Depletion of focal adhesion kinase by antisense depresses contractile activation of smooth muscle

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Tang, Dale D., and Susan J. Gunst. Depletion of focal adhesion kinase by antisense depresses contractile activation of smooth muscle. Am J Physiol Cell Physiol 280: C874–C883, 2001.—Focal adhesion kinase (FAK) undergoes tyrosine phosphorylation in response to the contractile stimulation of tracheal smooth muscle. We hypothesized that FAK may play an important role in signaling pathways that regulate smooth muscle contraction. FAK antisense or FAK sense was introduced into muscle strips by reversible permeabilization, and strips were incubated with antisense or sense for 7 days. Antisense decreased FAK expression compared with that in untreated and sense-treated tissues, but it did not affect the expression of vinculin or myosin light chain kinase. Increases in force, intracellular free Ca\(^{2+}\), and myosin light chain phosphorylation in response to stimulation with ACh or KCl were depressed in FAK-depleted tissues, but FAK depletion did not affect the activation of permeabilized tracheal muscle strips with Ca\(^{2+}\). The tyrosine phosphorylation of paxillin, a substrate for FAK, was also significantly reduced in FAK-depleted strips. We conclude that FAK is a necessary component of the signaling pathways that regulate smooth muscle contraction and that FAK plays a role in regulating intracellular free Ca\(^{2+}\) and myosin light chain phosphorylation.

Intracellular calcium; cytoskeleton; paxillin; smooth muscle contraction; myosin light chain phosphorylation

Focal adhesion kinase (FAK) is a widely expressed nonreceptor protein tyrosine kinase that localizes to the focal adhesion sites of cultured cells (19, 36). In cultured cells, FAK becomes activated by tyrosine phosphorylation in response to cell adhesion or receptor activation by growth factors or neuropeptides (1, 6, 41). FAK has been implicated in signaling pathways that regulate cytoskeletal organization, and it has been shown to be involved in cell motility and the spreading of cultured cells (15, 23, 34, 39, 41).

The transmission of force between the contractile apparatus of smooth muscle and the extracellular matrix is thought to occur at sites on the membrane referred to as membrane-associated dense plaques. These sites are structurally similar to the focal adhesion sites of cultured cells (4, 5). At these membrane plaque sites, transmembrane integrins engage with extracellular matrix proteins. Actin filaments anchor to transmembrane integrins via a series of linker proteins that include talin, vinculin, and α-actinin (4, 5, 10, 14, 42). These sites also contain a number of signaling molecules that include FAK and paxillin, a multidomain adapter protein that is a substrate for FAK in vitro (3, 36, 48). Membrane dense-plaque sites are likely to be important loci for the integration of signal transduction pathways that regulate many cellular processes (4, 5, 31).

FAK is phosphorylated on tyrosine residues during the contractile activation of tracheal smooth muscle tissues with muscarinic agonists (44). In tracheal smooth muscle tissues, contractile agonists also stimulate the tyrosine phosphorylation of paxillin (32, 44, 51), which is a substrate for FAK in vitro (3). The time course of FAK and paxillin phosphorylation closely follows that of force development (44, 51). Tyrosine phosphorylation of these proteins can also be stimulated by the muscarinic activation of Ca\(^{2+}\)-depleted tracheal smooth muscle strips (29, 44), indicating that the FAK and paxillin phosphorylation can be mediated by a Ca\(^{2+}\)-independent signaling pathway. However, it is not known whether FAK plays a role in the cellular processes that regulate the contractile activation of smooth muscle.

In the present study we have investigated the role of FAK in tracheal smooth muscle contraction by using antisense oligonucleotides to selectively suppress the expression of FAK protein. We evaluated tension generation, intracellular Ca\(^{2+}\), and MLC phosphorylation in FAK-deficient smooth muscle tissues. Because paxillin may be a substrate for FAK, we also evaluated the effects of FAK depletion on paxillin tyrosine phosphorylation. We found that the depletion of FAK protein reduces force, intracellular Ca\(^{2+}\), and MLC phosphorylation in response to the contractile stimulation of tracheal smooth muscle strips and that the depletion of FAK also suppresses paxillin tyrosine phosphorylation. Our results indicate that FAK plays an important role in regulating the contractile activation of smooth muscle.
METHODS

Preparation of tissue. Mongrel dogs (20–25 kg) were anesthetized with pentobarbital sodium and quickly exsanguinated. A 12- to 15-cm segment of extrathoracic trachea was immediately removed and immersed in physiological saline solution (PSS) at 22°C (composition in mM: 110 NaCl, 3.4 KCl, 2.4 CaCl₂, 0.8 MgSO₄, 25.8 NaHCO₃, 1.2 KH₂PO₄, and 5.6 glucose). The solution was aerated with 95% O₂-5% CO₂ to maintain a pH of 7.4. Rectangular strips of tracheal muscle 0.8–1 mm diameter and 8–10 mm long were dissected from the trachea after removal of the epithelium and connective tissue layer. The use of an appropriately sized strip was critical for maintaining muscle contractility during the incubation period and for successfully introducing oligonucleotides into the muscle strips. Each muscle strip was placed in PSS at 37°C in a 25-mL organ bath and attached to a Grass force transducer. At the beginning of each experiment, the optimal length for muscle contraction was determined by increasing muscle length progressively until the force of active contraction in response to a contractile stimulus reached a maximum.

Oligodeoxynucleotides (ODNs) dissolved in Tris-EDTA buffer were introduced into muscle strips according to experimental procedures described below. Muscle strips were incubated for 7 days with ODNs in DMEM. The strips were then incubated for 7 days in DMEM containing 5 mM Na₂ATP, 100 U/ml penicillin, 100 μg/ml streptomycin, and 8 μM antisense or 8 μM sense oligonucleotides, which were kept at 37°C and 5% CO₂. The media were changed every other day.

Extraction of proteins. Muscle strips were freeze-clamped and then pulverized under liquid nitrogen. The pulverized tissue was transferred to dry ice-cooled centrifuge tubes. The while tubes were on dry ice, 50 μL of extraction buffer were added to each of the tubes, and the tubes were quickly mixed. The extraction buffer contained 20 mM Tris HCl at pH 7.4, 2% Triton X-100, 2% SDS, 2 mM EDTA, phosphatase inhibitors (2 mM sodium orthovanadate, 2 mM molybdate, and 2 mM sodium pyrophosphate), and protease inhibitors (2 mM benzamidine, 0.5 mM aprotinin, and 1 mM phenylmethylsulfonyl fluoride). Each sample was boiled for 5 min in inactivate phosphatases and proteases and then maintained at 4°C for 1 h. The supernatant was collected after centrifugation at 14,000 rpm for 25 min at 4°C. For the extraction of paxillin, the concentration of SDS in the extraction buffer was decreased to 0.2%. The concentration of protein in each sample was determined using a standard bicinchoninic protein assay kit (Pierce).

Analysis of protein expression. Muscle extracts were boiled in sample buffer (1.54% dithiothreitol (DTT), 2% SDS, 80 mM Tris-HCl (pH 6.8), 10% glycerol, and 0.01% bromphenol blue) for 5 min and then separated by SDS-PAGE. Proteins were transferred to nitrocellulose, blocked with 2% gelatin for 1 h, and probed with monoclonal antibody to FAK (clone 77, Transduction Laboratories) and then with horseradish peroxidase (HRP)-conjugated anti-mouse Ig (Amersham Life Sciences). Nitrocellulose membranes were then stripped of bound antibodies. The blots were cut into two parts, one containing MLC kinase (MLCK) and the other containing vinculin. MLCK was probed using a monoclonal antibody to MLCK (courtesy of Dr. P. Gallagher, Indiana University) and then with HRP-conjugated anti-mouse Ig (Amersham Life Sciences). The blots for vinculin detection were incubated with polyclonal antibody and then with HRP-conjugated anti-rabbit IgG (Sigma Chemical). Proteins were visualized by enhanced chemiluminescence (ECL) and quantitated by scanning densitometry. Densitometric values of FAK, vinculin, and MLCK were determined for sense- and antisense-treated strips and normalized to those of untreated strips. The ratios of FAK to vinculin and to MLCK were calculated to verify that changes in protein expression were selective for FAK.

Immunoprecipitation of paxillin. Muscle extracts containing equal amounts of protein were precleared for 30 min with 50 μL of 10% protein A-Sepharose to remove cellular proteins that associate nonspecifically with protein A. The precleared extracts were centrifuged at 14,000 rpm for 2 min. The extracts were incubated with monoclonal antibody against paxillin (clone 349, Transduction Laboratories) overnight and then for 2 h with 125 μL of a 10% suspension of protein A-Sepharose beads conjugated to rabbit anti-mouse Ig. Immunocomplexes were washed four times in a buffer contain-
ing 50 mM Tris·HCl (pH 7.6), 150 mM NaCl, and 0.1% Triton X-100. All procedures of immunoprecipitation were performed at 4°C.

Analysis of paxillin phosphorylation. Whole muscle extracts or immunoprecipitates of paxillin were boiled in sample buffer (see above) for 5 min and separated by SDS-PAGE. Proteins were transferred to nitrocellulose, blocked with 2% gelatin, and probed with antibody to phosphotyrosine (PY-20, ICN Pharmaceuticals) and then HRP-conjugated anti-mouse Ig (Amerham Life Sciences) for visualization by ECL. Blots were then stripped of bound antibodies and reprobed with monoclonal antibodies against paxillin to confirm the location of paxillin and normalize for minor differences in protein loading. Phosphotyrosine and paxillin were quantitated by scanning densitometry after visualization by ECL. The tyrosine phosphorylation of paxillin was analyzed from immunoblots of whole muscle extracts for selected points from immunoblots of paxillin immunoprecipitates. No differences were observed in results obtained by analysis of immunoblots of whole muscle extracts and analysis of immunoblots of paxillin immunoprecipitates. Changes in the tyrosine phosphorylation of paxillin were expressed as a magnitude increase over paxillin phosphorylation in unstimulated tissues.

Measurement of intracellular Ca$^{2+}$ concentration. Tracheal smooth muscle strips were pinned in a dish at a slightly stretched length (1.2 times slack length) and incubated in PSS containing 20 μM fura 2-AM, which was dissolved in 0.5% DMSO premixed with 0.01% Pluronic 127. Sonication was used to generate suspended micelles of fura 2-AM, which facilitate the entry of fura 2 into the extracellular space of smooth muscle tissues. The tissues were incubated in the fura 2 solution for 3.5 h at room temperature. They were then washed in PSS for 30 min to remove extracellular fura 2-AM and allow time for the hydrolytic conversion of intracellular fura 2-AM to fura 2. Tissues were mounted in a cuvette and attached to a force transducer for the simultaneous measurement of force and fura 2 fluorescence using a ratio fluorescence spectrophotometer (system model C-14, Photon Technology International). The muscle was illuminated alternately at excitation wavelengths of 340 and 380 nm at a frequency of 2 Hz. Emitted light was collected through a single long-pass filter (510 nm) and detected with a photomultiplier tube. The ratio of fluorescence at 340 nm to fluorescence at 380 nm was continuously computed by a dedicated computer.

Analysis of MLC phosphorylation. Muscle strips were rapidly frozen 1 or 5 min after contractile stimulation and then immersed in acetone containing 10% (wt/vol) TCA and 10 mM DTT (acetone-TCA-DTT), which was precooled with dry ice. Strips were thawed in acetone-TCA-DTT at room temperature and then washed four times with acetone-DTT. Proteins were extracted for 60 min in 8 M urea, 20 mM Tris base, 22 mM glycine, and 10 mM DTT. MLCs were separated by glycerol-urea PAGE and transferred to nitrocellulose. The membranes were blocked with 5% BSA and incubated with polyclonal affinity-purified rabbit MLC-20 antibody. The primary antibody was reacted with $^{125}$I-labeled recombinant protein A (New England Nuclear). Unphosphorylated and phosphorylated bands of MLCs were detected by autoradiography. Bands were cut out and counted in a gamma counter. Background counts were subtracted, and MLC phosphorylation was calculated as the ratio of phosphorylated MLCs to total MLCs.

Permeabilization of muscle strips. After 7 days of incubation, muscle strips were dissected to a smaller size (0.5 mm wide × 5 mm long) to accommodate to the small size (700-μl volume) of the tissue bath used for the measurement of contractile force in permeabilized muscle strips. These strips were pinned in petri plates and incubated at 22°C in a relaxing solution composed of (in mM) 8.5 Na$_2$ATP, 4 K$^+$-EGTA, 1 DTT, 10 sodium creatine phosphate, 20imidazole, 8.9 magnesium acetate, 100.5 potassium acetate, and 1 mg/ml creatine phosphokinase (pH 7.1). After 10 min the strips were incubated in the same solution with the addition of α-toxin (30 μg/ml; Calbiochem), 1 μM leupeptin (a protease inhibitor), and 1 μM carbonyl cyanide p-trifluoromethoxyphenylhydrazone (a mitochondrial blocker) for another 20–25 min. An algorithm of Fabiato and Fabiato (12) was used to calculate the composition of relaxing or contracting solutions containing free Ca$^{2+}$ from pCa 9 to pCa 5. For the measurement of isometric force, permeabilized muscle strips were mounted in tissue baths and attached to force transducers (model GM-2, Gould). In each experiment, permeabilization of the strips was verified by contracting the muscles with 10 μM Ca$^{2+}$. In addition, at the end of the experiment, the permeabilized strips were returned to normal PSS and stimulated with ACh. No contractile response was observed under these conditions.

Statistical analysis. All statistical analysis was performed using SigmaStat software. Comparison among multiple groups was performed by one-way ANOVA or Kruskal-Wallis one-way ANOVA. Differences between pairs of groups were analyzed by Student-Newman-Keuls test or Dunn’s method. Values of n refer to the number of experiments used to obtain each value. P < 0.05 was considered to be significant.

RESULTS

Inhibition of expression of FAK by FAK antisense ODNs. We evaluated the effect of treatment with FAK antisense ODN on the expression of FAK in tracheal muscle strips. Extracts of smooth muscle strips that had been treated with antisense or sense ODNs for 7 days were analyzed by Western blot to compare the expression of FAK with that of vinculin and MLCK.

FAK expression was lower in muscle strips treated with antisense ODNs than in strips treated with sense ODNs or untreated strips (Fig. 1). Expression of vinculin and MLCK was not affected by antisense treatment. This indicates that the decrease in FAK expression was a selective effect of the antisense treatment and that it did not result from general deterioration of the tissue during the incubation period or from nonselective effects of the antisense on protein synthesis. Ratios of FAK to vinculin and FAK to MLCK were significantly lower in antisense-treated strips than in untreated or sense-treated strips (Fig. 1B; P < 0.05). There were no significant differences in the ratio of MLCK to vinculin in untreated, sense-treated, and antisense-treated muscle strips (Fig. 1B).

Effect of FAK antisense oligonucleotides on contractile force. We evaluated isometric force development in response to ACh in muscle strips treated with FAK sense and FAK antisense and in untreated strips (Fig. 2). Force in response to 10$^{-5}$ M ACh was compared before and after the 7-day incubation period. Without ODN treatment, isometric force in response to ACh declined to 88.9 ± 4.0% of the preincubation force (n = 14). Force in strips treated with FAK sense declined similarly to 85.3 ± 3.9% of preincubation force (n = 12). Contractile force in response to ACh in antisense-
Effect of treatment with FAK antisense on intracellular Ca$^{2+}$ sensitivity of the contractile proteins. Active stress at pCa 5 was 73.5 ± 3.8 mN/mm$^2$ in muscle strips without ODNs vs. 72.7 ± 4.2 mN/mm$^2$ in strips treated with FAK antisense (n = 10, P > 0.05). No significant differences in the Ca$^{2+}$-sensitivity were observed (Fig. 6).

Paxillin tyrosine phosphorylation in FAK-deficient muscle strips. The role of FAK in paxillin tyrosine phosphorylation in smooth muscle was evaluated by assessing paxillin tyrosine phosphorylation in response to ACh in FAK-deficient muscle strips. Paxillin tyrosine phosphorylation was compared in FAK sense-treated, FAK antisense-treated, and untreated strips. The tyrosine phosphorylation of paxillin in ACh-stimulated tissues was calculated as a magnitude increase over the level in unstimulated control tissues after treated strips was significantly reduced to 34.7 ± 4.4% of preincubation force (n = 16, $P < 0.05$; Fig. 2B).

**Effect of treatment with FAK antisense on intracellular Ca$^{2+}$**. Smooth muscle strips that had been treated for 7 days with FAK antisense or FAK sense ODNs were loaded with fura 2-AM and then stimulated with 10$^{-5}$ M ACh. The increase in intracellular Ca$^{2+}$ concentration and force generation in response to ACh was consistently lower in muscle strips that had been treated with FAK antisense ODNs than in strips treated with FAK sense ODNs (Fig. 3). In FAK-antisense treated tissues, the increase in the fluorescence ratio elicited by ACh was 26.2 ± 13.7% of the increase in the fluorescence ratio elicited by ACh in FAK sense-treated strips ($n = 3$, $P < 0.05$). The mean force in antisense-treated strips was 32 ± 11% of that in sense-treated strips ($n = 3$, $P < 0.05$).

**Effect of treatment with FAK antisense on MLC phosphorylation in response to stimulation with ACh.** Smooth muscle strips treated for 7 days with FAK antisense or FAK sense ODNs or with no ODNs were stimulated with 10$^{-5}$ M ACh for 1 or 5 min and then frozen for the analysis of MLC phosphorylation. In untreated strips, ACh stimulated a significant increase in MLC phosphorylation from the average resting level of 0.17 ± 0.05 to 0.49 ± 0.07 mol P/mol MLC after 1 min of stimulation and 0.41 ± 0.06 mol P/mol MLC after 5 min of stimulation (Fig. 4). MLC phosphorylation increased similarly in FAK sense-treated muscle strips to 0.40 ± 0.05 mol P/mol MLC after 5 min of stimulation. In contrast, the increase in MLC phosphorylation in response to ACh stimulation was significantly lower in FAK antisense-treated muscle strips: 0.26 ± 0.04 (n = 5) and 0.31 ± 0.03 (n = 4) mol P/mol MLC after 1 and 5 min of stimulation, respectively.

**Effect of treatment with FAK antisense on force and MLC phosphorylation in response to stimulation with KCl.** Tracheal smooth muscle strips treated with FAK antisense or with no ODNs were stimulated for 5 min with 60 mM KCl and then frozen for the determination of MLC phosphorylation. Increases in active force and MLC phosphorylation in response to stimulation with KCl were significantly depressed in muscle strips that had been treated with FAK antisense (Fig. 5).

**Effect of contractile activation with Ca$^{2+}$ on permeabilized FAK-depleted muscle strips.** Tracheal muscle strips were incubated for 7 days with FAK antisense or with no ODNs, and the strips were permeabilized with α-toxin. After permeabilization, in each experiment, two strips without ODNs and two strips treated with FAK antisense were stimulated in parallel with Ca$^{2+}$ at pCa 5. In some experiments, a complete dose-response curve to Ca$^{2+}$ was performed to evaluate the Ca$^{2+}$-sensitivity of the contractile proteins. Active stress at pCa 5 was 73.5 ± 3.8 mN/mm$^2$ in muscle strips without ODNs vs. 72.7 ± 4.2 mN/mm$^2$ in strips treated with FAK antisense (n = 10, $P > 0.05$). No significant differences in the Ca$^{2+}$-sensitivity were observed (Fig. 6).
normalization for differences in the amount of paxillin. The increase in tyrosine phosphorylation of paxillin in response to ACh was significantly lower in strips incubated with FAK antisense ODNs (1.94 ± 0.09) than in strips treated with FAK sense ODNs (2.99 ± 0.13) or untreated strips (2.88 ± 0.11, n = 6, P < 0.05; Fig. 7). We also evaluated the ability of ACh to stimulate the tyrosine phosphorylation of paxillin in FAK-deficient muscle strips that had been depleted of intracellular Ca²⁺. In the absence of Ca²⁺, paxillin tyrosine phosphorylation in response to ACh was also lower (2.17 ± 0.13) in strips treated with FAK antisense than in strips treated with FAK sense (2.92 ± 0.20, n = 6, P < 0.05).

DISCUSSION

Our results demonstrate that FAK plays an important role in the signaling pathways leading to contractile activation in tracheal smooth muscle. Tracheal tissues that are selectively depleted of FAK using antisense oligonucleotides develop significantly less active tension in response to stimulation with ACh or KCl than tissues subjected to comparable treatment with FAK sense ODNs or untreated tissues. The contractile response of untreated and sense-treated tissues declined by only 10–15% over the course of the 7-day incubation period, whereas the contractile response of antisense-treated muscles declined by 65–75% of the preincubation force during the same time period. Analysis of protein expression by Western blot confirmed that the antisense ODNs selectively suppressed the expression of FAK protein and that the expression of vinculin and MLCK was unaffected by the procedures used to introduce FAK antisense into the tissue or by the 7-day period of organ culture. Thus our results cannot be attributed to a general decline in

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**Fig. 2.** Effect of FAK depletion and 7 days of incubation on the contractile response to ACh. A: isometric force in response to 10⁻⁵ M ACh was compared in 3 muscle strips before and after 7 days of treatment with FAK sense, FAK antisense, or no ODNs. Strips were stimulated with ACh for 5 min, and the drug was then washed from the tissues. The contractile response to ACh was dramatically reduced in muscle strips treated with FAK antisense. Force development in strips treated with FAK sense was similar to that in untreated strips after 7 days of incubation. B: smooth muscle strips were contracted with 10⁻⁵ M ACh before and after 7 days of incubation with vehicle (no ODNs), FAK sense, or FAK antisense oligonucleotides. Active force in response to 10⁻⁵ M ACh was quantitated as percentage of ACh-induced force in each strip before incubation. Values are means ± SE (n = 12–16). *Significantly lower than sense and no ODNs (P < 0.05).
protein expression or tissue viability caused by the extended period of organ culture.

We found that the increase in intracellular Ca\(^{2+}\) elicited in response to ACh, as indicated by fura 2, was depressed in FAK-deficient tracheal tissues. MLC phosphorylation was also significantly depressed in the FAK-deficient tissues that were stimulated with ACh. FAK-deficient tissues also showed a reduced response to activation by K\(^{+}\) depolarization, indicating that the presence of FAK is also required for non-receptor-mediated contractile activation of tracheal smooth muscle. In contrast, however, no inhibition of contraction was observed in permeabilized muscle strips stimulated directly with Ca\(^{2+}\) after treatment with FAK antisense, indicating that the depression of contractile activation by FAK antisense treatment does not interfere directly with tension development by the contractile apparatus. Our results suggest that FAK plays a critical role in the regulation of Ca\(^{2+}\) signaling in tracheal smooth muscle and that the disruption of Ca\(^{2+}\) signaling in FAK-depleted tissues may account for the depression of MLC phosphorylation and tension development in response to ACh or KCl. The phosphorylation of Ser-19 at the NH\(_2\) terminus of smooth muscle myosin regulatory light chain 20 by Ca\(^{2+}\)/calmodulin-regulated MLCK is a major cellular event in the initiation of cross-bridge cycling and smooth muscle contraction (24). On the basis of previous measurements in this tissue (30), the level of force development observed in the FAK-depleted muscles in this study is appropriate for the reduced level of MLC phosphorylation.

There is accumulating evidence that tyrosine kinases are involved in mediating intracellular Ca\(^{2+}\) signaling in smooth muscle (9). Tyrosine kinase inhibitors have been shown to block receptor-activated increases in intracellular Ca\(^{2+}\) in a number of smooth muscles (9, 16). Several studies have demonstrated that tyrosine kinase inhibitors reduce L-type voltage-gated Ca\(^{2+}\) channel currents in smooth muscle cells (26, 53). However, there is little evidence as to which tyrosine kinase participates in the regulation of Ca\(^{2+}\) signaling.
signaling. The injection of pp60c-src into smooth muscle cells from rabbit ear artery increases voltage-dependent Ca\textsuperscript{2+} channel currents by a protein kinase C (PKC)-dependent mechanism, and a peptide that activates Src family kinases increases Ca\textsuperscript{2+} channel currents (54). In differentiated colonic smooth muscle, activated c-Src has been shown to interact with FAK to mediate the regulation of basal Ca\textsuperscript{2+} channel activity and the platelet-derived growth factor-induced enhancement of L-type Ca\textsuperscript{2+} currents (22). In these cells, the \( \alpha_1 \)-subunit of the voltage-operated Ca\textsuperscript{2+} channel coimmunoprecipitates with Src kinase and FAK, and it undergoes tyrosine phosphorylation after the application of platelet-derived growth factor. There is evidence that muscarinic receptors couple to membrane ion channels via phosphatidylinositol-3 kinase and atypical PKC (50). Phosphatidylinositol-3 kinase and PKC may also mediate FAK activation and thereby modulate voltage-dependent Ca\textsuperscript{2+} channels in smooth muscle cells (1, 13, 22, 45). Our observation that the contractile response to KCl is depressed in FAK-deficient muscle tissues is consistent with evidence that the presence of FAK may be essential for the regulation of voltage-dependent Ca\textsuperscript{2+} channels (22). However, our results do not rule out the possibility that FAK may also modulate the release of Ca\textsuperscript{2+} from intracellular stores.

In cultured fibroblasts, integrin activation caused by adhesion to extracellular matrix proteins or by anti-body-mediated integrin cross-linking leads to increased tyrosine phosphorylation of FAK and paxillin and the recruitment of Src to the integrin-associated complex (4, 20, 25, 31). The binding of integrins to extracellular matrix or the mechanical perturbation of beads bound to integrins also triggers a rise in intracellular Ca\textsuperscript{2+} (33, 52). Integrin activation by RGD peptides also triggers changes in intracellular Ca\textsuperscript{2+} in vascular smooth muscle (8). These effects could be mediated by the Src-FAK complex. We previously demonstrated that the tyrosine phosphorylation of FAK is strain sensitive in tracheal smooth muscle during contractile activation (44). The contraction of tracheal smooth muscles at long muscle lengths is associated with higher levels of FAK and paxillin phosphorylation, as well as higher levels of intracellular Ca\textsuperscript{2+} and MLC phosphorylation, than contraction of muscles at short lengths (30, 44). Evidence from many different cell types indicates that transmembrane integrins can function as mechanotransducers and that the regulation of cellular responses to mechanical stimuli is coordinated by the complex of cytoskeletal proteins that associate with the cytoplasmic domains of integrin molecules (40). Thus, in smooth muscle, FAK may function as part of an integrin-mediated signaling pathway for the mechanosensitive regulation of intracellular Ca\textsuperscript{2+} and contractile protein activation.

In tracheal smooth muscle, MLC phosphorylation can also be modulated by the small GTPase RhoA.
RhoA activates Rho kinase, which phosphorylates and inhibits MLC phosphatase, thereby increasing MLC phosphorylation (43). In cultured fibroblasts, the activation of Rho stimulates the tyrosine phosphorylation of FAK and paxillin, suggesting that Rho is a critical component of signaling pathways mediated by these proteins (2, 4). However, the inhibition of Rho or Rho kinase does not block FAK or paxillin tyrosine phosphorylation in tracheal smooth muscle (28); thus the pathway regulating FAK and paxillin tyrosine phosphorylation does not depend on Rho activation in this tissue. There is evidence that FAK may regulate the activity of a GTPase regulator associated with FAK (GRAF) (21, 46). Thus we cannot exclude the possibility that Rho-mediated signaling pathways are also disrupted in FAK-deficient tracheal smooth muscle and that this also contributes to the depression of contractility in these tissues. Although we found that FAK depletion did not alter the Ca\(^{2+}\) sensitivity of force in permeabilized tissues activated with Ca\(^{2+}\), FAK may still play a role in agonist-induced Ca\(^{2+}\) sensitization of smooth muscle contraction.

FAK and paxillin are coordinately phosphorylated on tyrosine residues during the contractile activation of tracheal smooth muscle (32, 44, 51). Structural and biochemical analysis of paxillin suggests that it functions as a multidomain adapter molecule that coordinates multiple signaling pathways (49). In addition, there is evidence that paxillin may mediate p21 GTPase-regulated actin cytoskeletal reorganization (47). In vitro, the tyrosine phosphorylation of paxillin can be catalyzed by FAK (3), and in cultured fibroblasts, the phosphorylation of paxillin requires subcellular localization and autophosphorylation of FAK (37). However, Src, Fyn, and Csk protein tyrosine kinases also interact with FAK in vitro and can phosphorylate paxillin in vitro (31, 37). Thus the role of FAK in the tyrosine phosphorylation of paxillin in vivo remains uncertain.

In the present study, the increase in paxillin phosphorylation in response to ACh was clearly lower in FAK-depleted tracheal smooth muscle strips. This suggests that FAK is a necessary component of the signaling pathway leading to paxillin tyrosine phosphorylation in tracheal smooth muscle. In tracheal smooth muscle, paxillin phosphorylation is mediated by Ca\(^{2+}\)-sensitive, as well as Ca\(^{2+}\)-insensitive, signaling pathways; thus the depression of paxillin phosphorylation in the FAK-deficient tissues could have resulted from the disruption of Ca\(^{2+}\) signaling. We measured paxillin phosphorylation in response to ACh in FAK-deficient tissues that had been depleted of intracellular Ca\(^{2+}\) to determine whether the reduction in paxillin phosphorylation was due to the disruption of Ca\(^{2+}\) signaling. In FAK-deficient muscle strips, ACh-induced paxillin tyrosine phosphorylation remained depressed in tissues that had been depleted of intracellular Ca\(^{2+}\), indicating that the effect of FAK depletion on paxillin phosphorylation in tracheal smooth muscle was not due to the depression of Ca\(^{2+}\) signaling. Thus, in tracheal smooth muscle, FAK acts as a direct catalyst of paxillin tyrosine phosphorylation, or it activates another Ca\(^{2+}\)-independent kinase that catalyzes paxillin tyrosine phosphorylation.

Receptor activation may initiate cellular processes in addition to the activation of contractile proteins that enable transmission of force generated by the contractile apparatus to the extracellular matrix (18, 32, 44).
In nonmuscle cells, FAK and paxillin have been implicated in the integrin-mediated signaling pathways that regulate cytoskeletal organization (4, 6, 38). The phosphorylation of FAK and paxillin has been correlated with the assembly of actin stress fibers and focal adhesion formation (1, 7, 31, 35, 39). We previously showed that the contractile activation of tracheal smooth muscle induces actin polymerization and that the inhibition of actin polymerization inhibits tension development without affecting the activation of contractile proteins (27). It is possible that FAK and its downstream substrate paxillin may be implicated in pathways that mediate these processes in tracheal smooth muscle.

Conclusions. We conclude that FAK plays an important role in the signaling pathways that mediate the activation of contractile proteins and tension development in tracheal smooth muscle. Our results indicate that the depletion of FAK protein depresses Cal2+ signaling and MLC phosphorylation in response to stimulation with ACh or KCl. FAK also appears to play a critical role in agonist-induced Cal2+-independent paxillin phosphorylation.

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