Calcium-independent contraction and sensitization of airway smooth muscle by p21-activated protein kinase


Calcium-independent contraction and sensitization of airway smooth muscle by p21-activated protein kinase. Am J Physiol Lung Cell Mol Physiol 284: L863–L870, 2003. First published January 3, 2003; 10.1152/ajplung.00068.2002.—In Triton-skinned phasic ileal smooth muscle, constitutively active recombinant p21-activated kinase (PAK3) has been shown to induce Ca2+-independent contraction, which is accompanied by phosphorylation of caldesmon and desmin (Van Eyk JE, Arrell DK, Foster DB, Strauss JD, Heinonen TY, Furmaniak-Kazmierczak E, Cote GP, and Mak AS. J Biol Chem 273: 23433–23439, 1998). In the present study, we investigated whether PAK has a broad impact on smooth muscle in general by testing the hypothesis that PAK induces Ca2+-independent contractions and/or Ca2+ sensitization in tonic airway smooth muscle and that the process is mediated via phosphorylation of caldesmon. In the absence of Ca2+ (pCa > 9), constitutively active glutathione-S-transferase-murine PAK3 (GST-mPAK3) caused force generation of Triton-skinned canine tracheal smooth muscle (TSM) fibers to increase by 40% of the maximal force generated by Ca2+ at pCa 4.4. In addition, GST-mPAK3 enhanced Ca2+ sensitivity of contraction by increasing force generation by 80% at intermediate Ca2+ concentrations (pCa 6.2), whereas it had no effect at pCa 4.4. Catalytically inactive GST-mPAK3K297R had no effect on force production. Using antibody against one of the PAK-phosphorylated sites (Ser657) on caldesmon, we showed that a basal level of phosphorylation occurs at this site in skinned TSM and that PAK-induced contraction was accompanied by a significant increase in the level of phosphorylation. Western blot analyses show that PAK1 is the predominant PAK isoform expressed in murine, rat, canine, and porcine TSM. We conclude that PAK causes Ca2+-independent contractions and produces Ca2+ sensitization of skinned phasic and tonic smooth muscle, which involves an incremental increase in caldesmon phosphorylation.

p21-activated kinase; monomeric G proteins; calcium sensitization; asthma; tonic smooth muscle

The primary mechanism of smooth muscle activation is mediated by an increase in intracellular Ca2+, which initiates a cascade of mechanisms utilizing calmodulin, myosin light chain (MLC) kinase (MLCK), and phosphorylation of the regulatory MLC (MLC20) to cause cross-bridge cycling and contraction (20). However, the sensitivity of the contractile apparatus to Ca2+ can be modulated (18, 19, 21). A number of mechanisms for Ca2+ sensitization of smooth muscle implicate the involvement of two groups of proteins. Those in the first group modulate the steady-state level of MLC phosphorylation at constant intracellular Ca2+ concentration; these proteins include Rho-associated kinase (ROK) (22), the MLC phosphatase inhibitor CPI-17 (12), protein kinase C (10), calmodulin-dependent kinase II (25), integrin-associated kinase (31), and mitogen-activated protein kinase (MAPK) (24). The proteins in the second group, including caldesmon and calponin, modulate the actin-myosin interaction and actin-activated myosin ATPase activity (15).

The Rho family, which forms a subgroup of the superfamily of Ras small-molecular-weight GTPases (21 kDa), consists of Cdc42, Rac1, Rac2, RhoA, RhoB, and RhoC (9). These monomeric GTPases act as molecular switches, which alternate between the “on” GTP-binding state and the “off” GDP-binding state. The equilibrium of the two states is regulated by guanine nucleotide exchange factors, which promote the exchange of bound GDP for GTP, and GTPase-activating protein, which enhances GTPase activity of the Rho GTPases. ROK is a well-characterized downstream effector of RhoA (9, 22), and it is believed to play a major role in mediating RhoA-induced Ca2+-independent contractions and Ca2+ sensitization in tonic and phasic smooth muscle (20, 21). ROK inactivates MLC phosphatase by phosphorylating its myosin-binding subunit, resulting in an increase in the steady-state level of MLC20 phosphorylation at a constant Ca2+ concentration. There is strong evidence that other members of the Rho family of small GTPases are also involved in regulating the Ca2+ sensitivity of smooth muscle contraction. For example, Foster et al. (6) and Van Eyk and coworkers (28) reported that the Cdc42/Rac-activated p21 Ser/Thr kinase (PAK) induced a Ca2+-independent contraction in skinned guinea pig ileal smooth muscle with concomitant phosphorylation of caldesmon and desmin, suggesting that PAK may play a significant role in the modulation of Ca2+ sensitivity in smooth muscle contraction. At least four PAK isoforms, PAK1, PAK2, PAK3, and PAK4 (62–68 kDa), have been identified in mammalian tissues, and they share ~70% identity in overall amino acid sequences and the costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
>90% identity within the kinase domain (1). Although PAK1, PAK2, and PAK3 are enriched in the mammalian brain, PAK2 is ubiquitously expressed in mammalian tissues including brain, heart and cardiac muscle, kidney, liver, lung, spleen, and testes (26). Recently, PAK1, PAK2, and PAK3 have been identified in tracheal smooth muscle (5), and PAK2 and PAK3 have been identified in guinea pig taenia coli and rat aortic smooth muscle (28). There is no systematic study of functional diversity and/or redundancy of the PAK isoforms, although it is likely that they have similar substrate specificities on the basis of the high degree of homology in the amino acid sequences in their kinase domains (1).

Airway smooth muscle (ASM) exhibits Ca\(^{2+}\) sensitization to a range of agonists and agents (3, 32, 33). Because abnormal contraction of airway and vascular smooth muscle may contribute to asthma and hypertension, respectively, the action of PAK in tonic smooth muscle is of considerable interest. In this study, we have examined whether PAK3 can produce Ca\(^{2+}\)-independent contraction as well as Ca\(^{2+}\) sensitization of contraction of skinned tonic ASM and whether PAK phosphorylation of caldesmon plays a role in these processes.

METHODS

All experimental procedures were approved by the Queen’s University Animal Care Committee and conform to the guidelines of the Canadian Council on Animal Care. Smooth muscle from the trachea of freshly killed (pentobarbital anesthesia followed by saturated KCl, administered intravenously) dogs, pigs, rats, and mice was dissected free of cartilage. Airway epithelium of rodents was denuded by rubbing the lumen; epithelium and connective tissue of nonrodents was removed by dissection.

Solutions and drugs. The tissue lysis buffer used to homogenize intact ASM had the following composition: 20 mM Tris (pH 7.4), 0.1 mM phenylmethylsulfonyl fluoride, 5 µg/ml leupeptin, 5 µg/ml pepstatin, and 3 mM NaF. For detection of caldesmon phosphorylation, 50 mM NaF and 2 mM Na\(_3\)VO\(_4\) were added to the lysis buffer. Laemmli sample buffer concentrate consisted of 60 mM Tris (pH 6.8), 2% SDS, 25% glycerol, 0.02% bromphenol, and 5% β-mercaptoethanol. The Western blot transfer buffer contained 2.5 mM Tris, 19 mM glycine, and 20% methanol.

Sucrose-potassium solution was used to deplete ASM of Ca\(^{2+}\) before skining and had the following composition: 150 mM sucrose, 50 mM KCl, 20 mM imidazole (pH 7.4), and 5 mM EGTA. Glycerol-storage solution had the following composition: 50% glycerol, 20 mM imidazole (pH 6.7), 10 mM MgCl\(_2\), 7.5 mM ATP, 4 mM EGTA, 1 mM NaN\(_3\), and 0.1 mM 1,4-dithioerythritole (DTE). Relaxing solution had the following composition: 20 mM imidazole, 10 mM MgCl\(_2\), 7.5 mM ATP, 4 mM EGTA, 1 mM NaN\(_3\), 10 mM phosphocreatine, 50 U/ml creatine kinase, and 0.1 mM calmodulin (pCa > 9; <1 × 10^{-9} M free Ca\(^{2+}\)), pH 6.7. Ca\(^{2+}\) contracting solution also contained 4 mM CaCl\(_2\) (pCa 4.4, 3.8 × 10^{-9} M free Ca\(^{2+}\)). Solutions with intermediate Ca\(^{2+}\) concentrations were made by mixing relaxing and Ca\(^{2+}\) contracting solutions, and 30% contracting solution-70% relaxing solution mix gave a solution with pCa 6.2 (5.9 × 10^{-7} M free Ca\(^{2+}\), 1.2 mM total Ca\(^{2+}\)). The solutions used to bathe skinned muscle fibers contained glutathione-S-transferase-murine PAK3 (GST-mPAK3), GST-mPAK3K297R, or an equivalent volume of imidazole buffer (10 mM, pH 6.7) from the final dialysis used in preparing PAK. The concentration of PAK bathing skinned fibers was ~0.3 mg/ml but varied with preparation; a concentration of 0.05 mg/ml is sufficient to contract skinned taenia coli (28). All glassware used to prepare solutions for use with skinned fibers was acid washed and rinsed multiple times in double-distilled water to remove residual Ca\(^{2+}\) deposits.

Detection of PAK in ASM. ASM was homogenized in tissue lysis buffer using a French press and centrifuged to remove cell particles. Total protein content of the clarified cell extract was measured (Bio-Rad detergent-compatible assay), and the samples were diluted 2:1 with Laemmli sample buffer concentrate. Samples were subjected to SDS-PAGE (10% acrylamide) at equal total protein loads (by changing the volume loaded between 5 and 20 µl). Proteins were transferred to nitrocellulose membranes by Western blot (1 h 15 min at 100 V and 250 mA) in Tris-glycine buffer. Western blots were blocked overnight in 10% skim milk or 1% bovine serum albumin (BSA) and probed with rabbit anti-PAK1 (α-PAK C-20) in gCruz Biotechnology blocking solution for up to 5 h (28), anti-PAK3 (β-PAK N-19, Santa Cruz Biotechnology, BSA blocked) antibodies diluted 1:500. The blots were washed and incubated with anti-rabbit or anti-goat secondary antibody linked to horseradish peroxidase diluted to 1:10,000. Enhanced chemiluminescence kits (Amersham Pharma) were used for detection. Specific binding of the primary antibody was assessed by adding the peptide used to generate the primary antibody (α-PAK N-20 or β-PAK C-19 blocking peptide, Santa Cruz Biotechnology) to the primary incubation (1:50 dilution, i.e., 10-fold excess over the primary antibody).

Detection of caldesmon phosphorylation. Phosphorylation of caldesmon at Ser\(^{657}\) was identified using phosphocaldesmon-specific antibodies. The antibodies were generated in rabbits against the synthetic phosphopeptide EGVNIKS(p) MWEKGC (a sequence based on Ser\(^{657}\) of chicken gizzard α-caldesmon) coupled to a carrier protein, keyhole limpet hemocyanin. Serum was first passed through an affinity column for the unphosphorylated peptide to remove antibodies directed against the unphosphorylated epitopes. The effluent was then passed through a second column coupled to the phosphorylated peptide. Specificity of the antibody for phosphorylated Ser\(^{657}\) (pSer\(^{657}\)) caldesmon was determined by Western blot using purified chicken gizzard α-caldesmon and an aliquot of this caldesmon that was phosphorylated by Western blot using purified chicken gizzard α-caldesmon and phosphorylated by mPAK3. Chicken gizzard caldesmon was phosphorylated and phosphorylated by mPAK3 as described previously (6). Western blots of intact canine trachealis were probed with pSer\(^{657}\) caldesmon antibody (diluted 1:300 in 1% BSA). Western blots were performed as described above.

Preparation of Triton-skinned ASM. Dog trachealis muscle was harvested from mongrel dogs as described above. Short (~2-cm-long) sections of trachealis were incubated in sucrose-potassium solution at 4°C twice for 30 min each to deplete the tissue of Ca\(^{2+}\) and relax the smooth muscle (23). Smooth muscle was skinned in sucrose-potassium solution supplemented with Triton X-100 (1%) and DTE (0.5 mM) for 24 h at 4°C (23). After it was skinned, the trachealis was washed for 1 h at 4°C in glycercyl-relaxing storage solution to remove Triton X-100. Skinned trachealis was stored at 20°C in glycercyl-relaxing solution for up to 5 weeks.

Skinned ASM protocols. Thin fiber bundles (~200–300 µm) were mounted on an optical force transducer connected to a computerized data acquisition program (Coda). Resting tension was set at ~200 µN using a micromanipulator attached to one of the mounting posts. Fibers were equilibrated.
RESULTS

PAK immunoreactivity in ASM. Western blot analysis (Fig. 1A) showed a band at ~68 kDa corresponding to PAK1 in mouse, rat, pig, and dog tracheal samples probed with anti-PAK1 N-20 antibody, which is specific for the NH2-terminal 20-amino acid sequence of PAK1. No immunoreactivity was observed when a 10-fold excess of the synthetic peptide used to generate the PAK1 antibody was included in the primary antibody incubation (data not shown). As reported by others, PAK1 is highly enriched in rat brain. In porcine and canine ASM, a few bands of low molecular weight were usually detected in the cell lysates, which likely represent degradation products. Surprisingly, we failed to detect PAK3 in dog (Fig. 1B) as well as mouse, pig, and rat tracheal smooth muscles (data not shown) using different batches of anti-PAK3 N-19 antibodies from a commercial supplier (see METHODS) and our laboratory, even when large amounts of lysate samples were loaded (up to 80 μg of protein load).

Contraction of skinned ASM by GST-mPAK3 in the absence of Ca2+. A constitutively active GST-mPAK3 induces Ca2+-independent contraction of Triton-skinned fibers of trachealis (Figs. 2 and 3). The canine skinned fibers contracted when bathed in the contracting buffer containing maximal Ca2+ (pCa 4.4) or when incubated with Ca2+-free relaxing buffer (pCa > 9) containing ~1 μg/ml of constitutively active GST-mPAK3 (Fig. 2, A and B). GST-mPAK3 produced a significant increase in force over basal tension (n = 10 fibers from 7 dogs, P < 0.005 by fixed-value t-test) that was ~40% of the force produced by maximal Ca2+ at pCa 4.4 (Fig. 3). The response to maximal Ca2+ had a rise time of 40 ± 5 min. Average rise time was approx-
approximately fivefold longer for contraction to GST-mPAK3 than for maximal Ca\(^{2+}\) (200 ± 26 min), although more rapid responses occasionally occurred (Fig. 2C). Variation in fiber thickness might account for the varied time course of GST-mPAK3 contractions by altering the diffusion time for large proteins into the fibers. Catalytically dead GST-mPAK3\(^{K297R}\) did not increase force in fibers, which also displayed normal responses to Ca\(^{2+}\) (Fig. 2, B and D).

**Fig. 2.** Typical responses of skinned tracheal fiber bundles. Representative traces show response of Triton-skinned tracheal smooth muscle fiber bundles. A and C: response to maximal Ca\(^{2+}\) (pCa 4.4) followed by pCa > 9 and then glutathione-S-transferase (GST)-mPAK3 (constitutively active mutant). GST-mPAK3 induced contractions at pCa > 9. Ca\(^{2+}\)-independent contractions to GST-mPAK3 generated less force than maximal Ca\(^{2+}\) and had a variable time course. B and D: control experiments carried out under the same conditions as A and C, but with GST-mPAK3 replaced with the inactive PAK mutant GST-mPAK3\(^{K297R}\). Inactive PAK mutant did not produce ASM contraction.

**GST-mPAK3 potentiates the response of ASM to Ca\(^{2+}\).** To determine whether PAK is capable of increasing Ca\(^{2+}\) sensitization of contraction in the skinned fibers, we studied the effect of mPAK3 on contraction at submaximal as well as maximal Ca\(^{2+}\) concentrations (Fig. 4). The canine skinned fibers were initially contracted in buffer containing a submaximal concentration of Ca\(^{2+}\) (pCa 6.2) and allowed to relax to baseline. The relaxed fibers were again incubated in the pCa 6.2 buffer with and without GST-mPAK3. mPAK3 increases force generation by ~80% at submaximal Ca\(^{2+}\) (n = 6 fibers from 5 animals, P < 0.01 by paired t-test), demonstrating that PAK enhances Ca\(^{2+}\) sensitivity of contraction (Fig. 4A). However, sensitivity to Ca\(^{2+}\) was not altered by GST-mPAK3 at maximal Ca\(^{2+}\) (pCa 4.4, n = 6 fibers from 5 animals; Fig. 4B).

To ensure that repeat exposures to Ca\(^{2+}\) alone have little effect on Ca\(^{2+}\) sensitivity, control experiments were performed on the skinned fibers with repeat exposures to Ca\(^{2+}\) (Fig. 4, C and D). At pCa 6.2, repeat exposure did not significantly alter force (n = 5 fibers from 3 dogs; Fig. 4C), whereas the force developed by a second maximal Ca\(^{2+}\) response was ~25% less than the initial response (n = 14 fibers from 9 dogs, P < 0.01 by paired t-test; Fig. 4D). Because GST-mPAK3 exposure always followed an initial Ca\(^{2+}\) contraction in our protocols, the above result suggests that, if anything,
we may have underestimated the effect of PAK on force development at maximal Ca\(^{2+}\).

**Phosphorylation of caldesmon in skinned trachealis.** mPAK3 phosphorylates h-caldesmon at two unique sites, Ser\(^{657}\) and Ser\(^{687}\), at the calmodulin-binding sites A and B, respectively (based on chicken gizzard sequence) (6). Neither site can be phosphorylated by other kinases that are known to phosphorylate caldesmon, including MAPK, cdc2, casein kinase II, calmodulin kinase II, and protein kinase C. To determine the level of caldesmon phosphorylation by PAK3 in the skinned fibers, we raised specific antibodies against a synthetic peptide corresponding to residues Glu\(^{651}\) and Gly\(^{662}\) (chicken gizzard h-caldesmon) containing the pSer\(^{657}\) residue. This antibody recognized GST-mPAK3-phosphorylated caldesmon but not its unphosphorylated counterpart (Fig. 5A). Western blots of skinned and resting intact canine trachealis showed low levels of phosphorylation of caldesmon at Ser\(^{657}\) (Fig. 5, B and C), indicating that caldesmon is phosphorylated at this PAK-specific site in intact ASM. The level of caldesmon phosphorylation at Ser\(^{657}\) increased significantly 60 and 90 min after mPAK3 treatment at pCa > 9 (Fig. 5D).

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Fig. 3. \(\text{Ca}^{2+}\)-independent contractions by PAK. Triton-skinned trachealis fiber bundles were contracted with maximal \(\text{Ca}^{2+}\) (pCa 4.4) solution, and after relaxation back to baseline at pCa > 9 the same fibers were exposed to GST-mPAK3 (pCa > 9). Exposure to GST-mPAK3 produced \(\text{Ca}^{2+}\)-independent contractions (\(P < 0.005\)) that generated less force than maximal \(\text{Ca}^{2+}\) (\(P < 0.001\), \(n = 10\) fibers from 7 dogs).

Fig. 4. \(\text{Ca}^{2+}\) sensitization by PAK. A: Triton-skinned trachealis fiber bundles were contracted with pCa 6.2, and, after relaxation to baseline, tissue was placed in pCa 6.2 solution containing GST-mPAK3. GST-mPAK3 at pCa 6.2 enhanced contraction compared with pCa 6.2 alone (\(P < 0.01\), \(n = 6\) fibers from 5 dogs). B: GST-mPAK3 at pCa 4.4 did not enhance contraction compared with pCa 4.4 alone (\(n = 6\) fibers from 5 dogs). C: repeat contraction with pCa 6.2 did not significantly change force produced (\(n = 5\) fibers from 3 dogs). D: force developed by a second pCa 4.4 contraction was less than initial response to pCa 4.4 (\(n = 25\) fibers from 11 dogs). **\(P < 0.01\) by paired t-test.
DISCUSSION

Interest in the effects of small GTP-binding proteins and their associated kinases on smooth muscle has increased after evidence of the Ca$^{2+}$-sensitizing properties of ROK (3, 8, 13, 15, 22). ROK and Rho have been the focus of several studies, whereas comparatively little work has examined other members of the Rho subfamily, Cdc42 and Rac, and their associated kinase PAK. In this study, we present evidence that 1) PAK1 is present in ASM across several mammalian species, whereas PAK3 protein is not detected by Western blot analyses; 2) mPAK3 produces Ca$^{2+}$-independent contraction as well as Ca$^{2+}$ sensitization of contraction in skinned ASM; 3) the level of mPAK3 phosphorylation of caldesmon increases after PAK-induced contraction in skinned fibers; and 4) mPAK3 phosphorylation of caldesmon is present in resting intact ASM. Given the homology of different PAK isoforms (1) and our use of the constitutively active catalytic domain of mPAK3, the presence of PAK-induced contraction in skinned ASM fiber is likely representative of the potential action of PAK1 within ASM. We speculate that PAK could have a significant role in ASM physiology and might affect nonspecific bronchial hyperresponsiveness.

Western blot analysis demonstrated the expression of PAK1 in the ASM of four species from three different mammalian orders (Rodentia, Ungulata, and Carnivora), suggesting that at least one PAK isoenzyme is ubiquitously expressed in ASM. Dechert et al. (5), who used an anti-PAK1 antibody that cross-reacts with PAK1, PAK2, and PAK3 in Western blot analysis, detected bands corresponding to the expected molecular weight of the three PAK isoenzymes in canine ASM, although PAK3 appears to be a minor component (see Fig. 1B in Ref. 5). However, we did not detect PAK3 in ASM using anti-PAK3-specific antibodies, although PAK3 has been shown to be expressed in guinea pig taenia coli and rat aortic smooth muscle (28). There is little information about the functional diversity of PAK1, PAK2, and PAK3 isoenzymes in various mammalian cell types, although it is likely that they share similar substrate targets considering the highly conserved sequence of the kinase domains. However, we do not rule out subtle differences in PAK isoenzyme functions in vivo as a result of more conspicuous sequence differences at their NH$_2$-terminal regulatory domains, which may lead to a difference in subcellular targeting and localization of the kinases and, therefore, substrate accessibility.

Addition of exogenous GST-mPAK3 produced Ca$^{2+}$-independent contractions of Triton-skinned dog ASM. Although previous studies have demonstrated Ca$^{2+}$-independent contraction of guinea pig taenia coli (a model phasic smooth muscle) in response to GST-mPAK3 (28), to our knowledge this is the first report of the effect of PAK on ASM (a model tonic smooth muscle). A role for PAK in control of ASM tone is of considerable interest because of the importance of smooth muscle in airway responsiveness and asth-

![Fig. 5. Detection of PAK phosphorylation in intact ASM. A: Western blot (from an 8% gel) showing specificity of phosphorylated Ser$^{657}$ (pSer$^{657}$) caldesmon (CaD) antibody for phosphorylated caldesmon. Caldesmon and GST-mPAK3-phosphorylated caldesmon were loaded at equal concentration (0.5 μg protein). pSer$^{657}$ caldesmon antibody only bound phosphocaldesmon and did not react with unphosphorylated caldesmon. B: Western blot (8% gel) of intact fresh (not skinned) canine ASM probed with anti-pSer$^{657}$ caldesmon. Total protein load (15 μg) was the same in all lanes. Binding of pSer$^{657}$ caldesmon antibody suggests that a pool of PAK-phosphorylated caldesmon exists in resting intact ASM and that PAK has access to the contractile machinery of intact muscle. C: Western blot identical to B but probed with anticaldesmon antibodies (not phosphospecific). All samples had similar levels of total caldesmon immunoreactivity. D: Western blot (8% gel) of skinned canine trachealis smooth muscle probed with anti-pSer$^{657}$ phosphospecific caldesmon antibody before GST-mPAK3 exposure and after 60 and 90 min of incubation with mPAK3 at pCa $> 9$. Total protein load (15 μg) was the same in all lanes. Incubation with GST-mPAK3 induced a time-dependent increase in phosphorylation of caldesmon.](https://ajplung.physiology.org/doi/10.1152/ajplung.00131.2002)
matic disease. This may be especially relevant given a recent report that shows that PAK induces migration of cultured canine tracheal smooth muscle in response to platelet-derived growth factor (5). If PAK is activated in the airway remodeling response associated with asthma, our data also raise the possibility that it may also contribute to enhanced contractile function.

In addition to Ca\(^{2+}\)-independent contractions, GST-mPAK3 enhanced the response to moderate Ca\(^{2+}\) concentrations (i.e., caused Ca\(^{2+}\) sensitization). This is a novel finding, because previous studies (using nonmuscle cells) have shown inactivation of MLCK by PAK in baby hamster kidney-21 and endothelial cells (8, 17), an action that would inhibit Ca\(^{2+}\)-calmodulin contractions. We found no evidence for reduced contractility. Endothelial cells and ASM cells express different isoforms of MLCK (29), and this may alter the actions of PAK in these cell types. Furthermore, the more organized contractile machinery in smooth muscle than in nonmuscle cells may prevent access of PAK to MLCK. The different effects of PAK on Ca\(^{2+}\)-independent contractions of skinned smooth muscle and permeabilized endothelial cells could easily be due to the different cell types studied and are suggestive of different roles for PAK in muscle and nonmuscle.

Although the mechanism of action for PAK-induced smooth muscle contraction is unknown, MLC\(_{20}\) is not believed to be involved, because its phosphorylation state does not change in skinned taenia coli exposed to PAK (28). Van Eyk et al. (28) showed that PAK phosphorylates several proteins in Triton-skinned fibers from guinea pig taenia coli, including desmon and caldesmon. Caldesmon is phosphorylated by PAK at two unique sites (Ser\(^{457}\) and Ser\(^{687}\) that are not phosphorylated by other known kinases (6). Phosphorylation of caldesmon by GST-mPAK3 reduces the inhibitory action of caldesmon on actin-stimulated myosin ATPase activity (6), which could produce contraction by allowing a greater interaction between actin and unphosphorylated myosin. PAK may also have a significant role in ASM cell migration and airway remodeling through a p38 MAPK pathway (5). The role of PAK in smooth muscle is unknown; however, the presence of PAK in ASM from several species and the actions of exogenous PAK on skinned fibers suggest a role for PAK in contraction or Ca\(^{2+}\) sensitization. In intact ASM, however, PAK could be compartmentally separated from its target in skinned fibers and unable to influence contraction. Our result showing phosphorylation of caldesmon at a unique PAK phosphorylation site (Ser\(^{457}\) in intact tissue indicates that endogenous PAK does gain access to the thin filaments and contractile machinery of intact ASM. To our knowledge, this is the only evidence implicating caldesmon as a target of PAK in vivo.

In addition to the normal physiological role of PAK in ASM, it could also be associated with the enhanced contractility and hyperresponsiveness associated with asthma. We clearly demonstrated the presence of PAK in ASM from several species and that PAK can induce contraction of ASM. Thus enhanced expression or activation of PAK in ASM could lead to enhanced smooth muscle responsiveness.

In conclusion, monomeric G proteins of the Rho subfamily and their associated kinases are a group of signal transduction proteins, the function of which has not been fully explored in ASM. We have found evidence of at least one PAK isoenzyme (PAK1) in ASM from many species, suggesting that PAK has a regulatory function in ASM. Exogenous mPAK3 induced Ca\(^{2+}\)-independent contractions and Ca\(^{2+}\) sensitization in skinned ASM fibers, which is associated with phosphorylation of a PAK-specific site of caldesmon. These findings implicate PAK in the control of contractile function of the airways. Caldesmon phosphorylation at a PAK-specific site in intact ASM suggests that endogenous PAK can and does phosphorylate contractile filament proteins. The mechanism of action of PAK and the physiological role of PAK in contraction remain to be explored. We speculate that PAK and other monomeric G protein-associated kinases have the potential to contribute to airway hyperresponsiveness or other pathological states of enhanced smooth muscle contraction.

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