Relaxin Stimulates Bronchial Epithelial Cell PKA Activation, Migration, and Ciliary Beating[1]

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Relaxin is an insulin-like serum protein secreted during pregnancy and found in many tissues, including the lung. Relaxin is reported to stimulate epithelial cell proliferation, but the effects of relaxin on airway epithelium are unknown. We tested the hypothesis that relaxin would stimulate the increased migration of bronchial epithelial cells (BEC) in response to wounding. Using monolayers of BEC in a wound-healing model, relaxin augmented wound closure with maximal closure occurring at 12 hr (1 μM). Unlike cytokines, relaxin did not stimulate increased BEC interleukin-8 (IL-8) release. Relaxin caused a significant stimulation of ciliary beat frequency (CBF) in BEC. Because protein kinase (PKA) activation increases CBF and relaxin can elevate intracellular cAMP levels, we measured PKA activity in BEC treated with relaxin. Relaxin increased PKA activity 3-4 fold by approximately 4 hr, with a return to baseline levels by 8–10 hr. Relaxin-stimulated PKA activity differs temporally from the rapid (1 hr) β-adrenergic activation of PKA in BEC. These data suggest that relaxin augments epithelial repair by increasing airway cell migration and CBF via PKA-dependent mechanisms. Exp Biol Med 227:1047–1053, 2002

Key words: airway epithelial; wound healing; relaxin; cAMP; cilia

Relaxin is a peptide hormone with structural homology to insulin (1). Originally identified as a pregnancy-related hormone, a number of other functions have now been identified for relaxin in a variety of tissues, including the lung (2, 3). In an ovalbumin-sensitized guinea pig model of asthma, relaxin reduced the severity of respiratory abnormalities, as well as histological alterations, mast cell degranulation, and leukocyte infiltration, suggesting an antiasthmatic property of relaxin (4). In a prospective clinical study, decreased asthma severity during pregnancy correlates with elevated relaxin levels (5). In addition to modulating inflammatory responses in asthma, relaxin has been shown to stimulate epithelial cell proliferation in rat cervix (6). Relaxin has also been proposed to inhibit the proliferation of human lung fibroblasts and to reduce fibrosis in a mouse lung model (7). Thus, by the dual action of promoting epithelial cell proliferation and inhibiting fibroblast proliferation, relaxin may play an important role in the repair of injured lung tissue.

In addition to inhibiting fibrotic events and stimulating epithelial cell growth, relaxin may promote effective mucociliary clearance. Ciliary beating is required for the proper maintenance of lung mucociliary transport. Cyclic nucleotides have been implicated in regulating ciliary motility, as intracellular increases in cAMP are associated with increased ciliary beat frequency (CBF) in mammalian cells (8). Our studies suggest that cAMP and the cAMP-dependent protein kinase (PKA) regulate CBF in bovine epithelial cells (BEC) and human airway epithelial cells (9). Relaxin has been shown to signal via cAMP and PKA in anterior pituitary cells (10) and atrial myocytes (11) of rat. We have also previously demonstrated that interleukin-8 (IL-8) is a major pro-inflammatory chemokine released by BEC in response to some agents capable of modulating CBF (12), connecting this mediator with chronic airways disease. The cellular mechanism(s) underlying relaxin function in the lung are not known. The lung has been shown to be a target tissue for the binding of exogenous injected relaxin in tracer studies (13).

We hypothesize that relaxin stimulates the migration of epithelial cells resulting in airway repair and that it improves clearance in the lung via a PKA-mediated mechanism. To test this hypothesis, we have evaluated the effects of relaxin on airway epithelial cell function using *in vitro* models of epithelial cell wound healing and ciliary beating. We have correlated these functional assays with the release of IL-8 as a marker for pro-inflammatory cytokine release

[1] This work was supported in part by the National Institutes of Health (grant 5 RO1 AA08769-07 to J.H.S.) and by Veterans Affairs Merit Review Grants to T.A.W. and J.R.S.

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Received April 1, 2002.
Accepted July 25, 2002.

1535-3702/02/22711-1047$15.00

and PKA activity as a marker for stimulated ciliary beating. Understanding the effects of relaxin on airway epithelial cells will help to discern its function in lung repair in response to injury.

Materials and Methods

Cell Preparation. Bovine BEC (BBEC) were prepared from bovine lung obtained fresh from a local abattoir. Bronchi were necropsied from the lung, cleaned of adjoining lung tissue, and incubated overnight at 4°C in 0.1% bacterial protease (type IV) in minimum essential media. After the overnight incubation, the bronchi were rinsed in Dulbecco's modified Eagles medium (DMEM) with 10% fetal calf serum repeatedly to collect the cells lining the lumen. These cells were then filtered through a 250-μm nylon mesh and then washed again in DMEM. This technique typically produces a high viability cell preparation of >95% epithelial cells (14). The cells were then plated in 1% type I collagen-coated 100-mm polystyrene petri dishes at a density of 1×10^4 cells/cm^2 in a 1:1 media mixture of LHC-9 and RPMI (15). Cell incubations were performed at 37°C in humidified 95% air/5% CO^2. Each dish contained approximately 2 mg of total cellular protein. Because of antibody specificity requirements in the IL-8 assay, human BEC (HBEC) were obtained by endobronchial brushings of patients undergoing flexible fiber optic bronchoscopy (16) and were used in IL-8 measurements for up to eight passages. Cell viability was determined by cell media assay of lactate dehydrogenase (LDH) release using a commercially available kit (Sigma, St. Louis, MO). An experiment consisted of homogenates prepared from three individual cell culture dishes and each of the three was assayed separately. Each experiment was repeated a total of three times for $n = 9$.

***In Vitro* Wound Closure (Migration) Assay.** Primary cultures of BBEC are grown to confluence in 96-well flat-bottomed tissue culture dishes. The cell monolayers are "wounded" with a small sterile scraper to remove a circular area of cells, approximately 1000 μm^2. The progress of migration is monitored with a phase contrast microscope outfitted with a video camera. The camera output is captured with image analysis software (NIH Image) on a Macintosh G-3 computer. Each wound is photographed with the video camera and image analysis software at specified time points and the area of the wound is measured. The dish is returned to the incubator between the measurements. As cells migrate into the wound, the open area of the wound is correspondingly reduced. The 96-well dish setup allows the assay of many different treatment conditions in triplicate, simultaneously. In this assay, control media-treated cells normally proliferate and migrate into the wounded area until wound closure is achieved at approximately 20–24 hr.

Kinase Activity Assay. PKA activity was determined in crude whole-cell fractions of BBEC. The assay used was a modification of procedures previously described (17) using 130 μ*M* PKA substrate heptapeptide (LRRASLG), 10 μ*M* cAMP, 0.2 m*M* IBMX, 20 m*M* magnesium acetate, and 0.2 m*M* [γ-^{32}P] ATP in a 40 m*M* Tris-HCl buffer (pH 7.5). PKG activity was assayed in a similar manner to PKA, with the substitution of the peptide RKRSRAE for the heptapeptide substrate, the addition of 10 μ*M* cGMP, and the presence of protein kinase inhibitor peptide (PKI). PKC activity was assayed by Biotrak kit (Amersham, Buckinghamshire, UK). Samples (20 μl) were added to 50 μl of the above reaction mixture and were incubated for 15 min at 30°C. Reactions were initiated by the addition of 10 μl of cell fraction diluted 1:10 with KPEM and 0.9 mg/ml bovine serum albumin (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 m*M*), washed once in ethanol, dried, and counted in nonaqueous scintillant. Kinase activity was expressed in relationship to total cellular protein assayed and is calculated in picomoles per minute per milligram. At least three individual culture dish samples were assayed in duplicate or triplicate per experiment, and no less than three separate experiments from three different animals were performed per unique parameter. Data was analyzed for significance using one-way analysis of variance (ANOVA).

Cytokine Release Assay. IL-8 levels in culture supernatants were quantified using a sandwich ELISA. Polystyrene microtiter plates (96-well flat-bottomed; Dynatec, Chantilly, VA) were coated with 200 μl/well of purified (goat) anti-human IL-8 antibody (R&D Systems, Minneapolis, MN) or IL-6 antibody (ICN Biomedicals, Costa Mesa, CA) diluted 1:2000 in Voller's buffer (pH 9.6) for 24 hr at 4°C. After washing plates three times in phosphate-buffered saline (PBS)-Tween 20, undiluted culture supernatants and human rIL-8 standards (Sigma) were applied to the plates and incubated at room temperature for 90 min. Plates were washed three times with PBS-Tween 20, followed by the addition of (rabbit) anti-human IL-8 antibody (UBI, Lake Placid, NY) or IL-6 antibody (Sigma) diluted 1:4000 in PBS Tween/BLOTTO (0.2% instant nonfat milk in PBS-Tween 20) for 60 min. After three washes, human serum-absorbed peroxidase-conjugated (goat) anti-rabbit IgG (ICN Biomedicals) was added at 1:2000 in PBS-Tween/BLOTTO for a 45-min incubation. The plates were again washed three times and 200 μl/well of peroxidase substrate (10 ng/ml orthophenylenediamine [Sigma] and 0.003% H_2O_2 in dH_2O) was added. The reaction was terminated with 27.5 μl/well of 8 *M* sulfuric acid, and plates were read at 492 nm in an automated ELISA reader (Bio-Rad, Hercules, CA).

CBF. Actively beating ciliated cells were observed, and their motion was quantified by measuring CBF using phase contrast microscopy, videotape analysis, and computerized frequency spectrum analysis. Ciliated cells in culture were maintained at a constant temperature (24° ± 0.5°C) by a thermostat-controlled heated stage. All images were visualized using an inverted phase-contrast microscope with a 20× objective lens with a 1.5× tube multiplier (IMT-2;

Olympus, Melville, NY). Video images were captured with a video camera (WV-D5000; Panasonic, Secaucus, NJ). Analog video recordings were made using an sVHS video cassette recorder (AG-1980; Panasonic). The analog video output sampled the image at 30 frames/sec (fps). Beat frequency analysis was later performed by analyzing videotaped experiments. For display and analysis, the analog video signal was split and modified by a custom made video signal processor (University of Nebraska Biomedical Instrumentation Department). This processor performed three tasks: an unmodified video image was routed to a television monitor for viewing and selecting of regions of interest (ROIs); a moveable sizable window was superimposed on the video image to indicate the ROIs to be analyzed; and the light intensity of the video signal contained within the ROI was averaged into an analog output light intensity signal that was routed into the computer for frequency analysis. A computer (Macintosh Iici; Apple Computer, Cupertino, CA) received the analog signal where an analog-to-digital converter board (A/D; National Instruments, Austin, TX) created a digital light intensity signal. This digital signal was then analyzed using customized software written in LabView v 2.1 (National Instruments). The software functioned as a virtual instrument to display a time versus amplitude waveform of the ROI as a virtual strip chart recorder; to perform a power spectrum analysis (using fast-Fourier transformation or FFT) and display the number of readings versus frequencies over a 0–15 Hz range present in the ROI; and to determine the dominant frequency among those displayed across the range of frequencies. All frequencies represent the mean ± 1 SEM from six separate cell groups or fields.

Materials. Porcine relaxin was obtained from A.F. Parlow and the National Hormone and Peptide Program (http://www.humc.edu/hormones). Type I collagen (Vitrogen 100) was purchased from Collagen Biomaterials (Palo Alto, CA). The LHC-9 basal medium was purchased from Biofluids (Rockville, MD). The RPMI-1640, DMEM, MEM, streptomycin-penicillin, and fungizone were purchased from GIBCO (Chagrin Falls, OH). Extraction of frozen bovine pituitary glands (Pel Freez, Rogers, AR), an essential component of the LHC-9 cell culture media, was performed as previously described and yielded an extract containing 10 mg/ml protein (15). The [γ^{32}P]-ATP was purchased from ICN Biomedicals; the phosphocellulose P-81 paper was purchased from Whatman (Clifton, NJ); and the peptides for kinase assays was purchased from Peninsula Laboratories (Belmont, CA). All other reagents not specified were purchased from Sigma.

Results

Depending upon the cell type, relaxin has been reported to either inhibit or promote cellular proliferation in wound healing models. To determine the effects of relaxin on airway epithelial cell wound repair, a wound repair model of cultured BBEC was used that models the rate of wound closure in serum-free monolayers of cells (18). Relaxin (1 n*M* and 1 μ*M*) accelerated the rate of wound healing several hours earlier (8–10 hr) versus control media (Fig. 1), resulting in earlier wound closure. The effects of relaxin were much more pronounced than those of equal concentrations of insulin, which had only a modest effect on promoting wound healing (data not shown). None of the concentrations of relaxin used in this study were associated with any significant cell toxicity as determined by cellular LDH release (data not shown). These data suggest that relaxin binds to and signals BEC to accelerate or augment their response to wound healing.

The mechanism of relaxin-mediated wound healing acceleration was studied by assaying various serine-threonine kinase activities in BBEC during wound repair. Recently, we established that BBEC migration in response to wound healing requires the elevation of intracellular cAMP levels and the subsequent activation of PKA (18). However, we have also observed that stimulated BBEC migration in response to tumor necrosis factor α (TNFα) is associated with the activation of PKCβ (19). Because intracellular kinases are critical for wound healing to occur, BBEC protein kinase activities were measured in response to relaxin treatment. Relaxin time dependently activated PKA with the maximal activity ratio observed at 1 μ*M* relaxin stimulation

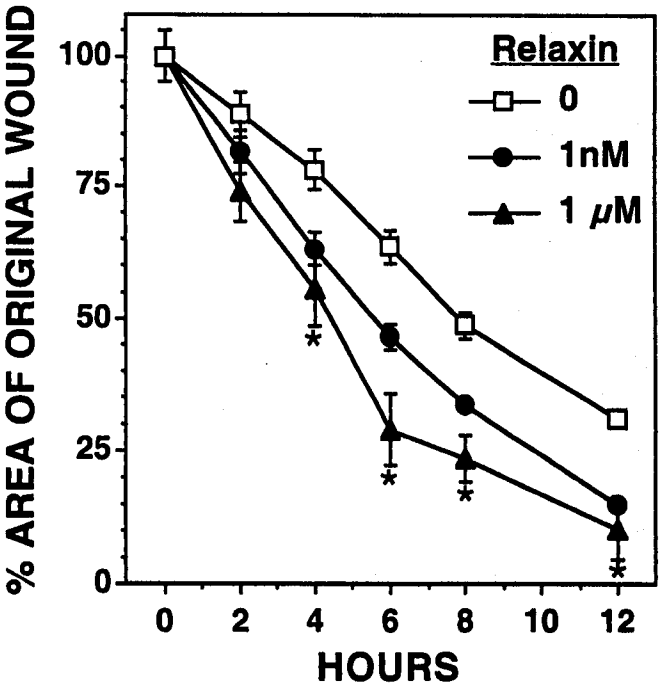

Figure 1. Relaxin accelerates wound repair in BBEC. Confluent monolayers of BBEC were wounded as described in "Materials and Methods." Cells were incubated in serum-free media (LHC9-RPMI) in the presence or absence of 1 n*M* or 1 μ*M* relaxin until wounds were closed as determined by wound area digital imaging. Increasing concentrations of relaxin accelerated the rate of wound closure (~12 hr) compared with media control wound closure (~22 hr). Bars represent SEM from three individual cell culture dishes assayed separately for each experiment. Each experiment was then repeated a total of three times for $n = 9$. Significance ($P \leq 0.05$) is indicated by an asterisk.

of BBEC (Fig. 2). As expected, relaxin stimulated a 3-fold increased production of cAMP levels in BBEC (data not shown). The maximal PKA activity profile occurred at 4 hr after relaxin exposure, which preceded the stimulated acceleration of wound healing. Similar to previous reports, PKA activity is not sustained in BBEC, but rather returns to baseline levels over time (9). As expected, preincubation of the cells for 1 hr with a specific inhibitor of PKA, KT5720 (1 μM), resulted in the inhibition of relaxin-stimulated PKA activity (data not shown). As a control, no change in the cGMP-dependent protein kinase (PKG) was observed in BBEC stimulated with any concentration of relaxin used (Fig. 2). Relaxin did not significantly alter PKC activity in BBEC over the same time course tested (Fig. 2). These data suggest that relaxin specifically signals the acceleration of wound healing in BEC via activation of PKA, but not PKC or PKG.

Relaxin-stimulated wound healing differs from PKC-mediated stimulation of BEC migration by TNFα. TNFα is also associated with a significant transcriptional production and secretory release of IL-8 in airway epithelial cells (20–22). To determine if relaxin treatment has an effect on the production of the pro-inflammatory chemokine, IL-8 release was measured in HBEC stimulated with the same concentrations that produced accelerated monolayer wound closure. No significant release of IL-8 was detected when HBEC were stimulated with any concentration of relaxin used to effect wound closure (Fig. 3). In contrast, TNFα elicited a significant release of IL-8 by HBEC. These data demonstrate that relaxin is not associated with epithelial cell release of proinflammatory mediators as compared with agents that signal wound healing via PKC (such as TNFα).

PKA activation regulates the stimulated increase in CBF observed in BBEC treated with β agonists (9) and ethanol (23). To examine the effect of relaxin on CBF, ciliated BBEC were stimulated with various concentrations of relaxin, and changes in the frequency of cilia beating were measured by video computer analysis. Baseline control media-treated cells demonstrated no change in CBF from 0 to 48 hr (Fig. 4). However, relaxin (1 μM) stimulated a slow elevation in CBF up to 6 hr. This elevated CBF was observed beyond 24 hr, with a return to baseline levels only by 48 hr (Fig. 4). This sustained elevation of CBF by relaxin differs dramatically from the CBF profile of β agonist-stimulated CBF. As demonstrated in Figure 5, isoproterenol (100 μM) stimulates increases in CBF very rapidly, but this elevation plateaus by approximately 2 hr, and returns to baseline CBF levels within 4–6 hr. Similarly, the activation of PKA is precedent to and temporally coincides with the observed elevations in CBF. The subsequent decrease in CBF tracks the declining PKA activity levels until both return to baseline. Unlike isoproterenol, relaxin elevates CBF at a much later time point (4 hr), and does not reach

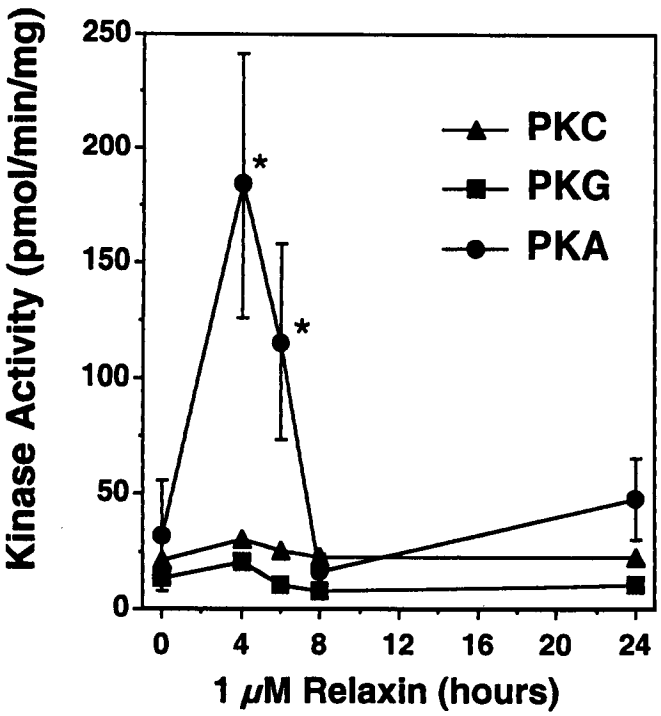

Figure 2. Relaxin activates PKA in BBEC. Confluent monolayers of BBEC were stimulated with 1 μM relaxin and flash frozen in cell lysis buffer (see "Materials and Methods"). Cell homogenates were assayed for PKA, PKG, and PKC activity. Relaxin stimulated a 3- to 4-fold activity increase in PKA at approximately 4 hr of treatment. No change in PKG or PKC activity was observed in cells treated with relaxin. Bars represent SEM of homogenates prepared from three individual cell culture dishes assayed separately for each experiment. Each experiment was then repeated a total of three times for $n = 9$. Significance ($P < 0.005$) is indicated by an asterisk.

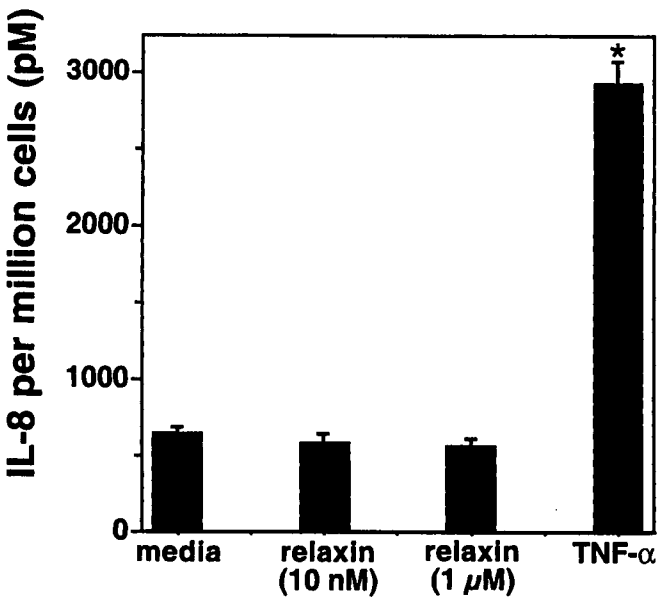

Figure 3. Relaxin does not stimulate IL-8 release in HBEC. Confluent monolayers of HBEC were stimulated with 10 nM or 1 μM relaxin for 18 hr, and cell culture supernatants were collected. IL-8 release was measured by ELISA. Relaxin did not stimulate a significant change in IL-8 release as compared with media controls. The positive control, TNFα (20 ng/ml), stimulated approximately a 6-fold elevated release of IL-8 in HBEC during the same incubation period. Bars represent SEM from three individual cell culture dishes assayed separately for each experiment. Each experiment was then repeated a total of three times for $n = 9$. Significance ($P \leq 0.0001$) is indicated by an asterisk.

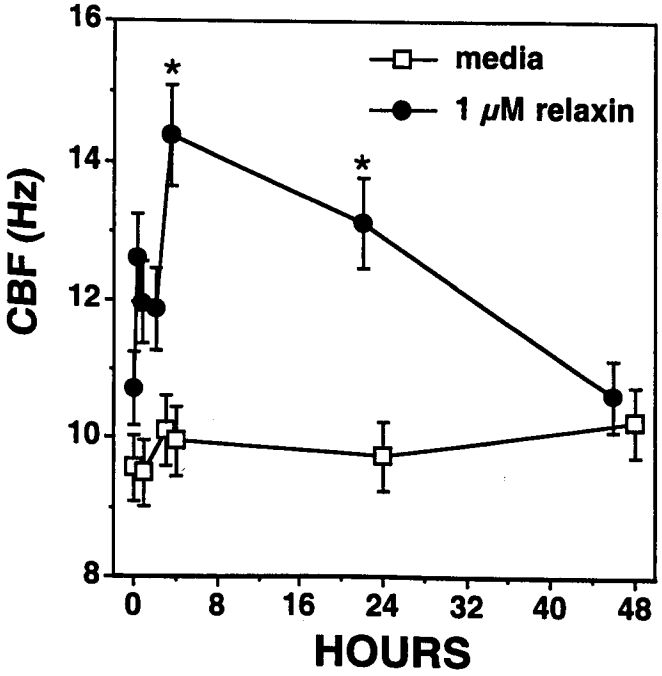

Figure 4. Relaxin increases ciliary beat frequency in BBEC. Ciliated BBEC were cultured as primary cells from a bovine bronchus and were analyzed for changes in CBF in the presence or absence of relaxin. Relaxin (1 μ*M*) stimulated an increase in CBF from baseline beating (~10 Hz) to a maximal CBF of over 14 Hz by 6 hr. This relaxin-elevated CBF was sustained beyond 24 hr before returning to baseline CBF at 48 hr. Bars represent SEM from three individual cell culture dishes assayed separately for each experiment. Each experiment was then repeated a total of three times for $n = 9$. Significance ($P \leq 0.02$) is indicated by an asterisk.

maximal CBF stimulation until 6–12 hr (Fig. 5). Likewise, the stimulation of PKA activity by relaxin is later and correlates with relaxin-stimulated CBF. Relaxin-stimulated PKA activity returns to baseline after 8 hr, whereas relaxin-stimulated CBF remains elevated to at least 24 hr (Fig. 4), suggesting a subsequent uncoupling of the direct regulation of CBF by PKA in relaxin-treated BBEC. These data suggest that relaxin stimulates a slower and sustained elevation of CBF in BEC that is initially triggered by PKA activation.

Discussion

Serum concentrations of relaxin are near the level of detection except during pregnancy. *In vivo*, local tissue concentrations are not well characterized. *In vitro* studies usually involve relaxin concentrations of 1–100 ng/ml, but some go as high as 1 μg/ml. Recently, two orphan G-protein-linked membrane receptors for relaxin have been shown to exist in several divergent tissues (24). Indeed, one of these receptors, LGR7, is expressed in lung, and functions to mediate a relaxin-stimulated cAMP pathway (24). Although evidence exists that relaxin can and does bind to receptors in lung tissue, the functional role that relaxin plays in lung is unclear. This study demonstrates that relaxin produces several functional changes in BEC. Similar to effects shown in cervical epithelium, relaxin stimulates *in vitro* wound closure of airway epithelium. Relaxin may function to stimulate the repair of damaged airway epithelium by increasing the ability of the epithelial cells to migrate in response to wounding. This concept is supported by the finding that the expression of a gene encoding a preprorelaxin-like protein occurs in the tracheal epithelial cells of rabbit when these cells are differentiated into the rapidly proliferative squamous phenotype (25). Multiple functions of relaxin in airway epithelium may be related to the presence of multiple relaxin genes present in lung (26).

Relaxin may differ from other promigratory agents in that no release of pro-inflammatory cytokines appears to be evoked. This is evidenced by the fact that relaxin treatment of HBEC is not associated with the increased release of IL-8. Our studies have shown that IL-8 release is regulated under many conditions by the activation of PKC in BEC (12, 27, 28). We have previously established that BBEC migration can be PKC mediated (19). However, relaxin-stimulated wound healing is associated with the activation of PKA, whereas TNFα-stimulated IL-8 release (see Fig. 3) is mediated via the action of PKC. Although a complete profile of cytokine release remains to be established, relaxin may represent a physiologic agent capable of augmenting airway epithelial repair without also promoting additional airway inflammation and remodeling. A recent study supporting this conclusion reports that relaxin does not alter lung resident macrophage cytokine expression at nonwound sites (29). This concept is also supported by recent studies demonstrating that relaxin inhibits the production and secretion of type I collagen in rat liver stellate cells, thus decreasing liver fibrosis (30). Therefore, relaxin may produce multiple signaling functions such as regulating cell proliferation and matrix release.

Relaxin may also aid in repair by promoting mucociliary clearance via an increase in ciliary beating. Although some agents (β agonists, bradykinin, and ethanol) very rapidly stimulate increased CBF via the activation of PKA, relaxin activates PKA and stimulates CBF between 4 and 6 hr after treatment. The timing of this CBF increase appears to have a later onset than that produced by β agonists, suggesting a regulation other than the cilia "flight response." Because both isoproterenol and relaxin can signal by activating adenylyl cyclase via G-protein-coupled receptors, it is not obvious as to why these two agents demonstrate different temporal regulation of CBF. Oftentimes, cyclic nucleotides regulate differential temporal effects of the same phenomenon. Clearly, multiple signaling messengers (such as NO, Ca^{2+}, cGMP, etc.) can regulate convergent pathways. Regulation by compartmentalization, phosphodiesterase activity, cross activation, and kinase autophosphorylation may each impact the utilization of cAMP on the timing of cell function. Our published studies concerning ethanol regulation of CBF have demonstrated that a dynamic interplay between NO and cAMP signaling exists in ethanol-stimulated cilia (31). Clearly, cAMP production is not the only pathway of cilia stimulation. Relaxin may in-

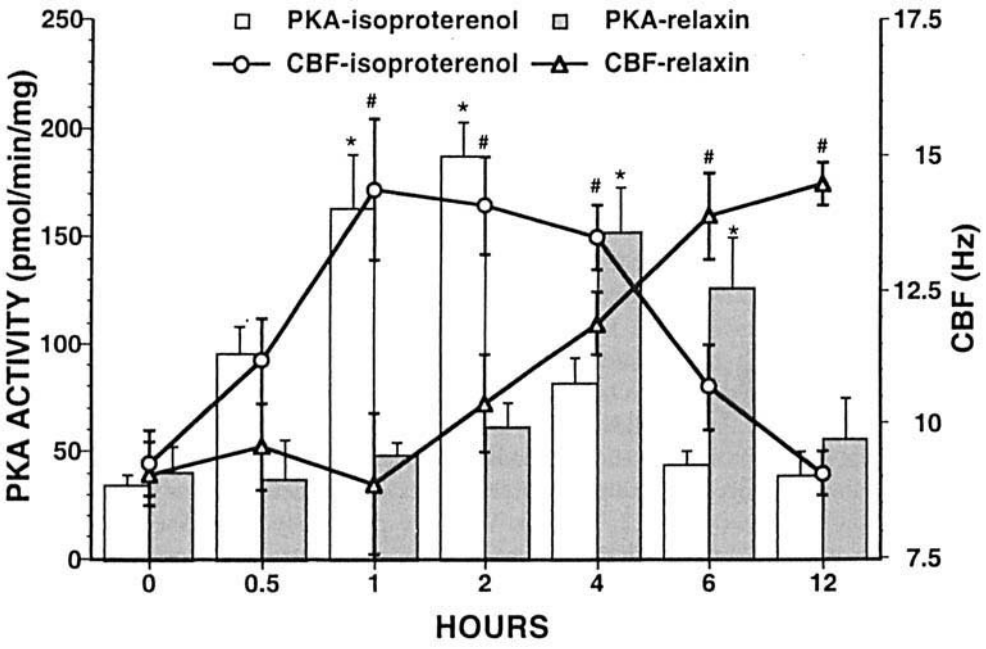

Figure 5. Relaxin stimulates PKA-mediated CBF later than isoproterenol. Monolayers of ciliated BBEC were stimulated with 1 μ*M* relaxin or 100 μ*M* isoproterenol and assayed for CBF over time. Cell homogenates of parallel BBEC culture dishes were flash frozen at CBF reading time points and were subsequently assayed for PKA activity. Isoproterenol rapidly activates PKA followed by a subsequent elevation of CBF up to 2 hr. Both isoproterenol-stimulated CBF and PKA returned to baseline by 12 hr. Relaxin did not stimulate PKA activity and CBF until later (4 hr) as compared with isoproterenol and CBF remained elevated beyond 12 hr, as shown in Figure 4. Bars represent SEM of homogenates prepared from three individual cell culture dishes assayed separately for each experiment. Each experiment was then repeated a total of three times for $n = 9$. Significance ($P < 0.005$) is indicated by an asterisk and ($P < 0.02$) is indicated by #.

volve additional regulatory pathways in addition to adenylyl cyclase that differentiates it from the action of isoproterenol on CBF.

The sustained CBF observed by relaxin stimulation also differs from that of β agonists. This slower onset and sustained stimulation of CBF by relaxin is consistent with that observed in BBEC treated with cytokines such as TNFα and IL-1β (32). Although PKA activation appears to function as a rheostat for isoproterenol-stimulated CBF, relaxin-stimulated CBF does not continue to correlate with PKA after 12 hr. Although the initial signal or trigger response for CBF elevation requires PKA activation, relaxin-mediated CBF elevations may involve a down-stream mediator that functions to maintain the CBF signal. One possibility for this may be a post-PKA substrate localized at the cilia that, once phosphorylated, maintains cilia beating after the kinase has inactivated. Such a mechanism would require that multiple substrate mediators are present at or near the cilia that have different responses to different kinases. This is supported by the findings that cAMP (33), PKA RI (34, 35), PKA RII (36), PKG (37, 38), Ca^{2+} (39), and PKC (40) have each been shown to promote phosphorylation of different cilia-localized substrates. Identification of specific relaxin-mediated kinase phosphorylation of ciliary proteins remains to be determined.

Recently, we determined that PKA activation can regulate wound healing (18). In our model of BBEC migration in response to wounding, we observed that agents that elevate intracellular cAMP levels promote accelerated wound healing. This response is also associated with the increase in PKA activity. Inhibitors of PKA, such as KT5720, Rp-cAMPS, and 4-cyano-3 methylisoquinoline, block PKA-mediated wound healing (18). PKA may be regulating the modulation of cell migration via Rho kinase. Relaxin shares the common signaling pathways of cAMP production and PKA activation observed in other stimulatory wound-healing agents. The wound closure assay used is virtually all cell spreading and migration. Somewhat surprisingly, there is not that much proliferation responsible for the wound closures. A study by Erjefalt *et al.* (41) suggests that migration occurs quickly and implies that proliferation is not so important in the early stages of the wound healing model. Kim *et al.* (42) use a technique similar to our wound model and support the idea that proliferation does not take place initially.

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