Agonist binding to β-adrenergic receptors on human airway epithelial cells inhibits migration and wound repair

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Peitzman ER, Zaidman NA, Maniak PJ, O’Grady SM. Agonist binding to β-adrenergic receptors on human airway epithelial cells inhibits migration and wound repair. Am J Physiol Cell Physiol 309: C847–C855, 2015. First published October 21, 2015; doi:10.1152/ajpcell.00159.2015.—Human airway epithelial cells express β-adrenergic receptors (β-ARs), which regulate mucociliary clearance by stimulating transepithelial anion transport and ciliary beat frequency. Previous studies using airway epithelial cells showed that stimulation with isoproterenol increased cell migration and wound repair by a cAMP-dependent mechanism. In the present study, impedance-sensing arrays were used to measure cell migration and epithelial restitution following wounding of confluent normal human bronchial epithelial (NHBE) and Calu-3 cells by electroporation. Stimulation with epinephrine or the β-2-AR-selective agonist salbutamol significantly delayed wound closure and reduced the mean surface area of lamellipodia protruding into the wound. Treatment with the β-2-AR bias agonist carvedilol or isothiocyanate also produced a delay in epithelial restitution similar in magnitude to epinephrine and salbutamol. Measurements of extracellular signal-regulated kinase phosphorylation following salbutamol or carvedilol stimulation showed no significant change in the level of phosphorylation compared with untreated control cells. However, inhibition of protein phosphatase 2A (PP2A) activity completely blocked the delay in wound closure produced by β-2-AR agonists. In Calu-3 cells, where CFTR expression was inhibited by RNAi, salbutamol did not inhibit wound repair, suggesting that β-2-AR agonist stimulation and loss of CFTR function share a common pathway leading to inhibition of epithelial repair. Confocal images of the basal membrane of Calu-3 cells labeled with anti-β1-integrin (clone HUTS-4) antibody showed that treatment with epinephrine or carvedilol reduced the level of activated integrin in the membrane. These findings suggest that treatment with β-2-AR agonists delays airway epithelial repair by a G protein- and cAMP-independent mechanism involving protein phosphatase 2A and a reduction in β1-integrin activation in the basal membrane.

biased agonism; β-arrestin signaling; carvedilol; epinephrine; impedance sensing

EPITHELIAL RESTITUTION following injury is a dynamic process that is critical in sustaining transepithelial transport and maintenance of epithelial barrier function in normal and pathological states (7, 12, 41). Pulmonary epithelial tissues are especially susceptible to injury due to continuous exposure to noxious components (e.g., particles, allergens, and microorganisms) in the atmosphere that compromise wound closure and contribute to the progression of airway and alveolar diseases (8, 9, 13, 16–18, 36). The role of adrenergic receptors (ARs), specifically β2-ARs, and their downstream signaling pathways in epithelial restitution is of particular interest, because β-AR agonists are widely used in patients suffering from pulmonary disease (2, 23, 24, 44, 46). Airway epithelial cells expressing β1- and β2-ARs bind epinephrine to activate adenylyl cyclase and, subsequently, increase intracellular cAMP concentration. Agonists, such as isoproterenol, can also activate both of these receptor subtypes, whereas dobutamine and salbutamol selectively activate β1- and β2-ARs, respectively (1, 15). In the lungs, aerosolized β2-AR agonists, such as salbutamol (albuterol), terbutaline, and salmeterol, are used to target β2-ARs on airway smooth muscle to relieve bronchoconstriction and bronchospasms associated with asthma, cystic fibrosis, and COPD (5, 6, 28, 45). However, the effects of these drugs on airway epithelial migration and wound repair are not completely understood.

In a previous study, isoproterenol was shown to increase bovine bronchial epithelial cell wound repair between 4 and 6 h after wounding, similar to the effects of dibutyryl cAMP and various phosphodiesterase inhibitors, suggesting that increases in β-AR-mediated cAMP production were involved in stimulation of wound closure (47). Consistent with these findings, inhibition of protein kinase A (PKA) activity with KT-5720 or 4-cyano-3-methylsinoquinoline blocked stimulation of wound repair induced by isoproterenol, indicating that PKA-dependent phosphorylation was required to increase the rate of wound closure. Specifically, PKA-mediated phosphorylation was shown to reduce Rho activation and formation of focal adhesions, which appeared to be linked to enhanced cell migration and epithelial repair. Similarly, in later studies with A549 cells and primary distal lung epithelial cells, Perkins et al. (35) reported that salbutamol significantly enhanced epithelial restitution and that cotreatment with propranolol significantly blocked this effect. They speculated that β-AR agonist-dependent stimulation of PKA might enhance β-catenin phosphorylation and its subsequent accumulation within the cytoplasm and nucleus, resulting in activation of the Wnt/β-catenin signaling pathway and stimulation of epithelial restitution.

In contrast to the results of the limited number of studies using airway epithelia, experiments with corneal epithelial cells and keratinocytes from oral and dermal origins showed that stimulation with β-AR agonists significantly reduced the speed of cell migration and delayed the process of wound repair, whereas β-AR antagonists enhanced both migration speed and wound closure (11, 42, 48). The inhibitory effect of β-AR agonists on wound healing was shown to occur by a cAMP-independent mechanism involving protein phosphatase 2A (PP2A) activation and dephosphorylation of extracellular signal-regulated kinase (ERK), while stimulation of wound repair with β-AR antagonists was associated with an increase
in ERK phosphorylation. β2-AR agonists and antagonists were also shown to affect directional migration of keratinocytes and corneal epithelial cells. Another property of wound healing is the existence of an electrical field (EF) associated with epithelial disruption that serves as an important guidance cue for the establishment of directed cell migration toward the denuded area of the wound (30, 40, 49). Migration within this EF, termed galvanotaxis, was shown to be inhibited following treatment with low concentrations of β-AR agonists by a cAMP/PKA-dependent mechanism (39).

The notable difference in responses to β-AR agonists between pulmonary and other epithelial cell types suggests a novel mechanism for β-AR-dependent regulation of cell migration and wound repair in airways. Therefore, the objective of the present study was to investigate the effects of selected β-AR agonists and β-AR bias agonists and antagonists on human airway epithelial restitution using continuous impedance-sensing technology to measure cell migration. Unlike previous data reported for bovine airway epithelial cells, A549, and DLE cells, the results of the present study show that β-AR agonists effectively delay human airway epithelial restitution that is reproduced by stimulation with β-AR agonists known to activate the β-arrestin signaling pathway. These findings suggest that the development of β-AR agonists that induce G protein-dependent stimulation of adenyl cyclase while minimizing activation of cAMP-independent signaling pathways could be beneficial in reducing the inhibitory effects of β-AR agonists on airway epithelial wound repair.

MATERIALS AND METHODS

**Materials.** Eagle’s minimum essential medium with Earle’s salts (MEM), fetal bovine serum, nonessential amino acids, penicillin-streptomycin, and phosphate-buffered saline (PBS) were purchased from Life Technologies (Carlsbad, CA); normal human bronchial epithelial (NHBE) cells, bronchial epithelial cell growth medium [(BEGM) with bullet kits], and trypsin from Lonza (Allendale, NJ); bovine serum albumin (BSA), CFTR inhibitor (CFTRinh-172), 2-DEVD, epinephrine, salbutamol, and t-cysteine from Sigma-Aldrich (St. Louis, MO); the selective β2-AR antagonist ICI 118551 from Tocris Bioscience (Bristol, UK); bovine collagen type 1 from BD Biosciences (San Jose, CA); Texas Red-X-phalloidin and SlowFade Gold antifade from Invitrogen; and electric cell-substrate impedance-sensing (ECIS) arrays from Applied Biophysics (Troy, NY).

**Cell culture.** NHBE cells were maintained in complete BEGM with provided bullet kits. Human airway adenocarcinoma (Calu-3) cells were maintained in MEM containing 10% fetal bovine serum, 1% nonessential amino acids, 1% penicillin-streptomycin. All cells were maintained in a humidified 5% CO2 incubator at 37°C. Constitutive silencing of CFTR in Calu-3 cells was accomplished by expression of shRNAs (shCFTR cells) targeting the channel, as previously described (32, 33). Additionally, a non-CFTR-targeting, altered shRNA sequence was expressed in Calu-3 (shALTR) cells to produce a control cell line. Calu-3 cells expressing CFTR or ALTR shRNAs were grown under selection in the presence of 4 μg/ml puromycin.

**Cell migration experiments.** ECIS was used to measure NHBE and Calu-3 cell migration as previously described (43). Briefly, cells were grown on t-cysteine-coated 8W1E ECIS arrays and maintained in complete growth medium until fully confluent. Medium was then changed to serum-free (Calu-3 cells) or bullet kit-free (NHBE cells) conditions for 48 h prior to experiments to synchronize cells within the G1 phase of the cell cycle and minimize any effects of cell proliferation during the migration assays. A 250-μm wound was produced by electroporation (6 V, 30 kHz, 60 s) of cells in contact with the electrode. Cells were washed with PBS, and serum-free or bullet kit-free treatment medium was added. After the cells were wounded, the extracellular matrix initially laid down by the epithelium served as the substrate over which the epithelial cells migrated during wound closure. Impedance was measured using an alternating current (~1 μA, 15 kHz) that was continuously applied to the electrodes. The time to 50% of maximum impedance (Zmax) was used to assess differences in wound closure rate produced by the various treatment conditions used in the experiments. Cells were continuously treated with agonists, antagonists, or inhibitors starting 10 min prior to measurement of changes in impedance.

**ERK1/2 phosphorylation measurements.** ERK1/2 phosphorylation was determined using an In-Cell ELISA kit (Thermo Scientific). Calu-3 and NHBE cells were plated at 1 × 104 cells/well according to the manufacturer’s instructions. Cells were treated for 15 min with 10 μM carvediol or 10 μM salbutamol in serum-free or bullet kit-free medium; untreated cells served as controls. Cells were fixed using 4% paraformaldehyde for 15 min and then washed twice with 1× Tris-buffered saline (TBS) and incubated with 100 μl/well 1× permeabilization buffer for 15 min. Cells were washed once with 1× TBS, treated with 100 μl/well quenching buffer, washed with 1× TBS, and then incubated with blocking buffer for 30 min at room temperature. Blocking buffer was removed, and primary antibody (1:50 dilution) and phosphorylated antibody (1:2,000 dilution) were applied following the manufacturer’s instructions. Cells were incubated overnight at 4°C and washed three times with wash buffer; then they were incubated with 100 μl/well diluted horseradish peroxidase for 30 min and washed three times with wash buffer. Tetramethylbenzidine substrate (100 μl/well) was added to the cells at room temperature for 15 min, tetramethylbenzidine stop solution (100 μl/well) was added, and absorbency was measured at 450 nm.

**Lamellipodia area measurements.** A lamellipodia protrusion assay was performed using NHBE and Calu-3 cells under the following treatment conditions: 10 μM epinephrine, 20 μM CFTRinh-172, 20 μM CFTRinh-172 + 10 μM epinephrine, shALTR cells, shCFTR cells, and untreated control conditions using NHBE and wild-type Calu-3 cells. Chamber slides were coated with bovine collagen diluted in 0.01 N acetic acid. NHBE or Calu-3 cells were seeded on precoated slides and maintained using standard conditions and growth medium until wounded. Growth medium was changed to serum-free or bullet kit-free medium 48 h prior to experiments. Scratch wounds were made using a scalpel blade dipped in liquid N2; cells were washed in PBS and exposed to treatment conditions for 60 min, which has been shown to be an appropriate time for cells to produce lamellipodia and start the process of migration (43). Thereafter, cells were washed and fixed with 3.7% paraformaldehyde for 10 min at room temperature and then treated with 0.1% Triton X-100 for 4 min. Cells were then blocked with 1% BSA for 20 min and stained with Texas Red-phalloidin (5 U/ml) for 20 min. Images were taken with a fluorescence microscope using 590-nm excitation and 610-nm emission wavelengths. Lamellipodia pixel area was measured using ImageJ software (National Institutes of Health, Bethesda, MD) as previously described (43).

**Immunocytochemistry.** Calu-3 cells were fixed with 4% paraformaldehyde for 20 min, washed with PBS, and permeabilized with 0.1% Triton X-100 (Sigma-Aldrich). Nonspecific binding was blocked after 1 h incubation in 3% BSA. The apical surfaces were treated with primary antibody overnight at 4°C. The primary antibodies mouse anti-CFTR antibody (ab2784, Abcam, Cambridge, MA), goat anti-β2-AR antibody (ab40834, Abcam), and mouse anti-integrin-β1, activated, clone HUTS-4 monoclonal antibody (EMD Millipore) were used at 1:100 dilution. Cells were washed with PBS and incubated with secondary antibodies [donkey anti-mouse Alexa Fluor 568 (1:250 dilution); Life Technologies, Waltham, MA] and donkey anti-goat Alexa Fluor 647 (1:250 dilution; Life Technologies)] for 45 min. Nuclei were counterstained with 4’,6-diamidino-2-phenylindole. Monolayers were mounted on microscope slides using VECTASHIELD.
HardSet mounting medium (Vector Laboratories, Burlingame, CA). Images were captured using an Olympus FV1000 confocal microscope. In Fig. 7, fluorescence intensity was measured using ImageJ software.

**Statistical analysis.** Statistical significance was determined using an unpaired, two-tailed t-test or ANOVA followed by Dunnett’s test for multiple comparisons using GraphPad PRISM 6.0. \( P < 0.05 \) was considered significant.

**RESULTS**

**\( \beta\)-AR agonists inhibit airway epithelial cell migration.** The effect of \( \beta\)-AR agonists on airway epithelial cell migration was studied using impedance-sensing technology after wounding of confluent cell monolayers with a 60-s voltage pulse (6 V) applied at a frequency of 30 kHz. Images showing progress toward monolayer restitution over the surface of the electrode are shown in Fig. 1A. Impedance traces for control and \( \beta\)-AR agonist-treated NHBE cells are shown in Fig. 1B. In these experiments the plateau phase of the traces represents reestablishment of a confluent layer of cells, which was verified by visual inspection of the arrays. The impedance value at each time point was normalized to \( Z_{\text{max}} (Z/Z_{\text{max}}) \) obtained when the cells reached confluence. For untreated control monolayers, a significant difference was observed in the time required for complete restitution between NHBE (5.4 ± 0.4 h, \( n = 25 \)) and Calu-3 (7.2 ± 0.4 h, \( n = 15 \)) cells after wounding. Treatment of NHBE cells with the nonselective agonist epinephrine and the \( \beta_2\)-AR-selective agonist salbutamol delayed restitution.

Similarly, salbutamol also delayed Calu-3 cell restitution. Statistical comparisons between control and treatment conditions were performed by measurement of the time required to achieve 50% \( Z_{\text{max}} \). In both airway epithelial cell models, \( \beta\)-AR agonists significantly increased the time needed to reach 50% \( Z_{\text{max}} \). The \( \beta_1\)-AR-selective agonist dobutamine also increased the time to 50% \( Z_{\text{max}} \) in NHBE cells, but no effect was detected with Calu-3 cells. These results indicate that \( \beta_1\)-ARs contribute to the delay in restitution in NHBE, but not Calu-3, cells.

![Image](http://ajpcell.physiology.org/)

Fig. 1. \( \beta\)-Adrenergic receptor (AR) agonist stimulation of airway epithelial cells inhibits cell migration and epithelial restitution. A: serial images of Calu-3 cells during wound repair, beginning after wounding (0 min) and at 120 and 300 min as cells reestablished confluence over the electrode surface. Electrode disc, outlined in white, has a diameter of 250 \( \mu \)m. B: epinephrine (10 \( \mu \)M) and the \( \beta_2\)-AR-selective agonist salbutamol (10 \( \mu \)M) delay the normalized increase in impedance (\( Z/Z_{\text{max}} \)) that occurs as normal human bronchial epithelial (NHBE) cells migrate across the electrode surface and become confluent (\( n = 8 \) for each condition). C: salbutamol (10 \( \mu \)M) delays Calu-3 cell restitution following wounding (\( n = 6 \) for each condition). D: time needed to reach 50% \( Z_{\text{max}} \) (\( Z_{0.5} \)) for NHBE and Calu-3 cells. Epinephrine (10 \( \mu \)M) and salbutamol (10 \( \mu \)M) and the \( \beta_1\)-AR-selective agonist dobutamine (10 \( \mu \)M) significantly increased the time to 50% \( Z_{\text{max}} \) relative to control NHBE cells (\( n = 8 \) for each condition). Similarly, salbutamol (10 \( \mu \)M) significantly increased the time to 50% \( Z_{\text{max}} \) compared with control Calu-3 cells (\( n = 6 \) for control and salbutamol treatment conditions), whereas dobutamine (10 \( \mu \)M) had no effect on Calu-3 cell migration (\( n = 8 \) for control and dobutamine treatment conditions). ANOVA and Dunnett’s test were used to analyze NHBE cell comparisons between control, epinephrine, and salbutamol treatment conditions. Unpaired t-tests were used for analysis of Calu-3 cell data and for NHBE cell dobutamine experiment. *Significant differences between treatment and control conditions.
Additional pharmacological evidence in support of a role for \( \beta_2 \)-ARs as mediators of epinephrine-dependent inhibition of NHBE cell restitution is reported in Fig. 2. NHBE cells were treated with the nonselective \( \beta_2 \)-AR antagonist propranolol or the \( \beta_2 \)-AR-selective antagonist ICI 118551 prior to addition of epinephrine. As shown in Fig. 2A, epinephrine delayed wound repair; however, pretreatment with propranolol blocked the effect of epinephrine. Moreover, ICI 118551 also inhibited the epinephrine-induced delay in wound closure, although the effect was not as complete as that of propranolol (Fig. 2B). Statistical comparisons of the time to 50% \( Z_{\text{max}} \) (Fig. 2C) between control and the two antagonist treatment conditions demonstrate that \( \beta_2 \)-ARs and, in particular, the \( \beta_2 \)-AR mediate the inhibitory actions of epinephrine on NHBE cell wound closure.

Bias ligands known to activate \( \beta_2 \)-arrestin signaling inhibit NHBE and Calu-3 cell restitution. Carvedilol and isoetharine treatment delayed NHBE cell restitution, as demonstrated in Fig. 3A. The responses to these agonists were similar to those of epinephrine and salbutamol. Statistical analysis revealed that carvedilol and isoetharine significantly increased the time to reach 50% \( Z_{\text{max}} \), similar to the effects of \( \beta_2 \)-AR agonists. Treatment of Calu-3 cells with carvedilol also significantly increased the time to achieve 50% \( Z_{\text{max}} \) (Fig. 3, B and C). These results indicate that bias agonists that fail to induce G protein activation effectively reproduce the effects of epinephrine and salbutamol on airway epithelial cell restitution.

\( \beta_2 \)-AR agonists did not alter ERK1/2 phosphorylation. ELISA of ERK1/2 phosphorylation showed that stimulation of NHBE cells or Calu-3 cells with salbutamol or carvedilol did not significantly change the level of phosphorylation relative to untreated controls: 340 ± 32, 301 ± 38, and 325 ± 45 pg/mg protein for untreated control, salbutamol-treated, and carvedilol-treated NHBE cells, respectively (\( n = 6 \) for each treatment condition) and 173 ± 13, 156 ± 30, and 201 ± 30 pg/mg protein for untreated control, salbutamol-treated, and carvedilol-treated Calu-3 cells, respectively (\( n = 6 \) for each treatment condition).
Phosphatase inhibition with cantharidin and CFTR silencing blocks the effects of β-AR agonists on restitution. Figure 4A shows the results of experiments examining the effect of the PP2A inhibitor cantharidin on epinephrine-dependent inhibition of NHBE cell restitution. Cantharidin pretreatment blocked the delay in restitution induced by epinephrine. Measurements of the time needed to reach 50% restitution showed that cantharidin treatment alone had no effect on restitution and that epinephrine in the presence of cantharidin was unable to produce a significant delay in restitution (Fig. 4B). These findings indicate that phosphatase activity is necessary to observe the inhibitory effect of epinephrine on NHBE cell restitution. The effects of CFTR silencing on the inhibitory effect of salbutamol are shown in Fig. 4C. Treatment of shALTR cells with salbutamol increased the time to 50% Zmax to the same extent as in wild-type cells, but no effect of salbutamol could be detected in shCFTR cells. These results suggest the existence of a common mechanism that produces the previously documented decrease in cell migration caused by CFTR silencing (43) and inhibition of epithelial restitution associated with β-AR agonists.

Colocalization of β2-AR and CFTR in Calu-3 cells. Confocal images (×200 magnification) in Fig. 5A show that the β2-AR is expressed in wild-type, shALTR, and shCFTR cells, providing evidence that shCFTR cells continue to express the β2-AR while CFTR expression was reduced by RNAi. This result indicates that the absence of an effect of salbutamol on wound repair in shCFTR cells is not the result of a loss of β2-AR expression. Figure 5, B and C, shows β2-AR and CFTR labeling at higher magnification (×600) in wild-type, shALTR, and shCFTR cells, and merged images in Fig. 5D show colocalization of the β2-AR and CFTR in the apical membrane of wild-type and shALTR cells.

Lamellipodia area during migration is reduced following treatment with epinephrine. Treatment of NHBE and Calu-3 cells with β-AR agonists reduced lamellipodia area during migration. Figure 6A shows NHBE cells in the absence or presence of epinephrine, where cells were labeled with Texas Red-phalloidin to detect actin filaments within the lamellipodia 60 min after wounding. Figure 6B shows images obtained from untreated control and salbutamol (10 μM)-treated Calu-3 cells. In Fig. 6C, pixel areas associated with lamellipodia are shown. Treatment with salbutamol significantly reduced lamellipodia area in shALTR Calu-3 cells but had no effect in shCFTR cells. Additionally, epinephrine treatment decreased lamellipodia area in NHBE cells to a level comparable to that in cells treated with CFTRinh 172. These results suggest that the inhibitory effect of β-AR agonists and reduced CFTR expression on restitution are associated with reductions in the extent of lamellipodia protrusion into the wound.

Epinephrine and carvedilol inhibit β1-integrin activation in the basal membrane. The effect of epinephrine (10 μM) and carvedilol (10 μM) on activation of β1-integrin was determined by confocal imaging of the basal membrane of Calu-3 cells labeled with anti-β1-integrin antibody (clone HUTS-4) that selectively binds to the activated conformation of the integrin (Fig. 7). Measurements of mean fluorescence intensity within eight 150 × 150 pixel sections of control, epinephrine-treated, and carvedilol-treated images revealed significant reductions in fluorescence intensity following 30-min exposures to epinephrine and carvedilol compared with untreated control conditions. No significant difference in fluorescence intensity was detected between epinephrine and carvedilol treatment.

DISCUSSION

In contrast to previous findings with bovine bronchial epithelial cells, data from the present study show that activation of β-ARs with epinephrine, an endogenous catecholamine agonist, or the selective β1-AR agonist dobutamine or the selective β2-AR agonist salbutamol significantly inhibits NHBE cell migration and wound repair. Salbutamol produced a similar result in Calu-3 cells, but no effect was observed in response to dobutamine, suggesting that β1-ARs were either not available for activation or not coupled to signaling pathways that regulate cell motility. These results were supported by experiments with the nonselective β-AR antagonist propranolol and the selective β2-AR antagonist ICI 118551 demonstrating that the primary receptor subtype responsible for the inhibitory effect of β-AR agonists on migration and wound repair was the β2-AR. These findings were consistent with previous studies on keratinocyte and corneal epithelial cell restitution, which

Fig. 4. Inhibition of protein phosphatase 2A (PP2A) activity blocks the effect of epinephrine on airway epithelial cell restitution. A: pretreatment with the PP2A inhibitor cantharidin (10 μM) blocked epinephrine-dependent inhibition of NHBE cell restitution (n = 4 for each condition). B: time to 50% Zmax for treatment conditions described in A. Cantharidin (Can) treatment alone had no effect on time to 50% Zmax (n = 4 for each condition). C: effects of salbutamol (10 mM) on time to 50% Zmax in Calu-3 cells expressing a non-CFTR-targeting, altered (ALTR) shRNA sequence and CFTR shRNA (n = 8 for each condition). An unpaired t-test was used for comparisons between control and treatment conditions. *Significant differences between control and treatment conditions.
showed that β-AR activation significantly delayed migration and wound closure (41). Furthermore, these experiments revealed that, at higher concentrations of β2-AR agonists, decreases in migration rate and wound closure involved a cAMP-independent mechanism. To explore this possibility in airway epithelial cells, the effects of two β2-AR bias agonists, carvedilol and isoetharine, were examined. Carvedilol, a third-generation β2-AR antagonist, blocks β1- and β2-ARs while inhibiting β1-ARs to produce vasodilation (1). It is used in the treatment of chronic heart failure and functions as a bias ligand to induce an altered conformational state of β2-ARs that inhibits G protein activation and subsequent adenylyl cyclase stimulation while facilitating β-arrestin-dependent signaling and receptor desensitization (10, 14, 25, 27, 34). Treatment of airway epithelial cells with carvedilol or isoetharine produced significant delays in migration that were comparable to the effects of epinephrine and salbutamol. These responses suggest that inhibition of airway epithelial wound repair by β2-AR agonists is also cAMP-independent and may be associated with activation of β-arrestin-mediated signaling.

Previous investigations have demonstrated that carvedilol binding to β2-ARs results in ERK1/2 phosphorylation and EGFR transactivation (25, 51). This effect on ERK and EGFR activation parallels results with the β2-AR antagonist timolol, which increased ERK phosphorylation two- to fourfold in murine corneal epithelial cells (11). Increased ERK phosphorylation
ylation was associated with enhanced migration speed, whereas the β-AR agonist isoproterenol reduced migration speed and ERK phosphorylation. In this study, ELISAs to detect ERK phosphorylation showed that salbutamol and carvedilol stimulation did not affect ERK phosphorylation, unlike the actions of isoproterenol or timolol in corneal epithelial cells (11). Although these negative findings are not completely conclusive, they do suggest that β-AR ligands are capable of inhibiting airway epithelial cell restitution by mechanisms that may not involve changes in ERK phosphorylation.

A key enzyme that was activated following β-AR agonist stimulation of keratinocytes and corneal epithelial cells was the PP2A, and inhibition of its activity has been shown to block the actions of β-AR agonists on epithelial wound healing (37–39, 41). PP2A activation was associated with ERK dephosphorylation and a decrease in kinase activity, ultimately resulting in reduced activation of specific ERK substrates that regulate cell migration, including myosin light chain kinase, focal adhesion kinase, and calpain (19). These substrates participate in processes such as lamellipodia protrusion and focal adhesion dynamics that are critical for cell motility. Experiments reported in the present study also showed that treatment with a selective PP2A inhibitor completely blocked the effect of β-AR activation on wound closure. Some possible candidate proteins known to interact with PP2A are identified in Fig. 8.

Previous studies have shown that Akt (protein kinase B), cofilin, and Nedd9 (Cas-L or HEF1) are PP2A substrates that play key roles in integrin signaling and cell migration (4, 21, 22).
Akt activation following phosphatidylinositol 3-kinase-mediated phosphorylation or integrin binding to collagen, for example, has been shown to enhance migration through phosphorylation of the actin-binding protein girdin, which is thought to be important in regulating actin reorganization (21, 22). Activation of PP2A decreases Akt phosphorylation and kinase activity, leading to a decrease in lamellipodia protrusion and motility. Similarly, dephosphorylation of coflin by PP2A inhibits migration in a variety of cell types by promoting actin depolymerization, thus destabilizing the actin filament network essential for lamellipodia and filopodia protrusion (31). Additionally, Nedd9 is an adhesion docking protein that is part of the signaling platform that forms at focal adhesion sites. Serine/threonine and tyrosine phosphorylation modulate Nedd9 interactions with other proteins associated with these sites, thus promoting its signaling function. Dephosphorylation by PP2A alters integrin adhesion dynamics by disrupting these interactions and reducing cell spreading (4). Consequently, PP2A functions as a pleiotropic regulator of signaling pathways linked to cell motility, and it would not be surprising that disruption of one or more of these pathways could lead to a significant decrease in cell migration and wound closure.

Studies of β-AR-dependent regulation of anion secretion by human airway epithelial cells have shown that β2-ARs and CFTR interact through scaffolding proteins to form a signaling complex in the apical membrane (26, 29), allowing luminal β-AR agonists to directly activate receptors without having to access the basolateral surface of the epithelium. This colocalization of β2-ARs and CFTR was confirmed in the present study by immunocytochemistry. Furthermore, it was shown that constitutive silencing of CFTR by expression of shRNAs targeting the channel blocks the inhibitory effects of β-AR agonists on epithelial restitution. In prior studies, treatment with CFTRinh 172 or silencing of CFTR by RNAi significantly delayed wound closure by at least two mechanisms shown in Fig. 8, one involving inhibition of galvanotaxis and the second through depletion of GM1 ganglioside and cholesterol levels within the plasma membrane with an associated decrease in β1-integrin, p130 Cas, and focal adhesion kinase phosphorylation (20, 49). Moreover, measurements of wound currents in an ex vivo primate trachea preparation showed that anion transport by CFTR significantly contributed to the injury-evoked EF, providing a guidance cue for directed migration into the wound (49). In the present study, inhibition of epithelial restitution following carvedilol stimulation occurs in the absence of G protein activation; therefore, it is unlikely that direct inhibition of CFTR channel activity was responsible for the delay in wound closure. However, an earlier study showed that PP2A was recruited to GM1 ganglioside-containing non-caveolar lipid raft microdomains that are located at the leading edge of lamellipodia (3). Interestingly, cholesterol depletion was found to inhibit PP2A recruitment into these lipid raft domains. Additionally, PP2A was shown to form a complex with β1-integrin, where it catalyzes dephosphorylation and facilitates interactions with actin filaments to stabilize cell adhesion (50). These earlier findings suggest a possible explanation for why loss of CFTR function blocks the inhibitory effect of β-AR agonists and carvedilol on airway epithelial repair. We speculate that β1-integrins in CFTR-deficient cells are already dephosphorylated as a consequence of GM1 ganglioside and cholesterol depletion from the plasma membrane and that PP2A activation following stimulation with β-agonists cannot further decrease integrin activation. Under these conditions, cell adhesion at the leading edge of lamellipodia is stabilized, thus limiting the dynamic regulation of cell attachments necessary for lamellipodia protrusion. This idea is consistent with the results of experiments where lamellipodia area was measured following exposure to β-AR agonists and CFTRinh 172 or CFTR silencing. β-AR agonist stimulation significantly reduced lamellipodia area projecting into the wound to an extent comparable to CFTR inhibition. Furthermore, experiments showing reduced anti-β1-integrin labeling within the basal membrane of Calu-3 cells provided direct evidence for inhibition of integrin activation following epinephrine or carvedilol treatment.

Conclusions. The findings of this investigation indicate that the inhibitory actions of β-AR agonists on migration and wound repair are consistent with previous data from keratinocytes and corneal epithelium, supporting the concept of a common mechanism of β-AR regulation of migration and restitution in epithelial cells. The observation that β-AR bias ligands, such as carvedilol, reproduce the effects of epinephrine and salbutamol suggests that the delay in wound repair involves a cAMP-independent regulatory pathway. Furthermore, data showing that the effect of epinephrine on migration is completely blocked by pretreatment with cantharidin indicate that the decrease in cell migration was linked to increases in PP2A activity. Since PP2A regulates the phosphorylation state of multiple proteins involved in cell motility, we speculate that dephosphorylation of one or more of these proteins produced the decrease in lamellipodia protrusion and cell migration observed in this study. Overall, these results suggest that a potential negative side effect of β-AR agonist therapy for treatment of respiratory disease is delayed wound healing, which could potentially increase susceptibility to infection. The development of new β2-AR agonists capable of inducing G protein-dependent increases in cAMP without stimulating PP2A activity may provide a treatment that reduces bronchoconstriction without compromising airway epithelial repair.

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The development of new β2-AR agonists capable of inducing G protein-dependent increases in cAMP without stimulating PP2A activity may provide a treatment that reduces bronchoconstriction without compromising airway epithelial repair.

REFERENCES

β-ADRENERGIC RECEPTOR AGONISTS INHIBIT AIRWAY WOUND REPAIR

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