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Inhibition of contraction and myosin light chain phosphorylation in guinea-pig smooth muscle by p21-activated kinase 1

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The p21-activated protein kinases (PAKs) have been implicated in cytoskeletal rearrangements and modulation of non-muscle contractility. Little, however, is known about the role of the PAK family members in smooth muscle contraction. Therefore, we investigated the effect of the predominant isoform in vascular smooth muscle cells, PAK1, on contraction and phosphorylation of the regulatory light chains of myosin (r-MLC) in Triton-skinned guinea-pig smooth muscle. We also investigated which of the three putative substrates at the contractile apparatus – MLCK, caldesmon or r-MLC – is phosphorylated by PAK1 in smooth muscle tissue. Incubation of Triton-skinned carotid artery and taenia coli from guinea-pig with an active mutant of PAK1 in relaxing solution for 30–60 min resulted in inhibition of submaximal force by about 50 %. The mechanism of inhibition of force was studied in the Triton-skinned taenia coli. In this preparation, inhibition of force was associated with a respective inhibition of r-MLC phosphorylation. In the presence of the myosin phosphatase inhibitor, microcystin-LR (10 μM), the rate of contraction and r-MLC phosphorylation elicited at pCa 6.79 were both decreased. Because under these conditions the rate of r-MLC phosphorylation is solely dependent on MLCK activity, this result suggests that the inhibitory effect of PAK1 on steady-state force and r-MLC phosphorylation is due to inhibition of MLCK. In line with this, we found that MLCK was significantly phosphorylated by PAK1 while there was very little ^{32}P incorporation into caldesmon. PAK1 phosphorylated isolated r-MLC but not those in the skinned fibres or in purified smooth muscle myosin II. In conclusion, these results suggest that PAK1 attenuates contraction of skinned smooth muscle by phosphorylating and inhibiting MLCK.

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The family of p21-activated protein kinases (PAKs) has been implicated in various signal transduction pathways leading to cytoskeletal rearrangements in both muscle and non-muscle systems. PAKs are serine/threonine kinases with homologues in different lower eukaryotes like yeast (STE20, Leberer *et al.* 1992), *Drosophila* (DPAK, Harden *et al.* 1996) and *Dictyostelium* (myosin I heavy chain kinase, Lee *et al.* 1996). PAKs consist of two functional domains: an N-terminal regulatory domain containing the Ccd42/Rac-binding site and proline-rich motifs that are binding sites for SH3-domains and a C-terminal catalytic domain. At least six mammalian PAK isoforms (Manser *et al.* 1994, 1995; Teo *et al.* 1995; Abo *et al.* 1998; Dan *et al.* 2002; Lee *et al.* 2002) have been identified showing different tissue-specific expressions. PAK1, PAK2 and PAK3 appear to be expressed in differentiated tracheal smooth muscle (Dechert *et al.* 2001). Only PAK1 and PAK3 appear to be expressed in vascular smooth muscle with PAK1 as the predominant isoform (Schmitz *et al.* 1998). The three most closely related mammalian PAK isoforms, PAK1, PAK2 and PAK3, show 70 % identity in overall amino acid sequence and over 90 % identity in the kinase domain. Binding of Cdc42/Rac in the active GTP-bound form leads to autophosphorylation of both the regulatory and catalytic domain of PAK and kinase activation (Manser *et al.* 1997; Chong *et al.* 2001).

The p21-activated protein kinases may affect smooth muscle contraction through phosphorylation of one or more regulatory proteins of the contractile apparatus: myosin light chain kinase (MLCK; Sanders *et al.* 1999; Goeckeler *et al.* 2000), the regulatory light chains of myosin (r-MLC; Chew *et al.* 1998), myosin light chain phosphatase (MLCP; Takizawa *et al.* 2002), and caldesmon (Van Eyk *et al.* 1998). MLCK is a highly specific serine/threonine kinase that is activated by Ca^{2+}-calmodulin

complexes when the intracellular Ca^{2+} concentration rises due to electrical or chemical stimulaton of smooth muscle (for review see Arner & Pfitzer, 1999). Activated MLCK phosphorylates Ser19 on r-MLC, which switches on the actin-activated MgATPase activity of myosin II, crossbridge cycling and contraction. The reverse reaction is catalysed by MLCP, a type 1 phosphatase, that dephosphorylates r-MLC and leads to relaxation of smooth muscle. The level of r-MLC phosphorylation is determined by the ratio of the catalytic activities of these two enyzmes, which can both be modulated in a Ca^{2+}-independent manner by several intracellular signalling cascades (see Pfitzer, 2001, for review). Caldesmon is a thin filament-associated protein that inhibits the actin-activated MgATPase activity of myosin II (for review see Arner & Pfitzer, 1999) and based on skinned fibre studies it was suggested that caldesmon is involved in the regulation of tension development in smooth muscle (Katsuyama *et al.* 1992; Pfitzer *et al.* 1993; Malmqvist *et al.* 1996). However, the precise role of caldesmon in regulating smooth muscle contraction is not yet clear.

Phosphorylation of MLCK, r-MLC and caldesmon by members of the PAK family has been reported to have diverse and, sometimes, contradictory effects on cellular responses. Transfection of baby hamster kidney (BHK-21) cells with a constitutively active PAK1 impaired cell spreading most likely due to an inhibitory phosphorylation of MLCK and, hence, a decrease in r-MLC phosphorylation (Sanders *et al.* 1999). A loss of stress fibres was observed after expression of constitutively active PAK1 in HeLa cells and fibroblasts (Manser *et al.* 1997). The microinjection of PAK1 in quiescent Swiss 3T3 cells induced the formation of polarized filopodia (Sells *et al.* 1997) and the membrane targeting of PAK1 in PC12 cells led to neurite outgrowth that is inhibited by expression of dominant-negative mutants of Cdc42 and Rac (Daniels *et al.* 1998). PAK2 is reported to both attenuate (Goeckeler *et al.* 2000) and stimulate (Chew *et al.* 1998) the contraction of permeabilized endothelial cell monolayers by phosphorylating and inhibiting MLCK (Goeckeler *et al.* 2000) or phosphorylating and activating myosin II (Chew *et al.* 1998), respectively. In contrast, incubation of permeabilized guinea-pig taenia coli fibres with PAK3 induced a Ca^{2+}-independent contraction that was associated with phosphorylation of caldesmon and desmin but not of myosin II (Van Eyk *et al.* 1998). It is not known whether these diverging results are due to the different cell types and/or different isoenzymes of PAK.

PAK1 is activated by angiotensin II in vascular smooth muscle cells (Schmitz *et al.* 1998). However, the effect of PAK1 on contraction of smooth muscle is not known. Depending on which substrate of the contractile apparatus is phosphorylated it could activate or inhibit force. We, therefore, investigated which of the three substrates of the contractile apparatus was phosphorylated by PAK1 and how this affected smooth muscle contraction. The majority of experiments were performed in Triton-skinned taenia coli smooth muscle because the effect of PAK3 on smooth muscle contraction was investigated in this preparation (Van Eyk *et al.* 1998). Furthermore, Triton-permeabilized smooth muscle is more suitable for studying direct effects of PAK1 on the contractile apparatus and its associated regulatory proteins (Pfitzer & Boels, 1991). We show here that MLCK but not caldesmon or r-MLC is phosphorylated by PAK1. This was associated with a decrease in the Ca^{2+} sensitivity of force development and steady-state r-MLC phosphorylation. The rate of r-MLC phosphorylation under conditions where MLCP is inhibited by the phosphatase inhibitor microcystin-LR was also decreased. PAK1 also inhibited submaximal contraction in Triton-skinned carotid arteries.

METHODS

Tissue preparation and force measurements

Guinea-pigs of either sex (Dunkin Harley) were anaesthetized with halothane and killed by exsanguination with procedures approved by the Institutional Animal Care and Use Committee. The taenia coli and carotid artery were rapidly removed and fixed at their *in situ* length with stainless-steel pins in a Sylgard dish. The tissue was heavily permeabilized by chemical skinning with 1 % Triton X-100 for 4 h on ice, as described in Albrecht *et al.* (1997). The skinned fibre bundles were stored at −20 °C in relaxing solution (see below) containing 50 % (v/v) glycerol and used within 1 week. For force measurements thin fibre bundles (150–250 μm in diameter) or small rings (width ~500 μm) were mounted in a myograph using a KG7 (Scientific Instruments, Germany) force transducer and allowed to equilibrate in relaxing solution for 15–20 min. Contraction was elicited by increasing Ca^{2+} concentrations. All experiments were carried out at room temperature (21–24 °C) unless stated otherwise. Force was normalized to the first maximal contraction (F_{max}) elicited at pCa 4.3 and 1 μM calmodulin (CaM), if not stated otherwise. Following the initial contraction–relaxation cycle, fibres were incubated in relaxing solution containing 10 nM okadaic acid and either PAK1 (72 μg ml^{-1}) carrying an activating mutation (T422E) or the appropriate amount of the dialysis buffer for 60 min. Recombinant PAK proteins are not fully active, even if they are carrying activating mutations and even if they are overexpressed in cultured cells containing endogenous Rac and Cdc42 (Manser *et al.* 1997). Moreover, the purified proteins are very sensitive to phosphatases (E. Manser, unpublished observations) and members of the PAK family can complex with type 2A phosphatases (Westphal *et al.* 1999). These points might explain why we detect low and variable effects in the absence of okadaic acid. Control experiments showed that okadaic acid, which at this low concentration has been reported to only inhibit type 2A phosphatases (Takai *et al.* 1989), had no significant effect on steady-state force and r-MLC phosphorylation (cf. Results).

Solutions

The relaxing solution for force measurements consisted of (mM): imidazole 20, EGTA 4, $MgCl_2$ 10, ATP 7.5, DTT 2, NaN_3 1, creatine phosphate 10, leupeptin 1 μM, calmodulin 0.5 or 1 μM, creatine kinase 140 U ml^{-1}. The pH was adjusted to 6.7 at room

temperature with KOH. The contracting solution contained in addition 4 mM $CaCl_2$. Alterations in the free Ca^{2+} concentration were obtained by mixing relaxing and contracting solution in the appropriate ratio and free [Ca^{2+}] was calculated as in Andrews *et al.* (1991). Rigor solution consisted of (mM): imidazole 20, EGTA 4, $MgCl_2$ 3, KCl 50, NaN_3 1, DTT 2, leupeptin 1 μM, pH 6.7 at room temperature.

Protein preparations

The GST-PAK1 used in these experiments was cloned, expressed and purified as described previously (Manser *et al.* 1997). In brief, the construct encoded rat α-PAK cDNA (this differs from human PAK1, which has one additional aspartic acid residue at position 180 in the polyacidic stretch). The autophosphorylation site in the activation loop of the catalytic domain of the enzyme was mutated (T422E) to increase the basal activity of the purified kinase. The cDNA was inserted in a pGEX-vector and the GST-fusion protein was expressed in *E. coli* BL21 strain. Purification followed the standard protocol (Pharmacia): elution from glutathione-Sepharose was with 10 mM glutathione in purification buffer (PBS containing 50 mM Tris-HCl pH 7.8, 0.5 mM $MgCl_2$). The purified protein (1.8 mg ml^{-1}) was dialysed into the following buffer (mM): KCl 100, imidazole 10 (pH 7.0), DTT 1. Calmodulin was purified from bovine testicle by using a modification of the procedure of Gopalakrishna & Anderson (1982). Caldesmon purified from chicken gizzard according to Chalovich *et al.* (1987) was used for immunization of rabbits (SeqLab, Göttingen, Germany). The antibodies were precipitated with 41 % ammonium sulphate and purified by affinity chromatography on a BrCN-Sepharose 4B column (Amersham Biosciences, UK) using 0.1 M glycin, pH 1.8, as elution buffer.

Quantification of myosin light chain phosphorylation

For determination of r-MLC phosphorylation, thin fibre bundles were mounted isometrically on loops of stainless-steel holders. At the desired time points the tissue was immersed in ice-cold 15 % trichloroacetic acid, 5 % sodium pyrophosphate for 10 min. The muscle strips were homogenized in the following buffer: urea 9.2 M, Tris-HCl (pH 7.5) 0.01 M, DTT 0.01 M, ampholine (pH 4.5–5.4) 3 %, bromophenol blue 0.0001 %. The proteins of the lysates were separated first by isoelectric focusing (pH gradient 4.5–5.4) followed by 15 % sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) as described previously (Lucius *et al.* 1998) using a mini-gel system (Biometra, Göttingen, Germany). The relative amounts of phosphorylated and non-phosphorylated r-MLC were determined by scanning the silver-stained gels (Bio-Rad) using a desktop scanner (GT-9600, Epson) and Phoretix software (Biostep, Jahnsdorf, Germany).

Protein phosphorylation experiments

For determination of phosphorylation of MLCK, caldesmon fibre bundles (6–7 or 2–3, respectively; diameter 150–250 μm, length 5–8 mm) of guinea-pig taenia coli were incubated in (mM): imidazole 20, EGTA 4, $MgCl_2$ 5.5, [γ^{32}P]-ATP 3 (specific activity 250 μCi (1.5 μmol)$^{-1}$), DTT 2, NaN_3 1, BSA 0.1 %, okadaic acid 10 nM, and GST-PAK1 72 μg ml^{-1} or an equal amount of dialysate for 1 h. The phosphorylation reaction was terminated by rapidly freezing the fibres in liquid nitrogen. The fibres were homogenized in 500 μl of the following buffer (mM): sodium pyrophosphate 50, NaF 100, NaCl 300, sodium molybdate 10, EGTA 10, EDTA 2, Na_2PO_4 25, and 10 nM okadaic acid and 1 % NP-40, pH 7.4. After addition of 7.2 μg of an affinity purified antibody to MLCK (de Lanerolle *et al.* 1991) the homogenates were incubated for 2 h at 4 °C on a mixing device. Thereafter 100 μl protein A-sepharose (Amersham Pharmacia, 50 % solution) were added and incubated for 1.5 h at 4 °C with gentle mixing. The beads were washed twice in homogenization buffer, twice in 1 M NaCl, 20 mM Tris, pH 7.5, and twice in 5 mM Tris, pH 7.5. With each solution change the beads were washed by gently inverting the tube back and forth for 1–2 min, pelleted in a microfuge and the supernatant removed. All procedures were carried out at 4 °C. For elution of the bound protein the beads were boiled in 150 μl of 0.25 % SDS and 50 mM DTT. This step was repeated two times and the pooled eluants were lyophilized. For electrophoresis the protein was resuspended in 10 % glycerol, bromphenolblue, 100 mM Tris, pH 6.7, and resolved on 7.5–20 % gradient gel. The gels were stained with Biosafe Coomassie (Biorad), dried on filter paper and exposed to X-ray films (Biomax, Kodak). ^{32}P incorporation was quantified by densitometrical scanning of the X-ray films and evaluated by using Phoretix software (Biostep).

For determining caldesmon phosphorylation, MLCK was first immunoprecipitated as described above, except that the amount of antibody was increased to 10.8 μg. The beads with the bound MLCK were removed and 41 μg of affinity-purified α-caldesmon antibody was added to the supernatant. Caldesmon was precipitated in the same way as MLCK, except that the beads were not washed with homogenization buffer. Eluting and processing of caldesmon for electrophoresis was carried out as described above.

For determining r-MLC phosphorylation *in vitro* myosin II, purified from bovine tracheal smooth muscle (6 μg), or purified bacterially expressed rat aorta smooth muscle r-MLC (2 μg), was incubated for 60 min at 30 °C with either PAK1 (0.5 μg) or purified chicken gizzard MLCK (50 ng) in (mM): $MgCl_2$ 10, DTT 2, [γ^{32}P]ATP 0.2 (specific activity ~2000 c.p.m. pmol^{-1}), Tris-HCl (pH 7.5) 20, $CaCl_2$ (0.5 mM) and calmodulin (100 nM) were also added to the MLCK assays. The reactions were terminated by boiling in SDS sample buffer and analysed by SDS-PAGE and autoradiography.

Determination of PAK content in taenia coli and Western blot analysis

Intact and Triton-skinned fibres taken immediately after skinning were homogenized in Laemmli buffer containing tris-(hydroxymethyl)-aminomethane (Tris)-HCl 50 mM, pH 6.8, urea 4.0 M, sodium dodecylsulphate (SDS) 1 % (w/v), DTE 20 mM. Proteins were separated with 10 % SDS-PAGE. Equal amounts of protein were loaded on each lane. The protein concentration was determined using the method of Bradford (1976) with bovine serum albumin (BSA) as standard. SDS-PAGE, transfer of the proteins to nitrocellulose and blocking of the membranes were carried out as in Pfitzer *et al.* (2001). PAK1 was detected with an α-PAK antibody (C-19, rabbit polyclonal, Santa Cruz Biotechnology, Inc.; dilution 1:500) that is partially cross-reactive with β-PAK and γ-PAK. MLCK was detected using the anti-MLCK clone K36 (mouse monoclonal) from Sigma (dilution 1:10 000). The anti-caldesmon IgG was a gift from Dr W. Lehmann (Boston University) and was used in a dilution of 1:2000. The affinity-purified caldesmon antibody was used in a dilution of 1:1000. All secondary antibodies were obtained from Jackson ImmunoResearch (Dianova, Hamburg, Germany). Immunoreactive protein bands were detected with enhanced chemiluminescence (ECL, Amersham) and quantified by densitometrical scanning of the autoradiograms using a desktop

scanner and Phoretix software (Biostep). The densitometric intensity of the PAK immunoreactive band was expressed relative to the intensity of the caldesmon immunoreactive band. To correct for slight variations in protein loading levels, the Ponceau Red-stained membranes were also scanned.

Materials and chemicals

[γ^{32}P]ATP (5000 Ci mmol^{-1}) was purchased from Hartmann Analytic (Braunschweig, Germany). Okadaic acid was purchased from Alexis Biochemicals, ML-9 (1-(5-chloronaphthalene-1-sulphonyl)-1*H*-hexahydro-1,4-diazepine), microcystin-LR (MC-LR) and wortmannin were from Sigma. Hexokinase was obtained from Boehringer-Mannheim.

Statistics

Values are shown as means ± S.E.M., and n is the number of observations. Difference of responsiveness among groups was analysed by ANOVA followed by the Newman–Keuls test. Student's t test was used when appropriate. A P value < 0.05 was considered to indicate statistically significant differences.

RESULTS

All Triton-skinned smooth muscle fibres from guinea-pig taenia coli were first subjected to a control activation at pCa 6.2 and pCa 4.3 in the presence of 0.5 μM CaM. The CaM concentration was then increased to 1 μM, which did not significantly increase force further (Fig. 1). All contractions were normalized to this contraction, denoted as F_{max}. Incubation with recombinant GST-αPAK (referred to as PAK1*) significantly inhibited subsequent tension development by about 50 % at pCa 6.2 and 29 % at pCa 4.3 (Fig. 1*B* and *C*). In the time control experiments (Fig. 1*A*), force elicited at pCa 6.2 was not significantly different from the preceding control contraction, while force at pCa 4.3 decreased by about 13 % irrespective of the concentration of CaM (Fig. 1*A* and *C*). Thus, force under maximally activating conditions in PAK1*-treated fibres

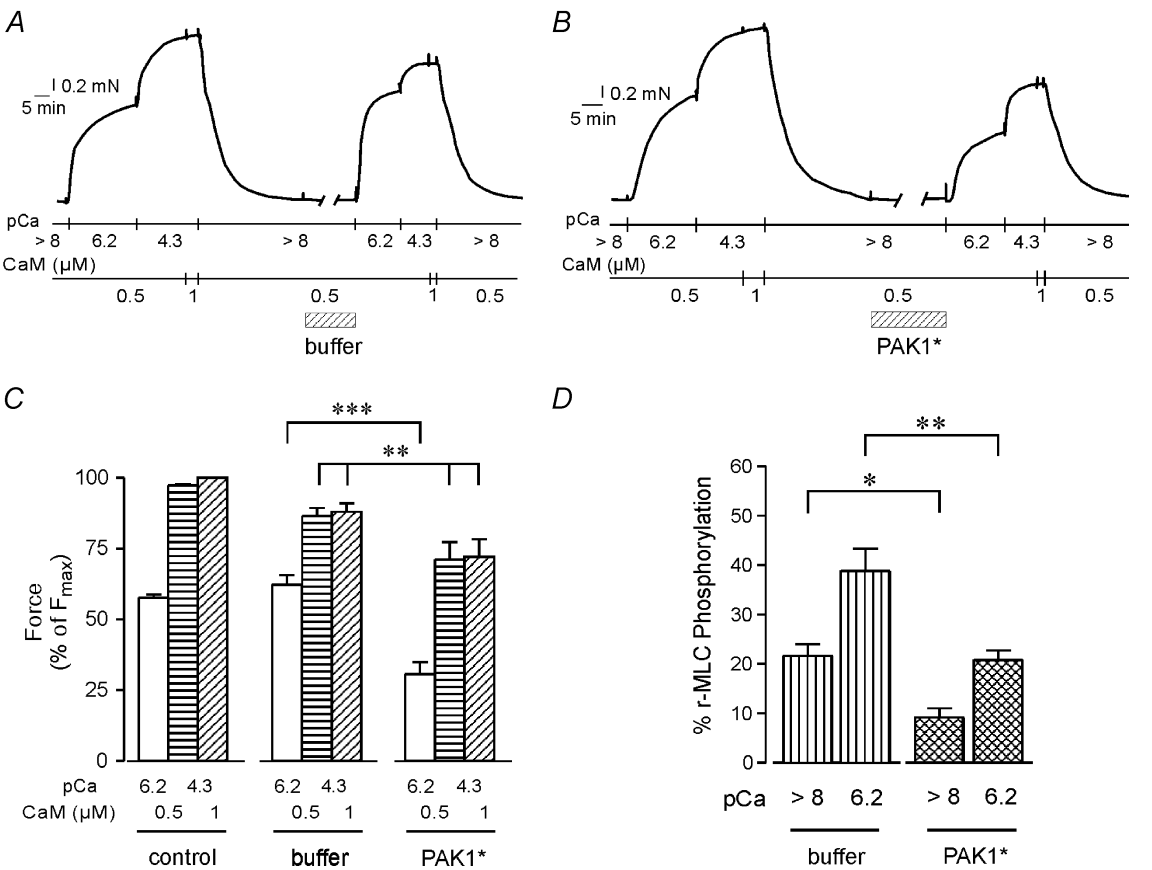

Figure 1. Activated GST-PAK1 (PAK1*) inhibits force development in guinea-pig taenia coli skinned muscle fibres

After control contractions at pCa 6.2 and pCa 4.3 with 0.5 μM CaM and pCa 4.3 with 1 μM CaM, fibres were incubated with buffer or PAK1* (72 μg ml^{-1}) for 1 h in the presence of 10 nM okadaic acid followed by a second stimulation with increasing [Ca^{2+}]. Force was normalized to F_{max} at pCa 4.3 with 1 μM CaM before incubation with PAK1* or buffer. Typical isometric force tracings of Triton X-100-skinned guinea-pig taenia coli fibres incubated with buffer (*A*) or PAK1* (*B*). *C* summarizes the results. Values are given as means ± S.E.M. for buffer control (n = 4–6) and PAK1* (n = 6–7); $^{**}P < 0.01$; $^{***}P < 0.001$. *D*, r-MLC phosphorylation was determined after incubation with PAK1* or buffer in relaxing solution containing 10 nM okadaic acid and in the plateau of the second contraction elicited with pCa 6.2. Values are given as means ± S.E.M. for buffer control (n = 4–5) and PAK1* (n = 4–5); $^{*}P < 0.05$; $^{**}P < 0.01$.

was significantly reduced compared with buffer-treated fibres. Reducing the incubation time to 30 min did not diminish the inhibitory effect of PAK1* on submaximal and maximal Ca^{2+}-activated force ($n = 3$, data not shown). Also, we never observed a contraction in relaxing solution, either in control or in PAK1*-treated fibres. Heat-inactivated PAK1* did not inhibit force.

The PAK1*-induced inhibition of force development at pCa 6.2 was associated with a significant decrease in steady-state r-MLC phosphorylation to ~50 % of control (Fig. 1*D*). Interestingly, PAK1* also inhibited resting r-MLC phosphorylation at pCa > 8 (Fig. 1*D*). To analyse further whether r-MLC is a substrate for PAK1* in intact smooth muscle myosin, *in vitro* phosphorylation experiments were carried out. Intact smooth muscle myosin II and recombinant r-MLC were incubated with MLCK or PAK1* and phosphorylation levels determined by autoradiography (Fig. 2). As expected, MLCK leads to phosphate incorporation both into r-MLC of intact myosin II and recombinant r-MLC. PAK1*, however, is only able to phosphorylate the isolated regulatory light chains. There is no detectable phosphorylation of intact smooth muscle myosin II by PAK1*, thus suggesting that r-MLC is not a physiologically relevant substrate for PAK1* in smooth muscle tissue.

The inhibitory effect of PAK1* on force generation was reversible. Experiments were carried out as in Fig. 1 but with an additional contraction–relaxation cycle in the absence of PAK1* or buffer and okadaic acid. As shown in Fig. 3*A*, the inhibitory effects of PAK1* on steady-state contraction were at least partially reversible, i.e. contraction in the fibres that had been treated with PAK1* was not significantly different from that of the buffer-treated fibres. Wash-out of PAK1* completely reversed the increase in half-time of submaximal tension development ($T_{½}$) seen after treatment with PAK1* (Fig. 3*C*). This was associated with a complete loss of recombinant PAK1* from the fibres ($n = 4$; Fig. 3*B*) indicating that the exogenous PAK1* does not bind tightly to proteins in the skinned fibres. Figure 3*B* also shows that the heavily Triton-permeabilized taenia coli fibres still contain some endogenous PAK immunoreactivity immediately after skinning and after a 30 min loading period with PAK1* in relaxing solution, but not in fibres that were subjected to several solution changes. The immunoreactivity of endogenous PAK was higher in the PAK1*-loaded fibres and we cannot exclude the possibility that this is due to cleavage of the fusion protein. In intact fibres the intensitiy of the PAK1 immunoreactivity is 2.4 ± 0.3-fold ($n = 6$, $P < 0.01$) higher than in the Triton-skinned fibres, which were analysed immediately after skinning (Fig. 4). In contrast, the content of caldesmon, which has binding sites for both actin and myosin (Pfitzer *et al.* 1993), is not different between intact and Triton-skinned fibres. We also expressed the intensity of the PAK band relative to that of the caldesmon band and found this ratio to be also significantly higher in the intact compared with the Triton-skinned fibres (Fig. 4), again indicating that the content of PAK is higher in the intact fibres. These experiments indicate that not only exogenous but also endogenous PAK does not bind tightly to the contractile apparatus or cytoskelelal structures.

To test whether the decreased steady-state r-MLC phosphorylation was due to a reduced MLCK activity we determined the rate of r-MLC phosphorylation in the presence of high concentrations of microcystin-LR, which completely inhibit MLCP activity, as described previously (Lee *et al.* 1997) with some modifications. In brief, following incubation with PAK1* or buffer as before, ATP was depleted by washing in rigor solution in the continuous presence of 10 nM okadaic acid, which at this concentration does not inhibit MLCP (Takai *et al.* 1989) to stabilize the phosphorylation of the PAK1* substrates. To this solution hexokinase and glucose were added to deplete endogenous ATP from the strips. This was followed by pretreatment with 10 μM microcystin-LR (MC-LR), an inhibitor of phosphatase PP1 and PP2A, in

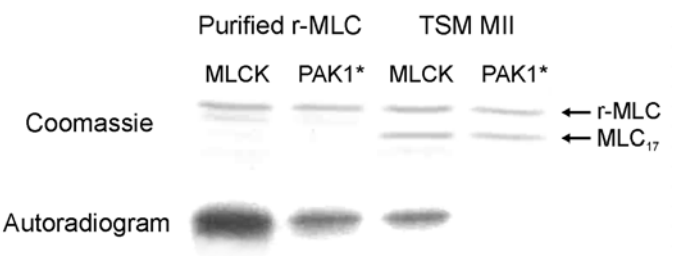

Figure 2. PAK1* does not phosphorylate intact smooth muscle myosin II *in vitro*

Intact myosin II (MII) purified from bovine tracheal smooth muscle (TSM) or recombinant regulatory light chains of myosin II (r-MLC) were incubated with MLCK or PAK1* at 30 °C for 60 min. Phosphorylation reactions were terminated and phosphorylation levels analysed by autoradiography. The upper panel shows the Coomassie-stained light chains of myosin II (r-MLC and MLC_{17}), and the lower panel shows the corresponding autoradiogram. Results are representative of two independent experiments.

rigor solution. The fibres were then activated in contracting solution (containing MgATP) at pCa 6.79. This low Ca^{2+} concentration produced no contraction in the absence of MC-LR. In PAK1*-treated fibres, the rate of rise of contraction and of r-MLC phosphorylation was significantly slowed down compared with the buffer-treated fibres (Fig. 5). As in this experiment the rate of r-MLC phosphorylation depends only on the activity of MLCK (Lee *et al.* 1997), the results are in line with the suggestion that phosphorylation of MLCK by PAK1* decreased the catalytic activity of MLCK (Sanders *et al.* 1999). However, inhibition of MLCK was incomplete as the MLCK inhibitor, wortmannin, produced a more pronounced inhibition of the rate of tension development (Fig. 5*A*).

We then investigated whether MLCK and/or caldesmon are phosphorylated by PAK1*. For these experiments, fibres were incubated with buffer or PAK1* in the presence of [γ^{32}P]ATP. Lysates were prepared from the fibres and MLCK and caldesmon were sequentially immunoprecipitated with specific antibodies (see Methods). As shown in Fig. 6*A*, ^{32}P incorporation was increased by 148 ± 20 % (n = 4) into a protein band with a molecular mass of approximately 130 kDa in PAK1*-treated fibres compared with time-matched control fibres incubated with buffer. Immunoblots (Fig. 6*B*) show that this band is immunoreactive with commercial MLCK antibodies but not with caldesmon antibodies. Figure 6 also shows that the caldesmon antibodies precipitated a protein band of similar mobility that was slightly phosphorylated. However, ^{32}P incorporation into the caldesmon immunoprecipitate was only 4 ± 0.8 % (n = 3) of that into the MLCK immunoprecipitate. There was also a slight reaction of the anti-MLCK antibody against the protein that was precipitated with the anti-caldesmon antibody (Fig. 6*B*). That was due either to a small amount of MLCK that co-precipitated with the anti-caldesmon or to a weak cross-

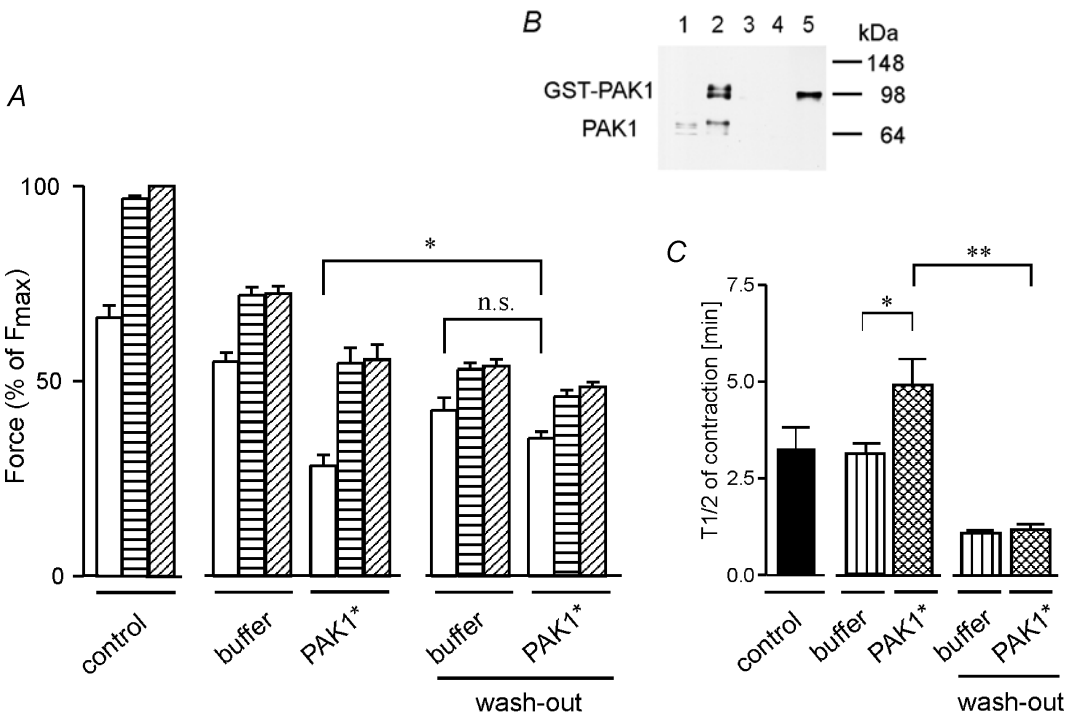

Figure 3. The inhibitory effect of PAK1* on force development and half-time of contraction is reversible

A, strips were incubated with PAK1* or buffer as in Fig. 1, which was followed by two contraction–relaxation cycles in the absence of PAK (pCa 6.2/CaM 0.5 μM, □; pCa 4.3/CaM 0.5 μM, ⊟; pCa 4.3/CaM 1 μM, ▨). Note that in PAK1*-treated strips submaximal force after wash-out is not significantly (n.s.) different from the buffer control and is significantly ($P < 0.05$) higher than before. Values are given as means ± S.E.M. for buffer control (n = 4) and PAK1* (n = 4). *B*, detection of endogenous PAK and PAK1* in Triton-skinned taenia coli. Strips were homogenized directly after permeabilization with Triton X-100 (lane 1), after incubation with PAK1* for 30 min in relaxing solution (lane 2), at the end of force measurements shown in Fig. 1 of PAK1*-treated strips (lane 3) or control strips (lane 4). Lane 5, purified GST-PAK1*. It should be noted that the PAK signal in lane 1 does not represent the content of PAK in intact smooth muscle. Results are representative of four independent experiments. *C*, half-times of contraction elicited at pCa 6.2. Experimental protocol as in panel *A* and Fig. 1. Bars represent means ± S.E.M. with n = 11–20 for control, buffer and PAK1* and n = 4 for the wash-out experiments. *$P < 0.05$; **$P < 0.01$.

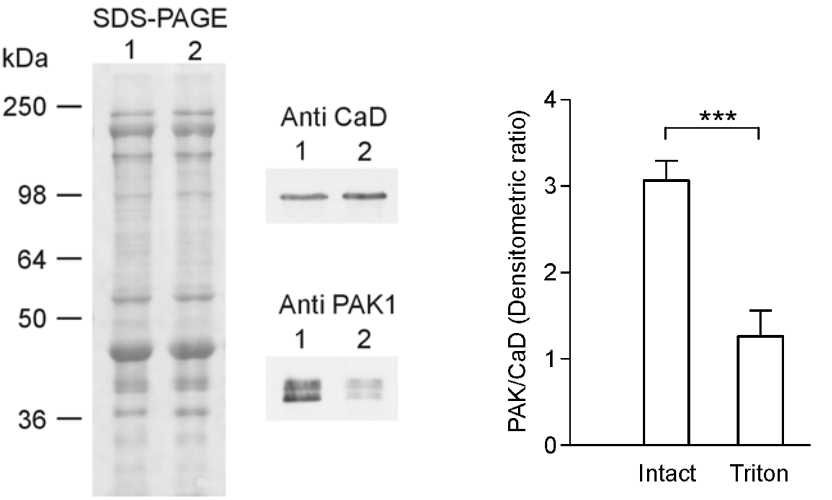

Figure 4. Content of PAK in intact and Triton-skinned taenia coli

Left panel shows Ponceau Red-stained SDS-PAGE of total muscle homogenates (20 μg/lane) taken from intact (1) or Triton-skinned (2) taenia coli. After transfer of the proteins to nitrocellulose the upper part of the blots was incubated with affinity-purified caldesmon antibodies (Anti CaD), and the lower part was incubated with anti-PAK1 antibodies (Anti PAK1). Immunoreactivity was visualized with enhanced chemiluminescence. The bars to the right represent means ± S.E.M. with $n = 6$ of the ratio of the densitometric intensities of the PAK1 to the CaD band. $^{***}P < 0.001$.

reactivity between the anti-MLCK antibody against caldesmon. Whatever the reason, the small background does not alter the conclusion that MLCK is much more heavily phosphorylated than caldesmon in response to PAK1* treatment of taenia coli fibres.

There was some increase in ^{32}P incorporation into MLCK in the buffer-treated fibres which was due to the presence of okadaic acid. Therefore, we tested whether this affected the activity of MLCK by the protocol shown in Fig. 5, which shows that omission of okadaic acid from the buffer control had no statistically significant effect on the rate of tension rise or r-MLC phosphorylation. After 2.5 min, r-MLC phosphorylation in the absence of okadaic acid was slightly higher ($P > 0.05$) suggesting that the ^{32}P incorporation into MLCK in the presence of okadaic acid may lead to a small, but not significant, inhibition of MLCK. Due to the complete inhibition of MLCP by microcystin in these experiments, inhibition of MLCP by okadaic acid cannot be detected. We therefore determined in a separate series of experiments with the experimental protocol shown in Fig. 1 whether omission of okadaic acid from the buffer would increase submaximal steady-state

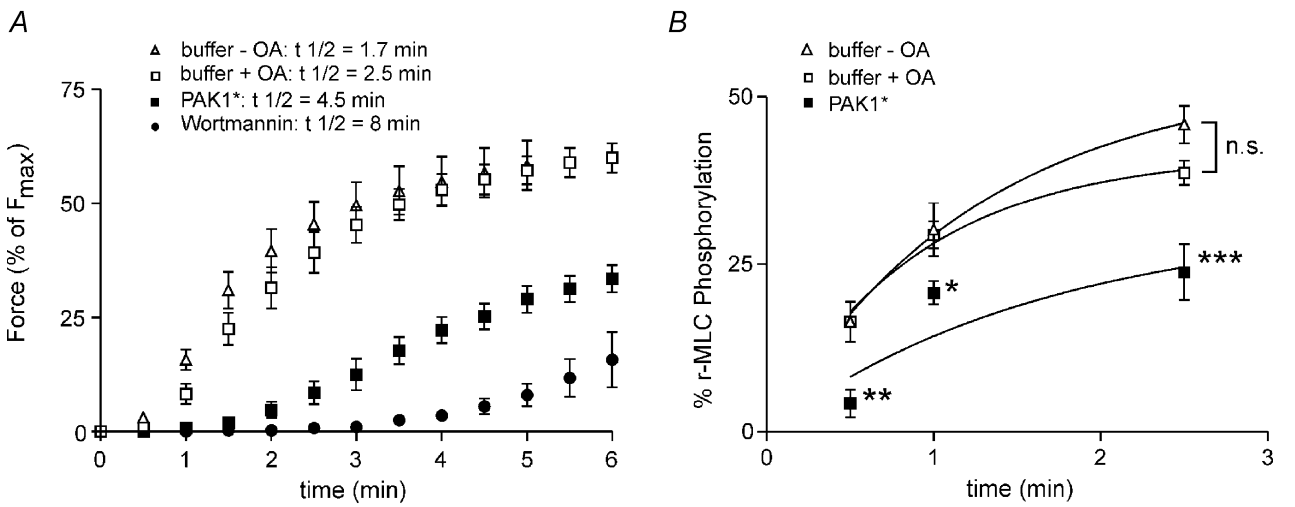

Figure 5. Effect of PAK1* on the time course of r-MLC phosphorylation and contraction after complete inhibition of MLCP by microcystin-LR

After eliciting maximal control contractions and incubation in PAK1* (■) or dialysis buffer (□), as described in Fig. 1, strips were incubated for 10 min in rigor solution in the continued presence of 10 nM okadaic acid with 10 mM glucose and 0.4 U (μl hexokinase)$^{-1}$ to deplete the fibres of ATP, followed by incubation for 5 min in rigor solution containing 10 μM microcystin-LR. The fibres were then activated with pCa 6.79 in ATP-containing contracting solution. Control experiments were carried out with 10 μM wortmannin (●) and in fibres in which okadaic acid was omitted from the dialysis buffer (△). *A*, time course of contraction. *B*, time course of r-MLC phosphorylation. Values are given as means ± S.E.M. for buffer control ($n = 4$–6), PAK1* ($n = 4$–7) and wortmannin ($n = 4$). $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$; n.s., not significantly different.

force and r-MLC phosphorylation. This is to be expected if okadaic acid inhibited MLCP activity. In fibres incubated in okadaic acid-free buffer, the second contraction elicited at pCa 6.2 and pCa 4.3 was 69.3 ± 4.7 % and 77 ± 5.7 % of F_{max} ($n = 3$), while in okadaic acid-treated fibres it amounted to 59 ± 5 and 75 ± 5 %, respectively ($n = 3$). The small (~15 %) inhibition of submaximal Ca^{2+}-activated force in okadaic acid-treated fibres was not statistically significant. It should also be noted that at this low concentration, okadaic acid did not induce a Ca^{2+}-independent contraction. Omission of okadaic acid from the buffer also had no statistically significant effect on steady-state resting and submaximal r-MLC phosphorylation, i.e. r-MLC phosphorylation amounted to 17.4 ± 2.5 % at pCa > 8 ($n = 11$), and 51.7 ± 4.7 % at pCa 6.2 ($n = 11$). These experiments show that we have no evidence for inhibition of either MLCP or MLCK by the low concentrations of okadaic acid used here. Rather, inhibition of force and r-MLC phosphorylation is due to treatment with PAK1*.

A decrease in steady-state r-MLC phosphorylation at a given $[Ca^{2+}]$ could also result from activation of MLCP. If this were the case we would expect an increase in the rate of relaxation in PAK1*-treated strips compared with the buffer control induced by inhibition of MLCK with ML-9 (200 μM) and rapidly removing Ca^{2+} and ATP (Lee *et al.* 1997). As shown in Fig. 7, there was essentially no difference in the rate of tension decline between PAK1*- and buffer-treated fibres.

We also tested whether PAK1 has the potential to modulate contraction in vascular smooth muscle. As this preparation is less stable than the Triton-skinned taenia coli, the first submaximal contraction and stimulation with pCa 4.3 in the presence of 1 μM calmodulin was omitted. Furthermore, the incubation period with PAK1* or buffer was reduced to 30 min. As shown in Fig. 8, PAK1* inhibited submaximal Ca^{2+}-activated force in Triton-skinned guinea-pig carotid artery to a similar extent as in taenia coli. In this preparation maximal force was not affected.

DISCUSSION

The data shown in this study suggest that recombinant PAK1* inhibits the contraction of Triton-skinned taenia coli at constant $[Ca^{2+}]$, most likely due to an inhibitory phosphorylation of MLCK. Inhibition of contraction was associated with inhibition of r-MLC phosphorylation. Because a decrease in r-MLC phosphorylation at constant $[Ca^{2+}]$ may be due to inhibition of MLCK or activation of MLCP, we determined MLCK activity in fibres by measuring the rate of r-MLC phosphorylation. This was

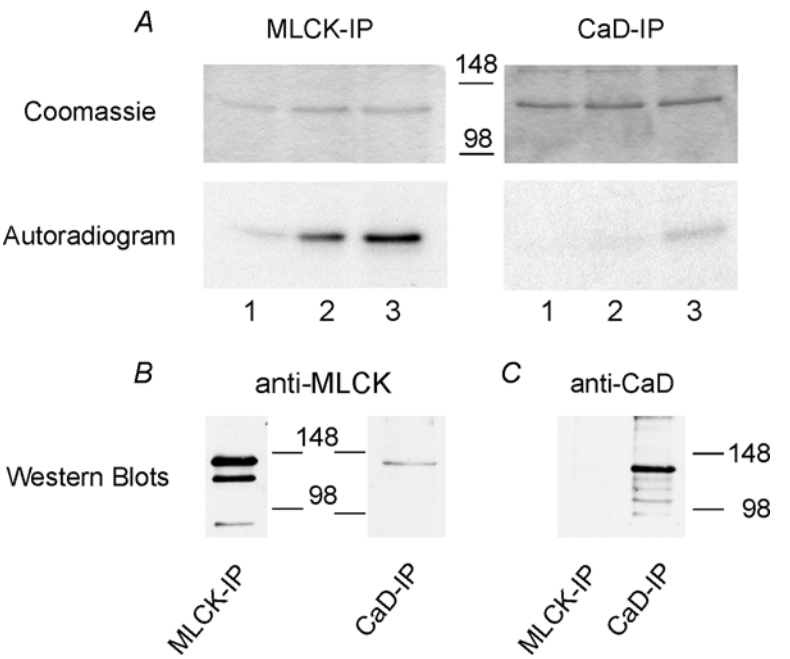

Figure 6. PAK1* phosphorylates MLCK rather than caldesmon

MLCK and caldesmon (CaD) were immunoprecipitated from strips incubated with PAK1* or buffer as described in Methods. *A*, Coomassie-stained SDS-PAGE of immunoprecipitates (IP) and corresponding autoradiograms. Lane 1, basal phosphorylation in the absence of okadaic acid; lane 2, buffer control; lane 3, PAK1*-treated fibres. *B* and *C*, MLCK and CaD immunoprecipitates were transferred to nitrocellulose membrane and probed with anti-MLCK (*B*) or anti-CaD (*C*) antibody as described in Methods. Note that there is a slight immunoreactivity with MLCK in the CaD immunoprecipitate but not vice versa. Results are representative of three to four independent experiments.

done in the presence of high concentrations of the phosphatase inhibitor microcystin-LR, which was shown to completely inhibit MLCP activity (Lee *et al.* 1997). Under this condition the rate of phosphorylation depends solely on the activity of MLCK. Both the rates of r-MLC phosphorylation and contraction were decreased in PAK1*-treated strips, which were associated with an increase of ^{32}P incorporation into MLCK. Omission of okadaic acid from the buffer-treated fibres had no effect on these rates suggesting that the ^{32}P incorporation into MLCK induced by okadaic acid has no effect on the activity of MLCK. However, we cannot exclude the possibility that this increase in phosphorylation facilitates the PAK-induced inhibition of force and r-MLC phosphorylation. In any case, our data support a mechanism in which PAK1* increases the phosphorylation of MLCK, which results in the inhibition of both r-MLC phosphorylation and force.

The effect of PAK1* is not confined to intestinal smooth muscle, as submaximal force was also inhibited in Triton-skinned carotid artery. We were, however, surprised to see that with carotid artery PAK1* inhibited contraction only at intermediate Ca^{2+} concentrations. Sanders *et al.* (1999) showed that in baby hamster kidney-21 (BHK-21) cells PAK phosphorylation of MLCK decreased its activity even in the presence of saturating concentrations of calcium and calmodulin. In line with this, we found inhibition of force under maximally activating conditions in taenia coli. This suggests that in the case of arterial smooth muscle, PAK1* phosphorylation may function by a different mechanism, e.g. by inhibiting Ca^{2+}–calmodulin binding. However, other explanations are possible. It will be interesting to investigate this further. We can say with some certainty that the inhibition of force and r-MLC phosphorylation in the presence of PAK1* at intermediate Ca^{2+} levels is real. We observed this consistently in more than 20 Triton-skinned taenia coli fibres that were derived from several animals and in the carotid artery. Furthermore, wash-out of PAK1* reversed its inhibitory effect while heat-inactivated PAK1* was without effect. So it appears that PAK1 has the potential to inhibit MLCK activity in both taenia coli and arterial smooth muscle.

Inhibitory phosphorylation of MLCK by both PAK1 (Sanders *et al.* 1999) and PAK2 (Goeckeler *et al.* 2000) has been demonstrated in non-muscle cells. This was associated with profound effects on the cytoskeleton and contractile activities of these cells. Thus, PAK1 impaired cell spreading of BHK-21 cells (Sanders *et al.* 1999) and PAK2 inhibited tension development by 75 % in saponin-permeabilized endothelial cells (Goeckeler *et al.* 2000). The observation that the activity of MLCK is inhibited at saturating concentrations of Ca^{2+} and calmodulin suggests that PAKs, unlike most other kinases that phosphorylate MLCK, have a direct effect on the maximum velocity (V_{max}) of MLCK (Sanders *et al.* 1999) rather than on the affinity for calmodulin (K_{CaM}; Gallagher *et al.* 1997).

While our data suggest that phosphorylation of MLCK by PAK1 is responsible for the decreased steady-state r-MLC phosphorylation, activation of MLCP could contribute to this effect. To determine whether MLCP activity was increased by PAK1*, we determined the rate of relaxation from maximally contracted fibres under conditions that

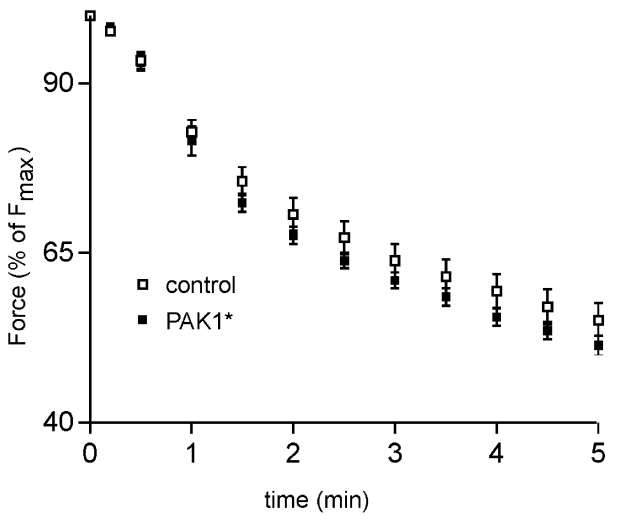

Figure 7. PAK1* does not affect the time course of relaxation

After eliciting a maximal control contraction (pCa 4.3) strips were incubated in PAK1* (■) or buffer (□) as shown in Fig. 1, followed by a second maximal contraction. The fibres were then quickly transferred to Ca^{2+}-free rigor solution containing the MLCK inhibitor ML-9 (200 μM). Force was normalized to the second maximal contraction. Values are given as means ± S.E.M. for buffer control (n = 8), and PAK1* (n = 9).

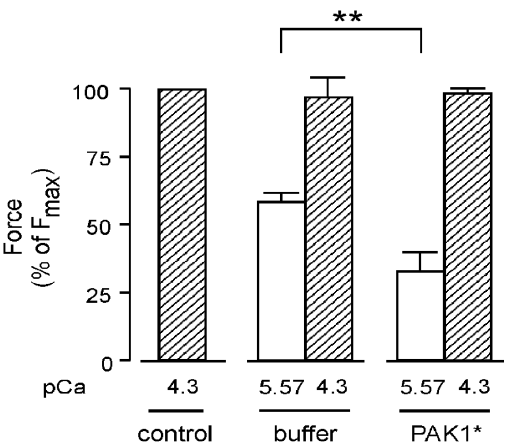

Figure 8. PAK1* inhibits submaximal force in Triton-skinned guinea-pig carotid artery

Experimental protocol as in Fig. 1 with the exception that the first submaximal contraction and contractions elicited at pCa 4.3 with 1 μM calmodulin were omitted and the incubation time with PAK1* was reduced to 30 min. Bars represent means ± S.E.M., n = 5, $^{**}P < 0.01$.

rapidly inactivate MLCK (Lee *et al.* 1997). As shown previously, the rate of relaxation increases or decreases under these conditions when the rate of dephosphorylation of r-MLC either increases (Lee *et al.* 1997) or decreases (Masuo *et al.* 1994) due to an altered MLCP activity. This experiment therefore may give a first indication of whether or not the activity of MLCP is altered. Because we did not see an increase in the rate of relaxation in PAK1*-treated fibres, we suggest that the inhibition of steady-state force and r-MLC phosphoryation is not due to inhibition of MLCP.

It is, however, surprising that we did not see a decrease in the rate of relaxation because during the reviewing process of this manuscript Takizawa *et al.* (2002) reported that PAK inhibits MLCP activity *in vitro* by two mechanisms: (i) phosphorylation of the regulatory subunit of MLCP (MYPT) at multiple sites including Thr641, the site that is responsible for Rho-kinase-induced decrease in MLCP activity *in vitro* (Feng *et al.* 1999), and (ii) phosphorylation of CPI-17 at Thr38, an endogenous inhibitory phosphopeptide of MLC phosphatase. Triton-skinned smooth muscle is devoid of CPI-17 (Woodsome *et al.* 2001). Nevertheless, we would have expected a decrease in the rate of relaxation because of the phosphorylation of MYPT. However, recent evidence suggests that the phosphorylation of this site does not change during agonist-induced Ca^{2+} sensitization in permeabilized smooth muscle and, hence, may not contribute to the regulation of MLCP activity in smooth muscle (Niiro *et al.* 2003; Kitazawa *et al.* 2003). This contrasts with the changes seen in CPI-17 phosphorylation upon agonist stimulation (Niiro *et al.* 2003). Clearly, further studies are required to investigate whether PAK1 affects phosphatase activity within the smooth muscle tissue.

Myosin II is also a putative substrate for members of the PAK family. Injection of endothelial cells with active PAK2 induced cell retraction together with a modification of the actin cytoskeleton, a result that was suggested to be due to the activation of endothelial non-muscle myosin II by direct phosphorylation of r-MLC (Zeng *et al.* 2000). In fibroblasts expression of constitutively active PAK1 also induced an increase in non-muscle r-MLC phosphorylation (Sells *et al.* 1999). Such r-MLC phosphorylation has also been reported for smooth muscle myosin II (Van Eyk *et al.* 1998). These authors described the *in vitro* phosphorylation of both intact myosin II, as well as purified r-MLC, by constitutively active PAK3. However, they also reported that PAK3 uncouples force generation from r-MLC phosphorylation. That is, there was no increase in r-MLC phosphorylation in PAK3-treated, permeabilized smooth muscle fibres and force appeared to correlate with the phosphorylation of caldesmon and desmin instead (Van Eyk *et al.* 1998). In contrast, using an approach very similar to Van Eyk *et al.*, we found a decrease in r-MLC phosphorylation under relaxing conditions (pCa > 8) in the presence of PAK1*, which is consistent with the phosphorylation and inhibition of MLCK. These results were supported by the observation that intact smooth muscle myosin II is not phosphorylated by PAK1*. A similar conclusion was reached by the study of Takizawa *et al.* (2002).

We also investigated the possibility that caldesmon is a substrate for PAK1* as the Ca^{2+} and r-MLC phosphorylation-independent contraction in Triton-skinned taenia coli induced by recombinant PAK3 was associated with phosphorylation of caldesmon (Van Eyk *et al.* 1998). Our investigations into PAK1*-induced phosphorylation of caldesmon showed that antibodies to caldesmon precipitated a protein band of ~130 kDa that was only slightly phosphorylated. The ^{32}P incorporation was about 4 % of the ^{32}P incorporation into the MLCK immunoprecipitates. Since this band was also immunoreactive with MLCK antibodies, there remains the possibility that the ^{32}P incorporation is due to MLCK contamination of the caldesmon immunoprecipitates. If one assumes that the signal represents phosphorylation of caldesmon and that the MLCK is phosphorylated up to a stoichiometric amount of 2 mol P_i (mol MLCK)$^{-1}$ (Goeckeler *et al.* 2000), then the caldesmon phosphorylation would still amount to < 0.1 mol P_i (mol caldesmon)$^{-1}$. Given the fact that stoichiometric *in vitro* phosphorylation of caldesmon (2 mol P_i (mol caldesmon)$^{-1}$) by constitutively active PAK3 resulted in only a partial attenuation (~50 %) of caldesmon's inhibition of actin-activated S1 MgATPase (Foster *et al.* 2000), the low level of phosphorylation observed in our studies is unlikely to be of functional significance.

It is known that in cultured smooth muscle cells PAK activity is increased in response to several agonists (Dechert *et al.* 2001; Schmitz *et al.* 1998, 2001). But it is not known at present whether PAK is involved in the regulation of smooth muscle contraction under physiological conditions. The incubation time of 60 min is rather long and could suggest that the inhibition of force is an *in vitro* phenomenon. We chose this time period in order not to miss a slowly developing Ca^{2+}-independent contraction (Van Eyk *et al.* 1998). But it should be noted that an incubation time of 30 min is sufficient to induce inhibition of similar extent. In a previous study we observed that this incubation period is necessary to homogenously load skinned fibres with large proteins such as caldesmon (G. Pfitzer, unpublished results). Thus, the time frame used in this study does not preclude a physiological function.

In β-escin-permeabilized smooth muscle, constitutively active Rac, which is an upstream activator of PAKs

(Manser *et al.* 1997), antagonized the Ca^{2+} sensitization induced by Rho (Gong *et al.* 2001). Thus, our results, together with those of the laboratory of Somlyo (Gong *et al.* 2001), suggest the interesting possibility that activation of the Rac/PAK pathway may oppose the Rho/Rho-kinase cascade. Activation of the latter pathway has been suggested to be required for tension maintenance during the tonic phase of smooth muscle contraction (for review see Somlyo & Somlyo, 2000; Pfitzer, 2001). However, the contractile agonist angiotensin II induced only a transient, phasic contraction in porcine carotid arteries (Adam *et al.* 1990). As angiotensin II increased Rac and PAK activity in cultured vascular smooth muscle cells (Schmitz *et al.* 2001), it will be interesting to see whether this transient contraction is due to inhibition of MLCK by PAK.

In conclusion, the results presented here analyse, to the best of our knowledge for the first time, the effect of PAK1 on contraction in skinned smooth muscle. In contrast to PAK3, PAK1, which is the predominant isoform in smooth muscle, inhibits Ca^{2+}-activated smooth muscle contraction and r-MLC phosphorylation, most likely due to an inhibitory phosphorylation of MLCK. Caldesmon and r-MLC, which are phosphorylated by other isoforms of PAK (Van Eyk *et al.* 1998; Chew *et al.* 1998; Zeng *et al.* 2000), are not phosphorylated to a significant extent by PAK1. However, our results do not exclude the possibility that PAK1 may also regulate MLCP activity. Future studies aimed at identifying the conditions under which PAK1 becomes activated in intact smooth muscle promise to provide new insight into whether and how PAK1 participates in the regulation of smooth muscle contraction.

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