Activation of protein kinase A accelerates bovine bronchial epithelial cell migration

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Spurzem, John R., Jitendrakumar Gupta, Thomas Veys, Kristen R. Kneifl, Stephen I. Rennard, and Todd A. Wyatt. Activation of protein kinase A accelerates bovine bronchial epithelial cell migration. Am J Physiol Lung Cell Mol Physiol 282: L1108–L1116, 2002.—Bronchial epithelial cell migration is required for the repair of damaged airway epithelium. We hypothesized that bronchial epithelial cell migration during wound repair is influenced by cAMP and the activity of its cyclic nucleotide-dependent protein kinase, protein kinase A (PKA). We found that, when confluent monolayers of bronchial epithelial cells are wounded, an increase in PKA activity occurs. Augmentation of PKA activity with a cell-permeable analog of cAMP, dibutyryl adenosine 3',5'-cyclic monophosphate, isoproterenol, or a phosphodiesterase inhibitor accelerated migration of normal bronchial epithelial cells in an in vitro wound closure assay and Boyden chamber migration assays. A role for PKA activity was also confirmed with a PKA inhibitor, KT-5720, which reduced stimulated migration. Augmentation of PKA activity reduced the levels of active Rho and the formation of focal adhesions. These studies suggest that PKA activation modulates Rho activity, migration mechanisms, and thus bronchial epithelial repair mechanisms.

Bronchi; cell movement; cAMP; epithelial cells

Similar to processes in other organs, repair of injured airway is accomplished by migration of epithelial cells from the edge of a wound and restitution of the epithelial barrier. Regeneration of the airway epithelium after injury has been evaluated at the light and electron microscopic levels in animal models (12). These morphological studies have shown that epithelial cells at the edge of a wound flatten and spread out before migration of the cells into the wounded area. The cells encounter a wound matrix that is rich in fibrinogen and fibronectin (12, 13). Regeneration of the columnar cell phenotype occurs subsequently.

Stimuli for bronchial epithelial cell migration have been described and include matrix proteins such as fibronectin and collagen (41), growth factors such as insulin and epidermal growth factor (EGF) (23, 43, 48), neuropeptides (24), and cytokines such as tumor necrosis factor-α (18). It is clear that a variety of stimuli potentially modulate the ability of a bronchial epithelial cell to disengage from its normal cell-cell and cell-matrix relationships and move into a wound area. The variety of stimuli suggests that there could be common pathways for control of cell movement. Candidate signaling systems that might be influenced by a number of stimuli include cyclic nucleotides and cyclic nucleotide-dependent kinases. Cyclic nucleotides such as cAMP and cGMP are known to modulate cell shape and attachment as well as movement of some cells (10, 36).

cAMP is synthesized on activation of adenylyl cyclase by many stimuli, including circulating hormone catecholamines that bind to adrenergic receptors on target cell membranes. Adenylyl cyclase activity has been measured in airway epithelial cells (22, 33). The major cellular receptor for cAMP, cAMP-dependent protein kinase (PKA), has been described in airway epithelial cells (3). cAMP levels and PKA are thought to modulate the migration of some tumor cell lines, epidermal cells, and endothelial cells. PKA is known to modulate the activity of the small GTP binding protein Rho, which regulates the formation of the cytoskeleton and focal adhesions (15, 27).

In the present study, we have hypothesized that bronchial epithelial cell migration during wound repair is influenced by cAMP and the activity of its major protein receptor, PKA. To address this hypothesis we used primary cultures of bovine bronchial epithelial cells in an in vitro model of wounding and cell migration. Mechanical wounding of cell monolayers modulated cAMP metabolism and caused an increase in PKA activity in the presence of a phosphodiesterase (PDE) inhibitor. Addition of a cAMP analog or agents that elevate cAMP levels accelerated cell migration in the wound closure assay, decreased the levels of active Rho, and reduced formation of focal adhesions. A PKA inhibitor was used to confirm a role of PKA activity in stimulated bronchial epithelial cell migration.

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MATERIALS AND METHODS

Reagents. LHC (Laboratory of Human Carcinogenesis) basal medium was purchased from Biofluids (Rockville, MD). RPMI 1640 (RPMI), DMEM, MEM, streptomycin-penicillin, and fungizone were purchased from Gibco (Chagrin Falls, OH). Bacterial type XIV protease, transferrin, insulin, EGF, hydrocortisone, sodium azide, sodium nitroprusside, epinephrine, isoproterenol, dibutyryl (DB) cAMP, 8-bromo-cGMP, and bovine fibronectin were purchased from Sigma (St. Louis, MO). Extraction of frozen bovine pituitaries from Pel-Freez (Rogers, AR) was as previously described and yielded an extract containing 10 mg/ml protein (28). Type I collagen (Vitrogen 100, 30 µg/ml) was purchased from Celtrix (Santa Clara, CA). PK-5720 was purchased from Alexis/LC Services (Waltham, MA). 4-Cyano-3-methylsoquinoline was purchased from Calbiochem.

Cell culture. Bronchial epithelial cells were obtained from bovine lungs by a modification (4, 45) of a method described by Wu and Smith (46). Briefly, bronchi were removed from bovine lungs, cut into large pieces, and trimmed of connective tissue. The bronchi were then put into MEM that contains 0.1% bacterial protease, Streptomyces griseus (type XIV, Sigma), and incubated at 4°C overnight. The bronchi were taken out, and lumens were washed with MEM containing 10% FCS to detach the bronchial epithelial cells. The bronchial epithelial cells were then washed once with MEM, 10% FCS, filtered through 100-µm Nitex mesh and resuspended in a 1:1 mixture of LHC9 and RPMI. The combination of LHC basal medium, supplements, and RPMI has been described (4). The usual LHC9 medium contains epinephrine (0.5 µg/ml), and this was used for initial isolation and growth of the cells. The cells were refed with fresh medium without epinephrine the night before all experiments where augmentation of PKA activity was studied.

PKA assay. We have previously used the assay in our cell system (47). Cell monolayers were flash-frozen in liquid N₂ after the addition of 1 ml of buffer containing 10 mM KH₂PO₄, 1 mM EDTA, and 25 mM 2-mercaptoethanol (KPEM). The cells were homogenized by sonication and centrifuged at 10,000 g for 30 min at 4°C. The assay employed is a modification of procedures previously described (19), with 130 µM PKA substrate hexapeptide (LRRA SLG), 10 µM cAMP, 0.2 mM IBMX, 20 mM MgCl₂, 0.5% sodium deoxycholate, 0.2 mM [γ-³²P]ATP in a 40 mM Tris·HCl buffer (pH 7.5). Samples (20 µl) were added to 50 µl of the above reaction mixture and incubated for 15 min at 30°C. Reactions were initiated by the addition of 10 µl cell fraction diluted 1:10 with KPEM and 0.9 mg/ml BSA. Incubations were halted by spotting 50 µl of each sample onto P-81 phosphocellulose papers. Papers were then washed five times for 5 min each in phosphoric acid (75 mM), washed once in ethanol, dried, and counted in nonaqueous scintillation fluid. Negative controls consisted of similar assay samples with or without the substrate peptide or cAMP. A positive control of 0.4 ng/ml purified catalytic subunit from type I bovine PKA (Promega) was included as a sample. Kinase activity was expressed in relationship to total cellular protein assayed and calculated in pmol·min⁻¹·mg⁻¹.

In vitro wound closure (migration) assay. Primary cultures of bovine bronchial epithelial cells were grown to confluence in 96-well flat-bottomed tissue culture dishes. The cell monolayers were “wounded” with a small sterile scraper to remove a circular area of cells. Because variation in wound size can affect closure rates, only wounds of similar size were used (23). The typical wound sizes were 782,500 ± 44,600 µm². The progression of migration was monitored with a phase contrast microscope outfitted with a video camera. The camera output was captured with image analysis software (NIH Image) on a Macintosh PowerMac 7600 computer. Each wound was photographed at specified time points, and the area of the wound was measured with the image analysis software. The dish was returned to the incubator between measurements.

Boyden chamber bronchial epithelial cell migration. After 2 days of primary culture, the cells were detached by MEM containing 0.05% trypsin and 0.53 mM EDTA and washed with MEM with 10% FCS to neutralize the trypsin. The cells were used for migration assay with the Boyden chamber technique using a 48-well multiwell chamber (Neuroprobe, Bethesda, MD) (43). Polycarbonate membranes with 8-µm pores (Neuroprobe) were coated with 0.1% gelatin (Bio-Rad, Richmond, CA) as previously described (43). Several fibronectin concentrations (0–90 µg/ml) were used in the bottom wells as attractants. Bronchial epithelial cells were placed into each of the top wells above the filter. The chambers were then incubated at 37°C, 5% CO₂ for 6 h. After incubation, cells on the top of the filter were removed by scraping. The filter was then stained with a 0.5% Wright stain (Leukostat; Fisher Scientific, Fairlawn, NJ). Epithelial cell migration activity was quantified as the number of migrated cells on the lower surface of the filter in 10 high-power fields (HPF) using a light microscope at ×400 magnification.

Quantitative cell attachment assay. Cell attachment assays in 96-well tissue culture dishes were adapted (1) from a previously described method (40). Bronchial epithelial cells at subconfluence were loaded with 5 µg/ml 2’,7’-bis(2-carboxyethyl)-5(6)-carboxyfluorescein (Calbiochem-Novabiochem, San Diego, CA) for 30 min at 37°C. Cells were trypsinized, and 10⁴ cells were plated on substrate-coated wells for 2 h at 37°C. Nonadherent cells and adherent cells were quantified using a spectrofluorimeter (Perkin Elmer luminescence spectrometer LS50).

Immunofluorescence analysis of focal adhesions and cell size. Bronchial epithelial cells at subconfluence were trypsinized as above, washed, and replated on fibronectin (30 µg/ml)-coated glass slides fitted with removable plastic chambers (Lab-Tek). After 30 min of incubation, the chambers were gently washed and fixed with 1% paraformaldehyde for 5 min. The fixed cells were permeabilized with 0.05% Triton X-100 in PBS for 5 min, followed by blocking with 1% goat serum in PBS overnight. Focal adhesions were detected with a monoclonal antibody to paxillin (Upstate Biotechnology), tetramethylrhodamine isothiocyanate-labeled goat anti-mouse IgG (Sigma), followed by confocal microscopy using a Zeiss LSM 410 laser scanning microscope. Phase contrast images of the cells were also captured on the video system described in In vitro wound closure (migration) assay for measurement of cell surface area on the slides.

Determination of Rho activity. Rho cycles between a GDP-bound inactive state and a GTP-bound active state. The assay employed was derived from that described by Ren and Schwartz (39) that uses affinity binding to the Rho-binding domain of Rhotein, which binds only to GTP-bound active Rho. Cells in the wounded monolayers and control dishes were lysed in 700 µl of immunoprecipitation assay buffer [50 mM Tris, pH 7.2, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 500 mM NaCl, 10 mM MgCl₂, 10 µg/ml each of leupeptin and aprotinin, and 1 mM phenylmethylsulfonyl fluoride (PMSF)]. A positive control lysate was prepared by treating 0.5 ml of a control lysate with 5 µl of 10 mM GTP·S (Upstate Biotechnology) in 50 mM Tris, pH 7.5, 1% Triton X-100, 200 mM NaCl, 10 mM MgCl₂, 0.1% β-mercaptoethanol, 10 µg/ml each of leupeptin and aprotinin, and 0.2 mM PMSF. The positive control was treated with 10 µl of 0.5
RESULTS

In vitro wounding and activation of PKA. To determine the effect of wounding on PKA activity a “cell rake” was used to remove cells in a grid-like pattern from confluent monolayers. The linear wounds averaged 325 μm in width and were 2 mm apart. This process removes ~14.1% of the total cells. In some dishes the cells were treated with a PDE4 inhibitor, rolipram, to allow accumulation of cAMP. Wounding alone did not have a significant effect on PKA activity in the cells (Fig. 1A). The addition of 80 μM rolipram had a small effect on PKA activity, consistent with a small increase in cAMP levels. The combination of wounding of the cell monolayers with rolipram had a marked effect on PKA activity, suggesting that wounding modulated cAMP production, which could be protected from PDE hydrolysis, thus allowing activation of PKA. In additional studies we evaluated a second model of wounding in which confluent monolayers were transiently exposed for 4 min to 0.05% trypsin (Fig. 1B). The majority of the cells were rounded but still attached. There was less cell-to-cell contact. After removal of the trypsin, the cells began to spread, and, similar to the direct wounding experiment in Fig. 1A, there was an increase in PKA activity in the presence of rolipram. PDE inhibitors are commonly used in cAMP assays to increase the sensitivity of the assay for elevations of cAMP levels (34).

Activation of PKA accelerates early bronchial epithelial cell migration in in vitro wound repair. To examine the effects of PKA activation in an in vitro model of wound repair, primary cultures of bovine bronchial epithelial cells were cultured to confluence in tissue culture dishes and then wounded. The rate of closure of the wound was quantified by phase contrast microscopy and video image capture (see MATERIALS AND METHODS). To stimulate PKA activation we used a β-receptor agonist, isoproterenol; a cell-permeable cAMP analog, DBcAMP; and inhibitors of PDE. We have previously characterized the minimum concentrations of these agents necessary to stimulate PKA activity in bovine bronchial epithelial cells (47). Isoproterenol was able to accelerate wound closure (Fig. 2A). PKA activation accelerated closure during the first 6 h. All of the wounds were nearly closed by 20–24 h after wounding. Subsequent experiments focused on wound closure during the first 6 h. There was some variation in the amount of wound closure for different preparations of bovine bronchial epithelial cells, so the wound closure assay was repeated nine times with isoproterenol at a concentration of 10−6 M. The mean percentage of original wound area left open at 6 h was 65.9 ± 3% vs. 77.2 ± 2% for control (P < 0.001 by paired t-test). To

Fig. 1. Wounding of cell monolayers activates protein kinase A (PKA). Confluent monolayers of bronchial epithelial cells were wounded with a “cell rake” (A). In separate experiments we wounded confluent monolayers with a 4-min exposure to 0.05% trypsin (B). At the indicated times the cells were lysed and assayed for PKA activity. In some dishes 80 μM rolipram (Rol) was added immediately after wounding. Data are means ± SE of triplicate dishes within a single experiment. *P < 0.05 for comparisons to all other conditions at that time point, by ANOVA. The experiments were repeated an additional 3 times with different batches of cells, and similar results were obtained.

A

Control
Wounded
Control + Rol
Wound + Rol

B

Control
Tryptsin
Control + Rol
Tryptsin + Rol

PKA activity pmol/min/mg
Time (min)

0 30 60 240
0 100 200 300

0 30 60
0 500 100

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ISOPROTERENOL AND BRONCHIAL EPITHELIAL CELL MIGRATION

M EDTA followed by a 30-min incubation and termination with the addition of 32 μl of 1 M MgCl2. Cell lysates were clarified by centrifugation at 10,000 g at 4°C for 10 min. GTP-bound Rho was affinity precipitated from cell lysates using the Rho-binding domain of Rhotekin, which was bound to beads (Upstate Biotechnology). Each cell lysate (400 μl) was incubated with 20 μg of beads at 4°C for 45 min. The beads were washed four times with Tris buffer containing 1% Triton X-100, 150 mM NaCl, 10 mM MgCl2, 10 μg/ml each of leupeptin and aprotinin, and 1 mM PMSF and resuspended in 50 μl of gel loading buffer. Twenty microliters of bead extract were loaded into a gel. Cell lysates (20 μl) were also loaded in the gels. Rho was detected by Western blotting using a monoclonal antibody against RhoA (Santa Cruz Biotechnology). Detection was achieved by chemiluminescence (ECL, Amersham Pharmacia Biotech). Densitometry analysis was performed, and the signals were adjusted for the relative amounts of cell lysates that were used. The amount of GTP-bound Rho is expressed as a percentage of the signal from the cell lysate from the corresponding condition.

Statistical analysis. The wound closure assays were performed with triplicate wounds (in separate wells). The data represent means ± SE for these triplicates. Other assays were also performed in triplicate wells. To pool data from multiple independent experiments, the effect was tested with paired t-tests to account for varying control results. For time course experiments, ANOVA was used to compare the data for multiple time points and different conditions. For post hoc comparisons within the time course experiments, the Scheffé method was used (Stat View, Abacus Concepts, Berkeley, CA).
determine whether direct stimulation of PKA would accelerate wound closure, DBcAMP was used (Fig. 2B). The wound closure assay was repeated five times with 10^{-7} M DBcAMP, and the mean percentage of original wound area left open at 6 h was 68.5 \pm 4\% vs. 76.9 \pm 4\% for control (P < 0.05 by paired t-test). The concentrations of DBcAMP and isoproterenol that were most effective were similar to the lowest concentrations that were capable of stimulating PKA activity in our prior study (47). To determine whether PDE inhibitors would augment the activity of cAMP during wound healing, the experiments were repeated with different PDE inhibitors (Fig. 2C). The concentrations used were based on previous studies (2, 7). Two nonspecific PDE inhibitors, as well as two inhibitors of type 4 PDE, accelerated wound closure. The experiments with PDE inhibitors were repeated four times with similar results.

Inhibitors of PKA suppress the effects of isoproterenol on wound closure. To confirm that the effects of isoproterenol were in fact mediated by PKA, selective and potent inhibitors of PKA, KT-5720 (21) and 4-cyano-3-methylisouquinoline (30), were used. The presence of inhibitors for 1 h before and during the assay suppressed the effect of isoproterenol on enhancing cell migration in the wound model (Fig. 3). Cell migration and wound closure in the isoproterenol-plus-inhibitor-treated dishes were similar to those in the control dishes. In a separate experiment, it was found that 1 \mu M KT-5720 is not toxic to the cells in that there is no increase in release of lactate dehydrogenase (LDH) (data not shown).

PKA activation stimulates bronchial epithelial cell migration in Boyden chamber migration assays. We previously demonstrated that a number of stimuli are capable of augmenting bronchial epithelial cell migration in Boyden blind well chamber migration assays (18, 41). The Boyden blind well chamber migration assay may assess different aspects of migration than the model described above, in that individual cells in suspension must attach to a gelatin-coated membrane and migrate through the pores of the membrane. Isoproterenol alone had a small effect on migration. Isoproterenol at 1 \mu M increased the cells migrated per HPF from 6 \pm 1 for control to 19 \pm 1 for treated cells. It did not matter if the isoproterenol was present in the
A

\[
\text{% of original wound area vs. hours after wounding.}
\]

B

\[
\text{% of original wound area vs. hours after wounding.}
\]

lower, upper, or both wells during the assay, suggesting a small chemokinetic effect on migration. When fibronectin (30 mg/ml) was present in the lower chambers to provide a gradient of protein for migration, isoproterenol had a more pronounced effect on migration (Fig. 4A). A checkerboard analysis showed similar effects when the isoproterenol was in both the upper and lower wells (data not shown), again suggesting a chemokinetic effect in addition to the chemokinetic effect in the lower wells for this effect. This concentration of fibronectin was similar to our earlier results, in which we found that higher concentrations of fibronectin were often less effective in inducing migration (41). In a separate experiment we evaluated the toxicity of 10^{-2} M isoproterenol and found an increase in the release of LDH from the cells (13% of the activity from a corresponding control cell lysate vs. only 2.3% LDH release for control cells). This may be due to the high concentrations of the isoproterenol counter ion bitartrate. To confirm the role of PKA activation in the Boyden chamber migration assay, we used KT-5720, an inhibitor of PKA in a separate experiment (Fig. 4B). KT-5720 at 1 μM did reduce migration.

To further evaluate the role of PKA activation in the Boyden chamber migration assay, the cAMP analog DBcAMP was used to directly stimulate PKA. Similar to the results with isoproterenol, DBcAMP stimulated fibronectin-induced bronchial epithelial cell migration (Fig. 5).

Evaluation of cell morphology and attachment. An important aspect of cell migration is the ability of cells to attach to the underlying matrix. It is possible that the positive effect of PKA activation on bronchial epithelial migration is due to altered ability of the cells to attach, spread, and form lamellipodia during the initiation of migration. To evaluate this possibility, we attached cells to fibronectin-coated slides for 30 min and assayed for cell surface area and the formation of focal adhesions (Fig. 6). We found that cells treated with isoproterenol and rolipram were able to attach, but their morphology was different from control cells. Focal adhesions were fewer, and there were smaller lamellipodia. The altered morphology was confirmed by direct measurement of the cell surface areas. The treated cells with elevated PKA activity had smaller surface area (568 ± 40 vs. 891 ± 91 μm² for control). When subconfluent 2-day-old cultures of bronchial epithelial cells were treated with isoproterenol to stimulate PKA activity or with a PKA inhibitor, we could not detect significant differences in cell morphology (data not shown). This would suggest that well-adhered, nonmoving bronchial epithelial cells are not as sensitive to the morphological effects of PKA. To determine whether isoproterenol could reduce the number of attached cells, 2-h cell attachment assays were performed using petri dishes precoated with fibronectin. Cells treated with concentrations of isoproterenol that increased migration did not have reduced attachment to fibronectin (Fig. 7). Other studies have shown examples of altered migration but not quantitative changes in attachment (25).

Wounding and PKA activation reduce Rho activity. One mechanism that has been described for the effects of PKA on cell adhesion and formation of focal adhesions is through inhibition of Rho activity (14, 27). We evaluated the levels of active Rho using an affinity-precipitation of GTP-bound Rho (Fig. 8). Wounding of cell monolayers in the presence of a PDE inhibitor, mimicking the conditions in Fig. 1, reduced the amount of active Rho relative to the total amount of Rho in the cell lysates. Inactivation of Rho early in the processes...
after wounding is similar to the findings of Ren et al. (37), in which there is a biphasic response of Rho activity after cell adhesion.

DISCUSSION

The present study demonstrates that mechanical and enzymatic wounding of cell monolayers modulates cAMP production and PKA activation and plays a role in wound closure. Activation of PKA by a β-adrenergic receptor agonist, isoproterenol, through direct activation by a cell-permeable analog of cAMP, DBcAMP, or by elevation of cAMP with PDE inhibitors can accelerate migration of bronchial epithelial cells and wound closure. PKA activation modulated the formation of focal adhesions and cell morphology during cell adhesion, consistent with an inhibitory effect on the small GTPase Rho.

Studies of cAMP and PKA in wound repair have shown variable results. In a study of newt epidermal cell migration it was found that wounded skin had much higher cAMP levels than nonwounded skin (10). In this same in vivo model, nonphysiological levels of DBcAMP (1 mM) plus a PDE inhibitor (1 mM theophylline) reduced migration. DBcAMP alone had no effect (11). Cholera toxin, which raises cAMP levels, enhanced the rate of wound closure in a corneal epithelial wound model (20). It has recently been shown that prostaglandin (PG) E2 can increase the migration of bronchial epithelial cells in an in vitro wound closure assay similar in design to the assay used in the present study (42). Because PGE2 can raise cAMP levels, this provides additional evidence that PKA activation can accelerate bronchial epithelial migration. PKA activation of cell migration has been studied in some tumor cell lines. It has been shown that metastatic clones of Lewis lung carcinoma tumors have significantly more PKA activity than do nonmetastatic clones. Elevation of PKA activity was associated with increased migration through matrix proteins and with decreased adhesion to matrix proteins (31). In contrast to these studies, an inhibitory role for cAMP has been described in migration of breast carcinoma cells (35). These studies provide evidence that cAMP can have a dual effect on cell-signaling events governing migration. Studies have established that cAMP produces different effects at different concentrations. Very high, nonphysiological concentrations may inhibit cell migration, whereas lower levels may be associated with stimulated migration.

There are several mechanisms by which PKA activation might influence cell migration. PKA activation is thought to modulate cytoskeletal assembly and focal adhesion formation. Potential targets for PKA include Rac/Rho GTPases that affect the assembly of actin stress fibers and cell migration (16, 17). Proteins that reside in focal adhesions (which facilitate signaling from and attachment to extracellular matrix proteins) are known targets for these GTPases (14). There is
evidence that PKA can phosphorylate and inhibit activation of Rho (9, 27). A new anchoring protein has also been described that appears to bind PKA and nucleate Rho-mediated signaling (8). Our studies are consistent with an inhibitory effect of PKA on Rho activation and focal adhesion formation in bronchial epithelial cells. Bronchial epithelial cells treated with isoproterenol and a PDE inhibitor attached to fibronectin-coated slides but had reduced formation of focal adhesions and reduced cell surface area on the slide. Downregulation of active Rho was confirmed by affinity precipitation. This is consistent with prior observations that downregulation of Rho promotes disassembly of focal adhesions or prevents their formation (14).

The regulation of Rho is complex and is influenced by several signaling systems. In addition to regulation by cell adhesion (37, 38), known stimuli of epithelial migration such as EGF, serum, and other growth factors also influence the activation of Rac/Rho GTPases. PKA may play a role in mediating the effects in these regulatory pathways. PKA can inhibit the activation of extracellular-regulated kinases (ERK) of the mitogen-activated protein kinase family (5, 29, 44). ERK are thought to mediate the effects of growth factors on cell migration (6).

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**Fig. 6**. Activation of PKA alters bronchial epithelial cell morphology during attachment. Subconfluent bronchial epithelial cells were trypsinized as in Fig. 4 and replated on fibronectin-coated glass slides. After 30 min of incubation in the indicated conditions, they were fixed and stained for paxillin, a component of focal adhesions, and observed by immunofluorescence microscopy. A: control cells where the majority of cells are well spread, with the majority of cells also having well-formed focal adhesions (arrows). B: cells that were also treated with 1 μM isoproterenol and 80 μM rolipram. These cells were more irregular in shape, and focal adhesions were uncommon. Cell surface areas were measured (see RESULTS).

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**Fig. 7**. Effects of isoproterenol on attachment of bronchial epithelial cells. Bronchial epithelial cells were grown for 2 days before trypsinization as in Fig. 4. The cells were replated onto fibronectin-coated dishes for a 2-h attachment assay. Isoproterenol at the indicated concentrations was added at the start of the attachment assay. The bars represent the means ± SE of 3 dishes. The experiment was repeated twice using different preparations of bovine bronchial epithelial cells with similar results.

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**Fig. 8**. Effects of wounding and PKA activation on activation of Rho. Confluent monolayers of bronchial epithelial cells were wounded as in Fig. 1A. At 10 min postwounding, the cells were lysed and evaluated for content of active Rho. A: a representative Western blot of Rho. B: the results of 3 separate experiments. In each experiment active Rho declined in the wounded cell monolayers. The results were statistically significant (P < 0.05, paired t-test).
Another mechanism for PKA modulation of migration and attachment may be through regulation of extracellular matrix receptors. It has recently been shown that PKA modulates cross talk between integrins (25). There is evidence that the fibronectin receptor $\alpha_5\beta_1$-integrin regulates the function of the vitronectin receptor $\alpha_v\beta_3$ in endothelial cells. The authors present evidence that antagonists of $\alpha_5\beta_1$-integrin raise PKA activity and decrease focal adhesion formation when the cells are attaching to vitronectin through the $\alpha_v\beta_3$-integrin. Similar to our studies of attachment and migration to fibronectin, there was a significant effect on migration over vitronectin but not on the number of attached cells to vitronectin. It is known that, in wounded or damaged epithelium, bronchial epithelial cells are exposed to an altered extracellular matrix environment in which there is more fibronectin and increased expression of the $\alpha_5\beta_1$-integrin (13). Thus it is possible that PKA activity plays a central role in mediating the balance of signals from adhesion receptors as adhesion changes during injury and repair.

Cell attachment and migration require a balance of adhesion forces. Disassembly and reassembly of strong attachments, focal adhesions, are required for the cells to move. Our studies are consistent with the idea that, under the conditions of cell culture, PKA activation alters the balance of focal adhesion and cytoskeletal formation toward bronchial epithelial cell migration and repair. The balance of stimuli for migration during in vivo repair may be different. The role of cAMP elevation by stimulants such as $\beta_2$-adrenergic agents during airway repair in chronic obstructive pulmonary disease and asthma is not known. We have previously shown that $\beta_2$-adrenergic agonists attenuate fibroblast contraction of collagen gels (32). This would imply that airway remodeling is altered, but it is not clear whether this is beneficial or not. Our observation that PKA activation is part of epithelial repair and the older observation that PKA activation protects epithelial cells from damage by endotoxin (26) would suggest a beneficial effect of PKA activation in airway repair. Ongoing studies to more clearly define the role of PKA activation in airway disease will be important for our understanding of the use of these important therapies in airways disease.

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