Abnormal smooth muscle contraction may contribute to diseases such as asthma and hypertension. Alterations to myosin light chain kinase or phosphorylase change the phosphorylation level of the 20-kDa myosin regulatory light chain (MRLC), increasing Ca$^{2+}$ sensitivity and basal tone. One Rho family GTPase-dependent kinase, Rho-associated kinase (ROK or p160ROCK) can induce Ca$^{2+}$-independent contraction of Triton-skinned smooth muscle by phosphorylating MRLC and/or myosin light chain phosphatase. We show that another Rho family GTPase-dependent kinase, p21-activated protein kinase (PAK), induces Triton-skinned smooth muscle contracts independently of calcium to 62 ± 12% (n = 10) of the value observed in presence of calcium. Remarkably, PAK and ROK use different molecular mechanisms to achieve the Ca$^{2+}$-independent contraction. Like ROK and myosin light chain kinase, PAK phosphorylates MRLC at serine 19 in vitro. However, PAK-induced contraction correlates with enhanced phosphorylation of caldesmon and desmin but not MRLC. The level of MRLC phosphorylation remains similar to that in relaxed muscle fibers (absence of GST-mPAK3 and calcium) even as the force induced by GST-mPAK3 increases from 26 to 70%. Thus, PAK uncouples force generation from MRLC phosphorylation. These data support a model of PAK-induced contraction in which myosin phosphorylation is at least complemented through regulation of thin filament proteins. Because ROK and PAK homologues are present in smooth muscle, they may work in parallel to regulate smooth muscle contraction.

In smooth muscle cells, elevation of [Ca$^{2+}$], in response to electrical or chemical stimulation causes calmodulin to activate myosin light chain kinase (MLCK). This highly specific serine/threonine kinase phosphorylates the 20-kDa regulatory light chain of myosin (MRLC) at serine 19, thereby increasing the actin-activated ATPase activity of myosin and inducing contraction. MRLC is dephosphorylated by myosin light chain phosphatases (MLCP) resulting in muscle relaxation. The properties and activities of MLCK or MLCP can be modified by phosphorylation, providing a means to link smooth muscle contraction to other signaling pathways (1, 2). Of particular interest is the recent finding that a Rho-associated kinase (ROK or p160ROCK) can induce Ca$^{2+}$-independent contraction of smooth muscle (3) by directly phosphorylating MRLC on serine 19 (4) and by phosphorylating and inhibiting MLCP (5). The serine/threonine protein kinases PAK and ROK are activated through interactions with the Rho superfamily of Ras-related low molecular weight GTPases (M$_{r}$ = 21,000) (for reviews see Refs. 6 and 7). ROK specifically binds RhoA, whereas PAK (M$_{r}$ = 62–68,000) associates with both Cdc42 and Rac but not RhoA.

Five closely related PAK isozymes have been identified in rat brain (8), human placenta and platelets (9), mouse fibroblast (10), and skeletal and vascular smooth muscle (9). In lower eukaryotes, PAK homologues include Ste20 in yeast (12) and the single-headed myosin I heavy chain kinases in Dictyostelium (13). PAK consists of two domains: an N-terminal regulatory domain that contains a Cdc42/Rac binding-domain and a C-terminal catalytic domain. The three mammalian PAK isoforms, PAK1, PAK2, and PAK3, share ~70% identity in overall amino acid sequence and over 90% identity within the kinase catalytic domain. Binding of GTP-Cdc42/Rac leads to autophos- 

There is substantial evidence linking activation and/or translocation of RhoA to Ca$^{2+}$ sensitivity of smooth muscle in some (16–19) but not all situations (18, 19). In addition, inactivation of RhoA (20) or ROK by a selective inhibitor (21) has been shown to correct hypertension in the spontaneous hypertensive rat model. However, the activation of RhoA and ROK cannot explain the increase in basal tone or Ca$^{2+}$ sensitization under all agonist-stimulated conditions (18, 19), indicating that alternate signaling pathways are likely to be involved. Because the PAK kinases have been implicated in the control of motile events in nonmuscle cells (6, 7), PAK is a potential modulator of smooth muscle contraction. In this manuscript it is shown that like ROK (GST-ROK), PAK (GST-mPAK3) causes Ca$^{2+}$-independent contraction of Triton-skinned smooth muscle fibers. However, PAK acts via a different molecular mechanism than ROK to induce Ca$^{2+}$-independent force in smooth muscle fibers.

**MATERIALS AND METHODS**

**Protein Preparations**—Intact smooth muscle myosin, MRLC, MLCK, and caldesmon were purified from chicken gizzard (22–24), whereas...
PAK3 was isolated from rat brain (13). Recombinant caldesmon fragments (CaD39 and CaD40) were prepared and purified as described in Ref. 25. Recombinant Cdc42 and Rac1 were expressed and purified as described in Ref. 8. Pseudomonas PA3-mPAK3 carrying mouse fibroblast mPAK3 fused to GST in pGEX-4G was expressed and purified according to the protocol of Ref. 32. GST-mPAK3 mutant with lysine amino acid residue 297 mutated to arginine (mPAK3K297R cDNA) was fused to GST by subcloning a BamHI fragment of pDK-mPAK3 into pGEX-4T. The solution used to bathe the fibers contained GST-mPAK3, GST-ROK, and 1 mM dithiothreitol. Following transfer to nitrocellulose, the azide, 7.5 mM ATP, and 20 mM imidazole, pH 6.7, and used within 1 month. Thin fiber bundles (0.5 to 5 μm in diameter) were mounted on an AMF 801 (Sensnor, Horten, Norway) force transducer for analysis (26, 27). “Relaxing” solution consisted of 10 mM magnesium chloride, 1 mM sodium azide, 7.5 mM disodium ATP, 4 mM EGTA, and 20 mM imidazole (pH 6.7), 10 mM sodium phosphocreatine, and 10 units/ml creatine kinase. The gel overlay assay was performed as outlined in Ref. 28. Intact and skinned t. coli samples were analyzed by 10% SDS-PAGE supplemented with 10% glycerol, 5 mM magnesium chloride, and 1% dithiothreitol. Following transfer to nitrocellulose, the proteins were denatured by incubating in a solution of 6M guanidine hydrochloride, 50 mM zinc chloride, 2.5 mM dithiothreitol, 25 mM MES, pH 6.5, 1.25 mM magnesium chloride, 50 mM zinc chloride, 1% bovine serum albumin, and 0.05% Triton X-100 (2 cm from acidic end of gel) to determine the pH gradient, blank gels resolved in the first dimension were compared with broad range protein markers (New England Biolabs, Beverly, MA). To determine the pH gradient, blank gels resolved in the first dimension were cut into evenly sized slices, each slice immersed in 1 ml of a solution containing 50% glycerol (v/v), 4 mM EGTA, 1 mM sodium azide, 7.5 mM disodium ATP, 4 mM EGTA, and 20 mM imidazole, pH 6.7, and used within 1 month. Thin fiber bundles (0.5 to 5 μm in diameter) were mounted on an AMF 801 (Sensnor, Horten, Norway) force transducer for analysis (26, 27). “Relaxing” solution consisted of 10 mM magnesium chloride, 1 mM sodium azide, 7.5 mM disodium ATP, 4 mM EGTA, and 20 mM imidazole (pH 6.7), 10 mM sodium phosphocreatine, and 10 units/ml creatine kinase (ionic strength of 110 mM, 2 mM free Mg²⁺). This series of experiments has been performed using four different guinea pig t. coli skin fiber preparations and two GST-mPAK3 preparations each yielding the identical pattern and alignments of autoradiography and Western blots.

Western Blot Analysis—10 and 12.5% SDS-PAGE and Western blot analysis were carried out as described (32). Detection of the PAK homologue in smooth muscle was achieved using an antibody raised against a synthetic peptide corresponding to 13 residues at the N-terminal end of mouse fibroblast mPAK3 (NT3 polyclonal antibody, gift from S. Pelech, Kinette, Vancouver, Canada; dilution 1:200). Cdc42, desmin, and MRLC were detected using the following antibodies: clone hHCD (dilution 1:2000), clone DEU-10 (dilution 1:100), and clone MY-21 (dilution 1:200), Sigma, respectively. For quantification of MRLC phosphorylation, skinned muscle fibers (2–3 fibers/lane) were subjected to one-dimensional isoelectric focusing (26), separating unphosphorylated MRLC from mono- and di-phosphorylated MRLC. The ratio of phosphorylated to total MRLC was quantified by densitometry (Sigma Gel, Jandel Scientific). overlay assays with Cdc42 and Rac1 provide a sensitive means to detect PAK kinases in smooth muscle. [35S]GTPyS-Cdc42 bound to bands of 62 and 65 kDa in extracts of guinea pig taenia coli smooth muscle, whereas [35S]GTPyS-Rac1 detected a single band of 62 kDa (Fig. 1, A and B). An antibody raised against the N-terminal 13 amino acid residues of mouse fibroblast mPAK3 reacted with a protein of 62 kDa in guinea pig smooth muscle and a protein of 65 kDa in skinned muscle in vitro (Fig. 1C). These results indicate that smooth muscle contains one and possibly two, PAK isoforms (Fig. 1A). PAK was absent from Triton-skinned smooth muscle fibers (Fig. 1, A and B), suggesting that, like ROK (3), PAK is either a cytoplasmic or membrane-bound enzyme.

Triton-skinned guinea pig taenia coli smooth muscle fibers were induced to contract in a Ca²⁺-independent manner when

² R. Cerione, personal communication.
incubated in the presence of recombinant, constitutively active GST-mPAK3 (Fig. 2A). The force induced by GST-mPAK3 (~5 μg/ml; 55 nM) in relaxing buffer (pCa < 8.0) reached a maximum level equivalent to 62 ± 12% (n = 10) of that achieved by addition of a calcium-containing activation solution (pCa 4.3). Under the same conditions, the inactive PAK mutant, GST-mPAK[K297R], was unable to induce force in the absence of Ca^{2+} (Fig. 2B). In previous studies, Ca^{2+}-independent smooth muscle contraction has been induced through the use of unregulated forms of MLCK (1, 2), by addition of phosphatase inhibitors (26, 33, 34), or most recently by another Rho family GTPase-dependent kinase, ROK (3). In all cases the degree of smooth muscle contraction correlates with an increase in the level of MRLC phosphorylation. In the case of ROK, contraction is promoted by the direct phosphorylation of MRLC on serine 19 (4) in addition to the phosphorylation and inhibition of MLCP (5). This dual effect of ROK was demonstrated by the use of wortmannin, which is a potent inhibitor of MLCK but does not affect the activity of either ROK (3) or PAK (Fig. 2C). The addition of a constitutively active GST-ROK catalytic domain to Triton-skinned smooth muscle fibers produces a wortmannin-sensitive contraction at pCa 6.5 (Ca^{2+}-dependent contraction) as well as a wortmannin-insensitive contraction at pCa < 8.0 (Ca^{2+}-independent contraction) (3). On the other hand, wortmannin at a concentration of 1 mM had little effect on the contraction of smooth muscle induced by GST-mPAK3 at low calcium (Fig. 2D), even though this concentration is sufficient
to completely inhibit MLCK-dependent contraction at elevated Ca\(^{2+}\) (data not shown). These results demonstrate that the Ca\(^{2+}\)-independent contraction promoted by PAK occurs without a requirement for MLCK activity. Furthermore, it seems unlikely that PAK promotes contraction by inhibiting MLCP because the Ca\(^{2+}\)-independent contractions achieved with phosphatase inhibitors are invariably dependent on MLCK activity and are abolished by MLCK inhibitors (e.g., 26, 33, 34).

Thus, PAK most likely works by direct phosphorylation of a contractile protein rather than altering either MLCK or MLCP.

These results prompted an investigation into whether PAK directly phosphorylates MRLC, thus achieving contraction in a "traditional" manner. *In vitro* analysis shows that GST-mPAK3 phosphorylates intact chicken gizzard smooth muscle myosin to 2 mol of phosphate/mol (Fig. 3A). Phosphate is incorporated into a single serine residue of MRLC (Fig. 3A). Furthermore, MLCK was unable to phosphorylate MRLC following GST-mPAK3 treatment (Fig. 3B), indicating that PAK and MLCK both phosphorylate serine 19. Indeed, identical two-dimensional tryptic phosphopeptide maps were obtained from MRLC phosphorylated by either MLCK or GST-mPAK3. These results predict that PAK, like MLCK and ROK, promotes smooth muscle force generation by increasing MRLC phosphorylation levels. However, under conditions where GST-mPAK3 induced Triton-skinned smooth muscle fibers to contract with ~70% of the maximal force obtained in the presence of Ca\(^{2+}\), no significant increase in the level of MRLC phosphorylation is observed (Fig. 3D). In fact, the level of MRLC phosphorylation remains similar to the level of relaxed fibers (absence of GST-mPAK3 and calcium, Fig. 3D, lane 4) even as force induced by GST-mPAK3 increases from 26 to 70% (Fig. 3D, lanes 1–3). The uncoupling between MRLC phosphorylation and force genera-
Fig. 4. PAK and ROK phosphorylate different proteins in the skinned muscle fibers. The protein substrates of PAK (A, C, and D) and ROK (B and E) in the skinned muscle fibers were identified by autoradiography and Western blot analysis. Skinned guinea pig taenia coli muscle fibers were incubated in the presence of [γ-32P]ATP in the absence (pCa 8.3, lane 1) and presence of calcium (pCa 4.3, lane 4). As well, fibers were incubated at pCa 8.3 in the presence of either constitutively active GST-mPAK3 (lane 2) or GST-ROK (lane 3). In vitro PAK-phosphorylated MRLC is included in A as a standard marker protein. A and B show autoradiographs of 12.5% SDS-PAGE analysis, whereas D and E show autoradiographs from 10% SDS-PAGE analysis generated from the same blots used for Western blotting with the anti-caldesmon or anti-desmin antibodies. C shows an autoradiograph of the two-dimensional gel electrophoresis of radioactively labeled 58- and 145-kDa PAK substrates in the Triton-skinned muscle fibers. The proteins were separated in the first dimension by a pH gradient produced by an ampholyte mixture of 10% pH 3.5 to 10.0 and 90% pH 4.0 to 6.5 and in the second dimension by 12.5% SDS-PAGE. Phosphorylation of MRLC by GST-ROK in the absence of Ca2+ is similar to that by MLCK at pCa 4.3 (B, compare lanes 3 and 4). Caldesmon is the only protein phosphorylated by GST-mPAK3 that is not phosphorylated by GST-ROK in the skinned muscle fibers (compare D, lane 2, and E, lane 3). F shows the time course of in vitro phosphorylation of chicken gizzard h-caldesmon by GST-mPAK3 (●) or GST-ROK (○). GST-mPAK3 phosphorylates hCaD to 2 mol of phosphate/mol of protein. G shows an autoradiograph of 12.5% SDS-PAGE of MRLC, C-terminal fragment of human fibroblast caldesmon (CaD39), and intact chicken gizzard h-caldesmon phosphorylated in vitro by GST-mPAK3. The methods are as outlined under "Materials and Methods."
tion implies that PAK does not directly or indirectly activate myosin but must employ an alternative and novel mechanism to contract the skinned muscle fibers.

To begin to define the molecular basis of PAK-induced contraction, it is critical to identify the proteins phosphorylated by mPAK3 in the skinned smooth muscle fibers. Protein substrates for mPAK3 were labeled with $^{32}$P under conditions where GST-mPAK3 produces $70\%$ of maximal Ca$^{2+}$-dependent force (Fig. 4). One- and two-dimensional gel electrophoretic analyses of the proteins labeled during a PAK-induced contraction were performed. With one-dimensional SDS-PAGE, two proteins with approximate molecular masses of 58 and 145 kDa are more highly phosphorylated in the presence than the absence of GST-mPAK3 (Fig. 4, A and D, compare lanes 1 and 2). Little if any, phosphorylation of MRLC is detected in the fibers contracted with GST-mPAK3 (Figs 3D and 4A).

The 58- and 145-kDa proteins were identified by Western blot analysis as desmin and caldesmon, respectively (Fig. 4D). Furthermore, the pI of the 58- and 145-kDa proteins were determined by two-dimensional gel electrophoresis using a pH gradient from 4.0 to 6.5 followed by 12.5% SDS-PAGE (Fig. 4C). The pI values for the 58-kDa protein are 5.59 $\pm$ 0.04 and 5.37 $\pm$ 0.04 for the mono- and diphosphorylated forms, respectively, and 5.62 $\pm$ 0.03 and 5.38 $\pm$ 0.04 for the mono- and di-phosphorylated forms, respectively. Again, the amino acid sequence of guinea pig desmin is not known, these pI are in the range expected for desmin (human and chick unphosphorylated desmin, Swiss P17661 and P02542, pI of 5.21 and 5.45, respectively). The 145-kDa protein, identified as caldesmon, has pI values of 5.63 $\pm$ 0.03 and 5.38 $\pm$ 0.04 for the mono- and di-phosphorylated forms, respectively. Amado (1997) demonstrated that caldesmon increases force of skinned gizzard smooth muscle fibers at low concentrations of Ca$^{2+}$ and has an approximate molecular mass of 158 kDa by SDS-PAGE.

Comparison of the protein substrates for ROK and PAK under conditions where GST-ROK and GST-mPAK3 induce similar amounts of Ca$^{2+}$-independent force (79.5 % versus 71.1%, respectively), indicated that GST-ROK incorporated more phosphate into MRLC than did GST-mPAK3 (Fig. 4, compare A and B). As well, GST-ROK did not phosphorylate caldesmon, which is one of the main substrates for GST-mPAK3 (Fig. 4, D and E). In vitro phosphorylation studies confirm that chicken gizzard h-caldesmon is a better substrate for GST-mPAK3 than GST-ROK (Fig. 4, D and E). In vitro, GST-mPAK3 phosphorylated h-caldesmon to 2 mol of phosphate/mol of protein (Fig. 4F). This explains the mono- and diphosphorylated states of caldesmon found in two-dimensional gel electrophoresis of the PAK phosphorylated Triton-skinned muscle fibers (Fig. 4C). Furthermore, the C-terminal domain of human fibroblast 1-caldesmon (corresponding to chicken gizzard caldesmon amino acid residues 458–752) is a substrate for GST-mPAK3 (Fig. 4G), but no phosphorylation of the N-terminal caldesmon domain was observed (data not shown). The C terminus of caldesmon contains multiple binding sites for actin, tropomyosin, and calmodulin.

Caldesmon inhibits the actin-activated Mg-ATPase of myosin (review see Ref. 35) and has been suggested to provide a basal inhibition of vascular tone. The force of contraction of Triton-skinned smooth muscle fibers increases upon the partial extraction of caldesmon (36) or decreases because of competitive binding of a 20-kDa actin-binding fragment of caldesmon (37). As well, a synthetic peptide of an actin-binding region of caldesmon increases force of $\beta$-escin skinned arterial muscle fibers at low concentrations of Ca$^{2+}$ (11), probably by competing with endogenous caldesmon for the actin filament. Taken together, these results suggest that reduction in caldesmon interaction with actin would increase force generation, resulting in contraction. Phosphorylation of the C terminus of caldesmon by PAK could release caldesmon inhibition of the ATPase activity resulting in augmented force development.

In conclusion, although two different Rho family-dependent kinases, PAK and ROK, are able to induce Ca$^{2+}$-independent contractions in smooth muscle, they do so via different molecular mechanisms. ROK increases the steady state level of MRLC phosphorylation. PAK, on the other hand, uncouples force from MRLC phosphorylation and likely acts by phosphorylating caldesmon. The data presented are consistent with a model of PAK-induced contraction in which myosin phosphorylation is at least complemented through the regulation of thin filament proteins.

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