Regulation of ciliary beat frequency by both PKA and PKG in bovine airway epithelial cells

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Wyatt, T. A., J. R. Spurzem, K. May, and J. H. Sisson. Regulation of ciliary beat frequency by both PKA and PKG in bovine airway epithelial cells. Am. J. Physiol. 275 (Lung Cell. Mol. Physiol. 19): L827–L835, 1998.—Ciliary beating is required for the maintenance of lung mucociliary transport. We investigated the role of cyclic nucleotide-dependent protein kinases in stimulating ciliary beat frequency (CBF) in bovine bronchial epithelial cells (BBECs). cAMP-dependent protein kinase (PKA) activity and cGMP-dependent protein kinase (PKG) activity were distinguished after DEAE-Sephadex chromatography of BBEC extracts. cAMP levels and PKA activity are increased in BBECs stimulated with 0.01–1 mM isoproterenol, with a corresponding increase in CBF. cGMP levels and PKG activity are increased in BBECs stimulated with 0.1–10 µM sodium nitroprusside, with a corresponding increase in CBF. Direct protein kinase-activating analogs of cAMP and cGMP (dibutyryl cAMP and 8-bromo-cGMP, respectively) also activate their specific kinases and stimulate CBF. Preincubation of BBECs with inhibitors of PKA or PKG [KT-5720 or Rp-8-(p-chlorophenylthio)-guanosine 3',5'-cyclic monophosphothioate] results in the inhibition of specific kinase activity as well as in the inhibition of CBF. These studies suggest that the activation of either PKA or PKG can lead to the stimulation of CBF in bovine airway epithelium.

NORMAL AIRWAY MUCOCILIARY CLEARANCE is an important host defense mechanism that clears the upper airways of inhaled particles such as dusts, aerosols, and bacteria. This defense mechanism is dependent on the production of mucus and the coordinated beating of ciliated cells that line the airway lumen. Cilia in the mammalian airway continually beat at a baseline frequency to sweep debris toward the glottis under normal breathing conditions. Importantly, during times of stress such as exercise or inflammation, cilia beat faster to increase clearance and rapidly clear the airway in response to an increased load of inhaled particles. In this manner, ciliary motility is a regulatable host defense that can be stimulated under a variety of circumstances, including the inhalation of β-agonists or noxious chemicals, the deposition of inhaled particles, or the release of cytokine mediators. It is likely that impairment of the regulation of ciliary motility is important in the pathogenesis of a number of airway diseases such as exercise-induced asthma, acute and chronic bronchitis, and cystic fibrosis.

Several mechanisms have been proposed to mediate ciliary beat frequency (CBF) stimulation. Intracellular calcium flux regulated by inositol trisphosphate has been correlated with CBF (45, 59). A nitric oxide-dependent mechanism of CBF has been proposed that can be regulated by exogenous β-agonists, tumor necrosis factor (TNF)-α, interleukin (IL)-1β, and ethanol (27, 28). In contrast, agents that activate protein kinase (PK) C have been reported to decrease CBF (47). However, most studies of ciliary motility focus on the cAMP pathway and the stimulation of beat frequency by β-adrenergic agents and analogs of cAMP (34).

Intracellular increases in cAMP are associated with increased CBF in some mammalian airway epithelial cells (12, 58). Subsequently, agents that induce a decrease in cellular cAMP levels (30, 57) have been suggested to reduce CBF. However, it has recently been reported that CBF can be stimulated by cGMP in human airway epithelium (16).

First identified by Sutherland and Robison (56), the second messenger cAMP is ubiquitous in all nucleated cell types investigated. cAMP is synthesized on activation of adenylyl cyclase by circulating hormone catecholamines that bind to adrenergic receptors on target cell membranes (33). Indeed, such adenylyl cyclase activity has been measured in airway epithelial cells in response to stimulation by prostaglandin E2 and the synthetic catecholamine isoproterenol (32, 42). The major cellular receptor for cAMP, the cAMP-dependent PK (PKA), was first identified as such in 1968 (61). The structure and function of PKA has been studied in detail (15), and PKA activity has been described in the airway epithelial cell (4). However, much less is known about the cGMP-dependent PK (PKG) in airway epithelial cells.

Although cGMP was discovered in 1963 (3), much less is known about its effects on cellular function. The concentration of cGMP in most tissues is generally 10- to 50-fold less than the concentration of cAMP. The challenges associated with the analysis and quantitation of the low tissue levels of cGMP account for the slow progress in this field (17). cGMP is synthesized on activation of guanylyl cyclase by cholinergic agents (23), vasoactive peptides (14), and nitric oxide (2). There are at least three major cellular receptors for cGMP: 1) PKG, 2) cGMP-regulated ion channels, and 3) cGMP-binding phosphodiesterases (37). First purified from crude bovine lung homogenates (38), PKG has demonstrated profound signaling effects in cells with only small amounts of the enzyme (67). cGMP has only recently been implicated in the regulation of CBF because it has been reported that C-type natriuretic peptide increases CBF via a cGMP-dependent mechanism in human airway epithelial cells (16). Likewise,
Jain et al. (27) have previously shown that CBF increases in response to isoproterenol stimulation are regulated by nitric oxide, which is known to stimulate cGMP production in many cells.

In this study, we have identified the PKA and PKG in the bronchial epithelial cell. Furthermore, we have correlated cyclic nucleotide elevation and cyclic nucleotide kinase activation with increases in CBF by utilizing an in vitro cell culture preparation of cultured airway epithelium. Our observations that the type I PKA is present in significant amounts in the bronchial epithelial cell represents the first report of this enzyme in bovine bronchial epithelial cells (BBECs).

**METHODS**

Cell preparation. As previously described (65), the cells 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 medium (MEM). After the overnight incubation, the bronchi were rinsed repeatedly in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal calf serum to collect the cells lining the lumen. These cells were then filtered through a 250-µm nylon mesh and washed again in DMEM. This technique typically produces a high-viability cell preparation of >95% epithelial cells (51). The cells were then washed in DMEM, counted with a hemocytometer, and plated in 1% collagen-coated 100-mm polystyrene petri dishes at a density of 1 × 10^6 cells/cm² in a 1:1 medium mixture of LHC-9 and RPMI 1640 media (35). Cell incubations were performed at 37°C in humidified 95% air-5% CO₂. Confluent monolayers of cells were obtained every 3 days. At this time, each 100-mm dish contained ~2 mg total cellular protein. Primary cultures of BBECs were used for these studies because it has been suggested that tissue culture artifacts may induce the down-regulation of certain enzyme activity in late-passaged cells (11). Additionally, we used primary cell monolayers of ciliated cells that were obtained from bovine trachea as described above, with the exception of the mesh-filtering step.

Chromatography of BBEC extracts. Cell monolayers as described above were grown to confluency in 100-mm collagen-coated tissue culture dishes. The cells (1 × 10^6) were added to 10 ml of buffer containing 10 mM KH₂PO₄, 1 mM EDTA, and 25 mM 2-mercaptoethanol (KPEM). The cells were homogenized by sonication (3 × 10 s) and centrifuged at 10,000 g for 30 min at 4°C. The supernatant was applied to a DEAE-Sephadex column (0.9 × 10 cm) equilibrated in KPEM buffer. The column was washed with 40 ml of KPEM buffer and developed with a 50-ml NaCl linear gradient (0–300 mM) and 1-ml fractions were collected. Individual fractions were analyzed for protein content by the technique of Bradford (7) and used to standardize each experiment. Protein in each sample was measured by the technique of Bradford (7), for NaCl concentration, and for kinase activity. Determination of cyclic nucleotide levels. Cyclic nucleotide levels were determined with a PK activation assay (10). The type I PKA was partially purified through a DEAE-cellulose chromatography step (43). Cell monolayers were flash-frozen in liquid N₂ after the addition of 1 ml KPEM/dish. The dishes were stored at −70°C until assayed. Monolayers were thawed, scraped, added to microfuge tubes, and boiled at 95°C for 5 min. Tubes were spun at 10,000 g for 30 min, and supernatants were collected. Samples were diluted 1:10 in 10 mM potassium phosphate (KP) buffer (pH 6.8) with 0.9 mg/ml of bovine serum albumin (BSA), and 20 µl were added to a 50-µl stock reaction mixture consisting of 40 mM Tris-HCl (pH 7.4), 20 mM magnesium acetate, 130 µM Kemptide (LRRASLG), and 0.2 mM [γ-β²P]ATP. In some studies, 0.2 mM 3-isobutyl-1-methylxanthine (IBMX) was added to the reaction mixture. Reactions were initiated by the addition of 10 µl of PKA diluted to 0.4 nM with KPEM and 0.9 mg/ml of BSA. After incubation at 4°C for 16–20 h, 50–µl aliquots were spotted onto phosphocellulose paper (Whatman P-81) and placed immediately into 75 mM phosphoric acid. The papers were then washed five times manually for 1 min with KPEM and rinsed in 1 M ethanol, dried, and counted in nonaqueous scintillant (44).

The assay for cGMP levels was performed similarly to that for cAMP (10). The PKG used was partially purified as previously described (39). Samples (10 µl) diluted 1:10 in K buffer with 0.9 mg/ml of BSA were added to 20 µl of the stock reaction mixture as above, except that a 150 µM heptapeptide substrate (RKRASRAE) specific for PKG was substituted for Kemptide. PK inhibitor peptide (5–24) (15 µM) was also added to the reaction mixture. Reactions were initiated with the addition of 10 µl of PKG (10 nM), incubated, and halted as described for the cAMP assay. All incubations were performed in duplicate, and each experiment was repeated three or more times. Cyclic nucleotide concentrations (in pmol/mg protein) were determined by comparison to a standard curve of cyclic nucleotide-activated kinase activities (in pmol/min/mg protein) that was performed concurrently with each experiment. Protein in each sample was measured by the technique of Bradford (7) and used to standardize each experiment.

Determination of cyclic nucleotide-dependent kinase activity. PKA activity was determined in both DEAE fractions as well as in crude whole cell fractions of bronchial epithelial cells. The assay employed is a modification of procedures previously described (29), with 130 µM PKA substrate heptapeptide (LRRASLG), 10 µM cAMP, 0.2 mM IBMX, 20 mM Mg(C₂H₃O₂)₂, and 0.2 mM [γ-β²P]ATP in a 40 mM Tris-HCl buffer (pH 7.5). Cyclic GMP kinase activity was assayed in a manner similar to that for PKA, with the substitution of the peptide RKRASRAE for the heptapeptide substrate, the addition of 10 µM cGMP, and the presence of PK inhibitor peptide. 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 of cell fraction diluted 1:10 with KPEM and 0.9 mg/ml of 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 scintillant as previously described (44). Negative control groups consisted of similar assay samples with or without the appropriate substrate peptide or cyclic nucleotide. A positive control group of 0.4 ng/ml of purified catalytic subunit from type I bovine PKA (Promega) was included as a sample. Kinase activity is expressed in relationship to total cellular protein assayed and was calculated in picomoles per minute per milligram. All samples were assayed in triplicate, and no less than three separate experiments were performed for each unique parameter. Data were analyzed for significance with Student's paired t-test.

CBF measurements. Actively beating ciliated cells were observed, and their motion was quantified by measuring CBF with 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 thermostatically controlled heated stage. The cells were maintained at room temperature during the time course of the CBF measurements because the temperature gradient is known to affect CBF (48). All observations were recorded for analysis with a Panasonic WV-D5000 video camera and a Panasonic AG-1950 videotape recorder. Beat frequency analy-
sis was performed on videotaped experiments with customized software written in LabView (National Instruments, Austin, TX) running on a Macintosh IId computer. The predominant frequency of a cilium or small group of cilia was determined by collecting data sampled at 40 Hz from 512 samples (12.8 s) and performing frequency-spectrum analysis. The CBF determined in this manner was deemed acceptable when a single dominant frequency was obtained with this technique. All frequencies are means ± SE from six separate cell groups or fields.

Materials. LHC basal medium was purchased from Biofluids (Rockville, MD). RPMI 1640, DMEM, MEM, streptomycin-penicillin, and Fungizone were purchased from GIBCO (Gaithersburg, MD). Extraction of frozen bovine pituitaries from Pel Freez (Rogers, AR) was performed as previously described (35) and yielded an extract containing 10 mg/ml of protein. [γ-32P]ATP was purchased from Amersham. DEAE and phosphocellulose P-81 paper were purchased from Whatman. Heptapeptide substrates for PKA and PKG and protein kinase inhibitor peptide-(5–24) were purchased from Peninsula Laboratories. The Rp diastereomer of 8-(p-chlorophenylthio)-guanosine 3',5'-cyclic monophosphothioate (Rp-8-pCPT-cGMPS) and KT-5720 were purchased from Alexis/LC Services. All other reagents not specified were purchased from Sigma (St. Louis, MO).

RESULTS

Identification of PKA and PKG in BBECs. To determine the presence of PKA in airway epithelial cells, crude homogenates of BBECs were fractionated, concentrated, and resolved by DEAE-Sephacel chromatography (see METHODS). As shown in Fig. 1, this procedure partially separated PKG and PKA activities, which were assayed with substrates specific to these enzymes. The cAMP-stimulated kinase activity in peak fractions (0.8–1.3 nmol·min⁻¹·mg⁻¹) was approximately fivefold greater than that in the same fractions measured in the absence of cAMP (100–200 pmol·min⁻¹·mg⁻¹). This peak activity eluted in the position of the type II isozyme (fractions 17–21), with only a small amount of activity eluting in the position of type I (fraction 11) or in the flow-through fractions (fractions 1–5). The PKG type Iα eluted before the major PKA peak (fractions 12–18) and demonstrated cGMP-stimulated kinase activity in the peak fractions (0.6–0.8 nmol·min⁻¹·mg⁻¹) approximately two- to threefold greater than that in the same fractions measured in the absence of cGMP (150–250 pmol·min⁻¹·mg⁻¹) and increased activity in the presence of cGMP. These data indicate that significant amounts of both PKA and PKG are present in BBECs.

BBEC cyclic nucleotide levels. To determine whether PKG and PKA activation was in response to elevated cyclic nucleotide, the cGMP and cAMP levels in BBECs were also determined. Cyclic nucleotide levels were determined in whole cell monolayer extracts of BBECs with a PK activation assay (10), with type I PKA partially purified through a DEAE-cellulose chromatography step (43). Cells were stimulated with 10 µM isoproterenol, which was found to be the optimal concentration eliciting maximal cAMP levels (data not shown). Some cells were treated with 0.1 µM sodium nitroprusside (SNP), an activator of soluble guanylyl cyclase. Additionally, some cells were preincubated with 0.2 mM IBMX, a nonspecific phosphodiesterase inhibitor. As demonstrated in Fig. 2, 10 µM isoproterenol stimulates a five- to sixfold increase in cAMP levels. This increase is augmented and sustained by preincubating the cells with 0.2 mM IBMX for 1 h before
stimulating the cells with isoproterenol (data not shown), suggesting that an active cAMP phosphodiesterase is present in the bronchial epithelial cells that functions to attenuate the isoproterenol-stimulated elevation of cAMP in the cell. IBMX alone had little or no effect on baseline levels of cAMP (data not shown). cAMP was increased in a concentration-dependent manner by isoproterenol, with the maximal levels of nucleotide being elevated by 1–100 µM isoproterenol (data not shown). Whereas it had only a small effect on baseline levels of cAMP, 0.1 µM SNP stimulated a three- to fourfold increase in cGMP levels (Fig. 2B). Likewise, 10 µM isoproterenol stimulated only a small increase in cGMP levels. This suggests that distinctive cyclic nucleotide pathway signaling can be maintained by β-adrenergic or cholinergic agents at specific concentrations.

Activation of PKA and PKG in response to cyclic nucleotide elevation. The activity of PKA in primary cultures of BBECs was assayed to compare the changes in PKA activity with the change in cAMP levels in cells treated with 10 µM isoproterenol. Kinase activity peaked by 60 min, remained elevated beyond 90 min compared with that observed for unstimulated cells, and gradually returned to baseline unstimulated levels by 4 h (Fig. 3A). Increased PKA activity was detected only after that time point (5 min) in which cAMP levels were most elevated (Fig. 2). Additionally, PKA activity was increased two- to threefold over baseline unstimulated cells by preincubating the cells for 10 min with 100 nM dibutyryl cAMP (DBcAMP), an analog that is specific only for PKA (Fig. 4). These findings suggest that the stimulation of cAMP accumulation by isoproterenol is sufficient to activate PKA in the BBEC.

Because phosphodiesterases may regulate the level of intracellular cyclic nucleotides in these cells, BBECs were treated with the nonspecific phosphodiesterase inhibitor IBMX. Preincubation of the cells with 0.2 mM IBMX for 1 h before isoproterenol stimulation resulted in an increase in maximal PKA activity and sustained PKA activity beyond the time points observed without IBMX (data not shown). Because DBcAMP is resistant to hydrolysis by any cAMP phosphodiesterase present in the BBEC, the presence of IBMX failed to augment the PKA activity increases stimulated by DBcAMP (data not shown).

The activity of PKG in primary cultures of BBECs was likewise assayed as above to compare the changes in PKG activity with the change in cGMP levels in cells treated with 1 µM SNP. Kinase activity peaked by 60 min, remained elevated beyond 4 h compared with that observed for unstimulated cells, and gradually returned to half-maximal stimulated levels by 6 h (Fig. 3B). Maximal PKG activity was detected at the same time point in which cGMP levels were most elevated (Fig. 2). Additionally, PKG activity was increased two- to threefold over baseline unstimulated cells by preincubating the cells for 30 min with 1 µM 8-bromo-cGMP (8-BrcGMP), an analog that is specific only for PKG (Fig. 4). These findings suggest that the stimulation of cGMP accumulation by SNP is sufficient to activate PKG in the BBEC.

![Figure 3](http://ajplung.physiology.org/)

**Fig. 3.** Cyclic nucleotide kinase activity in Iso- and SNP-treated BBECs. Monolayers were incubated in presence of Iso (A) or SNP (B) for up to 4 h. Cell supernatants were then assayed for cAMP-dependent protein kinase (PKA) activity (A) and cGMP-dependent protein kinase (PKG) activation (B) as described in METHODS. PKA and PKG activities were determined at 60 min for various concentrations of Iso (A, inset) and SNP (B, inset), respectively. Data are averages of triplicate measurements of 3 or more samples within an experiment. P ≤ 0.01 for 1 µM SNP-stimulated PKG activity and P ≤ 0.01 for 10 µM Iso-stimulated PKA activity vs. cells treated with medium only at 1 h.

![Figure 4](http://ajplung.physiology.org/)

**Fig. 4.** Cyclic nucleotide kinase activity in dibutyl cAMP (DBcAMP)- and 8-bromo-cGMP (8-BrcGMP)-treated BBECs. Monolayers were incubated in presence of various concentrations of DBcAMP for 60 min, and cell supernatants were assayed for PKA activity (A). Cells incubated in the presence of various concentrations of 8-BrcGMP for 60 min were then assayed for PKG activity (B). Data are averages of triplicate measurements of 3 or more samples within an experiment. P ≤ 0.01 for 1 µM DBcAMP and P ≤ 0.05 for 10 µM 8-BrcGMP vs. cells treated with medium only.
Preincubation of the cells with 0.2 mM IBMX for 1 h before isoproterenol stimulation resulted in an increase in maximal PKG activity and sustained PKG activity beyond the time points observed without IBMX. Because 8-Br-cGMP is resistant to hydrolysis by any cGMP phosphodiesterase present in the BBEC, the presence of IBMX failed to augment the PKG activity increases stimulated by 8-Br-cGMP.

Stimulation of CBF by cAMP and cGMP. To correlate kinase activity with ciliary motility, we determined CBF in the same BBECs assayed for cyclic nucleotide kinase activity. When intact monolayers of ciliated cells were treated with the cell-permeable analog to cAMP, DBcAMP, CBF steadily and significantly increased from 5 min to 1 h (Fig. 5A). This stimulation of CBF was sustained at up to 24 h of treatment with DBcAMP (data not shown). CBF was also stimulated within 30 min by treating the cells with 0.01–1 mM isoproterenol (Fig. 5B). These results suggest that increases in CBF can be elicited by increases in intracellular cAMP levels.

Similarly, CBF was stimulated in BBECs by the addition of 0.01–1 mM 8-Br-cGMP for up to 2 h (Fig. 5C). This increase in CBF remained for at least 24 h of treatment with 8-Br-cGMP (data not shown). CBF increased in BBECs treated with 1 µM SNP for up to 2 h (Fig. 5D) and remained at increased levels for over 24 h of stimulation (not shown).

Inhibition of CBF by inhibiting PKA or PKG activation. With the use of a selective and potent inhibitor of PKA, KT-5720 (31), we attempted to block increases in CBF by inhibiting the activation of PKA by isoproterenol. Preincubation of BBECs for 2 h with as little as 1 nM KT-5720 resulted in a significant decrease in 10 µM isoproterenol-stimulated PKA activity (isoproterenol without KT-5720, 336.3 pmol·min⁻¹·mg⁻¹; isoproterenol with KT-5720, 127.2 pmol·min⁻¹·mg⁻¹; P < 0.001; Fig. 6A). KT-5720 (100 nM) completely inhibited all stimulated PKA activity in BBECs (Fig. 6A). Similarly, 100 nM KT-5720 caused the inhibition of both isoproterenol- and DBcAMP-stimulated CBF up to 2 h compared with cells treated with these agents in the absence of KT-5720 (Fig. 6B). With the use of an antagonist analog of cGMP, Rp-8-pCPT-cGMPS (8, 9), we attempted to block CBF stimulation through the inactivation of PKG. Rp-8-pCPT-cGMPS binds to PKG at the cyclic nucleotide binding site(s) without activating the kinase, thus preventing cGMP from occupying the same site. Preincubation of BBECs for 1–2 h with 10 µM Rp-8-pCPT-cGMPS significantly lowered the activation of PKG observed in 1 µM 8-Br-cGMP-stimulated cells compared with BBECs not pretreated with Rp-8-pCPT-cGMPS (82.7 pmol·min⁻¹·mg⁻¹ for 8-Br-cGMP vs. 32.9 pmol·min⁻¹·mg⁻¹ for 8-Br-cGMP + Rp-8-pCPT-cGMPS; P < 0.01; Fig. 7A). A 1-h preincubation of BBECs with 10 µM Rp-8-pCPT-cGMPS completely inhibited CBF increases at 1 h of stimulation with 1 µM SNP and continued to partially inhibit SNP-stimulated CBF for up to 3 h compared with cells treated with these agents in the absence of Rp-8-pCPT-cGMPS pretreatment (Fig. 7B). No cytotoxicity due to either KT-5720 or Rp-8-pCPT-cGMPS was observed by trypan blue viability assay. The BBECs remained functional as tumor necrosis factor-α stimulated cell migration and PKC

![Fig. 5. Stimulation of ciliary beat frequency (CBF) by DBcAMP (A), Iso (B), 8-Br-cGMP (C), and SNP (D) in BBECs. CBF was measured over a period of 60 min in monolayers of BBECs. CBF in each experiment was derived from mean of at least 5 different cells from different areas of each plate. Various concentrations of each agent were added at time 0, and CBF was measured at 30 min and 1 h. Data are averages of triplicate measurements of 3 or more samples within an experiment.](image-url)
activation (66) was observed in the presence of KT-5720 or Rp-8-pCPT-cGMPS (data not shown).

**DISCUSSION**

We have chosen the BBEC as our model because of the large quantities of primary and low-passage cells required for obtaining reproducible cyclic nucleotide kinase assay data. In addition, large numbers of ciliated cells can be obtained from bovine trachea and bronchi. Similar to a previous report (4), we found significant PKA activity in bovine airway epithelial cells. Significant PKG activity was also measured by direct cyclic nucleotide kinase activity, which to the best of our knowledge has not been described previously in bronchial epithelial cells. A previous study (46) involving PKA was limited to the use of specific and nonspecific inhibitors of kinase activity. Due to the numerous reported side effects of kinase inhibitors and their lack of potency in vitro, we think that direct measurement of the cyclic nucleotide-mediated enzymes is a more meaningful assessment of enzyme activity (68).

Previous studies (12, 48, 59) concerning the duration of cAMP levels have differed from our results. These differences may involve species of the cell type, airway location of the cells, media, temperature conditions, and sampling methods. These studies have reported transient isoproterenol-stimulated cAMP increases in nonbovine ciliated cells. However, these studies do not report CBF measurements in cells stimulated with isoproterenol or cAMP beyond 1 h, and it appears that the extent of CBF increases are dependent on the concentration of isoproterenol employed in the assay. To correlate CBF with the activation of PKA, we assayed PKA activity in the presence of isoproterenol and the direct PKA-activating analog DBcAMP in intact monolayers of BBECs. The same studies were also performed in the presence of a PKA inhibitor (KT-5720). In addition, we assayed PKG activity in the presence of the soluble guanylyl cyclase activator SNP and the direct PKG-activating analog 8-BrcGMP and found that increases in CBF occur under conditions of PKG activation as well. Competitively blocking the activation of PKG by cGMP binding with the antagonist analog Rp-8-pCPT-cGMPS resulted in the decrease of stimulated PKG activation and CBF by SNP.

Although our results clearly show that CBF can be inhibited by cyclic nucleotide kinase inhibition, our
BBECs, whereas peak kinase activity and CBF were level increases very rapidly (5–10 min) in stimulated activation. We observed maximum cyclic nucleotide cAMP production by peak cyclic nucleotide protein kinase observations do not support a simple temporal regulation of CBF because increases in cAMP triggered by PKA and PKG can undergo autophosphorylation, which results in even greater measurable activity of these enzymes. In the case of PKG, kinase activity increases due to autophosphorylation occur significantly slower than cGMP-mediated activation (53). Although cyclic nucleotide kinase autophosphorylation has yet to be demonstrated in BBECs, this mechanism might explain the time lag between maximal cyclic nucleotide levels and maximal kinase activity.

We observed that isoproterenol significantly elevates measurable levels of cAMP in the bovine cell. This increase in cAMP is associated with a concomitant activation of PKA in the bovine cell. However, empirical intracellular cAMP levels may not be an accurate indicator of PKA activation in the airway epithelial cell because the concentration of cAMP produced in response to adrenergic agents is much greater than that required for PKA activation (54). This further emphasizes the importance of direct kinase activity measurements in these studies. The presence of a phosphodiesterase inhibitor such as IBMX enhances the magnitude of cAMP levels and the duration of PKA activity, suggesting cAMP-specific phosphodiesterase activity in the BBEC. Additionally, cell-permeable agonist analogs to cAMP, such as D8cAMP, have the same (if not augmented) effect as a β-agonist on the activation of PKA. Although the effects were lower in absolute magnitude than for cAMP, we identified detectable increases in cGMP and the activation of PKG in response to SNP. However, we failed to observe PKG activation in response to atrial natriuretic peptide (data not shown). This suggests that soluble guanylyl cyclase is primarily responsible for cGMP production in BBECs.

Earlier studies demonstrated that cyclic nucleotides have distinct effects on the regulation of cilia in non-mammalian cells. Both cAMP and cGMP increase forward swimming speed ciliary motility in Paramecium (5, 6). Type II PKA, type I PKA, and PKG, as well as a novel PKG, have each been purified from cilia of Paramecium (24, 40, 41). PKA causes significant phosphorylation of Paramecium dyneins (60). cGMP also stimulates in vitro phosphorylation of several proteins in isolated Paramecium cilia (11). Although a functional role for protein phosphorylation in cilia of Paramecium remains to be established, proteins associated with axonemal structures of the cilia were phosphorylated in vitro by cAMP and cGMP (13, 21). It has been suggested that the effect of cAMP on Paramecium cilia is due to changes in the mechanically coupled 22S dynein activity via phosphorylation or thiphosphorylation of a regulatory outer arm dynein light chain (20, 50). Calmodulin (CaM) kinase inhibitors have been used to block the stimulatory effects of calcium on CBF, thus suggesting a role for Ca2+/CaM kinase in the regulation of ciliary phosphorylation events (12, 49, 59). Several low-molecular-weight proteins in single-cell flagellates have been shown to be phosphorylated in a cAMP-dependent manner (13, 55). In vitro phosphoprotein substrate for PKA has been reported in sheep tracheal epithelial cells that can be blocked by the addition of a specific inhibitor to PKA (46). PKA has been implicated in the regulation of CBF because increases in ciliary motility due to elevated cAMP can be blocked by the nonspecific protein kinase inhibitor H-7 (12).

Existing evidence for the role of cAMP and cGMP in ciliary control appears to be contradictory because agents that elevate cAMP or cGMP, but not both, have a similar effect on stimulating cilia. This paradox cannot be explained by the bidirectional control hypothesis of cyclic nucleotide action as formulated some 25 years ago (18). This hypothesis led to the dogma that cAMP and cGMP always demonstrated distinct and usually opposing regulatory pathways in the cell (19, 26, 64). It has been assumed that PKA is specifically activated by cAMP and that PKG is specifically activated by cGMP (36). Since the bidirectional control hypothesis was first proposed, studies have suggested that the effector specificity of PKs may not be absolute. For example, the elevation of cAMP or cGMP can cause vasorelaxation, and it is uncertain if one or both of the cyclic nucleotide-dependent PKs mediate smooth muscle relaxation (22, 25).

Because of the structural homology that exists between PKA and PKG (62, 63), it would be tempting to speculate that these kinases may phosphorylate the same substrates compartmentalized within the cilia. However, it is much more likely that the similarities of cyclic nucleotide-binding regions on both PKA and PKG would be conducive to cyclic nucleotide cross-activation (29). Ongoing studies are directed at determining the unique and common phosphoprotein substrates for the cyclic nucleotide kinases as well as finding evidence for cyclic nucleotide cross-activation in BBECs.

In summary, our findings indicate that the stimulation of ciliary motility likely occurs through the generation of both adenosine 3′,5′-cyclic monophosphate (cAMP) and guanosine 3′,5′-cyclic monophosphate (cGMP) in BBECs, with the subsequent activation of their respective PKs. This indicates an interplay of second messengers during cilia activation that might result in synergistic effects on motility and enhanced airway clearance. It also suggests pharmacological strategies that might combine the known cilia-stimulatory effects of β-agonists with more innovative approaches toward cilia stimulation, such as inhaled nitric oxide donors or inducers of nitric oxide synthase. Such novel approaches may take advantage of the regulatable nature of ciliary motility by enhancing or restoring mucus clearance under cir-
cumstances in which ciliary regulation is blunted or impaired.

This study was supported by National Institute on Alcohol Abuse and Alcoholism Grant S-R01-AA-08769-07 to J. H. Sisson, American Cancer Society Grant IRG 165H to T. A. Wyatt, and a grant from the State of Nebraska Cancer and Smoking Diseases Program to T. A. Wyatt.

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Received 4 December 1997; accepted in final form 8 July 1998.

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