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Searching for Tryptase in the RBL-2H3 Mast Cell Model: Preparation for Comparative Mast Cell Toxicology Studies with Zebrafish

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Abstract

Mast cells comprise a physiologically and toxicologically important cell type that is ubiquitous among species and tissues. Mast cells undergo degranulation, in which characteristic intracellular granules fuse with the plasma membrane and release many bioactive substances, such as enzymes β -hexosaminidase and tryptase. Activity of mast cells in the toxicology model organism zebrafish has been monitored via tryptase release and cleavage of substrate BAPNA. An extensively-used *in vitro* mast cell model for studying toxicant mechanisms is the RBL-2H3 cell line. However, instead of tryptase, granule contents such as β -hexosaminidase have usually been employed as RBL-2H3 degranulation markers. In order to align RBL-2H3 cell toxicological studies to *in vivo* mast cell studies using zebrafish, we aimed to develop an RBL-2H3 tryptase assay. Unexpectedly, we discovered that tryptase release from RBL-2H3 cells is not detectable, using BAPNA substrate, despite optimized assay that can detect as little as 1 ng tryptase. Additional studies performed with another substrate, tosyl-Gly-Pro-Lys-pNA, and with an ELISA also revealed a lack of tryptase protein released from stimulated RBL-2H3 cells. Furthermore, none of the eight rat tryptase genes (*Tpsb2*, *Tpsab1*, *Tpsg1*, *Prss34*, *Gzmk*, *Gzma*, *Prss29*, *Prss41*) are expressed in RBL-2H3 cells, even though all are found in RBL-2H3 genomic DNA and even though β -hexosaminidase mRNA is constitutively expressed. Therefore, mast cell researchers should utilize β -hexosaminidase or another reliable marker for RBL-2H3 degranulation studies, not tryptase. Comparative toxicity testing in RBL-2H3 cells *in vitro* and in zebrafish mast cells *in vivo* will require use of a degranulation reporter different from tryptase.

Short Abstract

Mast cells degranulate, releasing substances such as β -hexosaminidase and tryptase. Mast cell activity in zebrafish is monitored via tryptase release. To align *in vitro* RBL-2H3 mast cell line studies to *in vivo* mast cell zebrafish studies, we aimed to develop an RBL-2H3 tryptase assay. However, tryptase protein is not released from stimulated RBL-2H3 cells. Also, no rat tryptase

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gene is expressed in RBL-2H3s. Comparative toxicity testing in RBL-2H3 cells and in zebrafish mast cells will require a non-tryptase degranulation reporter.

Keywords

Tryptase; RBL-2H3; Mast cell; degranulation; Zebrafish; BAPNA; tosyl-Gly-Pro-Lys-pNA

Introduction

Mast cells (MCs) are highly granulated cells that are typically recognized for their role in allergies and asthma (Kuby, 1997). However, they are also involved in many helpful immune functions such as host defense (Abraham et al., 2010; Galli et al., 2008), bacterial and parasitic clearance (Pawankar, 2005), and recruitment of neutrophils to sites of infection (Echtenacher et al., 1996; Malaviya et al., 1996). MCs possess additional immune-related functions that affect diseases such as cancer (Coussens et al., 1999; Gounaris et al., 2007), autoimmune disorders (Lee et al., 2002), and inflammatory bowel disease (Wilcz-Villega et al.). Interestingly, MCs also have roles in neurological processes and diseases such as autism (Theoharides et al., 2012), anxiety disorders (Nautiyal et al., 2008; Silver et al., 1996), and multiple sclerosis (Rozniecki et al., 1995). MCs, found in nearly all human tissues, are prominent in tissues in contact with the external environment, such as skin, blood capillaries, nerve terminals, gastrointestinal tract, respiratory mucosa, etc (reviewed in (Galli et al., 2005)). Also MCs are found in numerous different organisms (Baccari et al., 2011). Due to their physiological importance, ubiquity, and location near surface tissues, MCs are key toxicological targets.

MCs exhibit the unmistakably distinctive morphological feature of densely populated cytoplasmic granules, which get secreted upon MC stimulation: a process called degranulation (Kuby, 1997). Degranulation is typically initiated via multivalent antigen (Ag) crosslinking of immunoglobulin E (IgE) receptor-bound FcεRI receptors but can be stimulated in numerous ways, including via compound 48/80 (c48/80) or calcium ionophore application. The resulting signaling cascade culminates in degranulation, the release of granule-associated bioactive mediators, such as histamine, serotonin, β-hexosaminidase (β-hex), and tryptase (Schwartz et al., 1980). Assays for release of these mediators (and more) have been extensively utilized to test mast cell function.

Thus, the presence of granules containing tryptase is considered a canonical marker of MCs, and release of tryptase (into cell supernatant *in vitro* or into the bloodstream *in vivo*) has often been utilized as a test of mast cell stimulation and function (Irani et al., 1986). Mast cell tryptase is a family of trypsin-like serine proteases that cleave sites after basic amino acids such as Arg and Lys (Lehninger et al., 2005). Tryptase requires the glycosaminoglycan heparin for its activity (Hallgren et al., 2001). In general, tryptases can be grouped into three main types, α (I-II), β (I-III), and γ (transmembrane). Most tryptase purified from human tissues is a mixture of β isoforms which are the products of two genes, TPSB2 and TPSAB1 (Caughey, 2011). Numerous distinct roles of mast cell tryptase have been reported: some protective/anti-inflammatory, and some pro-inflammatory (reviewed in (Caughey, 2011)).

One example of a mechanism of action of tryptase is cleavage and inactivation of calcitonin gene-related peptide, thus regulating vasodilation (Tam et al., 1990; Walls et al., 1992). Tryptase acts upon many other substrates, as well. Mast cell tryptase is also involved in angiogenesis (Crivellato et al., 2004; Stack et al., 1994) and in development of multiple sclerosis (Rozniecki et al., 1995). In the laboratory, the presence of active tryptase in a solution can be detected by incubation with a chromogenic substrate such as N- α -benzoyl-DL-Arg-p-nitroanilide (BAPNA) or tosyl-Gly-Pro-Lys-p-nitroanilide: tryptase cleaves the bond between the Arg or Lys and the p-nitroaniline (pNA), releasing yellow-colored pNA, measurable in a plate reader (Lavens et al., 1993). Other chromogenic and fluorogenic tryptase substrates are also available.

Zebrafish (*Danio rerio*) (ZF) are a powerful, human-disease relevant (Lieschke et al., 2007; Ward et al., 2002) model organism for toxicology studies (Garcia et al., 2016). Zebrafish and humans share a highly conserved Fc ϵ RI receptor, and mast cells of both species share similarities in innate and adaptive immune responses (Da'as et al., 2015; Da'as et al., 2011; Dobson et al., 2008). Two methods for assessing mast cell function in zebrafish have recently been developed. Both methods use tryptase as the mast cell marker. First, a method has been developed to quantify mast cell degranulation in adult ZF, by assaying for tryptase in blood, using the substrate BAPNA (Da'as et al., 2011; Dobson et al., 2008). Intraperitoneal injection of adult zebrafish with an MC stimulant (such as c48/80) causes a large rise in blood plasma tryptase level (pNA production), as compared to saline-injected control animals (Dobson et al., 2008). Zebrafish have granzyme A (also known as granzyme K-like), a gene orthologue for human granzyme K (GZMK), which is conserved in rat (Gzmk), mouse, and ZF. Granzyme tryptases, including granzyme A and K, are serine proteases and shown to cleave after the basic residues Arg and Lys ((Plasman et al., 2014), so this ZF granzyme tryptase may be the enzyme cleaving BAPNA in this ZF assay. Also, Dobson et al. (Dobson et al., 2008) and Balci et al. (Balci et al., 2014) detected staining of ZF tissues with a monoclonal anti-human MC tryptase antibody specific for TPSAB1, although an orthologue to this specific gene has not yet been cloned from ZF. Second, a tryptase assay using ZF embryos also has been developed (Yang et al., 2015). In this method, all the drugs and reagents, including stimulant (e.g., c48/80) and substrate (e.g., BAPNA), are directly added to the fish water.

In addition to animal models for studies of mast cells, *in vitro* mast cell models have contributed enormously to scientists' understanding of the biochemical details of mast cell signaling and of drug and toxicant modes of action on MCs. Among mast cell models, the rat basophilic leukemia - clone 2H3 (RBL-2H3) cell line has been used widely as a well-accepted model of mast cell signalling and function (Barsumian et al., 1981). RBL-2H3 cells, used extensively for over 40 years, are an important mast cell model for *in vitro* studies of MC toxicology and pharmacology. Other experimental mast cells exist, but each has advantages and disadvantages, such as human HMC-1 cells which lack Fc ϵ RI (Nilsson et al., 1994), human LAD2 cells which contain Fc ϵ RI but which require >2 weeks for each doubling (Jensen et al., 2005), P815 cells which are largely non-adherent, and primary bone marrow-derived mouse mast cells which senesce after a brief time in culture. RBL-2H3 cells are easy to quickly and continuously culture in large quantities, contain the core signalling machinery of mature human mast cells (Fewtrell, 1979; Metzger et al., 1982), and are

functionally homologous to rodent mucosal mast cells (Seldin et al., 1985). Many molecular similarities between human and rodent mast cells have been detailed in (Abramson et al., 2007). The pathway leading to degranulation in RBL-2H3 cells is very well described, allowing for the identification of pathway targets for toxicants or drugs that affect this signaling system.

For many of the studies documented to date, RBL-2H3 degranulation has been quantified via measurement of released β -hexosaminidase (β -hex): β -hex in the supernatant over stimulated cells is incubated with fluorogenic substrate 4-methylumbelliferyl-N-acetyl- β -D-glucosaminide (4-MU), and the resulting fluorescent product is detected in a microplate reader (Naal et al., 2004). β -hex enzyme catalyzes degradation of GM2 ganglioside, and mutations in this enzyme lead to neurodegenerative diseases (Bayleran et al., 1987; Tiffet et al., 1997). β -hex has been successfully used as a MC function marker because it is released in parallel with canonical MC marker histamine (Schwartz et al., 1979). Of note, activated human primary lung mast cells secrete tryptase in parallel with histamine (Schwartz et al., 1981), but tryptase release has not typically been used as a RBL-2H3 degranulation marker.

Previously, we discovered that MC degranulation is disrupted by environmental toxicants, such as arsenic (Hutchinson et al., 2011; Shim, Kennedy, et al., 2016), and by antimicrobial drug triclosan (TCS) (Palmer et al., 2012; Weatherly et al., 2013). Along with other cell types, these studies utilized RBL-2H3 cells and β -hex detection to quantify MC degranulation. However, all published studies of mast cells in ZF have, instead, utilized tryptase as the biochemical marker of mast cell function. Thus, in order to align our cell culture-based toxicological studies in this proven RBL-2H3 mast cell model to *in vivo* toxicological studies of mast cells in the ZF model organism, we became interested in using tryptase as a marker for degranulation of RBL-2H3 cells. Measurement of tryptase release from RBL-2H3 cells would lay the groundwork for comparative/confirmative high-throughput toxicology assays of mast cell function and signalling that could use both ZF and this canonical mast cell model, RBL-2H3 cells. For example, we are interested in studying and comparing effects of TCS on mast cells in ZF as a whole organism follow-up to our findings that TCS inhibits function of RBL-2H3 cells (Palmer et al., 2012) and of human HMC-1 mast cells (Weatherly et al., 2016). Parallel studies of RBL-2H3 mast cells and ZF have already proven to be instructive in toxicology: for example, ZF mitochondrial function is inhibited by TCS (Shim, Weatherly, et al., 2016) at similar doses and time frames that also depress mitochondrial function in RBL-2H3 cells (Weatherly et al., 2016). Thus, we were motivated to develop a tryptase release assay from RBL-2H3 cells in order to design parallel assays of toxicant effects on MC function in both ZF and RBL-2H3, for applied toxicology investigations linking *in vitro* and *in vivo* systems. Background studies showing that TCS does not directly affect activity of purified tryptase (Kennedy, 2013) further bolstered our belief that it would be possible to use this degranulation marker for comparative mast cell toxicology studies of TCS.

In this study, we have optimized the conditions of a substrate-based assay for tryptase activity and have applied it to assess degranulation of RBL-2H3 cells. We report the results of three separate assay methods for detecting tryptase protein secreted from degranulated

RBL-2H3 cells, as well as of PCRs performed to detect mRNA transcripts of eight distinct tryptase genes within these cells.

Materials and Methods

Chemicals and reagents

Purified human lung tryptase (E.C. Number 3.4.21.59; corresponds to human gene symbols TPSD1, TPSB2, TPSAB1) was purchased from Calbiochem /EMD Millipore (Billerica, MA, USA) and was stored as aliquots wrapped in aluminum foil at -20°C . BAPNA ($\text{N}\alpha$ - Benzoyl -D,L-arginine-*p*-nitroanilide • HCl) tryptase substrate (Bachem, Bubendorf, Switzerland) was stored at -20°C in an amber bottle and wrapped in aluminum foil. Anti-dinitrophenyl (DNP) mouse Immunoglobulin E (IgE) (monoclonal, clone SPE-7), Ca^{2+} ionophore A23187, heparin, dimethyl sulfoxide (DMSO) were purchased through Sigma (St. Louis, MO, USA). Triton-X 100 (TX) was obtained from Thermo Scientific (Rockford, IL, USA).

Tryptase from human lung (TFHL) buffer was prepared to match the buffer contained in the purified tryptase purchased through Calbiochem, and contained 1 M NaCl, 50 mM acetate buffer, and 0.05 mM heparin (0.09% heparin), which was titrated to pH 5 with 10 N sodium hydroxide. 15-mL aliquots were frozen at -20°C , and thawed as needed.

The components of Tyrodes buffer are that of (Hutchinson et al., 2011) and (Weatherly et al., 2013). Modified Tyrodes buffer (mT), pH 7.4, contained lower NaCl and lower CaCl_2 than typical Tyrodes, and was made by combining 100 mM NaCl, 5 mM KCl, 0.9 mM CaCl_2 , 1 mM MgCl_2 , 20 mM HEPES, and 5.6 mM glucose. As needed, bovine serum albumin (BSA) at 1 mg mL^{-1} was added to mT on the day of an experiment. BAPNA buffer solution I was prepared by first combining 1 mM MgCl_2 , 0.1 M Trizma base (Sigma, St. Louis, MO, USA), and 1 M glycerol (Promega, Madison, WI, USA). After mixing well, 0.02% heparin (w/v) was added, and the pH of this solution was titrated to either 9 or 8, depending on the experiment. Similarly, BAPNA solution II was prepared in the same manner as solution I, only it contained the added component of 0.8 mM BAPNA (in assays, final concentration of BAPNA was 0.4 mM). These two buffer solutions were made fresh daily.

A23187 Ca^{2+} ionophore was prepared by dissolving A23187 powder into 100% DMSO for a final concentration of 2.5 mg mL^{-1} and stored as aliquots wrapped in aluminum foil at -20°C . On the day of an experiment, this stock was diluted directly into Tyrodes-BSA solution or mT-BSA to create necessary ionophore concentrations.

Cell harvest buffer (10X) was prepared with 1.35 M NaCl, 0.05 M KCl, 0.17 M HEPES, and 0.02 M EDTA, which was titrated to pH 7.6.

Chemicals of the highest possible purity were used. All buffers and media were sterile filtered using $0.2\text{ }\mu\text{M}$ VacuCap filters (Pall Corporation, Port Washington, NY, USA).

Purified tryptase assay

Purified tryptase working solutions containing 1-500 ng tryptase were prepared by making dilutions in TFHL buffer. Using a clear 96-well half-area plate (Grenier Bio-One, Monroe, NC, USA), 50 μ L of 0.02% heparin (w/v) mT-BSA was added to each sample well. From all wells, 1 μ L was removed, and tryptase (1 μ L) at the varying levels indicated replaced this volume. To these 50- μ L samples, 50 μ L BAPNA solution II (containing BAPNA substrate) was added; controls lacking BAPNA substrate contained 50 μ L BAPNA solution I (containing no BAPNA substrate). Tryptase activity was detected spectrophotometrically in a Synergy 2 plate reader (Biotek, Winooski, VT, USA) by measuring the colorimetric cleavage product p-nitroaniline (pNA) at 410 nm. The plate was read immediately ($t = 0$), as well as periodically, up to 72 h (Lavens et al., 1993). During incubation at 37 °C/5% CO₂, the plate was tightly sealed with parafilm, and the plate lid was weighted down by a lead ring; these methods, along with glycerol, aided in preventing sample loss over the 72-h time period.

Cell culture

RBL-2H3 culture methods were those of (Hutchinson et al., 2011) and NIH-3T3 mouse fibroblast culture methods were those of (Weatherly et al., 2016).

Stimulation of RBL-2H3 cells induced via IgE-receptor crosslinking by antigen or Ca²⁺ ionophore

Cells were stimulated with either DNP-BSA multivalent antigen (Ag) (following IgE sensitization) or A23187 Ca²⁺ ionophore, essentially as described (Weatherly et al., 2013) except that mT-BSA buffer was used in place of BT buffer. DMSO (0.004% final) was also used in spontaneous, 0.2% TX, and background wells to control for vehicle effects.

Following cell stimulation for 1h at 37°C, resulting β -hexosaminidase or tryptase released into cell supernatant was quantified as described in (Weatherly et al., 2013) or tryptase assay described in “RBL-2H3 cell degranulation assay for tryptase release, measured by BAPNA substrate,” below. Additionally, to test the effects of heparin on this process, 0.02% heparin was added to A23187, spontaneous, 0.2% TX, and background samples.

RBL-2H3 cell degranulation assay for tryptase release, measured by BAPNA substrate

Following treatment for cell stimulation described above, cell supernatant (50 μ L) was transferred to a 96-well half-area plate (Grenier Bio-One, Monroe, NC, USA) that contained 50 μ L of BAPNA solution II. The absorbance of the colorimetric cleavage product pNA was detected over a 72-h period as with the purified tryptase experiments.

RBL-2H3 cell degranulation assay for tryptase release, measured by commercial kit utilizing tosyl-Gly-Pro-Lys-pNA substrate

All the reagents were prepared following manufacturer's protocol (EMD Millipore, Billerica, MA, USA) unless otherwise indicated in individual figures' legends. RBL-2H3 supernatant were obtained as described in previous section. Lysates from RBL-2H3 cells were obtained in 2 steps: first, RBL-2H3 cells were harvested using 1X cell harvest buffer

(diluted from 10X with cell culture water) and centrifuged for 8 min at 500xg. After removal of supernatant, cell pellet was washed with PBS and resuspended in 1X assay buffer. Next, the cell supernatant from the lysate was obtained as described under Syk ELISA assay in (Shim, Kennedy, et al., 2016) and incubated with tosyl-Gly-Pro-Lys-pNA substrate. For statistical analysis, first raw absorbance data from each treatment were processed by subtracting average background values (1X assay buffer with substrate) from each sample, and the background-adjusted triplicates were then averaged. These values were normalized to the unstimulated (no Ag or no ionophore) control for each experimental day when compared among supernatants. For comparison between RBL-2H3 supernatant and RBL-2H3 lysates, values were normalized to the RBL-2H3 supernatant.

Lysates from NIH-3T3 cells were obtained in the same manner as the RBL-2H3 cell lysis methods as described above. To determine total protein concentration from the RBL-2H3 and NIH-3T3 cell lysates, the bicinchoninic acid (BCA) assay (Pierce BCA protein assay kit [Thermo]) was performed, following the manufacturer's protocol. Raw tryptase degranulation absorbance data obtained from lysate samples were averaged after subtracting average background values (1X assay buffer with substrate) from each sample. Background-subtracted values were then divided by the total protein concentration of that particular sample. Finally these values were normalized to RBL-2H3 lysates for each experimental day.

Tryptase (TPSAB1) ELISA

Tryptase ELISA kit (Cell Signaling Technologies, Beverly, MA, USA) was used to detect levels of rat (*Rattus norvegicus*) TPSAB1 protein according to the manufacturer's instructions. For this assay, RBL-2H3 were cultured in 6 well plates, stimulated and lysed as described under Syk ELISA assay in (Shim, Kennedy, et al., 2016). A volume of 300 μ L of cell lysate supernatant was placed into an appropriate ELISA well in duplicates per treatment. The remaining steps were carried out according to the manufacturer's directions.

RNA extraction and cDNA synthesis from RBL-2H3 cells

Total RNA was isolated from RBL-2H3 cells using Trizol® reagent (Life technologies, Grand Island, NY, USA) following the manufacturer's instructions. The concentration and purity of the total RNA were determined by Nanodrop-1000 UV-Vis spectrometer (Thermo scientific, Rockford, IL, USA). Total RNA (10 μ g per sample) was treated with TURBO DNA-free kit (Ambion, Grand Island, NY, USA) to remove any contaminating genomic DNA. The RNA preparation was reverse-transcribed to cDNA using a iScript cDNA synthesis kit (Bio-Rad, Hercules, CA, USA) according to the manufacturer's protocol.

Genomic DNA extraction from RBL-2H3 cells

DNeasy Blood & Tissue Kit (Qiagen, Valencia, CA, USA) was used for genomic DNA extraction from RBL-2H3 cells by following the manufacturer's instructions. The concentration of genomic DNA was measured using Nanodrop-1000 UV-Vis spectrometer (Thermo scientific, Rockford, IL, USA).

Polymerase chain reaction (PCR) for rat tryptase genes

Primers for each *Rattus norvegicus* tryptase genes were inferred from NCBI (<http://www.ncbi.nlm.nih.gov>). Primers were designed using NCBI Primer Blast (<http://www.ncbi.nlm.nih.gov/tools/primer-blast>) and IDT Oligoanalyzer software (<https://www.idtdna.com/analyzer/Applications/OligoAnalyzer>), and synthesized by Integrated DNA Technologies (Coralville, Iowa, USA). Sequences for PCR primer pairs, annealing temperatures, and size of the transcripts for tryptase genes are shown in Fig 6C and Fig 7B. PCR was carried out in a 50uL reaction volume, utilizing 100ng of the cDNA or genomic DNA as a template and 0.4 μ M of each primer. The amplicons were generated using a Phusion High-Fidelity DNA polymerase from New England Biolabs (Ipswich, MA, USA) and following PCR conditions: initial one cycle of 98°C for 30sec, then 35 cycles of 98°C for 10sec, 51.5-58.5C (depending on the genes) for 30sec, and 72°C for 30sec and followed by one cycle of a final 72°C extension for 5 min. A mock PCR, in which the DNA template was excluded, was also performed as a negative control. Amplification of rat β -actin, rat β -hexosaminidase A and B genes were used as positive controls. For gel electrophoresis, Seakeam LE agarose (Cambrex, Rockland, ME, USA) and 1Kb plus DNA ladder (Life Technologies, Grand Island, NY, USA) were purchased. The PCR products were analyzed in a 1% agarose gel and stained with 0.5 μ g/mL of ethidium bromide (Sigma, St Louis, MO, USA).

Sequencing analysis

To confirm the identity of amplified sequences, the PCR products were separated in a 1% agarose gel to extract the single specific band, which was subsequently purified using a Qiaquick Gel extraction kit (Qiagen, Valencia, CA, USA). Each purified product was then sequenced at the University of Maine sequencing facility using an ABI 3730 DNA sequencer (Applied Biosystems, Foster City CA, USA). The obtained DNA sequences were confirmed by NCBI BLAST search (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>) and ClustalW2 alignment program (<http://www.ebi.ac.uk/Tools/msa/clustalw2>).

Statistical analysis

Unless otherwise indicated in individual figures, results from degranulation experiments are reported as mean $\pm$ SEM, with significant differences determined by either a One-Way ANOVA (Analysis of Variance) followed by Tukey's *post-hoc* test or One-sample t-test, using Graphpad Prism software (San Diego, CA, USA).

Results

The tryptase activity assay is optimized to detect as low as 1 ng of purified human lung tryptase using BAPNA (N α -Benzoyl-D, L-Arg-p-nitroanilide) substrate.

Eight rat tryptase or tryptase-like genes were found in the rat genome database (RGD: <http://rgd.mcw.edu>) and were compared to their human orthologs (HGNC: <http://www.genenames.org>) (Table 1). Before testing RBL-2H3 mast cells, we optimized the tryptase assay in cell-free system, based on the protocol developed by Lavens et al. (Lavens et al., 1993), which is able to detect tryptase by measuring the cleavage product (*p*-

nitroanilide [pNA]) of a known tryptase substrate BAPNA and purified human lung tryptase. The purified human lung tryptase that was utilized contains three tryptase species: TPSAB1, TPSB2 and TPSD1. When various doses of purified tryptase (0-500ng) were incubated with BAPNA and absorbance at 410 nm was read in a plate reader, detection of pNA plateaued within 72 hours (Fig. 1A). Following optimization of both timing (Fig. 1A; including modifying assay parameters to minimize sample loss) and of buffer components (pH, heparin concentration, levels of Ca^{2+} and Na^{+} , addition of heparin and bovine serum albumin, etc), we successfully detected pNA production from as little as 1ng of purified tryptase tested (Fig. 1B). Previously, the lowest level of pNA detected by Lavens et al. was 10ng (Lavens et al., 1993).

Tryptase is not detectable in the supernatant from degranulated RBL-2H3 mast cells, using BAPNA substrate.

Following our purified tryptase experiments, our next goal was to detect tryptase released from a mast cell model line RBL-2H3 using the same tryptase substrate, BAPNA, incubated for a period of 72 h. Because the plan was to stimulate RBL-2H3 to degranulate for one hour with Ca^{2+} ionophore A23187 and to collect the cell supernatants to test for the presence of tryptase by measuring pNA production, we first tested the conditions of a modified Tyrodes buffer with no BSA on A23187 ionophore dose response degranulation curve in RBL-2H3. We monitored the release of β -hexosaminidase from these cells using 4-MU (4-methylumbelliferone) substrate. As seen in Fig. 2A, we detected robust increases in β -hexosaminidase released from RBL-2H3 due to ionophore stimulation. Then we used the same exact conditions for stimulating RBL-2H3 to degranulate, with the exception that now we assayed the cell supernatant for tryptase activity (pNA) using BAPNA substrate. Surprisingly, we were unable to detect any significant release of tryptase from RBL-2H3 cells (Fig. 2B). Importantly, we performed control studies in which 25 ng purified tryptase was incubated with BAPNA in a background of various reagents used in the degranulation assay (A23187 ionophore, DMSO [ionophore vehicle], TX, and BSA), and found that these variables do not significantly affect the ability of purified tryptase to cleave BAPNA substrate (Fig.S1). Taken together, these data indicate that RBL-2H3 cells likely do not release active tryptase protein that is able to cleave the BAPNA substrate.

Tryptase is not detectable in the supernatants from degranulated RBL-2H3 mast cells, using tosyl-Gly-Pro-Lys-pNA substrate.

After observing no tryptase detection from degranulated RBL-2H3 cells using the BAPNA tryptase substrate, we utilized the commercially available tryptase degranulation kit from EMD Millipore, which contains another known tryptase substrate: tosyl-Gly-Pro-Lys-pNA, instead of BAPNA. First, a high dose of antigen (1 $\mu\text{g/mL}$) in modified tyrodes buffer (following IgE sensitization) was applied for RBL-2H3 cell stimulation, but no tryptase was detected from the supernatants of stimulated RBL-2H3 cells (Fig. 3A). Then we examined the release of tryptase after stimulating RBL-2H3 cells with 1 $\mu\text{g/mL}$ antigen in the proprietary assay buffer which was provided in the kit, and, again, no tryptase was detected (Fig. 3B). Lastly, we stimulated RBL-2H3 cells with A23187 ionophore, strictly following the manufacturer's protocol provided by EMD Millipore, and measured the production of

pNA from the cell supernatant. There was no significant difference compared to unstimulated control cells in this condition, either (Fig. 3C).

Lysates from RBL-2H3 mast cells and from NIH-3T3 fibroblast cells contain a substance that can cleave the tosyl- Gly-Pro-Lys-pNA substrate.

Since tryptase release from RBL-2H3 cells was not detectable using either of those tryptase substrates, we next tested RBL-2H3 cell lysates. Compared to the signal from RBL-2H3 cell supernatant, lysates from RBL-2H3 cells displayed significantly higher readings when incubated with tosyl-Gly-Pro-Lys-pNA substrate (Fig. 4A). To test whether the signals obtained from the RBL-2H3 lysates were due to the presence of tryptase, we also examined NIH-3T3 cell lysates because NIH-3T3 cells are non-granulated fibroblasts which do not contain mast cell markers. We found that there was no significant difference between NIH-3T3 lysates and RBL-2H3 lysates (Fig. 4B).

Tryptase is not detectable in the supernatants from degranulated RBL-2H3 mast cells, using rat TPSAB1 ELISA.

Using two different tryptase substrates, BAPNA (Fig. 2) and tosyl- Gly-Pro-Lys-pNA (Fig. 3), no active tryptase protein (capable of cleaving either of these substrates) was detected in the supernatants of degranulated RBL-2H3 mast cells. Those data suggest that no active tryptase protein is present in the RBL-2H3 cells' granules. To further investigate whether degranulated RBL-2H3 cell supernatants contain tryptase, regardless of protein activity level, we utilized a sandwich ELISA kit which detects one of the key rat tryptase protein types, TPSAB1. This ELISA kit detects as little as 0.3 ng/mL tryptase (as shown in a standard curve generated using purified rat TPSAB1 protein [provided in the kit], Fig. S2). Again, no tryptase was detected in the supernatants of Ag-stimulated, degranulated RBL-2H3 cells (compare "RBL-2H3 control supernatant" to "RBL-2H3 Ag supernatant" in Fig. 5), another indication that tryptase is not present in the granules of RBL-2H3 cells. However, the phenomenon of apparent tryptase signal found in RBL-2H3 lysates, as in Fig. 4, was also found in RBL-2H3 lysates via this ELISA measurement (Fig. 5). Again, though, the TPSAB1 ELISA signal from RBL-2H3 lysates was equivalent to the ELISA signal arising from NIH-3T3 cell lysates (Fig. 5). Thus, the apparent TPSAB1 signal in RBL-2H3 cell lysates is not a granule-associated protein.

No rat tryptase mRNA was detected in RBL-2H3 cells using PCR.

Because three distinct methods of tryptase protein detection (Figs. 2, 3, and 5) failed to find tryptase protein released from degranulated RBL-2H3 cells, we next used PCR to investigate whether tryptase mRNA transcripts are present in RBL-2H3 cells. To generate cDNA template, total RNA was extracted from antigen-stimulated RBL-2H3 cells and was reverse-transcribed. Numerous cell treatment experimental conditions prior to the RNA extraction are listed in supplementary material Table S1, including several variations of IgE sensitization, antigen stimulation, and timeframe conditions. Primer pairs and the size of amplicons for eight rat tryptase genes are shown in figure 6C. In addition, mRNA of two rat β -hexosaminidase genes (*Hexa* and *Hexb*) and of β -actin (*Actb*) were amplified as positive controls. In fifteen separate testing conditions, from unstimulated controls to cells treated with high Ag doses to various RNA collection timepoints up to 24 hours post Ag

stimulation, none of the eight tested tryptase genes' mRNA was detected by PCR (Fig. 6A). This lack of tryptase transcript detection stands in contrast to consistent robust detection of actin transcripts (Fig. 6A) and of both β -hexosaminidase mRNAs (Fig. 6B), over all fifteen cell treatment conditions listed in Table S1. Therefore, these findings show that no tryptase genes are expressed in RBL-2H3 cells.

Genomic DNA of all rat tryptase genes was detected using PCR, in RBL-2H3 cells.

Because none of the tryptase genes' mRNA was detected, we next questioned whether tryptase genomic DNA is present in this cell line. To determine whether these eight tryptase genes exist in the RBL-2H3 genome, we extracted genomic DNA from RBL-2H3 cells and amplified with the gene-specific primer pairs shown in figure 7B. Genomic DNA from all eight tryptase genes was detected by PCR (Fig. 7A), and each band was excised, cleaned, sequenced, and confirmed as the correct gene (sequence data not shown). Overall, our results suggest that RBL-2H3 cells do not transcribe and translate tryptase genes even though all tryptase genes exist in the genomic DNA of RBL-2H3 cells.

Discussion

In this study, we optimized an assay, based upon cleavage of BAPNA substrate, for detection of as little as 1 ng active tryptase per sample. While this assay is 10-fold more sensitive than the BAPNA-based method published by Lavens et al. (which could pick up 10 ng tryptase) (Lavens et al., 1993), we were unable to find any tryptase released from stimulated, degranulated RBL-2H3 cells. In this assay, ~100,000 RBL-2H3 cells per well of a 96-well plate were stimulated under conditions causing robust degranulation and β -hex release, and one-quarter of the resulting cell supernatant (50 μ L out of 200 μ L supernatant in each well; equivalent to the contents released from ~25,000 RBL-2H3 cells) was incubated with BAPNA substrate. According to Lavens et al. (1993), human lung mast cells contain 11.2 ± 0.7 pg tryptase per cell, so, if RBL-2H3 cells contained similar tryptase levels, we would expect ~280 ng tryptase per sample from fully-degranulated cells or perhaps 28 ng tryptase per sample from modestly-degranulated cells—levels far above the assay's sensitivity, 1 ng. Therefore, our results with the BAPNA substrate suggest that RBL-2H3 cells contain very little or no active tryptase protein. Additional studies with an alternative tryptase substrate, tosyl-Gly-Pro-Lys-pNA, and with ELISA assays for TPSAB1 protein also revealed a lack of tryptase protein released from RBL-2H3 cells stimulated via multiple means.

Thus, we also investigated whether tryptase mRNA transcripts are found in RBL-2H3 cells. We found none of the mRNA transcripts of the rat tryptase genes (*Tpsb2*, *Tpsab1*, *Tpsg1*, *Prss34*, *Gzmk*, *Gzma*, *Prss29*, *Prss41*) in RBL-2H3 cells, whereas genomic DNA of all the tryptase genes was readily detected by PCR. Since these genes were found in genomic DNA, we looked for tryptase gene expression following a wide range of RBL-2H3 stimulation conditions, from 1 to 24 hours post antigen stimulation (Table S1), but no mRNA transcripts were found under any condition. The reason for testing at varied time points is that mast cells undergo recovery and regeneration/refilling of the granules after degranulation (Dvorak et al., 1988; Dvorak et al., 1986), so we had hypothesized that tryptase gene expression may fluctuate over time, before and after degranulation. Also, we performed clonogenic

cytotoxicity assays and found that RBL-2H3 cells survive post-degranulation (data not shown), a necessary control for Table S1 experiments. We did find that mRNA levels of another MC granule component, β -hexosaminidase (both forms A and B), are constitutively robust in RBL-2H3 cells, regardless of time point or stimulation method: further evidence that, if any of these eight tryptase genes were expressed in RBL-2H3 cells, we would have been able to find its transcript in at least one of the sixteen cell stimulation conditions tested.

There were occasional, faint bands in gels assessing mRNA expression of tryptase genes (Fig. 6A), though these were not the correct product sizes of the expected tryptases (Fig. 6C). For example, from one gel, a faint band was excised from the Prss34 lane, purified, sequenced, and determined to be not tryptase but, rather, carboxypeptidase A3 (data not shown). The nucleotide sequence homology between rat Prss34 and carboxypeptidase A3 is significant, so, most likely, the primers used for Prss34 accidentally amplified a small amount of this off-target gene after multiple rounds of PCR. Another time, a faint band from the Tpsab1 lane was sequenced and found to correspond to a histone deacetylase. Attempts to isolate and sequence a faint band, positioned below the expected Gzma size, from the Gzma lane were unsuccessful, likely because there was very little material present. Also, other researchers reported a lack of Gzma mRNA in RBL-2H3 cells (Nakajima et al., 1995). Overall, no tryptase mRNA was detected for any of the eight tested tryptase genes.

Other researchers have previously reported a lack of certain tryptases in RBL-2H3 cells. Similar to our Fig. 2 results, two other studies also observed a lack of tryptase via BAPNA assay in supernatants of degranulated RBL-2H3 cells, even while β -hex release was readily measured in the same supernatants (Peng et al., 2011; Tang et al., 2012). Mass spectrometry analyses of cell supernatants from degranulated RBL-2H3s also found no tryptase proteins (Sadroddiny, Ai, et al., 2012; Sadroddiny, Moir, et al., 2012). Furthermore, Ide et al. reported that no mRNA of *Tpsb2* (noted as rMCT [rat mast cell tryptase] in the article) was located in RBL-2H3 cells (Ide et al., 1995). However, one publication did report high levels of tryptase (as measured by BAPNA reaction) released into the supernatants of unstimulated RBL-2H3 cells, with further increases due to hypoxia (Maxova et al.). This result is puzzling because, typically, very low levels of spontaneous degranulation occur in unstimulated (no antigen, no ionophore, etc) RBL-2H3 cells.

Two additional studies have reported detection of tryptase in RBL-2H3 cells (Small-Howard et al., 2005; Song et al., 2016). However, these two studies measured tryptase (via ELISA in Song *et al.* and via substrate activity assay in Small-Howard and Turner) within cell lysates, not from the supernatants of stimulated intact cells. Since our current study demonstrated that lysates from both RBL-2H3 and NIH-3T3 cells processed tosyl-Gly-Pro-Lys-pNA substrate (Fig. 4) and yielded tryptase ELISA absorbance (Fig. 5), these signals are likely not from MC granule tryptases. Rather, other, non-granule-associated serine proteases may be picked up by these two studies and in Figs. 4 and 5. For example, retinoid-inducible serine carboxypeptidase 1 (also known as carboxypeptidase C) is found in RBL-2H3 cells (Sadroddiny, Ai, et al., 2012). Also, the substrate tosyl-Gly-Pro-Lys-pNA can be cleaved by enzymes other than tryptase, including plasmin/plasminogen (Drinane et al., 2006; Vakili et al., 2001; Wiedow et al., 1997). The key mast cell function is release of granule contents from safe storage in intracellular granules, into extracellular fluids, via plasma membrane

fusion; thus, analyzing the contents of MC whole-cell lysates is an erroneous measure of MC function.

Because tryptase is known to be localized within granules and exclusively expressed in mast cells (da Silva et al., 2014; Schwartz et al., 1987), we had anticipated to find tryptase released into the supernatants of degranulated RBL-2H3 cells in this study. One possible reason for our findings is that RBL-2H3 cells are physiologically similar to rodent mucosal mast cells (Seldin et al., 1985). MCs display a high degree of heterogeneity in contents of granules, as a consequence of their ubiquitous location and a wide range of microenvironments they encounter (da Silva et al., 2014). Human MCs are divided into two subsets based on protease content: MC_{TC}, containing tryptase, chymase, and carboxypeptidases, or MC_T, containing only tryptase (Irani et al., 1986). Rodent MCs are subcategorized into mucosal mast cells (MMCs) and connective tissue mast cells (CTMCs) (Metcalf et al., 1997). While tryptase is present in all human MCs (Irani et al., 1986), rodent MMCs do not express tryptase but, rather, predominantly express chymase (Huang et al., 1991; Le Trong et al., 1987; Lutzelschwab et al., 1997; Reynolds et al., 1990; Serafin et al., 1990). Thus, RBL-2H3 cells' similarity to MMCs might explain the lack of tryptase expression.

In summary, this current study is the first comprehensive report on detection of tryptase protein and transcripts from RBL-2H3 cells. Multiple lines of experimental evidence indicate that mRNA from eight tryptase genes is not present and that tryptase protein either is not present or is very difficult to find in supernatants from degranulated RBL-2H3 cells. Instead, mast cell researchers should utilize β -hex, histamine, serotonin, or other reliable markers for RBL-2H3 degranulation studies. Comparative studies of toxicant effects on RBL-2H3 mast cells *in vitro* and in zebrafish *in vivo* will require use of a degranulation reporter different from tryptase.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Measurement of interaction of purified human lung tryptase (TPSAB1/TPSB2/TPSD1) with N-benzoyl-DL-arginine-p-nitroanilide (BAPNA) substrate.

(A) As described in Materials & Methods, purified tryptase was incubated at the concentrations indicated over 72 hours with BAPNA. A_{410} is plotted against the time in h. Data presented are means $\pm$ SD of a representative experiment; duplicates per treatment per experiment. In (B), tryptase concentration is detected at a single, 72-h time point; tryptase (ng) is depicted versus the pNA signal. Data presented are means $\pm$ SD of a representative experiment; duplicates per treatment per experiment. Statistical significance was determined GraphPad Prism using a one-way ANOVA followed by a Tukey's post test; *** $p < 0.001$, ** $p < 0.01$.

Figure 2. Measurement of β -hexosaminidase and tryptase in the supernatant of RBL-2H3 mast cells stimulated with calcium ionophore A23187.

RBL-2H3 cells were incubated for one hour with A23187 ionophore in modified Tyrodes buffer, and degranulation was quantified after incubation with (A) 4-Methylumbelliferone (4MU) substrate for β -hexosaminidase or with (B) BAPNA substrate for tryptase. Data are shown from one representative experiment; values represent means $\pm$ SD of triplicate samples. Statistical significance was determined by one-way ANOVA followed by Tukey's post test. ** $p < 0.01$.

Figure 3. Measurement of tryptase in the supernatant of RBL-2H3 mast cells stimulated with antigen or A23187 ionophore using tosyl-Gly-Pro-Lys-pNA tryptase substrate. RBL-2H3 cells were sensitized for one hour with $0.1\mu\text{g mL}^{-1}$ anti-DNP mouse IgE in RBL-2H3 media (A and B). In (A), plated cells were treated with DNP-BSA Ag ($1\mu\text{g mL}^{-1}$) for 15 minutes in modified Tyrodes buffer. In (B), cells in suspension were treated with DNP-BSA Ag ($1\mu\text{g mL}^{-1}$) treatment for 1 hour in 1X Assay buffer provided in the Mast cell degranulation assay kit (EMD Millipore). In (C), cells in suspension were stimulated with 200nM A23187 ionophore for 1 hour. Degranulation was quantified with tosyl- Gly-Pro-Lys-pNA tryptase substrate provided by the Mast cell degranulation assay kit (EMD Milipore). Error bars represent the standard error of the mean of the data from three experiments; triplicates per sample were done on each experimental day. No statistical significance was determined by one-sample t-tests.

Figure 4. Measurement of tryptase from RBL-2H3 and NIH-3T3 cell lysates using tosyl-Gly-Pro-Lys-pNA tryptase substrate.

Lysates from RBL-2H3 cells (A) and NIH-3T3 cells (B) were collected as described in Materials & Methods and incubated with tosyl-Gly-Pro-Lys-pNA tryptase substrate for 72 hours. Error bars represent the standard error of the mean of the data from three experiments; triplicates per sample were done on each experimental day. (A) Statistical significance, as compared to the RBL-2H3 supernatant sample, was determined by one-sample t-tests. $**p < 0.01$ (B) No statistical significance was determined in comparing RBL-2H3 and NIH-3T3 lysates samples by one-sample t-test.

Figure 5. Measurement of Tpsab1 tryptase from RBL-2H3 and NIH-3T3 cells by ELISA. RBL-2H3 cells were sensitized with anti-DNP IgE ($0.1\mu\text{g mL}^{-1}$) for 1 hr before being treated with DNP-BSA Ag ($1\mu\text{g mL}^{-1}$) for 15 min. Rat tryptase protein (TPSAB1) was measured using a sandwich ELISA kit (Biomatik). Data are from one representative experiment; values represent means $\pm$ SD of duplicate samples. Statistical significance was determined as compared to the RBL-2H3 control supernatant sample, by one-way ANOVA followed by Tukey's post test. *** $p < 0.001$

Figure 6. Measurement of rat tryptase and β -hexosaminidase mRNA from RBL-2H3 cells using PCR.

Eight target rat tryptase genes' mRNA (A) and two β -hexosaminidase genes' mRNA (B) were analyzed. Total RNA was extracted from RBL-2H3 cells, and reverse transcription was performed as described in Materials & Methods in order to generate cDNA, which was amplified by PCR. Actin was used as a positive control. The primer pairs and the size of PCR products are listed in the table (C). The PCR amplicons were subjected to electrophoresis in 1% agarose gels, and the target bands were extracted for sequencing to be confirmed (data not shown). Data shown are representative of all cell stimulation conditions and timeframes (supplement table S1). None of tryptase genes' mRNA was found in any of the stimulation conditions whereas actin and β -hexosaminidase genes' mRNA were always detected. NTC is no template control; MW is molecular weight marker.

Figure 7. Measurement of rat tryptase genomic DNA extracted from RBL-2H3 cells using PCR.

A) Eight target tryptase genes' genomic DNA were amplified by PCR as described in Materials & Methods. The primer pairs and the size of PCR products are listed in the table (B). PCR amplicons were subjected to electrophoresis in 1% agarose, and the target bands were extracted for sequencing confirmation (data not shown). MW is molecular weight marker.

Table 1.

Tryptase and tryptase-like proteins in rats and human

Rat Gene Symbols	Alternative Names	Human Orthologs
<i>Tpsab1</i>	Mast cell protease-7; Tryptase β I; Tryptase α/β 1; Skin tryptase	TPSAB1
<i>Tpsb2</i>	Mast cell protease-6; Tryptase- β 2; Tryptase-2	TPSB2
<i>Tpsg1</i>	Transmembrane tryptase; Tryptase γ 1	TPSG1
<i>Gzmk</i>	Granzyme K; Natural Killer (NK)-tryptase-2	GZMK
<i>Prss29</i>	Serine protease 29; tryptase δ I	PRSS29P/TPSD1
<i>Prss41</i>	Serine protease 41; testis serine protease 1	PRSS41
<i>Gzma</i>	Granzyme A	GZMA
<i>Prss34</i>	Mastin; Serine Protease 34	-