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Reduction in the bioelectric properties of swine tracheal submucosal gland cells in culture after daily short-term exposure to cocaine

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Abstract

Chronic use of cocaine has been associated with respiratory complications. In this study, we investigated the effects of daily short-term cocaine exposure on epithelial bioelectric properties and chloride secretion in response to secretagogues in primary culture of swine tracheal submucosal gland cells grown on microporous inserts. Cell cultures exposed continuously to cocaine for 24 h or intermittently for 30 min daily for up to 3 consecutive days, resulted in a concentration-dependent reduction in transwell voltage and transepithelial resistance. Cocaine (300 μM) treatment for 24 h decreased the voltage and resistance by 87 and 75%, respectively. The voltage and resistance were also substantially decreased after 3 days of intermittent cocaine (10–30 μM) exposure. Cocaine exposure protocols used here did not enhance lactate dehydrogenase (LDH) release. Chloride secretion was measured as short-circuit current utilizing Ussing chamber methodology. Cocaine exposure did not change the decreases in short-circuit current caused by amiloride (10 μM), but reduced the increases in short-circuit current induced by acetylcholine and isoproterenol. After 3 days of intermittent cocaine (30 μM) exposure, the maximal acetylcholine and isoproterenol responses were reduced by 67 and 71%, respectively. Therefore, cocaine exposure continuously for 24 h or intermittently for 30 min daily for up to 3 days decreased basal transepithelial voltage as well as resistance and reduced the responses to cholinergic and β-adrenoceptor agonists. These results suggest that alterations in epithelial function can occur even after daily transient cocaine exposure. © 1997 Elsevier Science B.V.

Keywords: Cocaine; Cl^- secretion; Short-circuit current; Trachea; Gland cell

1. Introduction

Cocaine acting as a psychomotor stimulant produces an elevation of mood and a sense of increased self-esteem, well-being, and mental and physical capacity (Das and Laddu, 1993). The principal mechanism is through inhibition of the monoaminergic neuron uptake transporter, resulting in alterations of neurotransmitter homeostasis (Ritz et al., 1987; Calligaro and Eldefrawi, 1988; Pitts and Marwah, 1989). These properties of cocaine lead to drug abuse. Several medical complications also occur, for example, hyperthermia, cardiovascular dysfunction, hepatotoxicity, and neurologic and respiratory toxicity (Warner, 1993). Long-term use of cocaine has been linked to dysfunction in pulmonary diffusion, pulmonary hemorrhage, chronic sinusitis, atrophy and necrosis of nasal mucosa, and loss of the sense of smell (Itkonen et al., 1984; Schweitzer, 1986). The function of airway epithelium is altered after cocaine exposure. Cocaine reduces the rate of mucous clearance and paralyzes the cilia of the surface epithelium (Barton and Gray, 1979). Acute cocaine also decreases basal and secretagogue-stimulated epithelial ion transport by whole tissue (Farley et al., 1991) and submucosal gland cells in culture (Chan et al., 1996).

Airway epithelial secretions, including ion transport, mucous secretion and fluid movement, play an important role in pulmonary clearance and defense of the respiratory tract. Surface epithelium and submucosal gland cells are the major cell populations (Marin, 1986) in airway epithelium, with submucosal glands contributing the largest source of airway secretions (Reid, 1960; Rhodin, 1966). Gland cells in culture, grown in primary culture on microporous inserts at an air interface, generate transepithelial potential differences and currents, and retain their differentiation and polarity similar to intact tracheal epithelium (Sommerhoff and Finkbeiner, 1990; Yamaya et al., 1991; Chan et al., 1996). The isolation and culture of specific

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airway cells provide an important model to ascertain cell specific functions and study the toxic effects of chemicals. In our previous report, acute cocaine was shown to inhibit Cl^- secretion in cultured tracheal submucosal gland cells. However, the effects of repeated or chronic cocaine exposure on airway epithelium are not known. Therefore, this study was undertaken to investigate the effects of chronic cocaine treatment on airway epithelium. We propose that airway illness occurs when gland cell function is altered after daily cocaine use.

2. Materials and methods

2.1. Materials and solutions

Microporous membrane Millicell®-HA inserts with 0.45 μm pore size and 0.6 cm^2 area were obtained from Millipore (Bedford, MA) and 12-well tissue culture plates from Corning (NY). PC-1 serum free medium was purchased from Hycor Biomedical (Irvine, CA). Fetal calf serum and Glutamax were purchased from Gibco BRL (Grand Island, NY). All other reagents were obtained from Sigma Chemical (St. Louis, MO). The Ussing chamber system was purchased from Costar (Cambridge, MA) and a modified insert holder was built in the machine shop in the Department of Pharmacology and Toxicology, University of Mississippi Medical Center.

HEPES Ringer's solution contained (mM): NaCl, 140; KCl, 5.0; $CaCl_2$, 2.0; glucose, 5.5; HEPES, 10; pH was adjusted to 7.4. The solution used in the Ussing chamber had an osmotic pressure of 320 mOsm and was composed of (mM): NaCl, 113; KCl, 4.8; $CaCl_2$, 2.5; $NaHCO_3$, 18; KH_2PO_4, 1.2; $MgSO_4$, 1.2; glucose, 5.5; and mannitol, 30; pH was adjusted to 7.4.

2.2. Cell dissociation and culture

Male weaning swine (Yorkshire), 20 to 25 kg, were purchased from local suppliers. Swine were fed standard laboratory chow and tap water and were housed indoors in the animal facilities under automatically controlled temperature and light cycle conditions. Tracheal submucosal gland cells were isolated and cultured by methods identical to our previous report (Chan et al., 1996).

After isolation, cell viability was $>95\%$ as measured by trypan blue exclusion. The cell suspension was adjusted to a concentration of 5×10^6 cells/ml in PC-1 medium and plated at a density of 1×10^6 viable cells onto microporous-bottomed Millicell®-HA inserts coated with human placental collagen (20 $\mu g/cm^2$, Sigma C5533 Type IV). The inserts were incubated at 37°C in an atmosphere of $95\%O_2/5\%CO_2$ for 24 h to permit cell attachment. The culture medium was then removed from inside the inserts in order to grow the cells at an air-interface. Mucosal fluid was removed every day and the medium (0.5 ml) in the culture dish was changed every day. Confluent cell monolayers formed on the second day after plating.

2.3. Measurement of bioelectric properties

Transepithelial voltage and resistance were measured with a Millicell®-ERS (Millipore, MA) beginning the second day after plating and every day thereafter. In order to measure the bioelectric properties, about 0.3 ml of medium was added to the mucosal side of each insert. After measurements, the mucosal medium was removed to keep the cells at an air-interface. Transwell voltage is expressed in mV and transepithelial resistance in kΩ.

2.4. Cocaine exposure

Two to three days after plating the PC-1 medium on the serosal side of some inserts was replaced with the medium containing cocaine (30–300 μM) for 24 h. After exposure the bioelectric properties of submucosal gland cells in culture and lactate dehydrogenase (LDH) activity released into the culture medium were measured. In Ussing chamber study, the inserts after 24 h cocaine exposure were immediately washed free of cocaine to measure the short-circuit current. In other inserts cocaine (1–30 μM) was applied serosally for 30 min daily for up to 3 consecutive days to mimic the cocaine plasma levels expected in a cocaine abuser. This was termed intermittent treatment. After treatment the inserts were washed free of cocaine. Twenty-four hours after the last exposure, the bioelectric properties, LDH activity and short-circuit current in Ussing chamber were measured.

2.5. LDH assays

The determination of LDH activity was modified from the spectrophotometric method of Wroblewski and Ladue (1955). The activity of LDH was measured by monitoring the rate at which pyruvate was reduced to lactate. The LDH kits (340-LD) were purchased from Sigma Chemical (St. Louis, MO). Phosphate buffer (2.85 ml) was pipetted into the NADH vial followed by addition of 0.05 ml serosal medium collected from the cell cultures. The samples were vortex-mixed and left at room temperature for 20 min. Sodium pyruvate solution (0.1 ml) was then added to initiate the reaction. The rate of the decrease in absorbance of NADH was measured at 340 nm at 30 s intervals for 3 min vs. water as reference.

2.6. Measurement of short-circuit current

Short-circuit current was measured using the methods described by Farley et al. (1991) and Chan et al. (1996). The entire insert was mounted in the lucite chamber (Costar) which was adapted to accept the Millipore insert.

The chambers were maintained at 37°C. The solution in the chamber was bubbled continuously with $95\%O_2/5\%CO_2$ and circulated by a bubble lift device. Transepithelial potential difference across the confluent cell layer was measured using VCC 600 amplifiers (San Diego, CA). The transepithelial voltage was held at 0 mV by voltage clamp. The current required to maintain this potential has been termed the short-circuit current and results from Cl^- secretion and Na^+ reabsorption. Amiloride (10 μM) was added to the mucosal side to inhibit sodium reabsorption during all experiments. Therefore, short-circuit current primarily represents Cl^- secretion.

2.7. Data analysis

Data are presented as mean ± S.E.M., and n is the number of inserts examined. Concentration–response curves were fitted using the sigmoidal fit in Origin Microcal and EC_{50} values were estimated. Statistical significance was established using one-way analysis of variance (ANOVA) followed by Bonferroni post test for multiple comparisons. $P < 0.05$ was considered to be statistically significant.

3. Results

3.1. Effects of cocaine exposure on bioelectric properties of tracheal submucosal gland cells

In our previous report (Chan et al., 1996), tracheal submucosal gland cells cultured at an air-interface formed confluent monolayers that developed significant transwell voltage and resistance on day 2 that remained relatively stable through day 6 after plating. Thus the experiments performed here were done using inserts from day 3 to 6. Cultured gland cells maintain cell polarity and retain properties similar to intact tissue, as noted previously by others (Yamaya et al., 1991).

The transwell voltage (V) and transepithelial resistance (R) of tracheal submucosal gland cells in culture were reduced by continuous or intermittent cocaine exposure. Cocaine, applied in culture either at 30–300 μM for 24 h (Fig. 1) or at 1–30 μM for 30 min daily for 3 days (Fig. 2), significantly decreased the transwell voltage and transepithelial resistance of cultured tracheal submucosal gland cells in a concentration-dependent fashion. The average voltage and resistance for controls were 10.7 ± 1.0 mV and 2.4 ± 0.2 kΩ, respectively ($n = 6$) without cocaine exposure. However, the voltage and resistance were 1.4 ± 0.4 mV and 0.6 ± 0.1 kΩ after 300 μM cocaine exposure for 24 h ($n = 6$) giving 87 and 75% decreases, respectively. After intermittent treatment of cultures for 30 min daily for up to 3 days, cocaine (10 and 30 μM) also decreased both transwell voltage and resistance (Fig. 2A, B). Note that 3 μM cocaine reduced the calculated current ($I = V/R$) after one day exposure. However, 1 μM cocaine had no significant effect on the bioelectric properties of tracheal submucosal gland cells in culture.

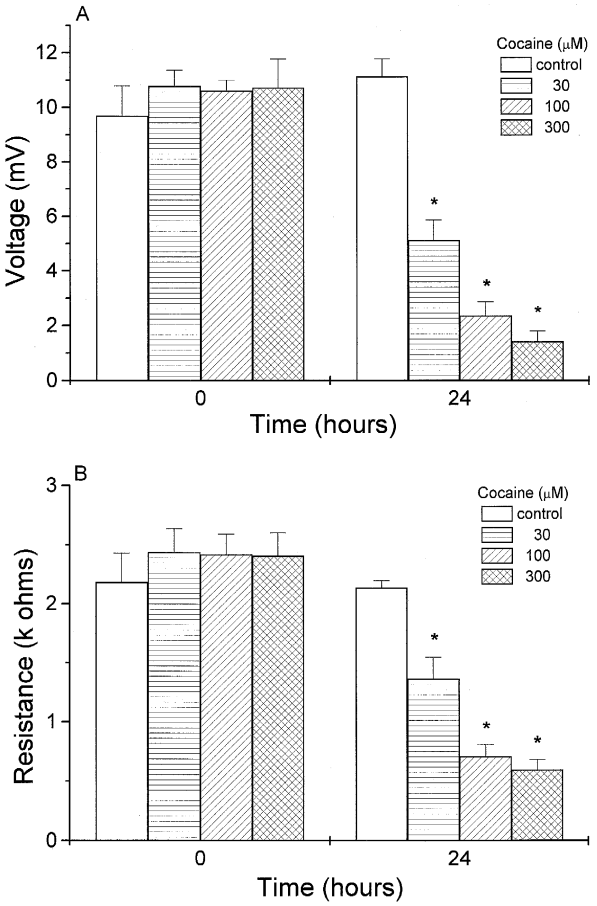

Fig. 1. Bioelectric properties of tracheal submucosal gland cells in culture after 24 h exposure to cocaine. Voltage (A) and resistance (B) were decreased by cocaine (30–300 μM) after 24 h treatment. Values are presented as mean ± S.E.M. ($n = 6$). The * indicates a significant ($p < 0.05$) difference from the respective control values.

3.2. LDH activity during exposure of tracheal submucosal gland cells to cocaine

Lactate dehydrogenase (LDH) activity released into the medium was measured in tracheal submucosal gland cells exposed to 30–300 μM cocaine for 24 h or 3–30 μM cocaine for 30 min daily for 3 consecutive days. Twenty-four hours after the beginning of cocaine exposure the serosal and mucosal medium were removed and assayed for LDH activity. LDH activity in control and continuous 24 h cocaine (30–300 μM) exposure was 16.4 ± 3.5 and 14.5 ± 1.3 to 21.1 ± 3.6 u/ml ($n = 3$), respectively. This cocaine treatment had no significant effect on the release of LDH when compared with control. The LDH activity increased from day 1 to day 3 both with and without cocaine exposure. However, the treatment protocol did not

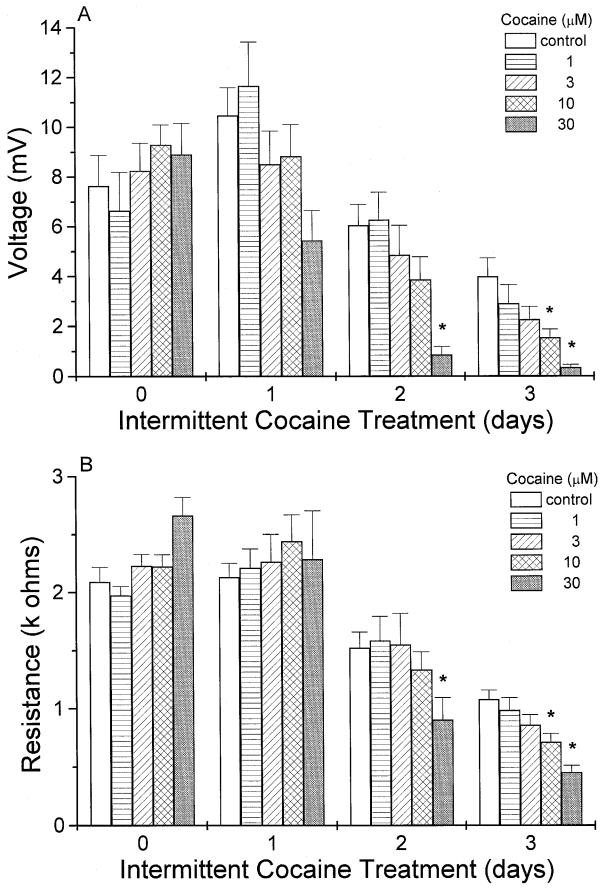

Fig. 2. Effects of daily intermittent cocaine exposure on bioelectric properties of tracheal submucosal gland cells. Cultures were exposed to cocaine (1–30 μM) for 30 min per day for 1–3 days. Both transepithelial voltage (A) and resistance (B) were reduced by cocaine in a concentration-dependent manner after 2–3 days exposure. Values are presented as mean $\pm$ S.E.M. ($n = 3$). The * indicates a significant ($p < 0.05$) difference from the respective control values.

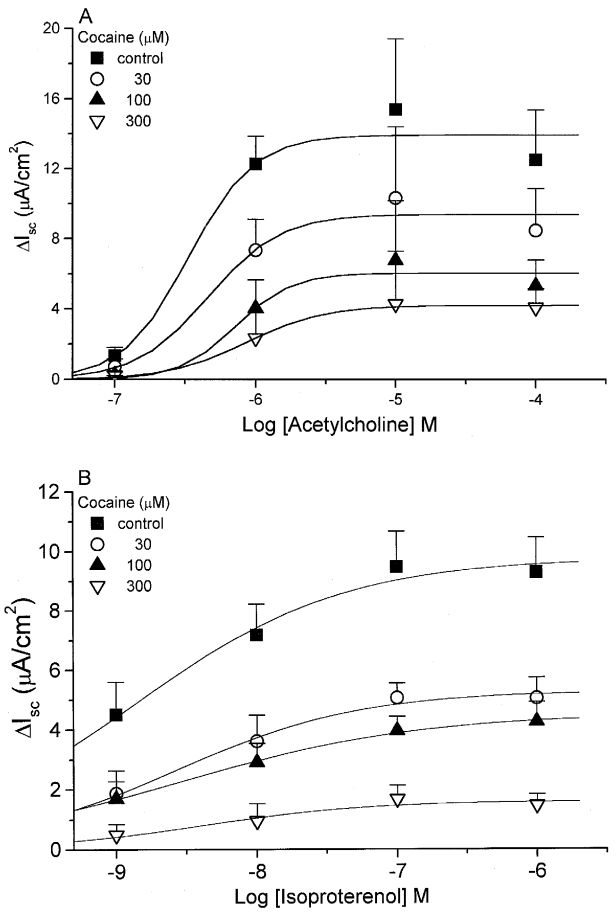

Fig. 3. Concentration-dependent effects of cocaine treatment for 24 h on the increases in short-circuit current induced by acetylcholine and isoproterenol. The curves are the best fit of data to the sigmoidal fit using Origin logistical equation. The initial parameter of the changes in short-circuit current (ΔI_{sc}) is set at 0 μA/cm^2. Cocaine (30–300 μM) decreased the increases in short-circuit current induced by cumulative addition of acetylcholine (A) and isoproterenol (B) in a concentration-dependent manner. The symbols represent the mean $\pm$ S.E.M ($n = 4$).

significantly increase LDH leakage when compared with timed controls (Table 1). Lysing the cells in the insert with PC-1 medium gave an LDH activity of $\sim$ 300 u/ml.

3.3. Effects of cocaine treatments on the increases in short-circuit current induced by acetylcholine and isoproterenol

Cocaine (30–300 μM) treatment for 24 h, caused a concentration-dependent reduction in the increases in short-circuit current induced by cumulative addition of acetylcholine (Fig. 3A) and isoproterenol (Fig. 3B). Note that the insert after 24 h exposure has been immediately washed free of cocaine before application of the neurotransmitters. The maximal responses of acetylcholine and isoproterenol were decreased by cocaine exposure.

The increases in short-circuit current induced by acetylcholine and isoproterenol were also reduced by intermittent cocaine (1–30 μM) exposure for 30 min daily for 3 consecutive days (Fig. 4). Amiloride (10 μM), applied to the mucosal side of the Ussing chamber, reduced the basal short-circuit current in all cases to a similar extent which was between -1.0 ± 0.1 and -1.4 ± 0.1 μA ($n = 6$). The maximal responses to acetylcholine and isoproterenol were also reduced after 3-day intermittent cocaine exposure in a concentration-dependent fashion (Fig. 4), but the EC_{50}s of acetylcholine and isoproterenol induced short-circuit current were not significantly changed (Table 2).

Table 1
LDH activity in control and 3-day intermittent cocaine exposure

Days	LDH activity (u/ml)			
	Control	Intermittent cocaine exposure		
		3 μM	10 μM	30 μM
1	14.3 $\pm$ 3.0	7.1 $\pm$ 1.8	10.1 $\pm$ 4.3	10.7 $\pm$ 1.6
2	24.7 $\pm$ 3.9	233.0 $\pm$ 0.5	23.8 $\pm$ 6.5	24.6 $\pm$ 3.1
3	27.4 $\pm$ 1.9	29.7 $\pm$ 4.6	32.3 $\pm$ 3.1	36.8 $\pm$ 3.2

Values are presented as mean $\pm$ S.E.M.

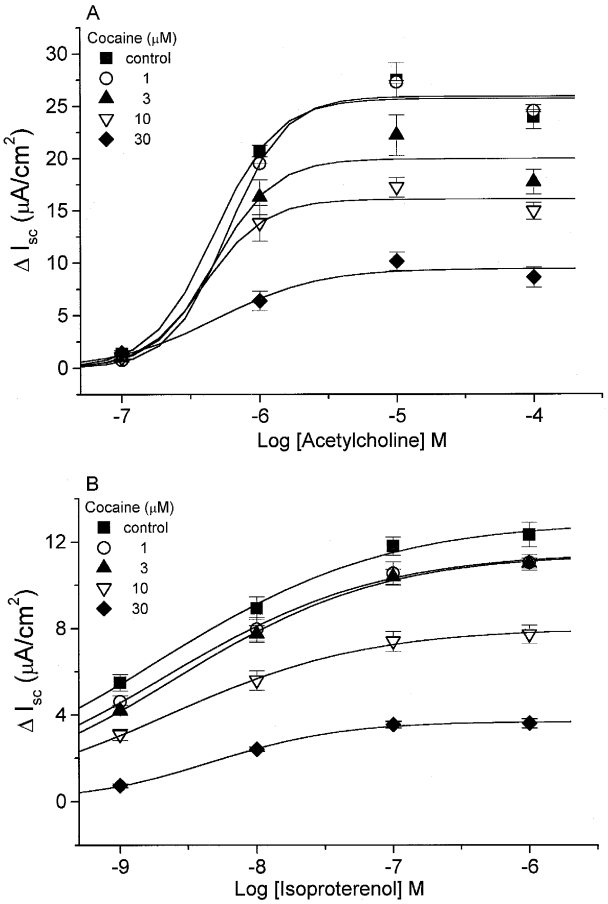

Fig. 4. Concentration–response relationships for intermittent cocaine exposure on the increases in short-circuit current induced by acetylcholine and isoproterenol. The plots are obtained by cumulative addition of acetylcholine (A) or isoproterenol (B) in the absence and presence of cocaine exposure. The curves are the best fit of data to the sigmoidal fit using Origin logistical equation. The initial parameter of the changes in short-circuit current (ΔI_{sc}) is set at 0 $\mu A/cm^2$. Cocaine (1–30 μM) inhibited the increases in short-circuit current induced by acetylcholine (A) and isoproterenol (B) in a concentration-dependent fashion. Note that cocaine at 3–30 μM reduced the maximal response to both acetylcholine and isoproterenol. The symbols represent the mean ± S.E.M ($n = 3$).

Cocaine (30 μM) decreased the maximal acetylcholine and isoproterenol responses by 67 and 71%, after 3 days intermittent treatment.

Table 2
EC_{50}s for acetylcholine and isoproterenol induced increases in short-circuit current in control cells and 3-day intermittent exposure to cocaine

Cocaine (μM)	EC_{50}	
	Log[acetylcholine] M	Log[isoproterenol] M
Control	−6.33 ± 0.20	−8.72 ± 0.12
1	−6.23 ± 0.17	−8.67 ± 0.09
3	−6.33 ± 0.33	−8.58 ± 0.07
10	−6.40 ± 0.21	−8.65 ± 0.08
30	−6.31 ± 0.24	−8.32 ± 0.06

Values are presented as mean ± S.E.M. ($n = 3$).

4. Discussion

When cocaine is abused via snorting or smoking, the luminal surface of airways is exposed to high concentrations that can alter the respiratory function. Snorting of cocaine can cause nasal collapse, septal perforation, palatal retraction, and pharyngeal wall ulceration (Deutsch and Millard, 1989). Septal mucosal abnormalities consisting of chronic crusting, irritation, and inflammation are commonly associated with nasal routes of cocaine administration (Snyder and Snyder, 1985; Schwartz and Grundfast, 1986). Acute respiratory symptoms, abnormalities in lung function and lung injury are also linked to crack smoking in the clinical population (Catravas and Waters, 1981; Jonsson et al., 1983; Harper et al., 1991). The heavy, habitual smoking of crack results in chronic airflow obstruction in large airways and respiratory tract injury manifested by acute respiratory symptoms (Tashkin et al., 1992). Long-term use of cocaine also causes chronic sinusitis, atrophy and necrosis of nasal mucosa, and loss of the sense of smell (Itkonen et al., 1984; Schweitzer, 1986). Other pulmonary complications induced by cocaine use include pulmonary edema, asthma, hemorrhage, and bronchial irritation (Cucco et al., 1987; Rubin and Neugarten, 1990). Our results suggest that even low level, intermittent cocaine exposure, more typical of recreational cocaine use, can reduce the secretory properties of gland cells.

The bioelectric properties of tracheal submucosal gland cells, transwell voltage and transepithelial resistance, in culture were reduced by 24 h cocaine (30–300 μM) treatment. When the cultures were continuously exposed to cocaine for 48 h, the transwell voltage dropped to zero and the transepithelial resistance was ~ 20% of control ($n = 4$; data not shown). The reduction in transepithelial resistance and potential by continuous 24 h treatment or intermittent exposure for 3 days, suggest that cocaine altered the ability of submucosal glands to transport ions, increased cell death, and/or reduced the cell proliferation rate in culture. The measurement of LDH release was used to assess cellular damage. The results show that LDH leakage was not significantly increased during the treatment protocols used. Thus, cocaine mediated alterations in bioelectric properties of submucosal glands in culture were not due to a large increase in cell death or to damaging the integrity of the confluent cell layer. Thus cocaine may alter cell cycling or cause alterations in the control or expression of ion channels.

Electrolyte transport in the airway epithelium is regulated by autacoids, hormones and neurotransmitters. Cholinergic and adrenergic agonists are some of the mediators found to stimulate airway secretion and have been the most extensively investigated (Welsh, 1987). The secretion of fluid by airway epithelium is driven by Cl^- transport (Na^+–K^+–$2Cl^-$ cotransport) across the serosal membrane and diffusion of Cl^- into the lumen through

Cl^- channels in the luminal membrane (Widdicombe et al., 1991). Potassium transport (Na^+–K^+–ATPase) and K^+ channels in the serosal membrane are also important in maintaining the ion gradients necessary for airway secretion (McCann and Welsh, 1990; Yamaya et al., 1993). Acute cocaine exposure has been shown to inhibit epithelial ion transport induced by neurohumoral agents by whole tissue (Farley et al., 1991) and submucosal gland cells in culture (Chan et al., 1996). The acute inhibitory effects require exposure of cells to cocaine at millimolar concentrations. These concentrations could be reached on the airway surface after snorting of cocaine powder (Farley et al., 1991; Chan et al., 1996). However, gland cells, due to their position in the submucosa, may not be exposed to such high concentrations, but will certainly be exposed to plasma levels of cocaine transiently after snorting. The concentrations of cocaine used in the current study are 10 to 1000 times lower than those used in our previous studies. These concentrations are in the range of blood concentrations (1–100 μM) that have been observed in those who abuse cocaine (Siegel, 1982; Mittleman and Wetli, 1984, 1987). The concentrations used are also lower than the K_i values for blockade of muscarinic receptors and β-adrenoceptors (Sharkey et al., 1988a,b; Farley et al., 1991). The concentrations of cocaine used here have minimal or no observable actions after acute application on either basal or stimulated increases in the short-circuit current (Chan et al., 1996). The question then remains, how do these low intermittent levels of cocaine exposure alter the properties of the monolayer?

The effects of daily 30 min exposure to cocaine (or continuous exposure at higher concentrations for 24 h) result in a reduction in the basal short-circuit current and in the maximal response to acetylcholine and isoproterenol with no significant change in EC_{50}. These data suggest that the affinities of the receptors for binding are not changed for agonists. However, changes in the density of receptors, density of ion channels or the activation properties of ion channels may occur during cocaine exposure. Although the mechanism by which these changes could be caused is unknown, it is obvious that cells grown in culture are rapidly dividing since they become confluent within two days after plating. Thus, any treatment that interferes with cell cycling could have profound effects on the expression of many cellular processes. Klein et al. (1991) have reported that cocaine suppressed the proliferation of cultured mouse splenocytes and human lymphocytes. Mitogenesis and proliferation are known to be inhibited by drugs which block potassium channels (Dubois and Rouzaire-Dubois, 1993; Nilius et al., 1993). The role of potassium channels in cell proliferation has been reviewed by Wonderlin and Strobl (1996). Thus cocaine, as a local anesthetic, may alter cell cycling and/or cause alterations in the control or expression of ion channels.

In summary, daily intermittent cocaine exposure decreases the transepithelial potential and resistance of tracheal submucosal gland cells in culture and inhibits their ability to transport ions and water in response to neurotransmitters. The inhibitory effects of cocaine are not due to increased cell death. The model used mimics the plasma/tissue levels of cocaine and the time course of exposure after drug abuse. These findings suggest that even snorting cocaine once per day for three days could lead to substantial reduction of fluid secretion and mucociliary clearance resulting in tenacious mucous secretions.

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