

# Airway Responses to Aerosolized Brevetoxins in an Animal Model of Asthma

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Florida red tide brevetoxins are sodium channel neurotoxins produced by the dinoflagellate *Karenia brevis*. When aerosolized, the toxin causes airway symptoms in normal individuals and patients with airway disease, but systematic exposures to define the pulmonary consequences and putative mechanisms are lacking. Here we report the effects of airway challenges with lysed cultures of *Karenia brevis* (crude brevetoxin), pure brevetoxin-2, brevetoxin-3, and brevetoxin-tbm (brevetoxin-2 minus the side chain) on pulmonary resistance and tracheal mucus velocity, a marker of mucociliary clearance, in allergic and nonallergic sheep. Picogram concentrations of toxin caused bronchoconstriction in both groups of sheep. Brevetoxin-tbm was the least potent, indicating the importance of the side chain for maximum effect. Both histamine H<sub>1</sub>- and cholinergic-mediated pathways contributed to the bronchoconstriction. A synthetic antagonist,  $\beta$ -naphthoyl-brevetoxin-3, and brevenal, a natural antagonist, inhibited the bronchoconstriction. Only crude brevetoxin and brevetoxin-3 decreased tracheal mucus velocity; both antagonists prevented this. More importantly, picomolar concentrations of the antagonists alone improved tracheal mucus velocity to the degree seen with mM concentrations of the sodium channel blocker amiloride. Thus, *Karenia brevis*, in addition to producing toxins that adversely affect the airways, may be a source of agents for treating mucociliary dysfunction.

**Keywords:** bronchoconstriction; mucus transport; natural therapies

Florida red tide is a harmful algal bloom caused by the dinoflagellate *Karenia brevis* (previously *Gymnodinium breve*). *K. brevis* produces at least nine structurally-related polyether brevetoxins (PbTx) (1–3), with PbTx-2 and PbTx-3 being the predominant forms (4, 5). As a group, the PbTx are lipid-soluble, fused polyethers with molecular weights of approximately 900 D. The toxicity of the PbTx is due primarily to binding at receptor site 5 on voltage-gated sodium (Na<sup>+</sup>) channels (6, 7), causing the channels to open at normal resting potentials with an increase in mean channel open time and inhibition of channel inactivation (8–10).

During red tide events, aerosolized toxin has been linked to both upper and lower airway symptoms, e.g., nonproductive cough, shortness of breath, rhinorrhea, and sneezing, in both normal individuals and in “susceptible populations”, i.e., those individuals with preexisting airway disease (11, 12). There is a suggestion that the frequency of these adverse respiratory events is increased in the latter group (12). A clinical survey indicated that 80% of patients with bronchial asthma were affected during

a red tide event, with some having overt asthma attacks (12). Studies using canine tracheal and human bronchial smooth muscle (12, 13) indicate the toxins stimulate parasympathetic postganglionic neurons, resulting in acetylcholine release and subsequent smooth muscle contraction. *In vitro*, PbTx-induced contractile effects could be blocked with atropine but not with an antihistamine (12, 13).

Although the experimental and epidemiologic data indicate that aerosolized PbTx are environmental irritants, systematic exposure assessment in humans characterizing the pulmonary consequences and the mechanisms responsible for these effects are lacking. Similarly, there is an absence of data from controlled aerosol exposures in animals using environmentally relevant PbTx concentrations (14). To address this problem, we studied the airway responses to inhaled PbTxs in nonallergic (normal) sheep and in our sheep model of asthma, which shares many characteristics of the disease in humans and so can serve as a surrogate for patients with compromised airways (15). For these studies, we determined the effects of controlled airway toxin challenges on pulmonary airflow resistance (RL) and tracheal mucus velocity (TMV), a marker of mucociliary clearance, before and after challenge with lysed cultures of *K. brevis* (crude PbTx, which contains all toxins and cell debris), two purified toxins (PbTx-2 and PbTx-3) present in the highest concentration during the organism’s growth phase (4), or PbTx-tbm, which is a PbTx-2 decomposition product missing the side chain (Figure 1). Our working hypothesis was that airway challenge with PbTx would induce pathophysiologic effects: i.e., bronchoconstriction (an increase in RL) and/or a decrease in TMV. The varied physiologic responses to the different toxin structures in combination with pharmacologic blockade of the toxin-induced effects were used to define mechanisms contributing to these responses.

Our results show that inhaled crude PbTx, purified PbTx-2, PbTx-3, and PbTx-tbm produce concentration-dependent bronchoconstriction in these animals, but the severity of the response is dependent on the presence of the side chain (functional “R” group). As predicted from *in vitro* studies, an anticholinergic agent inhibits the bronchoconstriction, but in contrast to the previous *in vitro* findings (13), we demonstrate that a histamine H<sub>1</sub> antagonist also inhibits the response. The combination of the two agents provides the most effective protection, suggesting that both cholinergic and histamine H<sub>1</sub>-mediated pathways contribute to the bronchoconstriction. We show for the first time *in vivo* that a synthetic PbTx derivative,  $\beta$ -naphthoyl-PbTx-3 (16), and brevenal (17), a newly described antagonist produced by the organism itself, inhibit the PbTx-induced bronchoconstriction. In contrast to the constrictor responses, we found that crude PbTx and PbTx-3, but neither PbTx-2 nor PbTx-tbm, cause mucociliary dysfunction in these animals. Both the synthetic and natural PbTx antagonists block this dysfunction. Finally, because of the putative Na<sup>+</sup> channel blocking action of these antagonists, we examined the effects of antagonists alone on mucociliary function. We show that the antagonists themselves provide the same increase in TMV as is seen with amiloride, a drug used to

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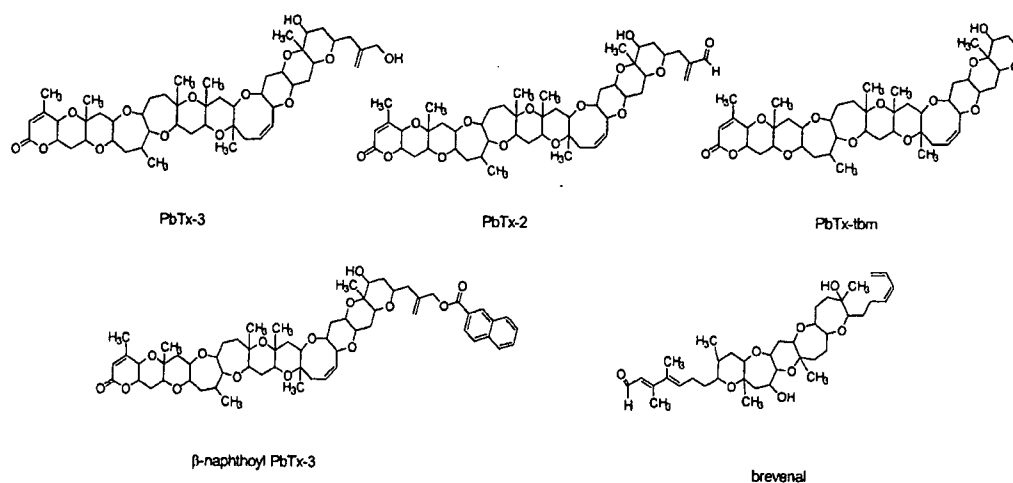


Figure 1. Structures of PbTx-2, PbTx-3, PbTx-tbm,  $\beta$ -naphthoyl-PbTx-3, and brevenal.

improve mucociliary clearance in diseases resulting from impaired epithelial ion transport, e.g., cystic fibrosis (18–20). Thus, *K. brevis*, in addition to producing toxins that cause adverse pulmonary effects, may be a source of agents that may be beneficial in the treatment of diseases associated with mucociliary dysfunction. Some of the results of these studies have been reported previously in the form of abstracts (21–25).

## METHODS

The majority of studies reported here were conducted in adult ewes that had demonstrated airway bronchoconstriction to *Ascaris suum* antigen (allergic sheep) (15). A small number of studies were conducted in animals that had never been exposed to allergen (nonallergic). The allergic animals were not exposed to allergen during the course of these studies. The animals were conscious and restrained in a cart in an upright position with their heads immobilized for the described studies. Instrumentation was performed under local anesthesia. The study was conducted at Mount Sinai Medical Center under the approval of the Mount Sinai Medical Center Animal Research Committee.

### RL

These methods have been reported in detail (26–28). Briefly, a balloon catheter was advanced through one nostril into the lower esophagus, and the animals were intubated with a cuffed endotracheal tube through the other nostril. Pleural pressure was measured via the esophageal catheter. Lateral pressure in the trachea was measured with a side-hole catheter advanced through and positioned distal to the tip of the endotracheal tube. Transpulmonary pressure, the difference between tracheal and pleural pressure, was measured with a differential pressure transducer. To measure RL, the proximal end of the endotracheal tube was connected to a pneumotachograph, and the signals of flow and transpulmonary pressure were recorded on a computer. Respiratory volume was obtained by digital integration of the flow signal so that RL was calculated from the transpulmonary pressure and flow at isovolumetric points. Analysis of 5–10 breaths was used for each determination of RL.

### TMV

These methods have also been reported in detail (29–31). The animal was secured as described previously, and then the sheep were nasally intubated with a shortened endotracheal tube (Mallinckrodt, St. Louis, MO). The cuff of the tube was placed just below the vocal cords to allow for maximal exposure of the tracheal surface area. After intubation, the animals were allowed to equilibrate for a period of ~20 minutes before beginning the TMV measurements. During the course of the experiment, the inspired air was warmed and humidified using a Bennett Humidifier (Puritan-Bennett, Lenexa, KS). To minimize possible im-

pairment of TMV caused by inflation of the endotracheal tube cuff, the cuff was deflated throughout the study.

### TMV Measurement

TMV was measured by a roentgenographic technique (29–31). Between 10 and 12 radiopaque Teflon/bismuth trioxide disks were insufflated onto the tracheal mucosa using a modified suction catheter connected to a source of continuous compressed air. The catheter remained within the endotracheal tube only, so that no contact with the tracheal surface was made. The cephalad-axial velocities of the individual disks were recorded on videotape from a portable image intensifier unit. Individual disk velocities were calculated by measuring the distance traveled by each disk during a 1-minute period. For each run, the mean value of all individual disk velocities was calculated. A collar containing radiopaque reference markers of known length was worn by the sheep and used as a standard to correct for magnification effects inherent in the fluoroscopy unit.

### Agents

Crude PbTx, purified PbTx-2, PbTx-3, PbTx-tbm,  $\beta$ -naphthoyl-PbTx-3 (16), and brevenal (17) were obtained from the Center for Marine Science, University of North Carolina at Wilmington. Crude PbTx was diluted in artificial sea-water medium (NH-15) buffer (32). PbTx-2, PbTx-3, and PbTx-tbm were first diluted in a small volume of Alkamuls (Emulphor EL-620: ethoxylated castor oil and water) (Chemtec Chemical Co, Chatsworth, CA) followed by suspension in phosphate buffered saline (PBS).  $\beta$ -naphthoyl-PbTx-3 and brevenal were first diluted in a small volume of acetone followed by PBS. Amiloride was diluted in distilled water. Atropine sulfate injection (Baxter Health Care, Deerfield, IL) was given at 0.2 mg/kg intravenously, and the histamine H<sub>1</sub> antagonist diphenhydramine hydrochloride (Elkins-Sinn, Inc., Cherry Hill, NJ) was diluted in PBS and given at a dose of 2 mg/kg intravenously. Cromolyn sodium (1 mg/kg) and histamine diphosphosphate were obtained from Sigma (St. Louis, MO) and diluted in PBS. As reported in detail by us (26–31), we used a dosimeter-piston ventilator system with a Raindrop nebulizer (Nelcor Puritan Bennett, Carlsbad, CA) to deliver aerosols directly into the endotracheal tube on inspiration at a tidal volume of 500 ml and a rate of 20 breaths/minute. The one exception was the amiloride study, where 4 ml of the drug were added to a Pari-LC Jet Plus nebulizer (Pari Respiratory Equipment, Midlothian, VA) (connected to the endotracheal tube), but the nebulizer was not controlled by the dosimeter-ventilator system. The animals breathed the aerosol until there was no drug remaining. Amiloride was obtained from Parion Sciences (Durham, NC). The amiloride data shown in this paper have been published previously and were included for comparative purposes only (33).

### Protocols

**Airway responses.** Baseline RL was measured, and then the sheep (either allergic or nonallergic depending on the experiment) were challenged

with 20 breaths of increasing concentrations of toxin: 0.1, 0.3, 1, 3, and 10  $\mu\text{g}/\text{ml}$  of PbTx-2, PbTx-3, and PbTx-tbm or 20 breaths of 0.1, 0.3, and 1  $\mu\text{g}/\text{ml}$  of crude PbTx. RL was measured within 5 minutes after each delivered concentration. Responses to the various toxins alone were compared with those obtained after treatment with the histamine  $\text{H}_1$  antagonist diphenhydramine or atropine (given 30 minutes before challenge), and the two PbTx antagonists:  $\beta$ -naphthoyl-PbTx-3 (20 breaths of 1, 10, 30, and 100  $\mu\text{g}/\text{ml}$ ) and brevenal (20 breaths of 3, 10, and 100  $\mu\text{g}/\text{ml}$ ). The PbTx antagonists were given 15 minutes before toxin challenge. Repeat challenges were separated by a minimum of 48 hours.

In a separate series of studies, aerosol challenges to 10  $\mu\text{g}/\text{ml}$  of PbTx-2 alone, 10  $\mu\text{g}/\text{ml}$  of PbTx-3 alone, or 10  $\mu\text{g}/\text{ml}$  of a combination of the two purified toxins were performed in six sheep. Baseline RL was measured and then the sheep were challenged with 20 breaths of the toxins. Repeat challenges were separated by a minimum of 48 hours.

Aerosol challenges with histamine were done in four sheep with and without atropine pretreatment. For these studies, baseline RL was measured and then the sheep received 20 breaths of 5  $\text{mg}/\text{ml}$  histamine diphosphate. RL was measured immediately after challenge (i.e., within 5 minutes).

**TMV studies.** TMV studies were done separately from the airway response (bronchoconstrictor) studies. Baseline TMV was measured and then the sheep were given 20 breaths of 10  $\mu\text{g}/\text{ml}$  of PbTx-2, PbTx-3, PbTx-tbm or 10 and 100  $\mu\text{g}/\text{ml}$  of crude PbTx. Repeat measures of TMV were obtained 30 minutes and 1 and 2 hours after challenge. These same studies were repeated with PbTx-3 except that the animals were pretreated with 20 breaths of 100  $\mu\text{g}/\text{ml}$  of  $\beta$ -naphthoyl-PbTx-3 or brevenal 30 minutes before PbTx-3 challenge. Repeat challenges were separated by a minimum of 48 hours.

The effects of the antagonists alone on TMV were studied. TMV was measured before, and then 15 and 30 minutes and 1, 2, and 3 hours after challenge with 20 breaths of 100  $\mu\text{g}/\text{ml}$  or 1,000  $\mu\text{g}/\text{ml}$  of the antagonists. The effects of the antagonists on TMV were compared with responses obtained with 3  $\text{mM}$  amiloride.

### Statistical Analysis

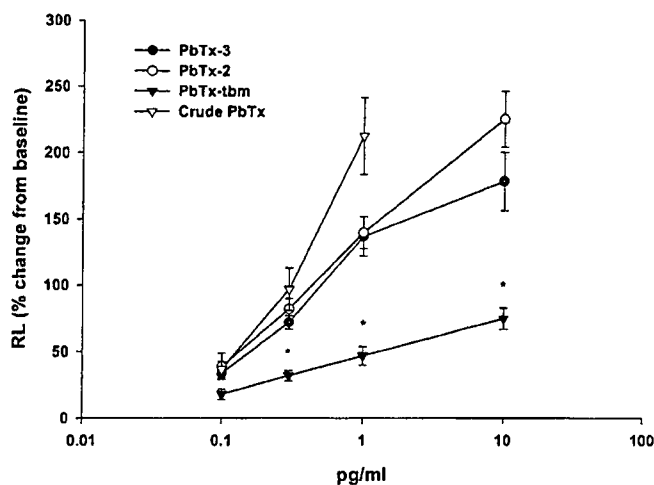
Data were analyzed with one-way analysis of variance followed by Tukey's post hoc test. When only two variables were compared, paired and unpaired *t* tests were used where appropriate. A *p* value of less than 0.05 using a two-tailed analysis was considered significant. Values in the text, tables, and figures are reported as mean  $\pm$  SEM.

## RESULTS

### Airway Responses

Figure 2 summarizes the airway responses of allergic sheep to the toxins used in this study: challenge with crude PbTx, PbTx-2, PbTx-3, and PbTx-tbm all induced concentration-dependent increases in RL (Figure 2). As illustrated in Figure 3, the maximum response occurred immediately after challenge (within 5 minutes) and then began to resolve, returning to prechallenge values by 1 hour. At the toxin concentrations used in this study, allergic sheep that demonstrate late bronchial responses to allergen do not develop late airway responses to inhaled toxin (data not shown). Therefore, for the remainder of the results, all airway responses reported represent the immediate effect of the toxin being studied.

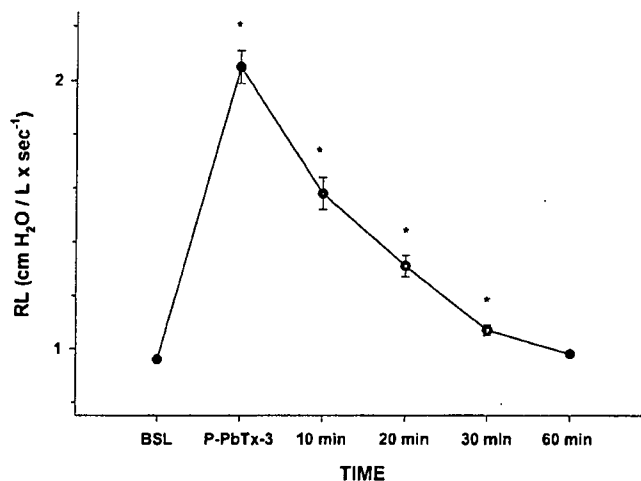
Table 1 gives the baseline and maximum values of RL for the highest concentrations of each toxin used (ending concentrations in Figure 2). Each animal was not exposed to every toxin, but all animals developed a bronchoconstrictor response to each toxin to which they were exposed. Although there were no differences in baseline values for the various challenges, there were differences in the severity of responses to the various toxins (Table 1, Figure 2). Overall, the response to crude PbTx used for these studies tended to be greater than that of either of the pure toxins. One  $\mu\text{g}/\text{ml}$  of crude PbTx produced a  $213 \pm 29\%$  increase in RL compared with  $140 \pm 12\%$  (*p* = not significant)



**Figure 2.** Effect of inhaled toxins on pulmonary resistance (RL) in allergic sheep. All toxins caused significant (*p* < 0.05, analysis of variance) concentration-dependent increase in RL. Values are mean  $\pm$  SEM for 6–12 sheep. \**p* < 0.05 versus all other toxins.

with PbTx-2 and  $137 \pm 15\%$  (*p* = 0.053) with PbTx-3. At 10  $\mu\text{g}/\text{ml}$ , PbTx-2 and PbTx-3 induced  $226 \pm 21\%$  and  $179 \pm 22\%$  increases, respectively, in RL, similar to that seen with the 1  $\mu\text{g}/\text{ml}$  of crude toxin. The response to a 10  $\mu\text{g}/\text{ml}$  mixture of PbTx-2 and PbTx-3 was the same as that for either toxin alone, which suggests that these toxins are acting at the same site. The response to PbTx-tbm was weaker (*p* < 0.05) than all other toxins (Figure 2, Table 1), suggesting that the presence of the side chain is important for maximum potency. Neither the NH-15 buffer nor the Alkamuls diluent affected RL.

To determine if the bronchoconstrictor response to inhaled toxin was limited to allergic sheep, we studied the effect of PbTx-3 in four nonallergic sheep. As was seen in the allergic animals, PbTx-3 caused a concentration-dependent bronchoconstriction in these nonallergic sheep (Figure 4). Although the concentration-response curves between the 2 groups appeared to separate at the highest concentration, the differences were



**Figure 3.** Time course of RL response to inhaled PbTx-3 in allergic sheep. Values are mean  $\pm$  SEM for 4 sheep. \**p* < 0.05 versus baseline (BSL). P-PbTx-3 = post-PbTx-3.

TABLE 1. EFFECT OF DRUGS ON TOXIN-INDUCED BRONCHOCONSTRICTION

Agent	Control*	Diphenhydramine	Atropine	Atropine + Diphenhydramine	Cromolyn
Crude PbTx (% inhibition) <sup>1</sup>	(n = 12)	(n = 4)	(n = 4)	(n = 3)	(n = 4)
Baseline	0.95 ± 0.01	(74 ± 6) <sup>‡</sup>	(72 ± 3) <sup>‡</sup>	(76 ± 1) <sup>‡</sup>	(65 ± 5) <sup>‡</sup>
1 pg/ml	2.97 ± 0.27	0.98 ± 0.01 <sup>‡</sup>	1.02 ± 0.03	0.98 ± 0.01	0.96 ± 0.02
Range:	1.79–4.33 <sup>‡</sup>	1.55 ± 0.02	1.93 ± 0.11	1.24 ± 0.02	2.02 ± 0.09
PbTx-2 (% inhibition)	(n = 7)	(n = 4)	(n = 3)	(n = 3)	
Baseline	0.95 ± 0.01	(74 ± 2) <sup>‡</sup>	(88 ± 1) <sup>‡,†</sup>	(93 ± 2) <sup>‡,†</sup>	ND
10 pg/ml	3.08 ± 0.16	0.95 ± 0.01	0.97 ± 0.02	0.97 ± 0.01	
Range:	2.65–3.91	1.48 ± 0.04	1.24 ± 0.05	1.13 ± 0.07	
PbTx-3 (% inhibition)	(n = 10)	(n = 5)	(n = 5)	(n = 3)	
Baseline	0.97 ± 0.02	(62 ± 7) <sup>‡</sup>	(76 ± 4) <sup>‡</sup>	(96 ± 1) <sup>**</sup>	ND
10 pg/ml	2.69 ± 0.19	0.96 ± 0.01	0.97 ± 0.01	0.97 ± 0.01	
Range:	1.93–3.55	1.59 ± 0.05	1.29 ± 0.02	1.02 ± 0.2	
PbTx-tbm (% inhibition)	(n = 6)	(n = 4)	(n = 3)	(n = 3)	
Baseline	0.97 ± 0.01	(23 ± 10) <sup>††</sup>	(63 ± 3) <sup>‡,†</sup>	(70 ± 3) <sup>‡,†</sup>	ND
10 pg/ml	1.69 ± 0.08	0.98 ± 0.01	0.96 ± 0.01	0.96 ± 0.02	
Range:	1.45–1.92	1.47 ± 0.04	1.22 ± 0.02	1.16 ± 0.01	

Definition of abbreviation: ND = not determined.

\* Values of pulmonary resistance (RL, cm H<sub>2</sub>O/L × sec<sup>-1</sup>) at baseline and for the highest concentration of toxin used for all animals challenged with the specific toxin.

<sup>1</sup> The % inhibition of the control response for only those control animals tested with the inhibitor; except for ranges, values are mean ± SEM.

<sup>‡</sup> p < 0.05 versus control response.

<sup>‡</sup> Baseline and maximum values of RL when toxin was given in the presence of drug for the specified number (n) of animals tested.

<sup>‡</sup> The range of the maximum response in these control studies; these two sets of values are given as reference because different animals from the control group for each toxin were used in the various pharmacologic studies.

<sup>†</sup> p < 0.05 versus H<sub>1</sub> antagonist alone.

<sup>\*\*</sup> p < 0.05 versus H<sub>1</sub> antagonist and atropine alone.

<sup>††</sup> p < 0.05 versus H<sub>1</sub> antagonist effect on other toxins.

not significant. At 10 pg/ml, PbTx-3 increased RL to 2.29 ± 0.07 cm H<sub>2</sub>O/L × second<sup>-1</sup> from a baseline value of 0.96 ± 0.01 cm H<sub>2</sub>O/L × second<sup>-1</sup>. This increase (139 ± 7%) was not significantly different from that seen in the allergic animals (179 ± 22%, see Table 1).

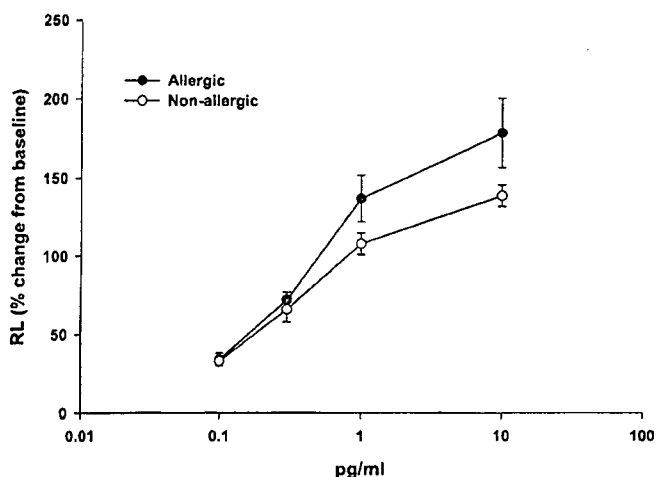


Figure 4. Effects of inhaled PbTx-3 on RL in allergic (n = 7) and nonallergic (n = 4) sheep. Values are mean ± SEM. There were no differences between the groups.

Atropine inhibited the crude PbTx-induced bronchoconstriction, confirming a neural (cholinergic) contribution to the response (Table 1). Unexpectedly however, significant inhibition of the crude PbTx-induced bronchoconstriction was seen with the antiallergic agent cromolyn sodium and the histamine H<sub>1</sub> antagonist diphenhydramine, suggesting that histamine also contributes to the response.

The effects of the H<sub>1</sub> antagonist were seen with the pure toxins as well. Pretreatment with the H<sub>1</sub> antagonist provided similar protection against the PbTx-2-induced (74 ± 2%) and PbTx-3-induced (62 ± 7%) constriction, but the drug was not very effective in blocking the response to PbTx-tbm (Table 1). This suggests that in addition to potency, the H<sub>1</sub>-mediated response is dependent on the presence of the side chain. As was seen with the crude toxin, atropine inhibited the response to PbTx-2 and PbTx-3 (Table 1). Atropine also inhibited the response to PbTx-tbm, but its effect was weaker than that against PbTx-2 (p < 0.01) and PbTx-3 (p < 0.07).

Interestingly, the combination of atropine and the H<sub>1</sub> antagonist was more effective than either drug alone in inhibiting the PbTx-2 or PbTx-3 response (Table 1). Atropine had no effect on histamine-induced bronchoconstriction (RL increased 137 ± 15% and 149 ± 10%, n = 4, over baseline, respectively, without and with atropine) in these animals. These results indicate that both a cholinergic and a histamine H<sub>1</sub>-mediated component contribute to the toxin-induced bronchoconstriction *in vivo*, but that histamine release may be the initial trigger for the response.

#### Effects of PbTx Antagonists

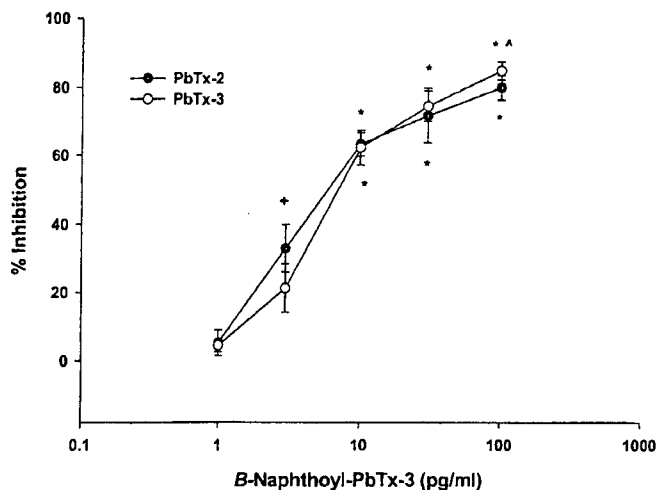
Both β-naphthoyl-PbTx-3 and brevenal competitively displaced brevetoxin in synaptosomal receptor binding assays (16, 17).

Therefore, we tested the effects of these agents on PbTx-induced bronchoconstriction.  $\beta$ -naphthoyl-PbTx-3 blocked PbTx-2- and PbTx-3-induced bronchoconstriction in a concentration-dependent fashion (data not shown). Using the maximum RL value obtained at each antagonist concentration, we plotted a mean inhibition curve for PbTx-2 and PbTx-3 (Figure 5) and from this calculated an inhibitory concentration of 50% ( $IC_{50}$ ) (by interpolation from the mean curve) for  $\beta$ -naphthoyl-PbTx-3 for PbTx-2 (6.9  $\mu$ g/ml) and for PbTx-3 (7.9  $\mu$ g/ml). Thus,  $\beta$ -naphthoyl-PbTx-3 was equally effective against the purified toxins.

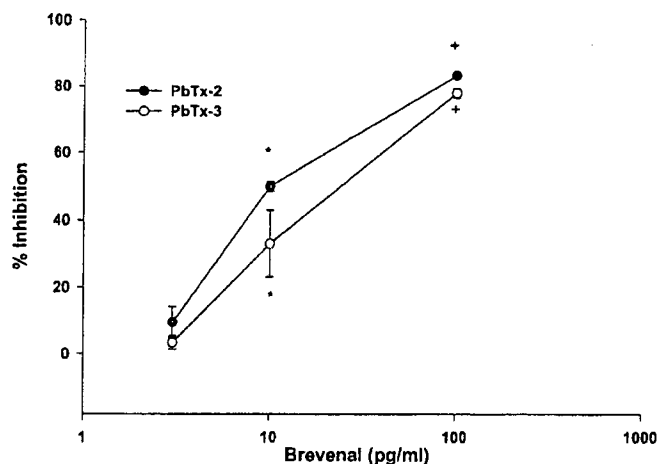
Brevalen also inhibited the constrictor responses to PbTx-2 and PbTx-3 in a concentration-dependent fashion (data not shown). However, we found that brevalen was less active against the PbTx-3-induced bronchoconstriction ( $IC_{50}$  = 44  $\mu$ g/ml) than against the PbTx-2 response ( $IC_{50}$  = 10  $\mu$ g/ml, Figure 6). The difference in potency between  $\beta$ -naphthoyl-PbTx-3 and brevalen was evident when comparing the antagonists' actions against the bronchoconstriction produced by crude PbTx. At 1 and 10  $\mu$ g/ml,  $\beta$ -naphthoyl-PbTx-3 provided  $65 \pm 5\%$  and  $94 \pm 2\%$  inhibition of the crude PbTx-induced response compared with  $20 \pm 2\%$  and  $46 \pm 9\%$ , respectively, with brevalen (both  $p < 0.05$  vs.  $\beta$ -naphthoyl-PbTx-3,  $n = 2-4$ ).

### TMV

Table 2 gives the baseline TMV values for the various studies. Figure 7 illustrates the effect of crude PbTx, PbTx-2, PbTx-3, and PbTx-tbm on TMV. PbTx-3 decreased TMV to  $59 \pm 12\%$  of baseline ( $p < 0.05$ ,  $n = 4$ ) 30 minutes after challenge, and TMV remained depressed for 2 hours. The lack of effect seen with PbTx-2 and PbTx-tbm differs from the bronchoconstrictor response, indicating the importance of both the presence and chemistry of the side chain. Challenge with 10  $\mu$ g/ml crude PbTx did not affect TMV, but at 100  $\mu$ g/ml crude PbTx reduced TMV to  $61 \pm 6\%$  of baseline, a response similar to that achieved with 10  $\mu$ g/ml of pure PbTx-3. The higher concentration of crude PbTx needed to affect TMV is consistent with the makeup of culture extracts that contain approximately 10% PbTx-3.  $\beta$ -naphthoyl-



**Figure 5.** Effect of  $\beta$ -naphthoyl-PbTx-3 on toxin-induced bronchoconstriction in allergic sheep. Data are presented as mean  $\pm$  SEM % inhibition of the maximum response to 10  $\mu$ g/ml PbTx-2 and PbTx-3. The  $IC_{50}$  of  $\beta$ -naphthoyl-PbTx-3 for PbTx-2 and PbTx-3 was 6.9 and 7.9  $\mu$ g/ml, respectively (calculated by interpolation from the mean curve). Values are for 4–7 sheep at each concentration. \* $p < 0.05$  versus 1 and 3  $\mu$ g/ml; ^ $p < 0.05$  versus 10  $\mu$ g/ml; +  $p < 0.05$  versus 1  $\mu$ g/ml.



**Figure 6.** Effect of brevalen on toxin-induced bronchoconstriction in allergic sheep. Data are presented as mean  $\pm$  SEM % inhibition of the maximum response to 10  $\mu$ g/ml PbTx-2 and PbTx-3. The  $IC_{50}$  of brevalen for PbTx-2 and PbTx-3 was 10 and 44  $\mu$ g/ml, respectively (by interpolation from the mean curve). Values are for 4–7 sheep at each concentration. \* $p < 0.05$  versus 3  $\mu$ g/ml; +  $p < 0.05$  versus 3 and 10  $\mu$ g/ml.

PbTx-3 and brevalen (both 100  $\mu$ g/ml) completely blocked the 10  $\mu$ g/ml PbTx-3-induced depression in TMV. Thirty minutes post PbTx-3 challenge, the respective TMV values of the  $\beta$ -naphthoyl-PbTx-3- and brevalen-treated sheep were  $94 \pm 8\%$  and  $95 \pm 2\%$ , respectively, of baseline (both  $p < 0.05$ ,  $n = 4$ ), and TMV values for both groups of treated animals remained near baseline values for the remainder of the study.

A potentially interesting finding was the stimulatory effect of the antagonists on TMV (Figure 8). At 100  $\mu$ g/ml, both  $\beta$ -naphthoyl-PbTx-3 ( $130 \pm 10\%$ ,  $n = 4$ ) and brevalen ( $119 \pm 4\%$ ,  $n = 4$ ) increased TMV 15 minutes after treatment. The effect of  $\beta$ -naphthoyl-PbTx-3 was still evident at 30 minutes ( $132 \pm 8\%$ ), whereas the response to brevalen waned.  $\beta$ -naphthoyl-PbTx-3 at 1,000  $\mu$ g/ml did not affect the peak increase in TMV at 30 minutes ( $126 \pm 16\%$  of baseline,  $n = 4$ ), but the response remained constant for 2 hours ( $125 \pm 17\%$ ). Increasing the brevalen dose had no additional effect. The magnitude and the time-course of the increase in TMV seen with the two antagonists was comparable to that achieved after treating the sheep with the sodium channel blocker amiloride (3 mM), a drug used in the treatment of cystic fibrosis (34).

**TABLE 2. BASELINE VALUES OF TRACHEAL MUCUS VELOCITY**

Agent	TMV (mm/min)
PbTx-2 ( $n = 4$ )	$8.2 \pm 0.8$
PbTx-3 ( $n = 4$ )	$9.0 \pm 1.0$
PbTx-tbm ( $n = 4$ )	$10.2 \pm 1.3$
Crude PbTx ( $n = 5$ )	$11.0 \pm 0.9$
Brevalen ( $n = 4$ )	$8.9 \pm 0.3$
$\beta$ -naphthoyl-PbTx-3 ( $n = 6$ )	$10.4 \pm 0.8$
Amiloride ( $n = 4$ )	$10.1 \pm 0.2$
Vehicle ( $n = 4$ )	$9.2 \pm 0.3$
Brevalen + PbTx-3 ( $n = 4$ )	$9.7 \pm 0.4$
$\beta$ -naphthoyl-PbTx-3 + PbTx-3 ( $n = 4$ )	$10.7 \pm 0.7$

Definition of abbreviation: TMV = tracheal mucus velocity.

Values are the mean  $\pm$  SEM for the number of sheep ( $n$ ) used in the study.

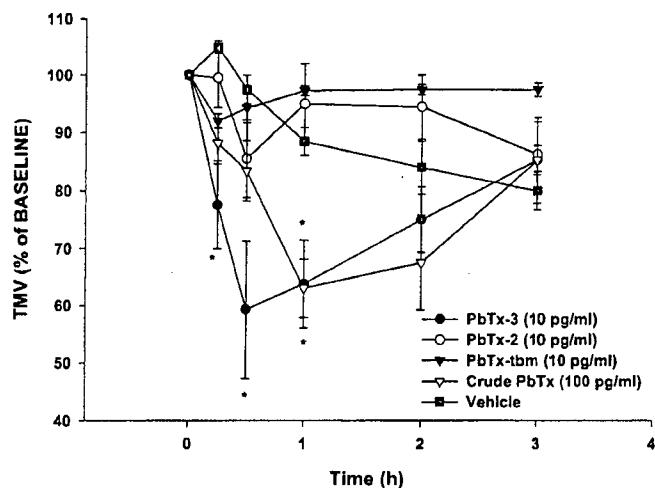


Figure 7. Effect of toxins on tracheal mucus velocity (TMV) in allergic sheep. Only PbTx-3 and crude PbTx reduced TMV. Values are mean  $\pm$  SEM for 4–5 sheep. \* $p < 0.05$  versus vehicle.

## DISCUSSION

These results provide several novel observations concerning the airway effects of aerosolized PbTx and PbTx antagonists. Using well controlled airway challenges we showed that brevetoxins caused bronchoconstriction and slowed TMV, responses indicative of pulmonary pathophysiology. The difference between the toxin-induced effects on  $R_L$  and TMV suggests that the molecular composition (side-chain chemistry) and receptor sites may be important factors in determining the severity of the responses. Unlike published reports, we show that histamine contributes to the toxin-induced bronchoconstriction, and we provide the first *in vivo* data demonstrating the protective effects of a synthetic and natural antagonist. Of greater import, however, is the finding that both antagonists stimulated TMV, and that this

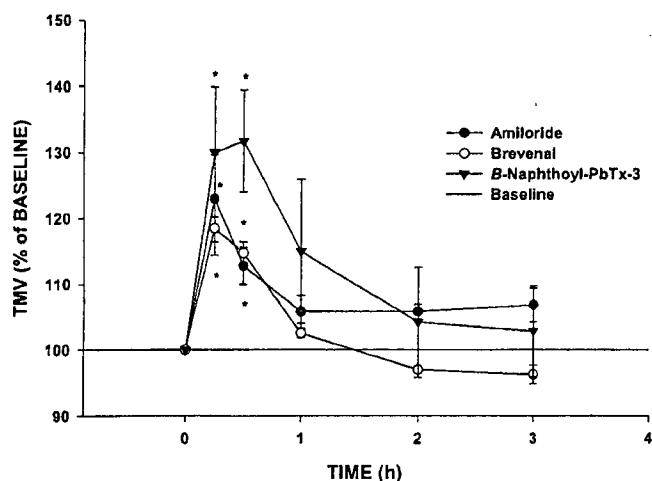


Figure 8. Effect of toxin antagonists and amiloride on TMV in allergic sheep. All agents increased TMV over the first 30 minutes after challenge. Amiloride was given at a dose of 3 mM;  $\beta$ -naphthoyl-PbTx-3 and brevenal were given at a concentration of 100 pg/ml. Values are mean  $\pm$  SEM for 4–6 sheep. \* $p < 0.05$  versus baseline (pretreatment value).

increase in mucociliary clearance was achieved at a  $10^{-6}$ -fold lower dose than that seen with amiloride. Thus, *K. brevis*, in addition to producing toxins, could also be a potential source of agents useful in treating diseases such as cystic fibrosis (34), whose etiology is related to abnormal epithelial ion transport (18–20).

Anecdotal reports that *K. brevis* induced asthma attacks prompted us to examine the bronchoconstrictor effects of inhaled PbTxs (12). Thus, whereas our observation that PbTxs cause bronchoconstriction was expected, quantifying the responses to environmentally relevant concentrations of crude and individual toxins under controlled challenge conditions *in vivo* and the pharmacologic modification of the responses are novel findings. Aerosols of crude PbTx, PbTx-2, PbTx-3, and PbTx-tbm caused concentration-dependent bronchoconstriction. The responses to the crude toxin and the individual toxins, except for PbTx-tbm, were similar in magnitude. Because the difference between PbTx-tbm, PbTx-2, and PbTx-3 is the absence of the side chain, our data suggest that the presence of this functional group is important for the maximum constrictor response. Furthermore, because PbTx-2 and PbTx-3 have different functional groups, the data indicate that the severity of the constrictor response is in part due to the presence and not the chemistry of the side chain. That the response to a mixture of PbTx-2 and PbTx-3 was the same as that for either toxin alone supports this hypothesis and suggests that PbTx-2 and PbTx-3 are acting at the same site.

The anecdotal survey reported by Asai and coworkers (12) indicated that 12 of 15 individuals with asthma were affected by contact with aerosolized toxin and that in some the toxin induced asthma attacks. These reports together with the knowledge that individuals with asthma have a heightened sensitivity to most noxious stimuli, led to the expectation that allergic sheep should have a more severe response to inhaled toxin than normal animals. Although not a primary endpoint of this specific study, we performed a limited number of experiments to address this question. Although we found some separation between the response curves of the 2 groups of animals (Figure 4), within the confines of the present study we were unable to demonstrate a significantly enhanced response to PbTx-3 in the allergic sheep when compared with the nonallergic sheep. Both groups of animals responded to inhaled toxin with a significant bronchoconstriction. That the normal animals respond to inhaled toxin is consistent with reports indicating that individuals with normal airways as well as those with compromised airways develop respiratory symptoms after an environmental exposure (11, 12). That we were unable to demonstrate a difference in the sensitivity to inhaled toxin between the two groups of sheep is not, however, entirely surprising. The inflammatory status of the airways at the time of toxin challenge could be one factor contributing to the similar response. Between antigen provocations the airway cellular characteristics of allergic sheep (as assessed by bronchoalveolar lavage) are relatively normal (15, 28). Thus, if the sensitivity to inhaled toxin is related to the inflammatory state of the lung, then similar responses between unchallenged allergic sheep and nonallergic sheep might occur, as seen in the present study. This explanation, although speculative, is based on our observations that the response to PbTx-3 is significantly enhanced in allergic sheep after antigen challenge (our unpublished observations) when the airways are visibly inflamed (27, 28). It is important, however, that the nonallergic animals responded to the toxin because it mimics what occurs with natural exposures (11, 12) and indicates that the airway responses reported here are not due to the allergic status of the animals.

An important finding was that the bronchoconstriction observed in these animals occurred after inhalation of pg concentra-

tions of toxin. Thus, our results are consistent with the reported environmental toxin levels associated with human respiratory symptoms (14). To put the present results in more perspective, using the same challenge conditions in this animal model (35), our findings with picogram concentrations of PbTx are equal in severity to those obtained with microgram concentrations of leukotriene D<sub>4</sub>, one of the most potent bronchoconstrictors in asthma (36).

The protective effects seen with the histamine H<sub>1</sub> antagonist diphenhydramine and the mast cell stabilizer cromolyn sodium suggest that histamine, presumably released from airway mast cells and/or basophils, contributes to the constrictor response. Our findings differ from *in vitro* studies that indicated antihistamines were ineffective (12, 13). The reason for the discrepancy between the *in vitro* and *in vivo* studies is not clear, but could include the fact that the animals used in the present study were allergic, thereby rendering their cells/receptors more responsive to toxin exposure. It is of interest that mRNA for the  $\alpha$  (1.8) subunit of site 5 voltage-gated Na<sup>+</sup> channels has been identified on human lung mast cells (37). This is different than the reported  $\alpha$  isoforms identified on skeletal (1.4) and cardiac (1.5) muscle that are responsive to PbTx-2 and PbTx-3 (7), but its presence on mast cells could provide a mechanism for direct stimulation. Alternatively, toxin may not stimulate mast cells/basophils directly, but could induce the release of histamine-releasing factors, which then cause histamine-containing-cell degranulation (38). Macrophages are one source of these peptides (39). Because macrophages are known to sequester toxin in the lungs (40) but are unlikely to be present in smooth muscle preparations, the contribution of an intermediary could also explain the different findings.

Atropine blockade of the PbTx-induced bronchoconstriction is consistent with previous *in vitro* studies using dog trachealis muscle (12) and *in vivo* studies in guinea pigs where PbTx was given parenterally (41). Thus, in addition to the histamine H<sub>1</sub>-dependent component, our findings confirm that a cholinergic, i.e., vagal component contributes to the bronchoconstriction. Because the dose of atropine used did not block histamine-induced bronchoconstriction in these animals, our collective findings suggest that both pathways (histamine H<sub>1</sub>- and vagally-mediated) contribute to the bronchial response. The involvement of both pathways is supported by our data showing that the combination of atropine and diphenhydramine provided complete protection against the PbTx-induced response. Furthermore, our data in conjunction with studies showing that histamine depolarizes airway ganglionic neurons via histamine H<sub>1</sub> receptors (42) could indicate that histamine release may be the initial trigger for the *in vivo* airway constrictor response to inhaled brevetoxins.

We show for the first time that the PbTx-induced bronchoconstriction can be blocked with a synthetic derivative,  $\beta$ -naphthoyl-PbTx-3 (16), or the naturally produced antagonist, brevenal (17). *In vitro*,  $\beta$ -naphthoyl-PbTx-3 was shown to competitively displace native toxin from Site 5 of voltage-gated Na<sup>+</sup> channels (16). Our data confirm this antagonist effect:  $\beta$ -naphthoyl-PbTx-3 blocked the crude PbTx-induced bronchoconstriction and was equipotent against PbTx-2 and PbTx-3 induced bronchoconstriction.

A similar pattern of response was seen with the natural antagonist brevenal, the first natural polyether ligand described that prevents natural toxin binding to receptor site 5 of the voltage-gated Na<sup>+</sup> channel (17). Brevenal was found to account for 2–10% of the total polyether biomass extracted from cell cultures (17). The exact physiologic relationship between brevetoxin and brevenal in crude toxin extracts has not been fully characterized, but our findings suggest that it can influence the airway irritancy of toxin. (17). That brevenal blocked the PbTx-induced constrictor

effects to crude and purified PbTxs is consistent with its *in vitro* antagonism. Although the two antagonists demonstrated similarities, there were also subtle differences. Unlike  $\beta$ -naphthoyl-PbTx-3, brevenal was more effective against PbTx-2 than PbTx-3 and was not as effective against crude PbTx as was the synthetic antagonist.

The sheep model is sensitive to inhaled agents that either increase (20, 30) or decrease mucociliary clearance (29, 31). On the basis of previous studies with other noxious agents, we expected that PbTxs would decrease TMV (31, 43). That only PbTx-3 and not PbTx-2 or PbTx-tbm decreased TMV is interesting given that the chemical structures of the side chains are different. Thus, unlike the constrictor responses that appear to be dependent on the presence of a functional group, the different TMV responses may be linked to both the presence and the chemical structure of the side chain. More importantly, these data suggest that the receptor sites inducing changes in TMV are different from the receptor sites responsible for the bronchoconstriction.

We studied the effects of TMV and airway mechanics separately because we did not want the tracheal catheter used in the measurement of airway mechanics to interfere with the TMV measurements. Furthermore, as indicated above, different mediators and/or agents can have different effects on the two parameters, e.g., the responses to PbTx-2 seen here, the effects of histamine, which increases TMV but causes bronchoconstriction or, conversely, the effects of albuterol, which causes bronchodilation but stimulates TMV (44). Thus, changes in TMV are not necessarily linked to changes in airway resistance and so studying the effects of unknown toxins on this parameter separately gave us the best opportunity for determining the effects of the different toxins on this parameter.

TMV reflects the functional interaction amongst the ciliated surface epithelium, the mucus layer, and the underlying periciliary fluid layer (44, 45). Abnormalities in any of these areas can negatively impact TMV. We did not dissect out the area(s) affected by the toxins responsible for the reduction in TMV. It is likely, however, that increased mucus secretion is the primary factor related to this depression, because both cholinergic stimulation and histamine increase mucus secretion (44), and such an increase is consistent with the reports of rhinorrhea and increased expectorated mucus resulting from human toxin exposure (11, 12).

Both antagonists blocked the PbTx-3-induced fall in TMV. The most significant observation, however, was that the antagonists themselves stimulated TMV. Our rationale for examining these agents was based on our previous work identifying compounds that enhance mucus transport (20, 30) and the therapeutic paradigm that blockade of epithelial Na<sup>+</sup> channels increases the periciliary layer, improving the hydration state of mucus and thereby improving transport (46, 47). The expectation was that because  $\beta$ -naphthoyl-PbTx-3 blocks Na<sup>+</sup> channel openings (16), and that brevenal is likely to have a similar mechanism of action; the two compounds would stimulate TMV. This is the putative rationale for the use of Na<sup>+</sup> channel blockers, such as amiloride, in the treatment of cystic fibrosis (34, 46). Our results supported this hypothesis. Furthermore, we demonstrated that pM concentrations of  $\beta$ -naphthoyl-PbTx-3 and brevenal increased TMV to the same degree as that achieved with mM concentrations of amiloride. These data not only reflect the potency of these molecules, but indicate that further modifications of these and/or newly identified PbTx derivatives could provide a source of potent compounds that can be used to combat diseases associated with mucociliary dysfunction.

It should be noted that we did not study the toxin-induced changes in TMV in nonallergic sheep. We do not think this

limitation significantly impacts our findings, however, because our airway response data suggest that the toxins act similarly in allergic and nonallergic airways. Furthermore, the reports of rhinorrhea and increased expectorated mucus in humans resulting from natural toxin exposure do not appear to be limited to individuals with asthma (11, 12).

In summary, we systematically studied the airway effects of inhaled PbTx<sub>3</sub> and showed that these toxins are potent bronchoconstrictors and can slow mucociliary clearance. Both effects would negatively impact normal individuals, but would be an added burden for patients with compromised airways. We describe the novel use of a synthetic and a natural antagonist to modulate these adverse airway events. Finally, we provide new data showing that these antagonists may be beneficial in the treatment of airway diseases whose etiology is related to abnormal epithelial ion transport, thus suggesting that brevetoxin derivatives may offer a new basis for disease-modifying therapies.

**Conflict of Interest Statement:** W.M.A. (with A.J.B. and D.G.B.) is included in the provisional patent application filed on behalf of the University of North Carolina at Wilmington by aaiPharma; A.J.B. has provisional patents filed with respect to treatment of mucociliary diseases for synthetic and natural products derived from cultured red tide, and has an interest in any licensing that might arise from patents and final patents that are not filed yet and, thus, have not been thoroughly reviewed by the U.S. Patent and Copyright Office; J.R.S. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript; A.A. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript; T.A.L. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript; I.S. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript; D.G.B. has provisional patents filed with respect to treatment of mucociliary diseases for synthetic and natural products derived from cultured red tide, and has an interest in any licensing that might arise from patents and final patents not filed yet and, thus, have not been thoroughly reviewed by the U.S. Patent and Copyright Office.

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