative MAP kinase phosphorylation site, was found to contain phosphate *in vivo* (Wu *et al.*, 1998; MacDonald *et al.*, 2000). However, these studies were limited to KRP in rabbit ileum and portal vein. Whether the pattern of KRP phosphorylation is the same in tonic (e.g., arteries) vs. phasic tissues (e.g., gizzard) has not been addressed.

Chicken KRP also has two additional serines positioned in between the PKA and MAP kinase (MAPK) phosphorylated residues. These serines fit the consensus requirements for hierarchical phosphorylation by acidotropic kinases such as glycogen synthase kinase 3 (GSK3) and casein kinases I and II. Acidotropic kinases utilize a negatively charged phosphoryl group as a prerequisite to phosphorylate residues located in $n+3$ (for casein kinase I), $n-3$ (for casein kinase CK2) and $n-4$ (for GSK3) positions from a phosphoresidue (n) (Roach, 1991). However, it is not known whether KRP is a substrate for these enzymes. Considering the potential functional implications of phosphorylation of KRP at this region (Wu *et al.*, 1998), and the lack of knowledge of how KRP phosphorylation correlates with tonic contraction and relaxation of intact smooth muscle, a more detailed analysis of KRP phosphorylation is required.

In the study reported here, we employed 2D phosphopeptide mapping to characterize multiple sited phosphorylation of KRP. We find that KRP can be phosphorylated by PKA, MAPK and GSK3 *in vitro*. MAPK and GSK3 phosphorylate KRP in an ordered fashion independent of PKA. The *in vitro* phosphorylation sites were identified in KRP and all phosphorylated forms of the protein were found *in situ*. Furthermore, phosphopeptide mapping of KRP from the resting arterial muscle showed a high level of phosphorylation at the PKA/PKG site, consistent with little change in the level of KRP phosphorylation upon forskolin-induced relaxation. These results suggest that there is not a straightforward relationship between phosphorylation of KRP and contraction or forskolin-induced relaxation in intact smooth muscle.

Materials and methods

Materials

[γ-^{32}P]ATP and [^{32}P]-trisodium orthophosphate were from the Institute of Physics and Energetics, Obninsk, Russia. PDBu was purchased from Sigma, USA and protein G-agarose from Calbiochem, USA. BL21 DE3 *Escherichia coli* strains was obtained from Gibco-BRL, UK. Catalytic subunit of PKA and protein kinase C (PKC) isoforms were a gift from Ms D.L. Silver and Dr J.R. Sellers (NHLBI, NIH, USA). GSK3 (β-isoform) was purchased from the Upstate Biotechnology, USA. Biological buffers and chemicals were from Sigma or BDH, UK. Earle's balanced salt solution (EBSS, Sigma) was prepared with addition of 24 mM HEPES, pH 7.4.

Tissue preparation and tension measurements

Four to six months old chickens were decapitated and carotid arteries were gently excised to avoid stretching, placed in EBSS and cut into rings of 3 mm width. The rings were placed into the 20-ml organ bath chamber and mounted onto the Harvard isometric force transducer and equilibrated at the initial loading 0.8 g for 1 h before registration. Gizzards were removed and cooled down for 10 min on ice to stiffen the muscle. The outer connective tissue was gently separated and slices were cut across the long axis of longer muscle body of gizzard, so that fibre bundles were aligned longitudinally. The inner basal support was then cut off to liberate $6 \times 4 \times 0.6$ mm tissue strips. These were clamped by stainless steel clips at shorter sides, mounted to the transducer and equilibrated after initial loading of 0.7 g for 1 h in 20-ml organ bath chamber. Tissue preparations were stimulated with 90 mM KCl without changing the medium and the maximum isometric tension achieved (usually about 0.2 g for arterial and 0.7 g for gizzard tissue) was calculated and used as a reference to normalize further contractile responses.

Tissue labelling and KRP phosphorylation in situ

Rings of chicken carotid artery prepared as above were mounted on stainless steel hooks either without stretching (no initial load), or stretched to obtain initial load similar to that in experiments for tension measurements. Then both sets of the tissue were immersed into 12 ml of EBSS containing 0.125 mCi/ml of [^{32}P]Na_3PO_4 and incubated for 3 h at 37°C with continuous carbogen administration. Unmounted strips of gizzard smooth muscle were freely placed in individual well of a separate custom made 12-ml chamber and similarly labelled with [^{32}P]Na_3PO_4 as described previously (Krymsky *et al.*, 1999). After removal of two gizzard strips or arterial rings for the unstimulated control, PDBu was added to achieve 2 μM. Individual strips were removed at the appropriate time intervals, then forskolin was added to remaining arterial rings and incubation continued for another 10 min. In other experiments, gizzard strips were treated for 45 min with 0.5 μM okadaic acid dissolved in water. After withdrawal tissue pieces were briefly rinsed in ice-cold EBSS, frozen in liquid nitrogen and pulverized while frozen with porcelain mortar and pestle. Extraction with buffer E (20 mM MOPS, pH 7.0, 1% (v/v) triton X100, 0.35 M NaCl, 25 mM $MgCl_2$, 2 mM EGTA, 1 mM dithiothreitol, 1 mM *o*-vanadate, and protease inhibitor cocktail) was performed as described previously (Krymsky *et al.*, 1999). The extracts were clarified and each supernatant was supplied with 25 μg of goat anti-chicken gizzard KRP affinity purified polyclonal antibody which specifically recognize only KRP and MLCK in smooth muscle extracts (Figure 1). Immune complexes formed by overnight incubation at 4°C were then precipitated by protein G-agarose. The agarose was washed three times with buffer E and twice with 20 mM Tris/HCl (pH 7.4) and 0.6 M NaCl. Immune complexes were eluted by boiling the agarose in SDS–PAGE sample buffer and subjected to 10% SDS–PAGE. Each sample was assayed in duplicate on separate gels. The gels were either directly stained with Coomassie R-250 and autoradiographed, or blotted onto Immobilon-P membrane (0.45 μm pore size, Millipore, UK) for 1 h in 10 mM CAPS (pH 11), 10% ethanol and 5 mM 2-mercapthoethanol. The membrane was autoradiographed and then developed for KRP using rabbit anti-chicken gizzard KRP affinity purified antibody 3007 described previously (Watterson *et al.*, 1995) and donkey anti-rabbit secondary antibody conjugated with peroxidase (Amersham, UK). Coomassie-stained gels, PVDF membranes stained with diaminobenzidine and corresponding autoradiograms were quantified by scanning densitometry. The relative level of KRP phosphorylation was calculated as the ratio of the values obtained from autoradiograms to those from protein bands on the gels or PVDF membrane. At least two autoradiograms with different exposure times were analysed to ensure linearity of the Kodak Biomax film (Sigma, UK) response.

Proteins

KRP was prepared from chicken gizzards according to Ito *et al.* (1989) and optical extinction coefficient, $A_{280}^{0.1\%} = 0.78$, was used to determine its concentration (Shirinsky *et al.*, 1993). Recombinant His-tagged KRP (recKRP) was expressed in BL21 (DE3) *E. coli* strain and purified as previously described (Silver *et al.*, 1997). Casein kinases 1 and CK2 were isolated from rabbit liver (Vorotnikov *et al.*, 1988), GST-tagged p44^{erk1} MAPK was expressed and purified as before (Krymsky *et al.*, 1999). To activate the kinase *in vitro*, serum-deprived subconfluent COS-7 cells grown on DMEM (Gibco, USA) were stimulated for 10 min with 50 nM EGF and activated MAPK kinase (MEK 1/2) was immunoprecipitated with anti-MEK 1/2 antibody (Transduction

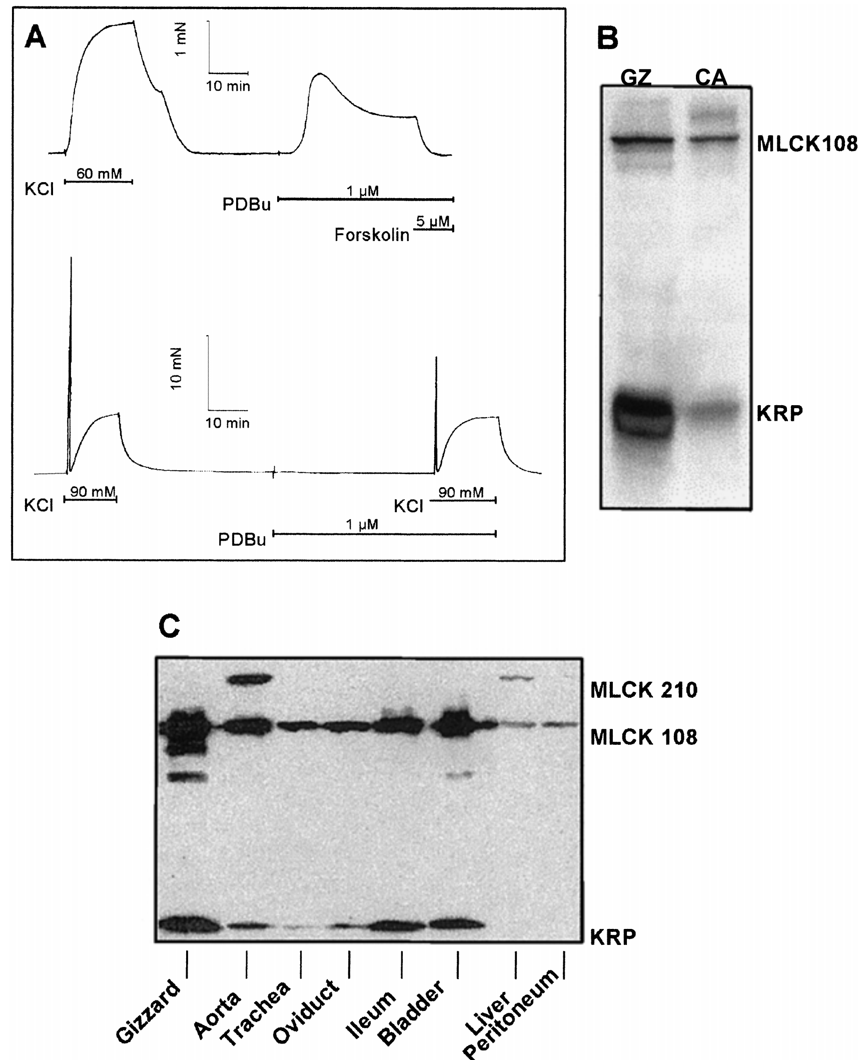

Fig. 1. Tonic and phasic smooth muscles display opposite contractile response to PDBu and different levels of KRP expression. (A) Isomeric contractions of chicken carotid artery rings (CA→uppertrace) and chicken gizzard strips (GZ→bottomout) were measured in EBSS as described in Materials and methods. Control contractions were stimulated by direct addition of 90 mM KCl which was washed after achievement of maximum tension. PDBu and forskolin were added as indicated. Each trace is representative of three independent experiments. (B) Western blot analysis of chicken carotid artery (CA) and gizzard (GZ) extracts (see Materials and methods). Ten micrograms of total protein was loaded on each lane and transferred onto PVDF membrane which was probed with goat anti-chicken gizzard KRP polyclonal antibody that detects both MLCK and KRP in these tissues. (C) Comparative content of protein products expressed from the MLCK gene locus in selected chicken tissues. Similar amounts of total protein were analysed as in (B), MLCK108 and MLCK210 denote the 108 and 210 kDa isoforms of MLCK (Watterson *et al.*, 1995).

Laboratories, USA) bound to protein G-agarose. The agarose was washed and incubated with 0.4 mg/ml GST-MAP kinase in 40 mM HEPES, pH 8.0, 5 mM $MgCl_2$, 0.4 mM ATP, 1 mM EGTA, 1 mM dithiothreitol, and 0.2 mM sodium *o*-vanadate. Alternatively, GST-MAP kinase was similarly activated by constitutively active His_6-tagged MEK1ca bacterially expressed from the plasmid kindly provided by Dr P.H. Sugden, Imperial College, London, UK. The activated GST-MAP kinase was stored at −20°C in 50% (v/v) glycerol.

Protein phosphorylation in vitro

Phosphorylation of 0.4 mg/ml KRP by casein kinases, PKC and Ca^{2+}-calmodulin-dependent protein kinase II was performed in the presence of [γ-^{32}P]ATP (0.5–1 × 10^6 cpm/nmol) as before (Vorotnikov *et al.*, 1988). One milligram per millilitre of KRP was phosphorylated with 2 µg/ml catalytic subunit of PKA or with 30 µg/ml GST-MAP kinase by incubation at 30°C for 1.5 h in the described above buffer used for the MAPK activation containing additional 0.6 mM ATP. Phosphorylated KRP was extensively dialysed to remove ATP and subjected to thin layer isoelectric focusing (1500 VH at 4°C) using pH 2.5–5 ampholines to determine the extent of phosphorylation (Vorotnikov *et al.*, 1996).

Phosphopeptide mapping

Following SDS–PAGE, ^{32}P-labelled bands were excised from the gel, destained and homogenized in 1 ml of

0.1 mM NH_4HCO_3, pH 8.0. Digestion was performed at 37°C by addition of 50 μg TPCK-trypsin (Worthington Biochem. Corp., USA) for 14 h and two more additions of 25 μg TPCK-trypsin for 3 h each. Resulting solution was lyophilized and pellet was dissolved in 10 μl of thin layer electrophoresis buffer (acetic acid/formic acid/H_2O, 15:5:80, by volume). The digest was spotted onto silica gel 60 × 20 × 20 cm^2 plates (Merck, UK) and subjected to electrophoresis at 1000 V for 75 min at 10°C in the first dimension followed by ascending chromatography in *n*-butanol/pyridine/acetic acid/H_2O (150:150:40:160, by volume) in second dimension. Phosphopeptides were visualized by autoradiography on Kodak Biomax film.

Purification and analysis of KRP phosphopeptides

KRP phosphorylated *in vitro* by PKA or MAPK was digested overnight at 37°C by TPCK-treated trypsin (50:1, w/w). Resulting peptides were separated on Vydac C18 monomeric (300 Å, 250 × 4.6 mm) HPLC column (Vydac, USA) using linear gradient 0–50% (v/v) acetonitrile in 0.05% (v/v) trifluoroacetic acid over 100 min at flow rate 1 ml/min. Fractions were collected at 1 min per fraction and analysed by Cherenkov counting. The fractions containing radioactivity were sequentially purified by HPLC in CH_3CN gradients on Aquapore RP300 C8 column (300 Å, 220 × 2.1 mm) (Perkin Elmer, USA) in 0.05% (v/v) heptafluorobutyric acid at 0.4 ml/min flow rate, and Aquapore OD300 C18 column (300 Å, 220 × 2.1 mm) (Perkin Elmer, USA) in 25 mM phosphate buffer, pH 6.5 at 0.4 ml/min flow rate. Aliquots of phosphopeptides were transferred onto PVDF membrane and subjected to microsequencing on an Applied Biosystems 492A sequencer (Applied Biosystems, USA).

Mass spectrometry was done on an Applied Biosystems (USA) Voyager DE-RP MALDI-TOF instrument. Peptide samples were dissolved in water and a 1 μl aliquot mixed with 9 μl of matrix solution (10 mg/ml α-cyano-4-hydroxy-cinnamic acid in 50% (v/v) acetonitrile–water containing 0.1% (v/v) trifluoroacetic acid). One microlitre of the resulting solution was then applied to a stainless steel sample plate and allowed to air dry. Mass spectra were obtained in reflector mode and calibrated to an external standard (angiotensin II).

Peptides corresponding to the Leu^6-Leu^{25} sequence of human KRP were synthesized on an Applied Biosystems solid-phase synthesizer using Fmoc amino acids and the manufacturer's recommended conditions for assembly and cleavage. Phosphoserine residues were introduced at selected positions using Fmoc-Ser(PO-(Obzl)-OH) (NovaBiochem, Germany). The crude peptides were purified by HPLC and verified by MALDI-TOF mass spectrometry (the details will be published elsewhere). MG1 is CLSGRKSS^{12}TGSPTS^{18}PLNAEKL, while peptides MG2 and MG3 are identical to MG1 except that they contain phosphoserine residues at Ser^{12} or Ser^{18}, respectively.

Results

Contractile behaviour of chicken phasic and tonic smooth muscles that have different levels of KRP

Intact strips of tonic carotid artery and phasic gizzard smooth muscles display different responses when exposed to 1 μM phorbol ester (PDBu). Under isometric conditions both muscles contracted in response to KCl and relaxed after washout (Figure 1). The subsequent addition of PDBu induced tonic contraction in arterial muscle 5 μM forskolin relaxed the tension. Consistent with results from other phasic tissues, no contraction was elicited by PDBu in gizzard strips (Figure 1). The relative contents of MLCK and KRP in the muscle extracts were estimated by an affinity purified anti-chicken gizzard KRP antibody that recognizes both proteins. Whereas the level of MLCK in carotid artery was comparable to that in gizzard, the amount of KRP was markedly higher in gizzard than in artery (Figure 1B). While the expression of KRP is generally restricted to smooth muscle tissues, similar differences between other tonic (aorta, trachea) and phasic (ileum, bladder) tissues were also found (Figure 1C) thus indicating that KRP is most abundant in phasic than in tonic smooth muscles. The different levels of KRP in these tissues, therefore, does not appear to be correlated with differences in contractile responses elicited by a common agent (KCl). Although gizzard tissue has not been investigated directly, the lack of response to PDBu in other phasic tissues is not due to the absence of sensitive PKC isoforms (Ohanian *et al.*, 1996; Walker *et al.*, 1998), but may be correlated with a secondary mechanism which employs a downstream substrate to module smooth muscle contraction (Walsh *et al.*, 1996).

KRP phosphorylation in chicken phasic and tonic smooth muscles

KRP phosphorylation in chicken gizzard and carotid artery was assessed in isolated tissue strips metabolically labelled with $[^{32}P]PO_4$. Representative autoradiograms of arterial and gizzard muscle extracts demonstrate that most of the radioactivity was associated with two bands corresponding to proteins of 85–95 and 26 kDa (Figure 2). The phosphorylation of these proteins was enhanced by 2 μM PDBu in a time-dependent manner. The 26 kDa protein was found to specifically cross-react with an anti-KRP antibody on Western blot and was immunoprecipitated by this antibody. Quantification of the phosphorylation levels by scanning densitometry gave similar results whether the total extracts or immunoprecipitated KRP were analysed (data not shown). Usually 2–2.5-fold stimulation by PDBu was observed in both resting and stretched arterial muscle and subsequent addition of 10 μM forskolin produced little further increase in KRP phosphorylation (Figure 3). In gizzard muscle the stimulation was about

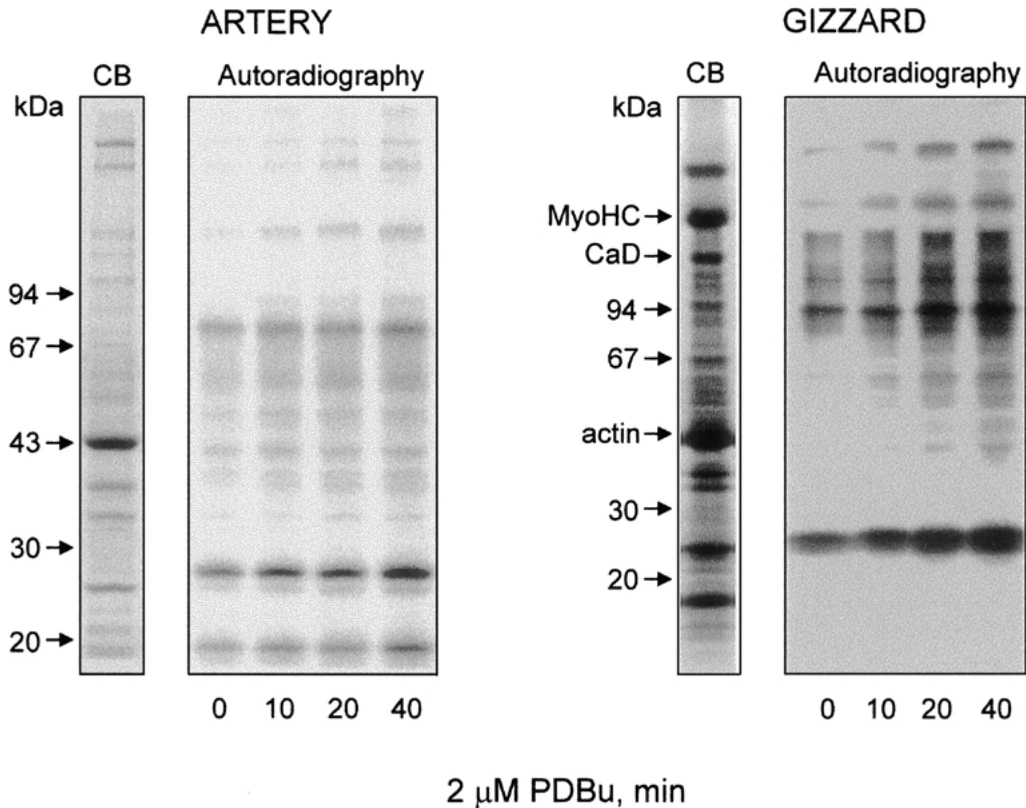

Fig. 2. Time-dependence of PDBu-stimulated protein phosphorylation in tonic arterial and phasic gizzards smooth muscles. Unloaded chicken carotid artery rings (left) and gizzard strips (right) labelled with $[^{32}P]PO_4^{3-}$ were treated with 2 μM PDBu for the indicated time intervals and extracted as described in Materials and methods section. Equal amounts of protein extracts were separated by gradient SDS–PAGE, stained with Coomassie R250 (CB) and autoradiographed. Migration of the molecular weight standards (in kDa) is shown on the left including actin, myosin heavy chain (MyoHC) and caldesmon (CaD). In separate experiments the phosphorylated M_r = 27 kDa protein was identified as KRP by Western immunoblots and immunoprecipitation.

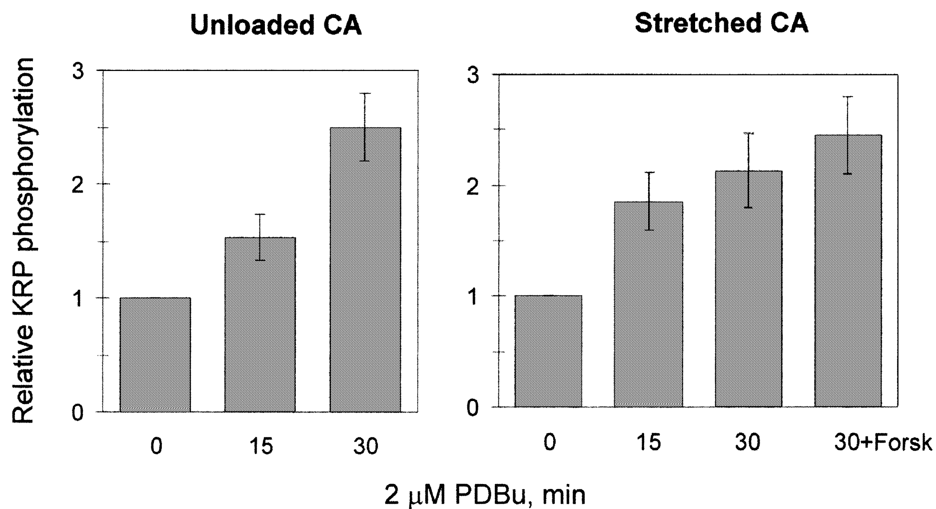

Fig. 3. Effect of PDBu and forskolin on KRP phosphorylation in stretched and unstretched carotid artery. Unloaded (left) or mechanically loaded (0.8 g, right) rings of carotid artery were stimulated for indicated times with 2 μM PDBu. The stretched rings were also treated for additional 15 min with 10 μM forskolin while PDBu was present (30 + Forsk). KRP was subsequently immunoprecipitated from tissue extracts and its phosphorylation level relative to unstimulated control ± SEM (n = 3) was quantified as described in Materials and methods.

1.5–2-fold. It should be pointed out that although PDBu activates PKC activity, PKC is probably not the kinase that phosphorylates KRP. In fact, KRP was found to be a poor substrate for PKC *in vitro* (Vorotnikov *et al.*, 1996; this report – see below). Activation of PKC can lead to downstream activation of other kinases. For

example, phorbol ester activation of PKC has been shown to lead to activation of the MAPK cascade in a mechanism where activation of c-RAF (the MAPK kinase kinase) occurs in a manner independent of Ras (Ueda *et al.*, 1996). A similar mechanism of phorbol ester activation of MAP kinases is also operative in both tonic (Adam *et al.*, 1992) and phasic (Gerthoffer *et al.*, 1996) smooth muscles. To demonstrate that PDBu stimulation does not lead to maximum phosphorylation of KRP, gizzard strips were also treated for 1 h with 0.5 μM okadaic acid, a Ser/Thr-phosphatase inhibitor. KRP phosphorylation was increased 3.7 ± 0.2-fold indicating that phosphorylation of at least 25–40% of the available sites was stimulated by PDBu.

Treatments with PDBu increased the level of KRP phosphorylation in both phasic and tonic muscles, while forskolin-induced relaxation of intact carotid artery was not apparently associated with a significant enhancement of KRP phosphorylation (Figure 3). These results suggest that PDBu stimulation followed by forskolin-induced KRP phosphorylation in avian carotid artery may differ from that observed in the rabbit ileum where it has been correlated with enhanced relaxation induced by cyclic nucleotides (Wu *et al.*, 1998). Therefore, we mapped the sites phosphorylated in KRP from chicken carotid artery and gizzard that had been stimulated by PDBu. To identify the major phosphopeptides and corresponding phosphorylation sites we first studied the phosphorylation of KRP *in vitro* and mapped the phosphopeptides for comparison to maps of the *in vivo* phosphorylated protein.

Phosphorylation of KRP by PKA and MAPK in vitro

Phosphorylation of KRP *in vitro* by PKA (Ito *et al.*, 1989) and MAPK (Vorotnikov *et al.*, 1996) has been previously described and some sites mapped from *in vivo* phosphorylation (MacDonald *et al.*, 2000). Because we wanted to compare maps of KRP phosphorylation from different chicken tissues, we used chicken KRP (produced in bacteria) as a standard. Recombinant KRP (recKRP) was phosphorylated to a stoichiometry of 0.8 mol of phosphate per mole of protein by either catalytic subunit of PKA, or recombinant GST-p44^{erk1} MAPK. Similar phosphorylation kinetics and stoichiometry were observed when purified gizzard KRP was used as a substrate, indicating that for these kinases, the presence of post-translational modifications (both phosphorylation and other modifications) in the tissue isolated KRP does not alter the phosphorylation profile.

Tryptic 2D phosphopeptide maps were then generated from the *in vitro* phosphorylated KRP. A single phosphopeptide 1 was observed on 2D map of KRP phosphorylated with PKA (Figure 4A) and two phosphopeptides 1 and 1′ were detected for KRP phosphorylated by recombinant MAPK (Figure 4B). These were purified by HPLC and analysed by mass spectrometry and microsequencing. A mass of 4370.4 Da and N-terminal sequence KAXGSSP… were determined for phosphopeptide 1 (X is the absence of serine derivative at this cycle of Edman degradation) confirming that Ser^{12} in the KRP peptide Lys^{10}-Lys^{48} is the

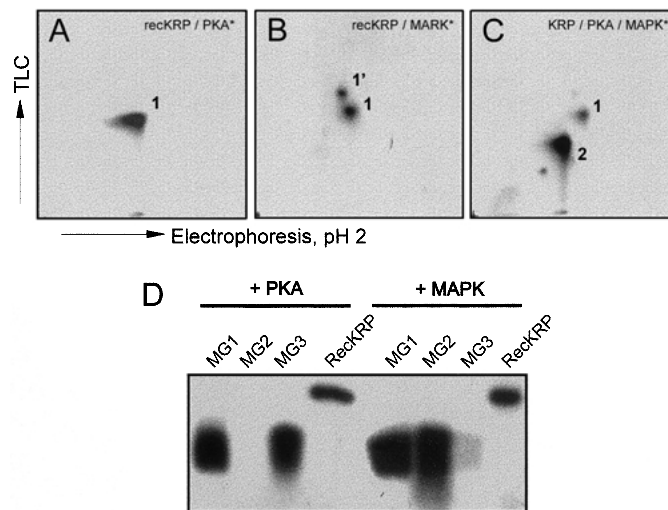

Fig. 4. Phosphopeptide mapping of KRP phosphorylated by PKA and MAPK *in vitro*. Bacterially expressed KRP (recKRP) was phosphorylated by PKA (A) or activated GST-MAP kinase (B) to stoichiometry of 0.8 mol of phosphate per mole of protein. Tissue purified KRP (C) was doubly phosphorylated to 1.5 mol Pi per mole with PKA and GST-MAP kinase. Phosphorylated proteins were resolved by SDS–PAGE and tryptic phosphopeptides were subjected to 2D phosphopeptide mapping on silica gel 60 plates as outlined in the Materials and methods section. Resulting autoradiograms are shown and identical phosphopeptides are correspondingly numbered following the co-mapping (co-maps are not shown). Arrows indicate the directions of electrophoresis at pH 2 and chromatography. Origins are at the left bottom corners. D: identification of PKA and MAPK phosphorylation sites. Hundred and twenty micromolar of synthetic peptides MG1–MG3 corresponding to Leu^{6}-Leu^{25} sequence of human KRP or 40 μM of recombinant chicken gizzard KRP were phosphorylated with PKA (+PKA) or GST-MAP kinase (+MAPK) in the presence of [γ-^{32}P]ATP and resolved by 25% SDS–PAGE. The autoradiograms are shown. Note that MG2 contains phospho-Ser^{12} and MG3 contains phospho-Ser^{18} while MG1 has no associated phosphate.

only PKA-phosphorylated site. The mass of phosphopeptide 1′ was about 130 Da smaller than 1 suggesting that it represents the phosphorylated Ala^{11}-Lys^{48} peptide of KRP consistent with the Lys^{10} cleavage site and Ser^{18} phosphorylation site by MAPK determined previously (Wu *et al.*, 1998; MacDonald *et al.*, 2000). Cleavage at Lys^{10} within the Arg^{9}-Lys^{10}-Ala^{11} sequence of KRP is disfavoured by phosphorylation of Ser^{12} by PKA, a common feature of trypsin specificity described previously (Boyle *et al.*, 1991b). Thus, both PKA and MAPK phosphorylate distinct, but single sites in KRP. Radioactive spot 2 (Figure 4) contains an apparent diphosphorylated peptide (Lys^{10}-Lys^{48}) of KRP phosphorylated *in vitro* with both PKA and MAPK (Figure 4C).

Although the mapping data are consistent with phosphorylation of Ser^{12} and Ser^{18} of KRP, to confirm the phosphorylation sites, synthetic peptides MG1–MG3 representing the phosphorylation site sequences Leu^{6}-Leu^{28} (LSGRKSS^{12}TGSPTS^{18}PLNAEKL) of human KRP were prepared (see Materials and methods). The peptides were phosphorylated by PKA (Figure 4D, left panel) or MAPK (Figure 4D, right panel) in the presence of [γ-^{32}P]ATP. The presence of phosphoserine at KRP at the Ser^{12} site in the MG2 peptide or at the Ser^{18} site in the MG3 peptide completely prevented their phosphorylation by PKA and MAPK, respectively. Taken together, our results indicate that PKA and MAPK independently phosphorylate Ser^{12} and Ser^{18} in KRP and thus our assignments of the phosphopeptide spots (Figures 4 and 5) to various mono and diphosphorylated peptides is valid.

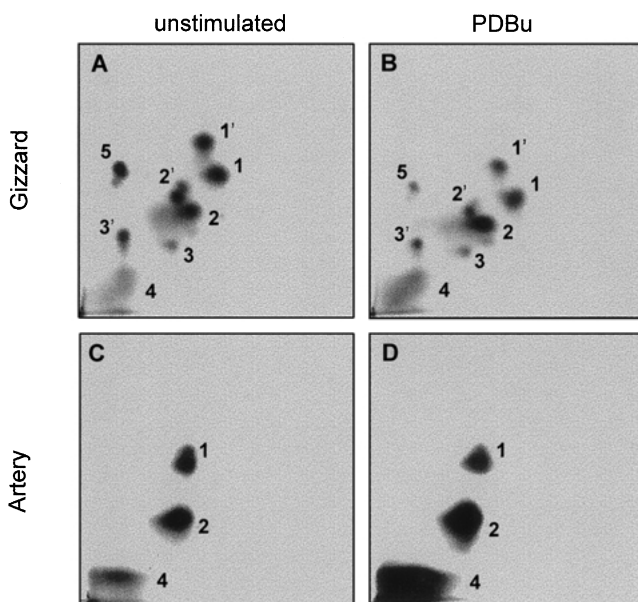

Fig. 5. Phosphopeptide mapping of KRP phosphorylated in gizzard and arterial chicken smooth muscle. Gizzard strips (A, B) and carotid artery rings (C, D) were labelled with [^{32}P]PO_4 and incubated for 30 min with either vehicle (A, C) or 2 μM PDBu (B, D). KRP was routinely immunoprecipitated and subjected to 2D tryptic phosphopeptide mapping as described in the legend to Figure 4.

Phosphopeptide mapping in situ *phosphorylated KRP*

To investigate the array of KRP sites that undergo PDBu-simulated phosphorylation in phasic vs. tonic muscles and whether there are site-specific differences, KRP was immunoprecipitated from ^{32}P-labelled muscle strips and subjected to 2D phosphopeptide mapping. Multiple phosphopeptides were obtained and numbered according to their mobility (Figure 5). Stimulation of gizzard (Figure 5A and B) or arterial (Figure 5C and D) strips with PDBu did not result in the appearance of additional phosphopeptides, however, in gizzard, PDBu caused a relative increase in the intensities of phosphopeptides 2 and 4 (Figure 5B) in line with an increase of KRP phosphorylation level by about 1.5-fold in this experiment. A similar distribution of radioactivity between the phosphopeptides was detected in carotid artery where the total phosphorylation of KRP was stimulated at least 2-fold consistent with our observations in Figure 3. Particularly striking is the increase in intensity of the phosphorylated peptides in spot 4.

Regardless of PDBu-dependent changes, two differences between gizzard and artery in KRP phosphorylation were noted (cf. Figure 5A–D). First, several minor phosphopeptides 2′, 3, 3′ and 5 were characteristic only for gizzard KRP and absent in the arterial protein. Secondly, as noted above, more radioactivity was associated with the spot associated with phosphopeptide 4 of arterial KRP than with its gizzard counterpart. Because of the diffuse and electronegative nature of spot 4, we tentatively assigned this spot to one or more multiple phosphorylated peptides that may or may not be related to the amino-terminal phosphorylation sites. This observation suggested that KRP was a substrate for other, as yet unidentified, protein kinases.

Phosphorylation of KRP by other protien kinases in vitro

To determine if KRP was a substrate for other kinases, several enzymes were assayed for their ability to phosphorylate KRP *in vitro*. Individually, Ca^{2+}-calmodulin-dependent protein kinase II, the $\beta1/\beta2$ or α isoforms of PKC, CK2 and GSK3 incorporated no more than 0.05–0.15 mol of phosphate per mole of the protein even after 24 h of incubation at 30°C. However, a higher stoichiometry of 0.4 mol/mol was obtained with KRP phosphorylated by casein kinase I. However, the maps (not shown) of casein kinase I generated phosphopeptides of KRP were distinct from any of the spots representing phosphopeptides obtained from KRP *in vivo*. Therefore, the sites represented in phosphopeptide 4 (Figure 5) from the KRP phosphorylation *in vivo* are likely the result of the activity of multiple kinases that may or may not include casein kinase I. Although, phosphorylation by CK2 and GSK3 alone was poor, it was also likely that these acidotrophic kinases required prephosphorylation by other enzymes such as PKA and/or MAPK.

Ordered phosphorylation of KRP in vitro

One possible explanation for the presence of phosphopeptide 4 in the maps of *in vivo* phosphorylated KRP (Figure 5) is that it may be the result of conditional phosphorylation by acidotropic kinases such as casein kinases I and CK2, or GSK3 following the primary phosphorylation by PKA and MAPK. To test this hypothesis, KRP was incubated with either PKA or GST-MAP kinase or vehicle in the presence of unlabelled ATP and then analysed for the phosphorylation-dependent shift of mobility during isoelectrofocusing (Vorotnikov *et al.*, 1996). In this analysis, apparently essentially all of the KRP was phosphorylated by PKA, but only 50% phosphorylated by MAPK. These preparations were desalted to remove residual ATP and a second phosphorylation using acidotropic kinases was performed in the presence of [γ-^{32}P]ATP. Phosphorylation by casein kinase I did not depend on the modification of either MAPK (not shown) or PKA (Figure 6A) sites. Phosphorylation of KRP by CK2 was only slightly enhanced by prior phosphorylation of KRP with MAPK (not shown). However, the low stoichiometry and slow rate of phosphate incorporation achievable under favourable *in vitro* conditions (including the presence of polylysine) indicates that CK2 is probably not a relevant KRP kinase.

In contrast, phosphorylation of KRP by GSK3 was strongly potentiated by primary phosphorylation with MAPK (Figure 6A). Quantitatively, 0.5 mol of [^{32}P]-phosphate was incorporated per mole of KRP when 30 μg of tissue purified protein, 50% prephosphorylated by GST-MAP kinase, was incubated with 0.3 units of GSK3 for 2 h at 30°C. 2D mapping revealed accumulation of major phosphopeptides 2 and 2′ (Figure 6B) correlated with diphosphorylated peptide fragments Lys^{10}-Lys^{48} and Ala^{11}-Lys^{48} of KRP, respectively. Also present in this map (Figure 6B) is residual monophosphorylated peptide 1 consistent with incomplete phosphorylation of KRP by either MAPK or GSK3.

Phosphopeptide 4 was detected on a 2D map when KRP prephosphorylated by GST-MAP kinase was then sequentially phosphorylated by GSK3 in the presence of unlabelled ATP, followed by PKA in the presence of [γ-^{32}P]ATP (Figure 6C). Recall that about half of the KRP preparation was not phosphorylated by MAPK, and therefore, when phosphorylated by PKA, only monophosphopeptide 1 is generated, while the remaining KRP underwent sequential phosphorylation by all of the kinases resulting in multiple phosphorylated peptide 4. Thus, GSK3 phosphorylates KRP at a site(s) different from PKA and MAPK and gives a peptide map consistent with phosphorylation within the same amino-terminal region as PKA and MAPK. Both Ser^{14} and Ser^{15} of avian KRP fulfill the consensus requirements for GSK3 assuming that Ser^{18} is phosphorylated by MAPK. The former is located four residues N-terminal from Ser^{18} (n-4 position), an ideal site for GSK3 recognition (Roach, 1991). The latter is in an n−3

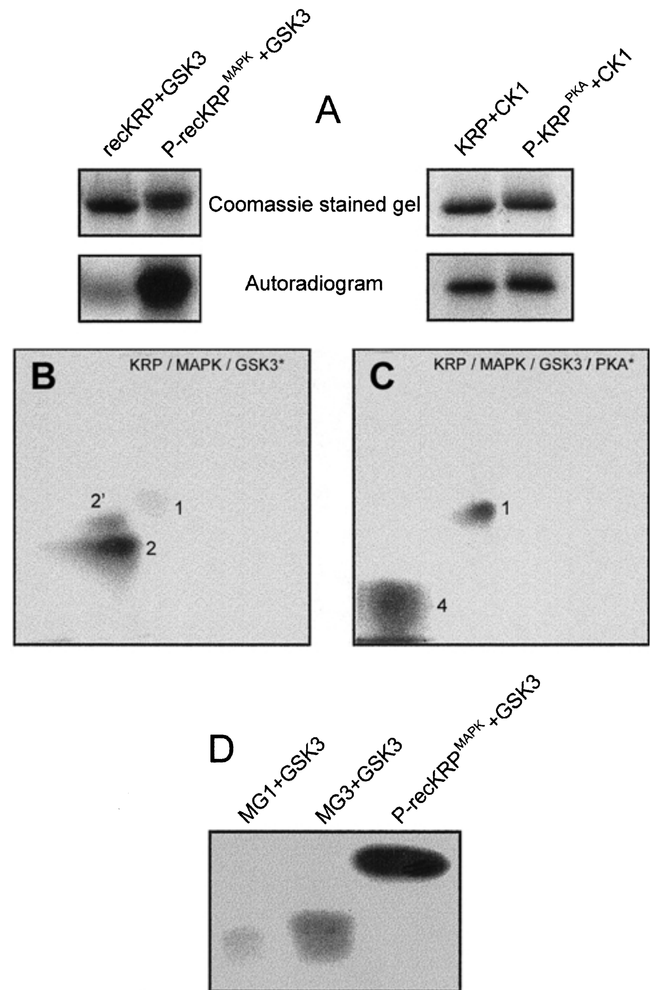

Fig. 6. Ordered phosphorylation of KRP *in vitro* by MAPK and GSK3 and mapping of multiple phosphorylated peptides. (A) KRP phosphorylation by GSK3 requires prior modification of the MAPK phosphorylation site, while phosphorylation of KRP with casein kinase I is independent on the phosphorylation state of the PKA site. Left panels: unphosphorylated recKRP and that phosphorylated with GST-MAP kinase using unlabelled ATP (P-recKRP^{mapk}) were incubated with GSK3 in the presence of [γ-^{32}P]ATP and analysed by SDS–PAGE and autoradiography. In control experiments no residual MAPK activity was found in P-recKRP^{mapk} preparation. Right panels: similarly to (A), tissue purified KRP and that phosphorylated with PKA using unlabelled ATP (P-KRP^{pkA}) were incubated with casein kinase I in the presence of [γ-^{32}P]ATP and analysed as above. (B) and (C) Autoradiograms of 2D tryptic phosphopeptide maps of tissue purified KRP diphosphorylated with GST-MAP kinase and GSK3 and tissue purified KRP sequentially phosphorylated with GST-MAP kinase (to 50%, see the text), GSK3 and PKA, respectively. The directions of electrophoresis and chromatography, and origins are as in Figure 4. Asterisks denote protein kinases with which phosphorylation was carried out in the presence of [γ-^{32}P]ATP. Due to incomplete phosphorylation of KRP with GST-MAP kinase at the first step, the final phosphorylation with PKA resulted in the appearance of both monophosphorylated and triple phosphorylated peptides 1 and 4, respectively. (D) Phosphorylation of synthetic peptides of human KRP by GSK3. Phosphorylation of the synthetic Leu^{6}-Leu^{25} peptide (MG1) and that containing phospho-Ser^{18} (MG3) was compared to phosphorylation of KRP fully prephosphorylated by GST-MAP kinase (P-recKRP^{mapk}). Phosphorylated polypeptides were resolved by 25% SDS–PAGE and the resulting autoradiogram of the gel is shown.

position followed by a proline, a sequence recognized by GSK3 in amyloid precursor protein and c-Jun (Boyle *et al*., 1991a; Aplin *et al*., 1996). To evaluate which of these serines may be a preferred site for GSK3, we compared *in vitro* phosphorylation of the synthetic peptide analogs (MG1–MG3) of human KRP that have a Ser corresponding to Ser^{15} with that of avian KRP which has both Ser^{14} and Ser^{15} (Figure 6D). Although KRP that has been prephosphorylated with MAPK is an excellent substrate for GSK3, the peptide, MG3, containing phospho-Ser^{18} (the MAPK site) was phosphorylated poorly, but better than the unmodified MG1 peptide (Figure 6D). Pilot experiments have also demonstrated that GSK3 phosphorylates the recombinant human and avian full-length proteins that have been prephosphorylated at Ser^{18} (data not shown). Taken together, these results suggest that Ser^{15}, common to both the avian and mammalian KRP, is a preferred GSK3 phosphorylation site. Although GSK3 is a ubiquitous kinase and phosphorylates other substrates such as tau protein (Moreno *et al*., 1996), dystrophin (Michalak *et al*., 1996) and insulin receptor substrate 1 (Eldar-Finkelman and Krebs, 1997), it may not be the acidotropic kinase that phosphorylates KRP. However, the available data suggest that a kinase with substrate selectivity properties similar to GSK3 phosphorylates KRP *in vivo*.

In summary, many of the phosphopeptides derived from KRP phosphorylated *in vivo* can be reproduced by phosphorylation *in vitro*. The phosphorylation sites are clustered in the amino-terminal region of KRP that appears to exist in both tonic and phasic avian smooth muscle in multiple phosphorylated forms (Figures 4 and 5). In resting gizzard muscle KRP is predominantly mono- and diphosphorylated. However, the presence of phosphopeptides 1′, 2′ and 3′ (Figure 5A) are indicative that the PKA site (Ser^{12}) is not completely phosphophorylated. In contrast, the absence of phosphopeptides 1′ and 2′ in either stimulated or unstimulated arterial KRP suggests almost complete phosphorylation of the PKA site. This is in agreement with a larger fraction of KRP being phosphorylated in resting arterial muscle (Figure 5C) and is consistent with the finding that the level of KRP phosphorylation was not greatly enhanced by further forskolin treatment (Figure 3).

Discussion

Several lines of evidence are consistent with a model of that KRP being involved in regulation of smooth muscle contraction. First, KRP is genetically related to MLCK, the key Ca^{2+}-calmodulin-dependent activator of smooth muscle myosin. Second, studies of KRP *in vitro* demonstrate its ability to regulate both smooth muscle myosin structure and myosin phosphorylation by MLCK (Shirinsky *et al*., 1993). Third, KRP is expressed at high levels in phasic smooth muscles which normally undergo deep relaxation, but at lower levels in tonic smooth muscles that always display intrinsic tone *in vivo* (Herring and Smith, 1996; Wu *et al*., 1998; and this work). Finally, KRP has been demonstrated to promote relaxation of submaximally contracted skinned smooth muscle fibres (Sobieszek *et al*., 1998; Wu *et al*., 1998).

The molecular mechanisms of KRP action remain unclear. One suggestion based on *in vitro* studies is that KRP increases the K_m of MLCK for the regulatory light chain (Silver *et al*., 1997) and thus indirectly antagonizes myosin phosphorylation. Based on the assumption of effective infusion into skinned muscle fibres, Wu *et al*. (1998) found no influence of KRP on the rate of endogenous myosin regulatory light chain thiophosphorylation, which argues against competition of KRP with MLCK for myosin binding *in vivo* (Vorotnikov, 1997). As an alternative mechanism for KRP-dependent relaxation, activation of MLC phosphatase by KRP has been proposed (Somlyo *et al*., 1998; Wu *et al*., 1998). In this mechanism, KRP either directly or indirectly affects myosin phosphatase activity. The observation that KRP is a major phosphoprotein in unstimulated smooth muscle tissue (Vorotnikov *et al*., 1996; this work), raised the possibility that phosphorylation of KRP may modulate its ability to influence phosphatase activity. Indeed, the phosphorylation of MAPK and PKA/PKG preferred sites in KRP have been recently demonstrated *in situ* (MacDonald *et al*., 2000). Initial experiments suggested that phosphorylation of KRP potentiates its relaxing activity (Wu *et al*., 1998) and thus may participate in cyclic GMP-mediated relaxation in smooth muscle (Somlyo *et al*., 1998). However, later studies using phosphorylation site mutants of KRP indicated that phosphorylation was not required for relaxing activity, and the addition of MAPK to permeabilized tissue did not potentiate relaxation (MacDonald *et al*., 2000).

To investigate the importance of KRP phosphorylation in intact smooth muscle we challenged aortic (tonic) and gizzard (phasic) smooth muscle with PDBu. This elicited significant contraction only in the former tissue (Figure 1). These results are in agreement with earlier studies where phorbol esters caused tonic contraction in swine carotid artery (Singer, 1990), rabbit (Singer *et al*., 1989; Miura *et al*., 1997) and rat aortas (Ozaki *et al*., 1990; Itoh *et al*., 1993), but did not produce contraction in phasic canine colonic muscle (Sato *et al*., 1994), guinea-pig taenia caeci (Mitsui and Karaki, 1993) and rat anococcygeus muscle (Shimizu *et al*., 1995). Overall, the results indicate that this difference is a general feature for the effect of PDBu among tonic and smooth muscle. In contrast to the different contractile responses, PDBu increased phosphorylation of KRP in both tissues (Figure 2), and similar phosphopeptide patterns of KRP were obtained (Figure 5). Although PDBu has no direct contractile effect on phasic smooth muscles, it has been earlier shown to affect Ca^{2+}-sensitivity of contractile responses to other stimuli. For example it potentiated both KCl-induced (Sato *et al*., 1994; Gerthoffer *et al*., 1996) and agonist-induced (Sato *et al*., 1994) contraction in colon smooth muscle, suggesting that the

responsive PKC isoforms are present and functional in phasic tissues. Thus, PDBu-induced phosphorylation of KRP may be mediated by PKC activation and downstream pathways common to phasic and tonic smooth muscles, rather than linked to Ca^{2+}-sensitizing contractile effects of PDBu.

Subsequent addition of forskolin to the PDBu-precontracted arterial strips produced their complete relaxation but this event was not associated with large changes in KRP phosphorylation (Figure 3). Moreover, the absence of specific phosphopeptides 1′ and 2′ on the maps of arterial KRP suggests a significant level of phosphorylation at Ser^{12} of KRP even before forskolin treatment (Figure 5). Therefore, PDBu-induced phosphorylation of KRP occurs during contraction and this phosphorylation is not apparently correlated with the potential of subsequent relaxation. In contrast, other investigators found an increase in phosphorylation of Ser^{12} in permeabilized phasic rabbit ileum or portal vein and associated this with cyclic GMP-mediated relaxation (Wu *et al.*, 1998; MacDonald *et al.*, 2000). Consistent with this published data, phosphopeptide mapping of phasic (gizzard) muscle KRP (Figure 4A and B) revealed a lower level of KRP phosphorylation at Ser^{12}, which could be augmented upon PKA/PKG activation. However, our study on intact tonic smooth muscle (carotid artery) suggests that its forskolin-induced relaxation is not associated with specific phosphorylation of KRP. However, further analysis will be necessary to validate the role of KRP in cyclic nucleotide-mediated relaxation of other muscle types, especially considering that we have discovered multiple phosphorylated KRP isoforms in both tonic and phasic muscles.

Other sites of KRP also undergo phosphorylation independent of cyclic nucleotide-activated kinases. The treatment of gizzard muscle with okadaic acid increased phosphorylation of KRP about 4-fold, suggesting a simultaneous multiple sited phosphorylation. Evidence is now provided that PDBu stimulates phosphorylation of KRP at residues other than Ser^{12} in intact smooth muscle. This is consistent with direct triggering of PKC and following downstream activation of MAPK, which phosphorylates Ser^{18} of KRP. This may in turn initiate sequential phosphorylation by an acidotropic protein kinase such as GSK3, which phosphorylates $Ser^{14/15}$ (Figure 6). Noteworthy, nearly all of these phosphorylatable residues are clustered within the short amino acid stretch in the N-terminus of KRP (Ser^{12} to Ser^{18}). The conservation of the same sequence in MLCK isoforms (Watterson *et al.*, 1999) suggests that phosphorylation of some of these sites may also have an effect on smooth muscle MLCK activity. For instance, phosphorylation of site B in MLCK (equivalent to Ser^{12} of KRP) has been recently proposed to increase the affinity of MLCK for myosin without an effect on its catalytic activity (Samizo *et al.*, 1999), and phosphorylation of MLCK by MAPK (which may include Ser^{18} of the KRP sequence), enhances MLCK activity by changing the V_{max} (Morrison *et al.*, 1996).

In summary, we describe multiple and ordered phosphorylation of KRP *in vitro* and in smooth muscle achieved by protein kinases activated via distinct signalling cascades. We observed no correlation of KRP phosphorylation and contractile activity of tonic smooth muscle and the functional meaning of endogenous KRP phosphorylation, especially at multiple sites, awaits further elucidation.

While this manuscript was under review, Walker *et al.*, published a paper on the effects of selected phosphorylation site mutations on KRP relaxing activity. Consistent with our results, the mutant, S18D-KRP, which may mimic phosphorylation of Ser^{18} by MAPK, does not exhibit enhanced relaxation through phosphorylation induced by cyclic nucleotides. In fact, the relaxation effect of this mutant appears to be less than wild-type KRP. Thus, KRP relaxation effects appear to be dependent upon the external contractile stimulus that modulates its phosphorylation state at multiple sites.

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