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Marine brevetoxin induces IgE-independent mast cell activation

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Abstract Brevetoxins (PbTx) are sodium channel neurotoxins produced by the marine dinoflagellate *Karenia brevis* during red tide blooms. Inhalation of PbTx in normal individuals and individuals with pre-existing airways disease results in adverse airway symptoms including bronchoconstriction. In animal models of allergic inflammation, inhalation of PbTx results in a histamine H_1-mediated bronchoconstriction suggestive of mast cell activation. How mast cells would respond directly to PbTx is unknown. We thus explored the activation of mouse bone marrow–derived mast cells (BMMCs) following exposure to purified PbTx-2. Following in vitro exposure to PbTx-2, we examined cellular viability, mast cell degranulation (β-hexosaminidase release), intracellular Ca^{2+} and Na^{+} flux, and the production of inflammatory mediators (IL-6). PbTx-2 induced significant cellular toxicity within 24 h as measured by LDH release and Annexin-V staining. However, within 1 h of exposure, PbTx-2 induced BMMC degranulation and an increase in IL-6 mRNA expression independent of the high-affinity IgE receptor (FcεRI) stimulation. Activation of BMMCs by PbTx-2 was associated with altered intracellular Ca^{2+} and Na^{+} levels. Brevenal, a naturally produced compound that antagonizes the activity of PbTx, prevented changes in intracellular Na^{+} levels but did not alter activation of BMMCs by PbTx-2. These findings demonstrate that PbTx-2 activates mast cells independent of FcεRI providing insight into critical events in the pathogenesis and a potential therapeutic target in brevetoxin-induced airway symptoms.

Keywords Brevetoxin · Mast cell · Immune

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Introduction

Brevetoxins (PbTx) are toxins produced by *Karenia brevis* dinoflagellates that are capable of adversely affecting marine mammals and human health including adverse respiratory symptoms in allergic airways (Abraham et al. 2005a; Abraham et al. 2005b; Fleming et al. 2005). Human exposure to PbTx occurs via ingestion of contaminated shellfish or inhalation during red tide events in the Gulf of Mexico, off the coasts of Florida and North Carolina, and other coastal areas around the world. Although ingestion is associated with more severe effects due to exposure to larger doses, inhalation of aerosolized PbTx is significantly more widespread (Milian et al. 2007).

Inhalation of red tide aerosols is associated with both upper and lower respiratory symptoms including bronchoconstriction (Backer et al. 2005; Fleming et al. 2009; Fleming et al. 2005; Fleming et al. 2007). Persons with asthma are particularly susceptible to the respiratory effects of inhaled PbTx (Fleming et al. 2009; Fleming et al. 2007). During moderate to high levels of PbTx exposure during a red tide event in Florida, up to 30% of participants in a study on the effects of PbTx developed lower respiratory symptoms (tightness of chest, wheezing, and shortness of breath) and upper respiratory symptoms (cough, eye and nose irritation, and nasal congestion) (Backer et al. 2003). In addition, >33% of these participants developed a mild inflammatory response (Backer et al. 2003). In addition to the epidemiological data on human exposures to red tide

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and respiratory outcomes, the pulmonary effects of PbTx have been studied using several animal models including models of allergic asthma. In asthmatic and non-asthmatic sheep, instillation of PbTx causes significant and rapid increase in bronchoconstriction (Abraham et al. 2005b). Importantly, the PbTx-induced bronchoconstriction in sheep was effectively blocked by addition of the anticholinergic agent atropine, the glucocorticosteroid budesonide, the β_2-agonist albuterol, the mast cell stabilizing drug cromolyn, and a histamine H_1 blocker (Abraham et al. 2005a; Abraham et al. 2005b). In addition, a cutaneous injection of PbTx into skin of sheep resulted in an H_1-mediated hypersensitivity reaction (Abraham et al. 2005a). Both the pulmonary and skin data suggest the participation of mast cells in the adverse response to PbTx exposure. However, it remains to be determined if PbTx activates mast cells.

Mast cells represent an important component of the immune system, which influence both innate and adaptive immunity. They are well documented to be a critical effector cell in allergic reactions and release histamine as well as many other mediators including cytokines and chemokines that influence innate and adaptive immune responses (Brown et al. 2008). Mast cells reside within tissues, including the lung, and have the capability of reacting both within minutes and over hours to specific stimuli leading to local and systemic effects (Brown et al. 2008). Mast cells express the high-affinity IgE receptor (FcεRI) and upon aggregation of FcεRI by allergen-specific IgE, mast cells release and generate biologically active preformed and newly synthesized mediators involved in allergic inflammation, including asthma (Brown et al. 2008). The suggestive evidence that mast cells participate in the asthmatic-like responses in vivo following PbTx inhalation led us to hypothesize that PbTx activates mast cells in vitro independent of FcεRI. We thus entered into experiments to determine the ability of PbTx to induce bone marrow–derived mast cell (BMMC) degranulation and cytokine production. Since the activity and toxicity of PbTx is due to binding of voltage gated sodium channels, we utilized brevenal, the naturally occurring antagonist to PbTx, to investigate if BMMC activation by PbTx is mediated through Na^+ channels (Bottein Dechraoui and Ramsdell 2003; Bourdelais et al. 2004; Purkerson-Parker et al. 2000; Purkerson et al. 1999).

Materials and methods

Brevetoxin preparation

Purified brevetoxin-2 and brevenal were generously provided by Dr. Daniel G. Baden at the University of North Carolina at Wilmington. The PbTx was prepared by dissolving 500 μg in a combination of 80% ethanol and Hanks Balanced Salt Solution (HBSS) then appropriately diluted to make 10^{-5} (9 μg/ml), 10^{-6} (900 ng/ml), 10^{-7} (90 ng/ml), 10^{-8} (9 ng/ml), 10^{-9} (900 pg/ml), 10^{-10} (09 pg/ml), 10^{-11} (9 pg/ml), and 10^{-12} (0.9 pg/ml) molar concentrations in RPMI-1640 media. Brevenal was initially dissolved in a combination of 80% ethanol and HBSS, then prepared for cell culture in RPMI-1640 at 0.5 μM, a concentration that was determined not to induce toxicity as measured by LDH release (data not shown), but still antagonizes PbTx interaction with voltage gated sodium channels.

Cell culture

Mouse BMMCs were cultured from femoral marrow cells of C57BL/6 mice as previously described (Jensen et al. 2006). Cells were cultured in RPMI-1640 supplemented with 10% FBS, 100 U/ml penicillin, 100 μg/ml streptomycin, 25 mM HEPES, 1 mM sodium pyruvate, nonessential amino acids (Sigma–Aldrich, St. Louis, MO), 0.0035% 2-mercaptoethanol, and 300 ng/ml recombinant murine IL-3 (PeproTech, Rocky Hill, NJ). BMMCs were used after 4–6 weeks of culture, a time at which >95% of the cells are mast cells as determined by granule content and FcεRI expression. Cells were treated with PbTx-2 in 96-well plates at 10^{-5} through 10^{-12} M for 1–24 h in the absence of IgE. Cell viability was determined by lactate dehydrogenase (LDH) release. The percentage of LDH release was measured in supernatants of BMMCs exposed to PbTx-2 (10^{-5}–10^{-12} M) for 1, 4, 8 and 24 h (BioVision, Mountain View, CA). Vehicle cells were used as a negative control, and 1% Triton X-100-treated BMMCs were used as a positive control, representing 100% LDH release. In addition, cellular apoptosis was examined by Annexin-V/propidium iodide staining of BMMCs exposed to 10^{-5} M PbTx-2 for 1, 4, 8, and 24 h. Annexin-V and PI staining was determined by flow cytometry (FACscan, BD Biosciences) according to manufacturer's recommendations (Biovision).

In Vitro BMMC degranulation and cytokine release

For degranulation experiments, BMMCs were seeded at 5×10^4 cells/well in a 96-well plate and sensitized overnight with 100 ng/ml mouse IgE anti-DNP (Sigma–Aldrich) to generate a positive control. For treated samples, PbTx-2 (10^{-5}–10^{-12} M) was added for 1 h, and for positive control samples, DNP-HSA was added to induce degranulation via FcεRI. After 1 h incubation with PbTx-2 or DNP-HSA (as a positive control) at 37°C, *p*-nitrophenyl-*N*-acetyl-β-D-glucopyranoside was added to cell supernatants and lysates (generated by addition of 0.1% Triton-X-100) for 90 min as a chromogenic substrate for *N*-acetyl-β-

D-hexosaminidase. The reaction was stopped with 0.2 M glycine. Optical density was measured at 405 nm using a Synergy HT microplate reader (BioTek, Winooski, VT). *β*-Hexosaminidase release was expressed as the percentage of total cell content after subtracting background release from unstimulated cells. Mouse IL-6 was measured in the cell culture supernatants of BMMCs seeded at 2×10^5 cells/well 6 h after addition PbTx-2 (10^{-5} M) using a mouse DuoSet ELISA system (R&D Systems, Minneapolis, MN).

Quantitative real-time PCR

Total RNA from BMMCs (1×10^6 total cells per treatment group) was isolated 2 h following treatment with PbTx-2 at 10^{-5} M or following stimulation of FcεRI via IgE anti-DNP followed by treatment with DNP-HSA for 2 h. RNA was isolated using Qiagen RNeasy Mini Kit (Qiagen, Valencia, CA). RNA was reverse transcribed using a QuantiTect reverse transcription kit (Qiagen). Quantitative real-time PCR was performed using QuantiTect primer assays for mouse IL-6 and SYBR green master mix (Qiagen). A Bio-Rad iCycler was used to obtain cycle threshold (Ct) values for target and internal reference cDNA levels. Target cDNA levels were normalized to GAPDH, an internal reference using the equation $2^{-[\Delta Ct]}$, where ΔCt is defined as $Ct_{target} - Ct_{internal\ reference}$. Values shown are the average of 5 independent experiments.

Calcium and sodium measurements

Intracellular Ca^{2+} and Na^{+} levels were measured in BMMCs following treatment with 10^{-5} M PbTx-2. BMMCs (5×10^5/sample) were treated for 5 min with 10^{-5} M PbTx followed by addition of 10 μM Calcium Green dye or CoroNa Green sodium dye (Molecular Probes, Eugene, OR). In addition, another set of BMMCs was sensitized overnight with IgE anti-DNP (Sigma) followed by stimulation with DNP-HSA (Sigma) for 5 min to generate a positive Ca^{2+} signal. Following addition of the fluorescent dye, BMMCs were washed and intracellular Ca^{2+} and Na^{+} levels were determined by flow cytometry. BMMCs (10,000 total events/sample) were analyzed using FACScan flowcytometer (BD Biosciences).

Statistics

Statistical analysis employed PRISM, version 5 (GraphPad, San Diego, CA). For in vitro studies, differences between untreated and PbTx-2-treated BMMCs were assessed using one-way ANOVA with Bonferroni post-test. All values are reported as mean ± SEM. In all cases, a P value of less than 0.05 was used to indicate statistical significance between groups.

Results

Effects of brevetoxin-2 on mast cell viability

To assess the effects of PbTx-2 on BMMC viability, cells were treated with PbTx-2 or brevenal for 1 h followed by treatment with PbTx-2 at 10^{-5} through 10^{-12} M for up to 24 h. As determined by LDH release, PbTx-2 induced BMMC cytotoxicity in a dose- and time-dependent manner (Fig. 1). Increased LDH release was observed at 10^{-5} and 10^{-6} M concentrations at 24 h; however, an increase in LDH release was not detected at concentrations below 10^{-6} M out to 24 h (Fig. 1). In addition to LDH release, 10^{-5} M PbTx-2 treatment induced apoptosis in approximately 30% of BMMCs after 24 h as measured by Annexin-V binding (Table 1). We did not observe an increase in Annexin-V staining at 1, 4, and 8 h after addition of PbTx-2 when compared to untreated cells (data not shown). A similar increase in propidium iodide staining was observed in PbTx-2-treated BMMCs suggesting the cells also die by necrosis (Table 1). Brevenal (0.5 μM) treatment of BMMCs for 1 h prior to addition of 10^{-5} M PbTx-2 had no effect on BMMC cytotoxicity (Table 1).

Activation of mast cells by brevetoxin-2

To assess the ability of PbTx-2 to influence BMMC activation, we examined *β*-hexosaminidase (*β*-hex) release as a measure of degranulation and IL-6 production as measure of inflammatory cytokine production. As shown in Fig. 2, 10^{-5} M PbTx-2 induced a modest but significant release of *β*-hex 1 h following treatment when compared to the amount of *β*-hex released when BMMCs are stimulated via

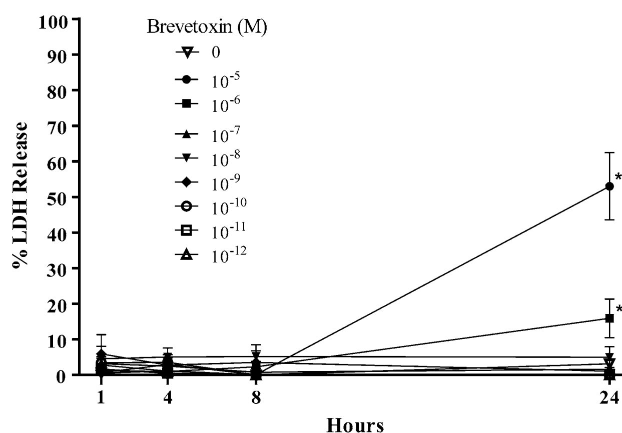

Fig. 1 PbTx treatment induces LDH release from BMMC. LDH release was measured in the supernatant of PbTx-2-exposed BMMCs. BMMCs were treated with 10^{-5}–10^{-12} M PbTx-2 for 1, 4, 8, and 24 h. Results are presented as the percentage of LDH released when compared to total cellular LDH content. Data are means ± SEM. *$P \leq 0.05$ when compared to vehicle-treated cells

Table 1 Annexin-V/Propidium iodide staining of brevetoxin-2 exposed BMMCs

Treatment	Annexin-V (% positive)	PI (% positive)
Vehicle	9.9 ± 5.8	4.3 ± 5.2
5-min UV Exposure (positive ctrl)	81.2 ± 3.9*	76.3 ± 4.7*
PbTx (10^{-5} M) (24 h)	29.0 ± 13.8*	31.8 ± 6.7*
PbTx (10^{-5} M) (24 h) + 0.5 μM Bvnl	28.7 ± 15.1*	28.9 ± 11.9*

24-h exposure. Values are mean ± SEM. $N = 3$ independent experiments

* $P < 0.001$ vs vehicle exposed BMMC

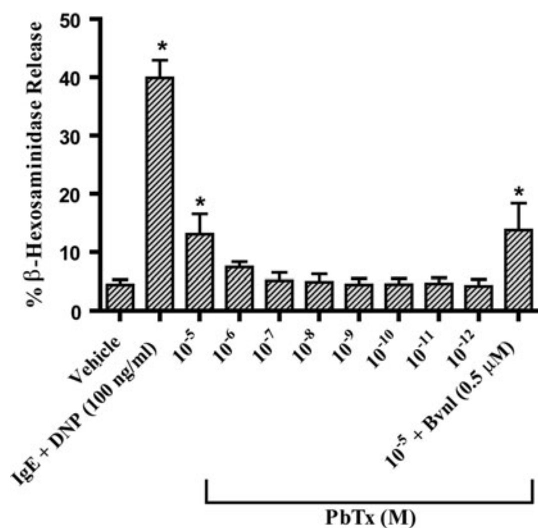

Fig. 2 PbTx induces BMMC degranulation. B-hexosaminidase release was measured as an indicator of degranulation in supernatants of PbTx-2 treated BMMCs 1 h following treatment. BMMCs sensitized with IgE anti-DNP and challenged with DNP-HSA were used as a positive control. In addition, BMMCs were pretreated with 0.5 μM brevenal prior to addition of 10^{-5} M PbTx-2. Data are presented as % β-hex release when compared to total cellular β-hex content. Data are means ± SEM. *$P \leq 0.05$ as compared to vehicle treated cells

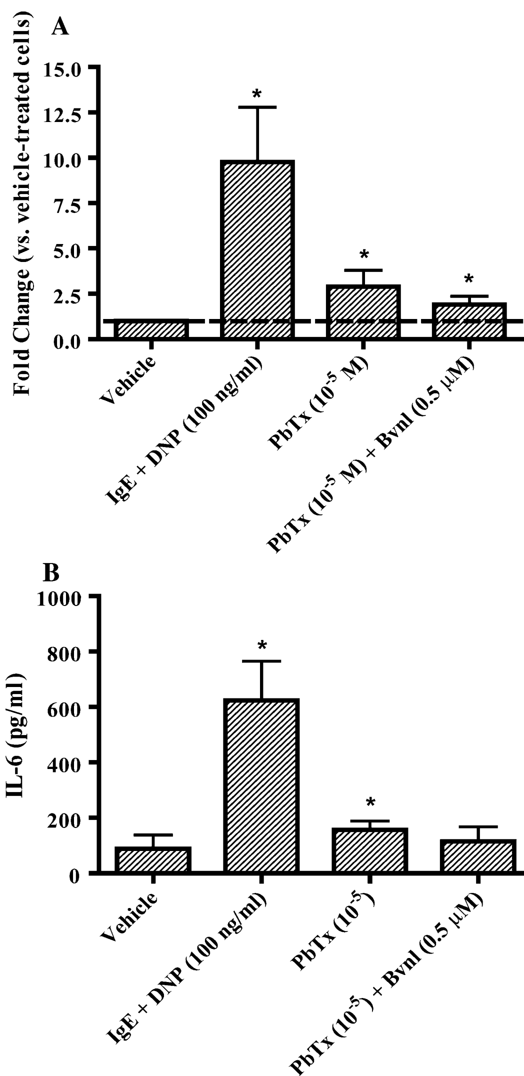

Fig. 3 BMMCs increase IL-6 following PbTx exposure. **a** IL-6 mRNA levels were measured by quantitative PCR in BMMCs treated with 10^{-5} M PbTx-2 for 2 h. **b** IL-6 protein levels were measured by ELISA in supernatant of PbTx-2 treated BMMCs. For both experiments, BMMCs sensitized with IgE anti-DNP and challenged with DNP-HSA were used as a positive control. In addition, BMMCs were pretreated with 0.5 μM brevenal prior to addition of 10^{-5} M PbTx-2. Data are means ± SEM. *$P \leq 0.05$ when compared to vehicle-treated cells

FcεRI. Addition of 0.5 μM brevenal 1 h prior to treatment with PbTx-2 had no effect on the ability of PbTx-2 to induce degranulation of BMMCs (Fig. 2). In addition to β-hex release, we observed a 3-fold increase in mRNA transcripts for IL-6 in BMMCs 2 h following addition PbTx-2 (Fig. 3a). PbTx-2 exposed BMMCs that were pretreated with 0.5 μM brevenal for 1 h displayed a ~2-fold increase in IL-6 mRNA levels (Fig. 3a). IL-6 protein levels were slightly increased in supernatants of BMMCs exposed to 10^{-5} M PbTx-2 for 6 h when compared to vehicle treated BMMCs (157 ± 31 pg/ml vs. 88 ± 49 pg/ml, respectively) (Fig. 3b). Pretreatment of BMMCs with 0.5 μM brevenal did not significantly alter IL-6 release from PbTx-2 treated BMMCs when compared to PbTx-2 BMMCs that were not pretreated with brevenal (115 ± 52 pg/ml vs. 157 ± 31 pg/ml, respectively) (Fig. 3b).

Intracellular Ca^{2+} and Na^{+} levels following brevetoxin-2 treatment

Since activation of mast cells by PbTx-2 was shown by β-hex release and induction of IL-6 expression and release, we investigated the involvement of Ca^{2+} and Na^{2+} homeostasis on cellular activation. We measured intracellular Ca^{2+} levels 5 min following treatment with PbTx-2. As expected,

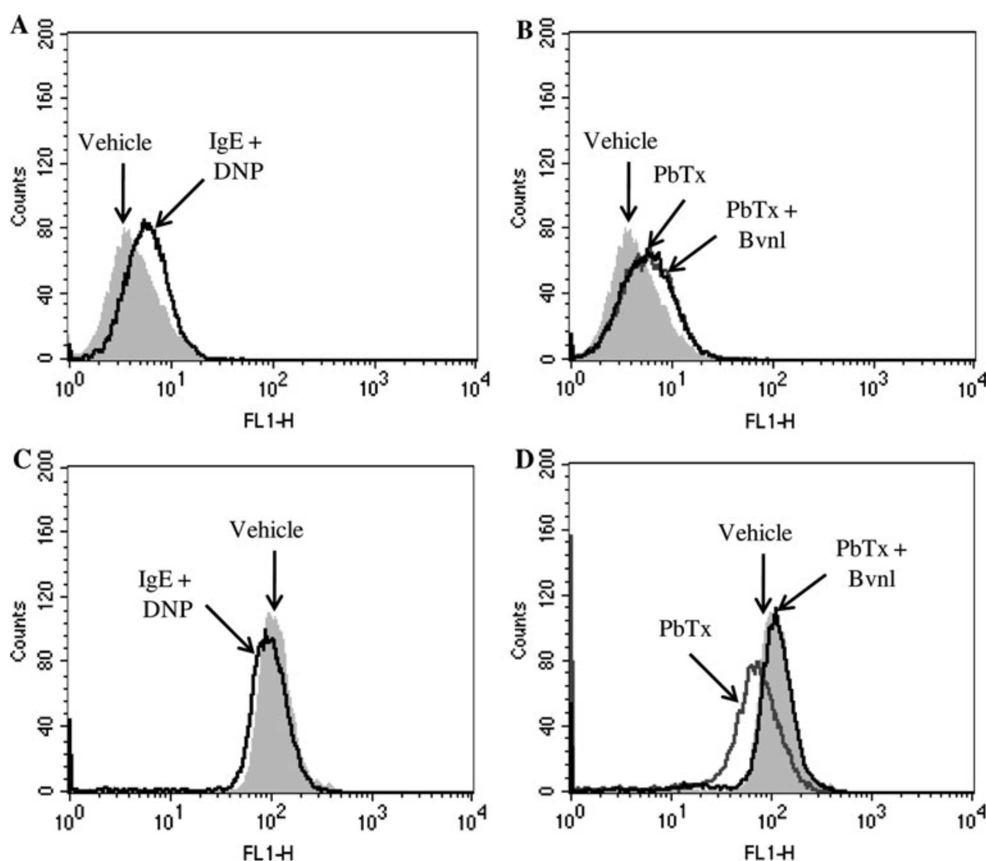

Fig. 4 PbTx influences intracellular Ca^{2+} and Na^{+} levels in BMMCs. **a** Intracellular Ca^{2+} levels in BMMCs treated with vehicle (*light gray shaded*) or sensitized with IgE anti-DNP and challenged with DNP-HSA (*black line*) for 5 min as measured by flow cytometry. **b** Intracellular Ca^{2+} levels in BMMCs treated with vehicle (*light gray shaded*) or 10^{-5} M PbTx-2 (*dark gray line*) for 5 min as measured by flow cytometry. Additionally, BMMCs were pretreated with 0.5 μM brevenal prior to addition of 10^{-5} M PbTx-2 (*black line*). **c** Intracellular Na^{+} levels in BMMCs treated with vehicle (*light gray shaded*) or sensitized with IgE anti-DNP and challenged with DNP-HSA (*black line*) for 5 min as measured by flow cytometry. **d** Intracellular Na^{+} levels in BMMCs treated with vehicle (*light gray shaded*) or 10^{-5} M PbTx-2 (*dark gray line*) for 5 min as measured by flow cytometry. Additionally, BMMCs were pretreated with 0.5 μM brevenal prior to addition of 10^{-5} M PbTx-2 (*black line*). Data is a representative example of three independent experiments

stimulation of BMMCs via FcεRI induced an increase in intracellular Ca^{2+} levels (Fig. 4a). Similarly, PbTx-2 induced an increase in intracellular Ca^{2+} levels within minutes following treatment (Fig. 4b). Pretreatment with 0.5 μM brevenal did not affect intracellular Ca^{2+} levels induced by PbTx treatment (Fig. 4b). Lastly, since PbTx exert their toxicity by opening voltage gated Na^{+} channels and brevenal antagonizes this interaction, we investigated intracellular Na^{+} levels in BMMCs exposed to PbTx-2. As shown in Fig. 4c, FcεRI stimulation did not influence intracellular Na^{+} levels (Fig. 4c). However, PbTx-2 treated BMMCs exhibited a decreased in intracellular Na^{+} levels, which was prevented by pretreatment of BMMCs with brevenal.

Discussion

A role for mast cells in the pulmonary symptoms observed in humans and animal models following inhalation of PbTx has been suggested; however, the ability of PbTx to directly activate mast cells has not been explored. In this study, we demonstrate in vitro that PbTx are cytotoxic to mast cells; induce degranulation and inflammatory cytokine production; and that these responses are not dependent upon changes in intracellular Na^{+} levels as the natural PbTx antagonist brevenal did not inhibit mast cell activation or cytotoxicity.

Mast cells have been implicated in the observed airway symptoms, including bronchoconstriction, following exposure to PbTx (Abraham et al. 2005a; Abraham et al. 2005b). In these studies, PbTx induced bronchoconstriction in an animal model of allergic- and non-allergic asthma. More importantly, administration of either histamine H_1 blockers or the mast cell–stabilizing drug cromolyn significantly abrogated the airway responses to PbTx exposure (Abraham et al. 2005a; Abraham et al. 2005b). This led us to hypothesize that PbTx has the ability to directly activate mast cells leading to degranulation and cytokine production.

The mast cell is a crucial effector cell in the pathogenesis of asthma, particularly asthma with an allergic basis (Brown et al. 2008; Galli et al. 2008; Hamid and Tulic 2009). Mast cell numbers are increased within the airway smooth muscle of asthmatic patients, and the location of mast cells within the smooth muscle is believed to facilitate hyperresponsiveness through localized mediator release and/or direct cell-to-cell contact (Amin et al. 2005; Begueret et al. 2007; Brightling et al. 2002). Many mast cell mediators such as histamine, tryptase, prostaglandins, and inflammatory cytokines (IL-4, IL-5, IL-6 and IL-13 for example) will induce contraction of airway smooth muscle cells (Berger et al. 1999; Berger et al. 2002; Bradding et al. 1994; Sekizawa et al. 1989). Mast cells are also found within the bronchial epithelium in asthmatic patients, thereby allowing increased access to allergens or potentially increased exposure to toxins including inhaled PbTx (Bradding et al. 1994; Pesci et al. 1993). Indeed, brevetoxins with a mean diameter of 6–12 μm are deposited within the upper airways, thereby localizing to an area of the respiratory tract where mast cells are common (Cheng et al. 2005). However, our observed effects occurred at the two highest concentrations of 10^{-5} and 10^{-6} M PbTx-2 that would be significantly higher than the ambient air PbTx levels reported to significantly induce changes in respiratory symptoms (Fleming et al. 2009).

One possible mechanism for mast cell involvement in PbTx-induced airway responses is through degranulation and/or alteration in cytokine and chemokine levels within the lung (Bradding et al. 1994). Therefore, the ability of PbTx to induce degranulation (β-hex release) and cytokine production (IL-6 release) was examined. Figures 2 and 3 demonstrate that PbTx exposure of BMMCs led to β-hex and IL-6 release. In comparison with a maximum antigenic stimulation of BMMCs via FcεRI (IgE + DNP), PbTx induced a minimal amount of β-hex release. However, the increase in β-hex induced by PbTx was significant and is likely sufficient to induce a physiological response in vivo. A similar response was observed with BMMC production of IL-6 mRNA and protein following exposure to PbTx. Stimulation of BMMCs via FcεRI induced a significant increase in IL-6 mRNA and protein, while PbTx induced a 3-fold increase in IL-6 mRNA and a nearly 2-fold increase in IL-6 protein. For the IL-6 experiments, we measured protein levels 6 h after addition of PbTx, a time at which the BMMCs did not display any signs of toxicity. However, by 24 h following exposure to PbTx, a significant percentage of cells (~50% by LDH assessment and ~30% by Annexin/PI staining) were dying or dead. This was not unexpected as PbTx has been shown to induce cytotoxic cell death in lymphocytes (Murrell and Gibson 2009; Sayer et al. 2005).

Calcium signals are essential for the activation of immune cells, including mast cells (Di Capite and Parekh, 2009). Stimulation of mast cells, particularly activation via FcεRI, stimulates release of intracellular Ca^{2+} ions from the endoplasmic reticulum. This is also accompanied by the entry of Ca^{2+} from the extracellular space through store-operated Ca^{2+} channels (Di Capite and Parekh, 2009). Within minutes of PbTx exposure, BMMCs exhibited an increase in cytosolic Ca^{2+} levels consistent with cellular activation and degranulation. The increase in intracellular Ca^{2+} by PbTx was similar to the level of Ca^{2+} following FcεRI stimulation confirming the ability of PbTx to induce mast cell activation.

Brevetoxins exert their cellular effects through opening of voltage gated Na^{+} channels (Kulagina et al. 2004; Lombet et al. 1987; Purkerson et al. 1999; Trainer et al. 1995). The role of Na^{+} channels in excitable, conducting cells such as nerves and cardiac cells is clear. In these cell types, as the cell membrane depolarizes, a threshold is reached, thereby opening up the channel resulting in rapid influx of Na^{+} followed by membrane depolarization. Such channels are rarely described in non-excitable cells such as immune cells. However, expression of several Na^{+} channels has been reported in mast cells including $Na_v1.8$, SCN12A, Na_vx, and $Na_v\beta1.1$ (Bradding et al. 2003). A role for these Na^{+} channels in mast cells has not been described. Therefore, we investigated if PbTx could influence intracellular Na^{+} levels in BMMCs. As shown in Fig. 4, PbTx did induce a decrease in intracellular Na^{+} levels. These results suggest that PbTx are opening Na^{+} channels on mast cells although as to which Na^{+} channel PbTx targets on the mast cell remains to be elucidated.

The ability to inhibit PbTx-directed mast cell activation was examined using brevenal, a natural antagonist to PbTx that prevents binding of PbTx to voltage gated sodium channels (Bourdelais et al. 2004). The addition of brevenal 5 min before treating BMMCs with PbTx had no effect on cytotoxicity or degranulation and had minimal effect on IL-6 mRNA and protein expression. In addition, brevenal did not influence the ability of PbTx to induce an increase in intracellular Ca^{2+} levels, but brevenal did prevent an alteration in intracellular Na^{+} levels as expected. These results suggest that (1) intracellular Na^{+} does not influence BMMC activation and (2) that PbTx is potentially activating mast cells independent of the voltage gated Na^{+} channel. The mechanism of mast cell activation by PbTx remains to be determined, but appears to involve Ca^{2+} signaling.

In total, the data demonstrate that PbTx can directly activate mouse mast cells leading to degranulation and inflammatory cytokine production and that the activation involves Ca^{2+} signaling. Through this process, we have demonstrated that activation of mast cells by PbTx might contribute to the adverse airway responses observed following

PbTx inhalation and thereby provide further justification for the use of mast cell–directed therapies for the treatment of PbTx-induced pulmonary symptoms.

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Conflict of interest statement The authors declare that there are no conflicts of interest.

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