Chronic ethanol downregulates PKA activation and ciliary beating in bovine bronchial epithelial cells

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Wyatt, T. A., and J. H. Sisson. Chronic ethanol downregulates PKA activation and ciliary beating in bovine bronchial epithelial cells. Am J Physiol Lung Cell Mol Physiol 281: L575–L581, 2001.—Previously, we reported that ethanol (EtOH) stimulates a rapid increase in ciliary beat frequency (CBF) of bovine bronchial epithelial cells (BBEC). Agents activating cAMP-dependent protein kinase (PKA) also stimulate CBF. EtOH stimulates BBEC CBF through cyclic nucleotide kinase activation. However, EtOH-stimulated CBF is maximal by 1 h and subsides by 6 h, returning to baseline by 24 h. We hypothesized that the loss of EtOH-stimulated CBF was a result of downregulation of PKA activity. To determine the PKA activation state in response to EtOH, ciliated BBEC were stimulated for 0–72 h with various concentrations of EtOH and assayed for PKA. EtOH (100 mM) treatment of the cells for 1 h increased PKA activity threefold over unstimulated controls. PKA activity decreased with increasing time from 6 to 24 h. When BBEC were preincubated with 100 mM EtOH for 24 h, the stimulation of PKA by isoproterenol or 8-bromo-cAMP was abrogated. EtOH desensitizes BBEC to PKA-activating agents, suggesting that EtOH rapidly stimulates, whereas long-term EtOH downregulates, CBF via PKA in BBEC.

CLINICAL STUDIES have demonstrated that airway host defenses are impaired in alcoholics (6, 17). Chronic alcohol consumption is associated with a high incidence of bronchitis, pneumonia, and aspiration (2, 15). The mucociliary escalator serves as the frontline defense for the lung against inhaled microorganisms, particles, and debris. This regulatable host defense is likely impaired by the excessive consumption of alcohol and contributes to the increased risk and presentation of lung disease in alcoholics. The exact mechanisms of impaired mucociliary function in response to ethanol consumption remain poorly understood.

Changes in mucociliary transport can be experimentally modeled by monitoring changes in ciliary beat frequency (CBF). CBF is likely increased during situations of airway epithelial cell “stress.” This can occur transiently as part of the “fight or flight” response to substances such as β-agonists, substance P, and bradykinin (10, 20, 35, 39). Alternatively, ciliary stimulation can be prolonged in response to inflammation via cytokines such as tumor necrosis factor-α or interleukin (IL)-1β released from inflammatory cells (9). Previously, we have studied the acute stimulation of CBF by demonstrating that agents that increase either cGMP or cAMP and subsequently activate either cGMP-dependent protein kinase (PKG) or cAMP-dependent protein kinase (PKA) lead to increased CBF in the bovine ciliated airway epithelial cell (39). We have also demonstrated that ethanol rapidly and transiently stimulates airway epithelial cell CBF in vitro and that a nitric oxide (NO)-dependent mechanism is involved in these ethanol-stimulated CBF increases (31, 32). Additionally, there appears to be a cAMP-regulatable component to acute ethanol-stimulated CBF increases as ethanol activates PKA in the airway epithelial cell (32). Thus we have found that ethanol signaling of CBF is directly linked to the activation of PKA and is indirectly linked to activation of PKG.

The rapid and transient stimulatory effects of acute ethanol exposure on ciliary motility in vitro are seemingly inconsistent with the impaired airway host defenses that ethanol is known to cause in alcoholics. Because independently regulated NO-dependent mechanisms have been described for acute vs. chronic activation of ciliary motility (9), we explored the long-term responses of airway cell CBF to ethanol. Because impaired host defenses are associated with chronic ethanol consumption, we hypothesize that chronic exposure to ethanol blunts NO-dependent ciliary stimulation through downregulation of PKA. This downregulation of the mechanism by which CBF is increased correlates with the ethanol-associated impairment in mucociliary clearance.

In this study, we show that the early stimulation in ciliary activity observed after acute ethanol treatment is followed by a return to baseline (unstimulated) CBF between 6 and 24 h in bovine bronchial epithelial cells...
(BBEC). Similarly, ethanol-stimulated PKA activity also declines to unstimulated levels in a parallel manner. We have observed that the BBEC become desensitized to further ethanol-stimulated increases in CBF and PKA at these later time points. Importantly, after long-term ethanol treatment, BBEC are desensitized to additional CBF increases because of agents that otherwise acutely stimulate CBF. Our observations suggest that chronic ethanol exposure of BBEC results in the desensitization of PKA-mediated CBF.

**MATERIALS AND METHODS**

**Cell preparation.** As previously described (36), 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 MEM. After overnight incubation, the bronchi were rinsed in DMEM with 10% FCS repeatedly to collect the cells lining the lumen. This technique typically produces a high-viability cell preparation of >95% epithelial cells (29). The cells were then washed in DMEM, counted with a hemacytometer, and plated in 1% collagen-coated 100-mm polystyrene petri dishes at a density of 1 × 10^4 cells/cm² in a 1:1 medium mixture of LHC-9 and RPMI (16). 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 60-mm dish contained ~2 mg of total cellular protein. Primary cultures of BBEC were used for these studies, as it has been suggested that tissue culture artifact may induce the downregulation of certain enzyme activity in the late-passaged cell (3).

**Determination of cyclic nucleotide-dependent kinase activity.** PKA activity was determined in crude whole cell fractions of BBEC. The assay employed is a modification of procedures previously described (11) using 130 μM PKA substrate heptapeptide (LRRASLG), 10 μM cAMP, 0.2 mM IBMX, 20 mM magnesium acetate, and 0.2 mM γ-32P[ATP in a 40 mM Tris-HCl buffer (pH 7.5). Samples (20 μl) were added to 50 μl of the above reaction mixture and incubated for 15 min at 30°C. Spotting 50 μl of each sample onto P-81 phosphocellulose papers halted incubations. Papers were then washed five times for 5 min each in phosphoric acid (75 mM), washed one time in ethanol, dried, and counted in nonaqueous scintillant as previously described (25). Negative controls consisted of similar assay samples with or without the appropriate substrate peptide or cyclic nucleotide. A positive control of 0.4 mg/ml purified catalytic subunit from type I bovine PKA (Promega) was included as a sample. Kinase activity is expressed in relation 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 per unique parameter. Data were analyzed for statistical significance using Student’s paired t-test.

**Determination of cyclic nucleotide levels.** Cyclic nucleotide levels were determined using a protein kinase activation assay as previously described (39). Briefly, cell monolayers were flash frozen in liquid N₂ after addition of 1 ml KPEM (10 mM KH₂PO₄, 1 mM EDTA, and 25 mM 2-mercaptoethanol) per dish. Cell protein extracts were transferred to microfuge tubes and boiled at 95°C for 5 min. After centrifugation (10,000 g for 30 min), the supernatants were diluted to 0.4 mM with KPEM and 0.9 mg/ml BSA. After incubation at 4°C for 16–20 h, 50-μl aliquots were spotted on phosphocellulose paper (Whatman P-81) and placed immediately in 75 mM phosphoric acid. The papers were then washed, dried, and counted in nonaqueous scintillant (25). The assay for cGMP levels was performed similarly as above, substituting partially purified PKG (18), and 150 μM heptapeptide substrate (RKRSSRAE) specific for PKG was substituted for kemptide. PKA inhibitor peptide (15 μM) was also added to the reaction mixture. All incubations were performed in duplicate, and each experiment was repeated three or more times. Cyclic nucleotide concentrations (pmol/mg protein) were determined by comparison with a standard curve of cyclic nucleotide-activated kinase activities (pmol·min⁻¹·mg⁻¹) that was performed concurrently with each experiment. Protein in each sample was measured by the technique of Bradford (1) and was used to standardize for each experiment.

**CBF measurements.** 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 thermostatically controlled heated stage. The cells were maintained at room temperature during the time course of the CBF measurements, since the temperature gradient was known to affect CBF (27). All observations were recorded for analysis using a Panasonic WV-D5000 video camera and a Panasonic AG-1950 videotape recorder. Beat frequency analysis was performed on videotaped experiments using customized software written in LabView (National Instruments, Austin, TX) running on a Macintosh G3 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 using this technique. All frequencies represent means ± SE from six separate cell groups or fields.

**Materials.** LHC basal medium was purchased from Biofluids (Rockville, MD). RPMI 1640 medium, DMEM, MEM, streptomycin-penicillin, and Fungizone were purchased from Gibco BRL (Chagrin Falls, OH). Extraction of frozen bovine pituitaries from Pel Freez (Rogers, AR) was performed as previously described and yielded an extract containing 10 mg/ml protein (16). γ-32P[ATP was from Amersham, phosphocellulose P-81 paper was from Whatman, peptide substrates were from Peninsula Laboratories, and absolute ethanol was from McCormick Distilleries. All other reagents not specified were purchased from Sigma Chemical (St. Louis, MO).

**RESULTS**

Ethanol-stimulated PKA activity decreases over time. To determine the duration of ethanol-stimulated PKA activity in airway epithelial cells, crude homogenates of BBEC were fractionated, and the soluble cytosolic fraction was assayed for PKA (see MATERIALS AND METHODS). BBEC treated with 10–100 mM ethanol for ~1 h demonstrate maximal activation of PKA (Fig. 1). This activity begins to diminish between 2 and 6 h, returning to baseline (unstimulated) PKA activity after 6–8 h. Concentrations of ethanol <10 mM fail to activate PKA. After ethanol-stimulated PKA activity returned...
to baseline levels, no further increase in PKA was observed from 18 to 64 h (data not shown). The cell medium was then exchanged each hour with a fresh dose of 100 mM ethanol in new medium so that the concentration of ethanol across all time points would remain constant at 100 mM. With the use of this medium replacement technique, the pattern of PKA activation was again observed to return from maximal activation back to baseline levels between 6 and 8 h (Fig. 2). This suggests that ethanol is not being consumed by metabolism or volatilization under our experimental conditions. These data indicate that prolonged exposure to ethanol results in a downregulation or autoinactivation of PKA activity to continued ethanol exposure.

**Ethanol-stimulated CBF decreases over time.** To determine the duration of ethanol-stimulated increases in CBF in airway epithelial cells, ciliated primary cultures of BBEC were treated with 100 mM ethanol, and ciliary motility was measured (see MATERIALS AND METHODS). With no pretreatment, ethanol stimulates CBF by 1 h with a return to baseline levels after 6 h (Fig. 3). When the cells were pretreated with 100 mM ethanol for 24 h and then reexposed to fresh medium containing 100 mM ethanol, no significant increase in CBF was observed, similar to BBEC exposed to control medium. This suggests that prolonged exposure to ethanol (≥6 h) causes desensitization of ciliary motility to ethanol and that long-term exposure to ethanol inhibits increases in CBF as a result of subsequent ethanol challenges.

**Ethanol desensitizes BBEC to other CBF stimuli.** To determine if the desensitization of ethanol-stimulated PKA activity in BBEC affects further stimulation of CBF by activators of PKA, cells pretreated with 100 mM ethanol (or medium) for 24 h were then exposed to 100 μM isoproterenol or 10 μM 8-bromo-cAMP (8-BrcAMP). BBEC pretreated with medium demonstrated significant elevations in CBF upon treatment with either isoproterenol or 8-BrcAMP (Fig. 4B). In sharp

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**Fig. 1.** Time course of cAMP-dependent protein kinase (PKA) activation in ethanol (EtOH)-treated bovine bronchial epithelial cells (BBEC). Monolayers of BBEC were treated with 0.01–100 mM EtOH from 0 to 16 h, and PKA activity was assayed. Kinase assays were performed as described (see MATERIALS AND METHODS) using specific heptapeptide substrates for PKA. Each data point represents the average of triplicate measurements of 3 or more samples within an experiment (P ≤ 0.05 for 10 and 100 mM EtOH-stimulated PKA activity (*) compared with cells treated with medium only at 1, 2, and 6 h). Vertical bars represent ± SE in Figs. 1–6.

**Fig. 2.** Time course of PKA activation in BBEC treated with repeated doses of fresh EtOH. Monolayers of BBEC were treated with fresh medium containing either 100 mM EtOH or control medium every hour from 1 to 8 h, and PKA activity was assayed. Each data point represents the average of triplicate measurements of 3 or more samples within an experiment (P ≤ 0.05 for EtOH-stimulated PKA activity compared with cells treated with control medium from 1 to 6 h. No significant difference exists between conditions at 7 and 8 h).

**Fig. 3.** Effects of long-term EtOH exposure on ciliary beat frequency (CBF) stimulation (stim) in BBEC. Monolayers of BBEC were pretreated (pretreat) for 24 h with either 100 mM EtOH or control medium followed by treatment with 100 mM EtOH or control medium from 0 to 6 h, and CBF was assayed. CBF was assayed as described (see MATERIALS AND METHODS) using videomicroscopy and frequency spectrum analysis. Each data point represents the average of triplicate measurements of 3 or more samples within an experiment (P ≤ 0.05 for cells pretreated with medium and stimulated with 100 mM EtOH for 1, 2, and 4 h (*) compared with cells treated with medium only or pretreated with EtOH for 24 h).
contrast, pretreatment of cells with 100 mM ethanol inhibited any increases in CBF resulting from isoproterenol or 8-BrcAMP (Fig. 4A). Interestingly, a prolonged exposure to 100 mM ethanol also inhibited the ability of 10 μM sodium nitroprusside (SNP) or 10 μM 8-bromo-cGMP (8-BrcGMP) to stimulate CBF in BBEC. These findings parallel the effects of chronic ethanol exposure on PKA activity (Fig. 5). BBEC pretreated with medium demonstrated significant increases in PKA activity upon treatment with either isoproterenol or 8-BrcAMP (Fig. 5A). However, pretreatment of cells with 100 mM ethanol inhibited any significant increases in PKA as a result of isoproterenol or 8-BrcAMP (Fig. 5B). As expected, neither 10 μM SNP nor 10 μM 8-BrcGMP stimulates PKA activity in BBEC.

Although the use of a phosphodiesterase-resistant cyclic nucleotide analog to directly activate PKA circumvents the problem of phosphodiesterase activation or adenylyl cyclase inactivation, we have observed the same chronic ethanol desensitization of forskolin-stimulated PKA in our system, suggesting that cellular impermeability to 8-BrcAMP is not occurring (Fig. 6). As depicted in Table 1, no decreases in isoproterenol-, forskolin-, or 8-BrcAMP-stimulated cAMP levels were detected in response to ethanol at any time point observed. Elevations in cAMP were consistently detected after 1–2 h of treatment with 100 mM ethanol, with a subsequent return to baseline levels as previously re-

Fig. 4. Effects of long-term EtOH exposure on agonist-stimulated CBF in BBEC. Monolayers of BBEC were pretreated for 24 h with either 100 mM EtOH (A) or control medium (B) followed by treatment with 10 μM 8-bromo-cAMP (8-BrcAMP), 10 μM 8-bromo-cGMP (8-BrcGMP), 100 μM isoproterenol (Iso), 10 μM sodium nitroprusside (SNP), or control medium from 0 to 6 h, and CBF was assayed. Each data point represents the average of triplicate measurements of 3 or more samples within an experiment (P ≤ 0.05 for cells pretreated with medium and stimulated with agonists for 1–6 h compared with cells treated with medium only or cells pretreated with EtOH for 24 h followed by agonist stimulation).

Fig. 5. Effects of long-term EtOH exposure on agonist-stimulated PKA activity in BBEC. Monolayers of BBEC were pretreated for 24 h with either 100 mM EtOH (B) or control medium (A) followed by treatment with 10 μM 8-BrcAMP (cAMP), 10 μM 8-BrcGMP (cGMP), 100 μM isoproterenol, 10 μM sodium nitroprusside, or control medium from 2 to 4 h, and PKA activity was assayed. Each data point represents the average of triplicate measurements of 3 or more samples within an experiment (P ≤ 0.05 for cells pretreated with medium and stimulated with cAMP or isoproterenol for 2–4 h compared with cells treated with medium only or cells pretreated with EtOH for 24 h followed by agonist stimulation).

Fig. 6. Effects of long-term EtOH exposure on forskolin (FSK)-stimulated PKA activity in BBEC. Monolayers of BBEC were pretreated for various times (0, 1, 6, or 24 h) with 100 mM EtOH followed by treatment with 100 μM forskolin for 30 min, and PKA activity was assayed. Each data point represents the average of triplicate measurements of 3 or more samples within an experiment (P ≤ 0.05 for cells pretreated with medium or 1 h EtOH and stimulated with forskolin for 30 min compared with cells pretreated with EtOH for 24 h followed by agonist stimulation).
Table 1. Ethanol effects on isoproterenol, forskolin, 8-BrcAMP, SNP, and 8-BrcGMP

<table>
<thead>
<tr>
<th>Agonist, 1 h</th>
<th>Cyclic Nucleotide, pM/mg protein</th>
<th>Preincubation With 100 mM Ethanol, h</th>
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</thead>
<tbody>
<tr>
<td>Medium 100 µM</td>
<td>cAMP 0.8 ± 0.12 cGMP 0.35 ± 0.1</td>
<td>0 1.1 ± 0.3 24 1.2 ± 0.2</td>
</tr>
<tr>
<td>Isoproterenol</td>
<td>cAMP 6.2 ± 0.85 cGMP 1.4 ± 0.3</td>
<td>0 6.6 ± 0.5 24 6.3 ± 0.3</td>
</tr>
<tr>
<td>Forskolin 10 µM</td>
<td>cAMP 8.4 ± 1.3 cGMP 1.1 ± 0.3</td>
<td>0 8.4 ± 0.4 24 8.3 ± 1.6</td>
</tr>
<tr>
<td>8-BrcAMP 10 µM</td>
<td>cAMP 11.2 ± 0.9 cGMP 0.38 ± 0.5</td>
<td>0 13.3 ± 1.7 24 13.7 ± 0.9</td>
</tr>
<tr>
<td>SNP 10 µM</td>
<td>cAMP 1.6 ± 0.2 cGMP 4.1 ± 0.6</td>
<td>0 1.3 ± 0.5 24 1.3 ± 0.2</td>
</tr>
<tr>
<td>8-BrcGMP 10 µM</td>
<td>cAMP 1.1 ± 0.3 cGMP 8.6 ± 1.3</td>
<td>0 1.1 ± 0.5 24 1.5 ± 0.3</td>
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8-BrcAMP, 8-bromo-cAMP; SNP, sodium nitroprusside; 8-BrcGMP, 8-bromo-cGMP.

ported (32). Ethanol alone did not alter the SNP- or 8-BrcGMP-stimulated levels of cGMP (Table 1). We also did not detect any decrease in BBEC baseline PKG activity resulting from chronic ethanol exposure (data not shown). These data indicate that a prolonged exposure to ethanol blunts airway epithelial cell responsiveness to additional challenges by agents that stimulate CBF increases in vitro.

**DISCUSSION**

In contrast to our earlier observations with acute ethanol exposure (31), the current findings suggest that chronic exposure of ciliated cells to ethanol results not only in a loss of the acute cilia stimulation effect but also desensitization of the ciliated cell to subsequent stimulation by ethanol. Not only does ethanol desensitize the ciliated cell to subsequent ethanol stimulation, but it also blocks stimulation by β-agonists. These observations indicate that chronic exposure to ethanol impairs the ciliated cell’s ability to increase motility, as might be required during infection or exercise. Closely associated with cilia desensitization, chronic ethanol exposure appears to downregulate PKA, suggesting a possible mechanism for cilia desensitization.

The tolerance of human tissues to high concentrations of ethanol is a peculiar and established quality of this compound. As we have previously reported (31, 32), the concentrations of ethanol required for CBF stimulation in BREC correspond to “legal intoxication” levels (~25 mM), with the maximum increases in CBF occurring at 100 mM. The concentration of ethanol used in this study (100 mM) has been demonstrated to be pathophysiologically relevant to the heavy consumption of alcohol by humans (14). This concentration is equivalent to the blood alcohol content of an individual who is legally intoxicated (8). Indeed, an established animal model of chronic alcohol disease, the Tsukamoto-French diet (5), uses levels of ethanol well beyond 100 mM for up to 8 wk before levels of toxicity are observed. This would suggest that the desensitization of PKA-mediated ethanol stimulation of CBF is not the result of cellular toxicity.

To control for a global toxicity effect of ethanol on the BBEC, we have assayed numerous cellular functions after 100 mM ethanol treatment. Our cell viability studies (lactate dehydrogenase release and trypan blue exclusion) show that 100 mM ethanol is not cytotoxic to the cells. Additionally, the protein kinase C-mediated release of IL-8 in these cells (37, 38) remains intact after ethanol washout, further supporting the fact that chronic ethanol treatment specifically desensitizes CBF (data not shown). Also, the baseline levels of CBF continued throughout extended ethanol treatment and did not decrease below medium-conditioned baseline CBF levels, as would be expected in a dying cell. Only the “flight response” as induced by agonist stimulation of cilia was affected by chronic treatment with ethanol. Salathe and Bookman (26) have suggested that this “idle speed” of cilia is regulated by calcium waves, not by cyclic nucleotides. Furthermore, 100 mM ethanol-treated BBEC can continue to proliferate and migrate beyond 24 h to close a wound in the in vitro wound-healing model of Kim et al. (13), normal cell functions that would not occur in the toxic dying cell. Finally, cyclic nucleotide measurements demonstrate that adenyl cyclase continues to be stimulatable after long-term ethanol treatment because both isoproterenol and forskolin can elevate cAMP, even in the 24-h ethanol-exposed cells (Table 1). The function of the cyclases are not diminished by chronic ethanol, as observed in our assays. Exposure to ethanol is reversible in washout studies of ciliary beating. This would also suggest that 100 mM ethanol is not toxic to the cells.

Our control studies indicate that this phenomenon is not the result of the evaporative loss of ethanol (Fig. 2) nor is this a function of the metabolic breakdown of ethanol by alcohol dehydrogenase (ADH). Indeed, we found little or no presence of ADH activity in the BBEC (data not shown). We have also found that this desensitization effect occurs with direct or vapor-phase ethanol exposure in vitro (data not shown). Stimulated increases in enzyme activity are generally accepted to be significant if the degree of the activity ratio is two or larger. Indeed, we consistently observed at least a twofold ethanol-stimulated increase in PKA beginning...
at ~1 h of treatment in all experiments. In Fig. 2, there is an actual increase in the PKA activity ratio, approaching threefold between 4 and 6 h. As speculated, this difference is most likely the result of the maximal ethanol stimulus of 100 mM being replaced every hour, producing a protocol-specific effect. This effect appears to manifest itself by sustaining the duration and magnitude of maximal PKA activation but not indefinitely. This would support our hypothesis that no decrease in ethanol signal could be occurring because of evaporative loss or cellular metabolism, as PKA activity subsides after 6 h in both cases.

The alteration of protein kinases by ethanol is well described in other systems (4, 7, 21, 22, 28, 34), further supporting an important role for kinase control of ciliary motility in the airway. Just as the increase in CBF appears to be tightly coupled to the activation of PKA in the BBEC, we also observed that prolonged exposure to ethanol downregulates PKA activity. Furthermore, once the BBEC have been exposed to desensitizing concentrations of ethanol, PKA-stimulated CBF remains unresponsive to activation by cAMP analogs and isoproterenol. This suggests that ethanol has uncoupled the regulatory pathway by which PKA activation leads to increases in ciliary motility. Interestingly, ethanol also desensitizes the cells to CBF increases stimulated by 8-BrcGMP and SNP (Fig. 4). This observation supports our previous findings that both cAMP- and cGMP-dependent pathways are responsible for stimulated increases in CBF (39) and that ethanol stimulation of CBF involves a dual activation of PKA and PKG (32). Certainly, there is an NO component to CBF as first presented by us (31) and supported by the findings of others (40). The lack of response of cellular CBF to cGMP elevation in the chronic ethanol model may also indicate that cGMP signals via PKA in the ethanol-stimulated airway epithelial cell. We cannot rule out that cGMP and cAMP increase CBF by a compound pathway instead of two independent pathways. The precise mechanism of interaction between these cyclic nucleotide pathways during acute ethanol treatment is currently under investigation.

The downregulation of β-adrenergic receptors in response to β-agonists has been well described (12, 23). However, it does not appear that ethanol-stimulated PKA and CBF involve the β-adrenergic receptor. We have found that pretreatment of the BBEC with propranolol does not inhibit ethanol-mediated increases in PKA or CBF (data not shown). Although receptor desensitization would explain the loss of isoproterenol-stimulated PKA activity after chronic ethanol exposure, it does not account for the loss of stimulated PKA and CBF by cAMP agonist analogs in the chronic ethanol-treated cell. In preliminary studies, we have observed a small activation of cAMP-phosphodiesterase in response to ethanol, but this does not explain our cAMP analog observation because 8-BrcAMP should be resistant to phosphodiesterase activity. Because PKA remains unresponsive to exogenous activators after chronic ethanol exposure, our findings suggest that the function of the kinase itself is altered in some way by long-term ethanol exposure.

There may be several possible explanations for the modulation of PKA by chronic ethanol. Chronic ethanol may induce the formation of a PKA inhibitory protein over time. The rapid nature of desensitization (hours vs. days) suggests that synthesis of a new PKA inhibitor is unlikely. Chronic ethanol may alter the binding of cAMP to PKA or may directly affect the active site of PKA. If cAMP binding is altered because of chronic ethanol treatment, then 8-BrcAMP would not be able to activate PKA after the cells have been desensitized to ethanol. Previous in vitro kinase activity studies with purified enzyme and substrate suggest that ethanol does not alter the functional structure of the enzyme (19). Chronic ethanol may interfere with the autophosphorylation of PKA, thus altering the “priming” of the kinase to acute ethanol. The autophosphorylation of PKA has been shown to increase the enzyme baseline catalytic activity in response to full activation by cAMP (33). Chronic ethanol may reduce the autophosphorylation potential of PKA, thus resulting in the kinase’s inability to bind cAMP in response to an agonist.

In our opinion, the most compelling explanation of PKA desensitization relates to the compartimentalization of PKA in the BBEC. Chronic ethanol may interfere with the targeting of PKA to its substrate. The specific compartimentalization of PKA may direct its ability to phosphorylate substrate resulting in increased CBF. If chronic ethanol blocks or alters the targeting of PKA to this substrate, it could result in no PKA activity or CBF increases. Therefore, it may be useful to localize PKA in the cell under conditions of ethanol treatment and identify potential cellular substrates for PKA.

In summary, we have found that chronic ethanol exposure downregulates PKA in airway epithelium. This is likely an important mechanism by which ethanol blunts the airway’s responsiveness to normal and pathological stimuli. Ethanol’s effect on signal transduction in the lung may cause the impaired host defenses observed after chronic ethanol ingestion. The seemingly beneficial effects of acute ethanol exposure (≤6 h) on ciliary motility we originally observed (31) appear to contradict what is known about host defenses in chronic alcoholics. Our current findings provide a more compelling explanation of how ethanol likelyimpairs rather than improves airway host defenses. In this way, ethanol may be a “two-edged sword” for the ciliated airway cell, since acute exposure has a very different impact on ciliary motility than does chronic exposure.

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REFERENCES


