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Antibacterial agent triclosan suppresses RBL-2H3 mast cell function

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ABSTRACT

Triclosan is a broad-spectrum antibacterial agent, which has been shown previously to alleviate human allergic skin disease. The purpose of this study was to investigate the hypothesis that the mechanism of this action of triclosan is, in part, due to effects on mast cell function. Mast cells play important roles in allergy, asthma, parasite defense, and carcinogenesis. In response to various stimuli, mast cells degranulate, releasing allergic mediators such as histamine. In order to investigate the potential anti-inflammatory effect of triclosan on mast cells, we monitored the level of degranulation in a mast cell model, rat basophilic leukemia cells, clone 2H3. Having functional homology to human mast cells, as well as a very well defined signaling pathway leading to degranulation, this cell line has been widely used to gain insight into mast-cell driven allergic disorders in humans. Using a fluorescent microplate assay, we determined that triclosan strongly dampened the release of granules from activated rat mast cells starting at 2 μ M treatment, with dose-responsive suppression through 30 μ M. These concentrations were found to be non-cytotoxic. The inhibition was found to persist when early signaling events (such as IgE receptor aggregation and tyrosine phosphorylation) were bypassed by using calcium ionophore stimulation, indicating that the target for triclosan in this pathway is likely downstream of the calcium signaling event. Triclosan also strongly suppressed F-actin remodeling and cell membrane ruffling, a physiological process that accompanies degranulation. Our finding that triclosan inhibits mast cell function may explain the clinical data mentioned above and supports the use of triclosan or a mechanistically similar compound as a topical treatment for allergic skin disease, such as eczema.

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Introduction

Mast cells are immune effector cells involved in the pathogenesis of allergic disease (including asthma and eczema) as well as carcinogenesis, and are widely distributed throughout the body (Blank et al., 2007; Dvorak, 1986; Farrell et al., 1995; Kuby, 1997). Through a well-characterized pathway, they can be activated by a range of stimuli to release the contents of their cytoplasmic granules (degranulation), producing the well-known symptoms of an allergic reaction.

Degranulation typically begins when multivalent antigen (such as an allergen like pollen) aggregates (crosslinks) IgE-bound Fc ϵ RI receptors, but mast cells can be activated to a similar extent by antibodies (Ortega et al., 1999). Mast cell activation, whether by antigen or anti-IgE antibody, results in a tyrosine phosphorylation cascade, leading to the activation of phospholipase C (Beaven and Metzger, 1993; Benhamou et al., 1992). In turn, inositol 1,4,5-triphosphate

(IP₃) is produced by hydrolysis of a membrane glycerophospholipid, and upon binding to its receptor in the endoplasmic reticulum, IP₃ activates Ca²⁺ efflux from the endoplasmic reticulum, into the cytosol (Ferris et al., 1989; Millard et al., 1989; Putney, 2009; Putney et al., 2001). Efflux leads to subsequent Ca²⁺ influx across the plasma membrane, and signaling culminates in degranulation, the release of allergic mediators (e.g., histamine, β -hexosaminidase, leukotrienes, and cytokines) from preformed granules.

Triclosan¹ (TCS) is a mild, yet effective broad-spectrum antimicrobial first introduced to the healthcare industry in 1972 as a surgical scrub ingredient at 1%. At low (sub-lethal) doses, TCS acts as a bacteriostatic agent by interfering with bacterial fatty acid biosynthesis (Levy et al., 1999; McMurry et al., 1998; Russell, 2004). For several decades, its favorable safety profile encouraged widespread application. Today, TCS is found in hundreds of medical, consumer, and personal care products (e.g., toys, bedding, deodorant, cosmetics, soap, and toothpaste) at concentrations up to 0.3% or 10 mM (Jones et al., 2000).

Clinically, TCS and TCS-containing lotions have been shown to alleviate symptoms of skin allergic disease, such as atopic dermatitis

Abbreviations: RBL, rat basophilic leukemia, clone 2H3; TCS, triclosan; DNP, dinitrophenyl; LDH, lactate dehydrogenase; PBS, phosphate buffered saline; Ag, antigen.

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¹ The chemical name for triclosan is 5-chloro-2-(2,4-dichlorophenoxy)phenol, and it is also known as Irgasan DP300 and Irgasan MP (CAS Number is 3380-34-5).

(Barkvoll and Rolla, 1995a, 1995b; Kjaerheim et al., 1995; Sporik and Kemp, 1997; Tan et al., 2010; Wohlrab et al., 2007), but the mechanism(s) by which it does this are unclear (Breneman et al., 2000). In a study by Kjaerheim et al. (1995), TCS was found to minimize the size of weals produced by intradermal histamine treatment of human skin. Barkvoll and Rolla (1995b) found that TCS reduces symptoms of an allergic patch test reaction in allergic females. Additionally, Tan et al. (2010) performed a randomized double-blind controlled trial to compare TCS ointment to control for the treatment of atopic dermatitis. They found that TCS treatment reduced the necessary amount of a topical steroid to effectively treat atopic dermatitis and was safely tolerated (Tan et al., 2010). In the current study, we further investigated the anti-allergy, anti-eczema potential and mechanism of TCS through the endpoint of mast cell degranulation.

In addition to IgE receptors, mast cells have a variety of other receptors involved in degranulation, including those for steroid hormones. In human and murine mast cells, recent exposure studies have demonstrated that endogenous and environmental estrogens exacerbate IgE-mediated degranulation (Narita et al., 2007; Zaitsu et al., 2007). Because TCS is similar in structure to non-steroidal estrogens, and because several studies are supportive of its having intrinsic endocrine disrupting activity (Ahn et al., 2008; Foran et al., 2000; Gee et al., 2008; Helbing et al., 2011; Ishibashi et al., 2004; Kumar et al., 2009; Matsumura et al., 2005; Veldhoen et al., 2006), we were additionally motivated to study the effects of TCS on mast cell degranulation. In fact, in light of data indicating TCS is an endocrine disruptor, the US Food and Drug Administration and other government agencies are working to further assess the toxicology of TCS (<http://www.fda.gov/forconsumers/consumerupdates/ucm205999.htm>).

In this study we have addressed the question of whether TCS affects mast cell degranulation by utilizing RBL-2H3 (RBL) cells, a well-accepted model for allergic signal transduction and which are functionally homologous to mucosal mast cells (Seldin et al., 1985). We have sensitized RBL cells with IgE (which binds to the FcεRI receptor) and have aggregated IgE-bound FcεRI receptors with either multivalent antigen or anti-IgE antibodies in the presence of non-cytotoxic doses of TCS. Other cells were stimulated with calcium ionophore ($\pm$ TCS), which brings Ca^{2+} across the plasma membrane, into the cell, thereby stimulating degranulation while bypassing FcεRI engagement and early molecular events (Siraganian et al., 1975). We have measured subsequent degranulation in a fluorescence assay based upon release of β -hexosaminidase (Naal et al., 2004), which is linearly related to release of histamine (Schwartz et al., 1979). Additionally, we assessed the effects of TCS on membrane ruffling (due to F-actin rearrangement), a process that normally accompanies degranulation in stimulated mast cells.

Materials and methods

Chemicals and reagents

To minimize the risk of contaminants causing toxicological side effects, ingredients of the highest possible purity were selected. Additionally, media and buffers were sterile filtered with ZapCap filters (0.2 μm ; Whatman, Florham Park, NJ, USA).

Triclosan (TCS) was purchased from Sigma-Aldrich (St. Louis, MO, USA) under the tradename “Irgasan” ($\geq 97\%$ by HPLC; CAS no. 3380-34-5). A starting TCS stock (3 or 30 mM, depending on the assay) was freshly prepared for each experiment, and contained 24% ethanol (v/v). TCS granules were first dissolved in 100% ethanol (ACS/USP Grade; PHARMACO-AAPER, Brookfield, CT, USA) before addition of sterile cell culture water (deionized, distilled water for cell culture applications; BioWhittaker Lonza, Walkersville, MD, USA). After 30-min sonication (Branson 1200 ultrasonic cleaner; Branson Ultrasonics, Danbury, CT, USA), this main stock was serially diluted in 24% ethanol to stocks of ‘100 $\times$ ’ TCS doses. Final TCS exposure up to 150 μM

(0.24% ethanol vehicle) was achieved by dilution in Tyrodes–BSA buffer, pH 7.4 (recipe given in Hutchinson et al. (2011)).

Calcium Ionophore A23187 (Sigma) was dissolved in 100% dimethyl sulfoxide (DMSO; Sigma) to generate a stock concentration of 2.5 mg mL^{-1} , and was kept at -20°C until needed. On the day of an experiment, ionophore stock was diluted in Tyrodes–BSA buffer to make ionophore stocks in 0.004% DMSO vehicle. Final DMSO vehicle for all TCS experiments was 0.004%.

Alexa 488-phalloidin (lyophilized; Invitrogen, Carlsbad, CA, USA) was reconstituted in 100% methanol (ACS grade; BDH) to 200 U mL^{-1} , and kept in the dark at -20°C until needed. As an added safety measure, this chemical was always handled with double-gloved hands.

Cell culture

RBL-2H3 (RBL) cells were a generous gift from D. Holowka (Cornell University, Ithaca, NY, USA). Cell culture methods were those of Hutchinson et al. (2011).

IgE-receptor crosslinking by antigen and TCS exposure

Cells were sensitized with 0.1 $\mu\text{g mL}^{-1}$ of anti-dinitrophenyl (DNP) mouse IgE (monoclonal, clone SPE-7, Sigma, St. Louis, MO, USA) in RBL cell media for 1 h at 37°C , as described in Hutchinson et al. (2011). Crosslinking of IgE-bound FcεRI receptors was achieved in a 1-h incubation of RBL cells with DNP-BSA multivalent antigen (at either 0.004 $\mu\text{g mL}^{-1}$ or 0.0004 $\mu\text{g mL}^{-1}$) $\pm$ TCS, essentially as described (Hutchinson et al., 2011). A variation on this protocol included a 15-min incubation with 0.004 $\mu\text{g mL}^{-1}$ DNP-BSA. Degranulation was quantified as described in “Degranulation assay: quantification of β -hexosaminidase release.”

Cytotoxicity

TCS cytotoxicity was assessed by lactate dehydrogenase (LDH) cytotoxicity and trypan blue exclusion (both 1-h TCS exposures) as described previously (Hutchinson et al., 2011). In minor modification of the LDH assay, the 15-min incubation of “high control” samples with lysis solution was included as part of the last 15 min of a 1-h TCS exposure, rather than an additional 15 min. Also, to control for TCS interference on LDH enzyme activity, 0.000625 U mL^{-1} purified LDH enzyme standard was used in place of the stated 0.000078 U mL^{-1} , in order to obtain a moderate, non-saturating A_{490} value.

Release conditions monitored in degranulation assays

Each experimental plate contained the following three conditions: (1) *stimulated*, which varied by assay (either antigen, anti-IgE antibody, or calcium ionophore stimulation as described below); (2) *spontaneous* release, in which cells in Tyrodes–BSA buffer, alone, were used to measure basal release of β -hexosaminidase; and (3) *Triton-X 100 detergent lysis* (Surfact-Amps X-100, 10%, low carbonyl and peroxide; Thermo scientific, Rockford, IL, USA), used to quantify the maximum release of β -hexosaminidase from cytoplasmic granules.

IgE-receptor crosslinking by anti-IgE antibody and TCS exposure

Crosslinking of IgE-bound FcεRI receptors was achieved in a 1-h incubation of RBL cells with anti-IgE antibody (at either 10 $\mu\text{g mL}^{-1}$ or 4 $\mu\text{g mL}^{-1}$) $\pm$ TCS, as described previously (Hutchinson et al., 2011). Resultant degranulation was quantified as described in “Degranulation assay: quantification of β -hexosaminidase release.”

Calcium ionophore stimulation and TCS exposure

RBL cells were stimulated to degranulate with calcium ionophore at concentrations chosen from preliminary dose response data (data not shown). Following overnight incubation in a black 96-well plate (Grenier Bio-One), spent medium was discarded, and cells were washed twice with 200 μ L Tyrodes–BSA buffer per well in a non-systematic fashion. Next, 200 μ L of ionophore (either 0.85×10^{-7} M or 1.5×10^{-7} M) $\pm$ TCS was added to each sample well, and was non-systematically approached, triplicate-by-triplicate. DMSO (0.004% final) was also used in spontaneous, Triton X-100, and background wells to control for vehicle effects. The plate was then left to incubate for 1 h at 37 °C and 5% CO₂. Resultant β -hexosaminidase released into cell supernatant was quantified as described in “[Degranulation assay: quantification of \$\beta\$ -hexosaminidase release](#).”

Degranulation assay: quantification of β -hexosaminidase release

In order to measure RBL cell degranulation upon stimulation with antigen, anti-IgE antibody, or calcium ionophore, the release of β -hexosaminidase from RBL mast cells was quantified using a spectrophotometric microplate reader (Synergy 2; Biotek, Winooski, VT, USA) following the fluorescence-based protocol detailed previously by [Hutchinson et al. \(2011\)](#), which is a modification of [Naal et al. \(2004\)](#). After subtracting out the average background fluorescence (Tyrodes–BSA buffer without cells) from all raw fluorescence readings, triplicate values were averaged, and these averages were expressed as a percentage of the average β -hexosaminidase released by Triton-X 100-lysed RBL cells (theoretical maximal release), as described in detail by [Hutchinson et al. \(2011\)](#). Then, each “percentage degranulation” response found in the presence of TCS was normalized to that of the 0 μ M TCS control; these normalized data from multiple days of experiments were averaged. Also following [Hutchinson et al. \(2011\)](#), we controlled for the possibility that TCS interfered with the enzyme–substrate reaction used to quantify degranulation.

Confocal imaging of membrane ruffling

For membrane ruffling experiments, 2 mL of a 0.5×10^6 cells mL^{−1} cell mixture was plated per MatTek dish (P35G-1.5-14-C, Na 1.5 cover glass, MatTek, Ashland, MA) and left to grow overnight in cell culture medium (at 37 °C and 5% CO₂).

The next day, spent medium was discarded and replaced by 2 mL fresh medium containing 0.1 μ g mL^{−1} monoclonal anti-DNP mouse IgE (Sigma #D8406, clone SPE-7), which was left on the cells for 1 h at 37 °C and 5% CO₂ for the purpose of IgE-receptor sensitization. Following this, IgE-containing medium was discarded and cells were washed twice with 2 mL Tyrodes–BSA buffer. RBL samples destined for antigen stimulation were incubated with 0.0004 μ g mL^{−1} DNP-BSA $\pm$ TCS in Tyrodes–BSA buffer for 1 h (at 37 °C and 5% CO₂). Along with these stimulated cells, unstimulated RBL cells were incubated in 2 mL Tyrodes–BSA buffer (no antigen) $\pm$ TCS. Controls received Tyrodes–BSA buffer ($\pm$ antigen) containing 0.24% ethanol as vehicle.

Afterwards, cells were washed with 2 mL phosphate buffered saline (PBS; Lonza, Rockland, ME), and a 3.7% formaldehyde–PBS solution was used to fix cells (in 10-min incubation at room temperature). Once the formaldehyde mixture was discarded, cells were washed twice with 2 mL PBS.

To permeabilize the cell membrane, 2 mL of a 0.1% dilution of Triton X-100 (Thermo scientific, Rockford, IL, USA) in PBS was added to each MatTek dish and incubated for 5 min at room temperature. After washing twice with 2 mL PBS per wash, cells were incubated for 20 min at room temperature in a 1% (w/v) dilution of BSA in PBS. Following this, cells were incubated with one unit of Alexa Fluor 488 conjugated phalloidin (diluted in PBS) for 20 min at room

temperature, during which time they were shielded from light. Then, dishes were washed three times in PBS (2 mL per wash), and PBS with 10 mg mL^{−1} BSA and 1 mM EDTA was added to the wells, which were stored at 4 °C until imaged.

Membrane ruffling was imaged using an Olympus FV-1000 laser scanning confocal system in conjunction with an Olympus IX-81 inverted microscope, using 488 nm excitation/520 nm emission and a confocal aperture of 110 μ m. An oil-immersion 100 $\times$ objective with an NA of 1.4 was used together with a 30 milliwatt multi-argon laser to image. Multiple fields of view were taken for each MatTek dish at 4 μ s/pixel, which were analyzed using FV-10 Olympus software.

Statistical analyses

Results from degranulation experiments are reported as mean $\pm$ standard error of the mean (SEM), with significant differences determined by Prism software (Graphpad, San Diego, CA, USA). For multiple-comparison testing, one-way analysis of variance followed by Tukey's post hoc test was used to test for significance ($\alpha = 0.05$). To compare antigen dose response differences in the presence and absence of 30 μ M TCS, a two-way analysis of variance followed by Bonferroni adjustment was used in [Fig. 1A](#). In cases where data required normalization to vehicle control (as was needed to combine degranulation responses from multiple days), significant differences were evaluated in comparison to the 0.1 μ M TCS average response, which represented a very low dose not significantly different from the 0 μ M TCS average. For the trypan blue exclusion cytotoxicity assay, 0.01 μ M TCS was used in the determination of significance. In the event that normalization was unnecessary (as in the case of data from the LDH cytotoxicity assay), significance was directly compared to the response for 0 μ M TCS. Significance is represented by *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$.

Results

TCS inhibits the degranulation of RBL cells activated by low and maximal multivalent antigen doses

To determine the effects of TCS on mast cell degranulation, RBL mast cells and a fluorescence-based assay ([Naal et al., 2004](#)) of β -hexosaminidase release were utilized. Multivalent DNP-BSA antigen (Ag) was used to laterally crosslink IgE-bound Fc ϵ R1 receptors, which initiates allergic signal transduction, culminating in the release of pre-formed allergic mediators. For these experiments, both a low and a maximal dose of Ag were employed. These doses were selected on the basis of relative degranulation responses that were elicited in the absence of TCS (ethanol vehicle was present). A representative curve for DNP-BSA dose response (with and without TCS present) in RBL cells is presented in [Fig. 1A](#). In the absence of TCS, the maximal Ag dose (0.004 μ g mL^{−1}) elicited an average absolute degranulation response of $40 \pm 2\%$ (SEM) for all degranulation experiments at this Ag dose; the low Ag dose (0.0004 μ g mL^{−1}) elicited an average absolute degranulation level of $10.0 \pm 0.5\%$. For comparison, spontaneous levels (those coming from unstimulated cells) elicited an average absolute degranulation of $2.5 \pm 0.1\%$. In the presence of 30 μ M TCS, the dose response curve is significantly dampened ([Fig. 1A](#)).

[Fig. 1B](#) presents the results for IgE-sensitized RBL cells that have been incubated for 1 h in Tyrodes–BSA buffer containing a maximal Ag dose, 0.004 μ g mL^{−1} DNP-BSA Ag, with TCS or ethanol vehicle. Statistically significant inhibition of degranulation began at 15 μ M, where degranulation levels were 0.79-fold $\pm$ 0.02 (SEM) of the 0 μ M TCS control levels. As TCS dose was increased, degranulation was further dampened in a clear dose-responsive manner. At 20 μ M TCS, degranulation was 0.55-fold $\pm$ 0.06, and by 30 μ M TCS, levels were 0.37-fold $\pm$ 0.02. We confirmed that this inhibition was neither the result

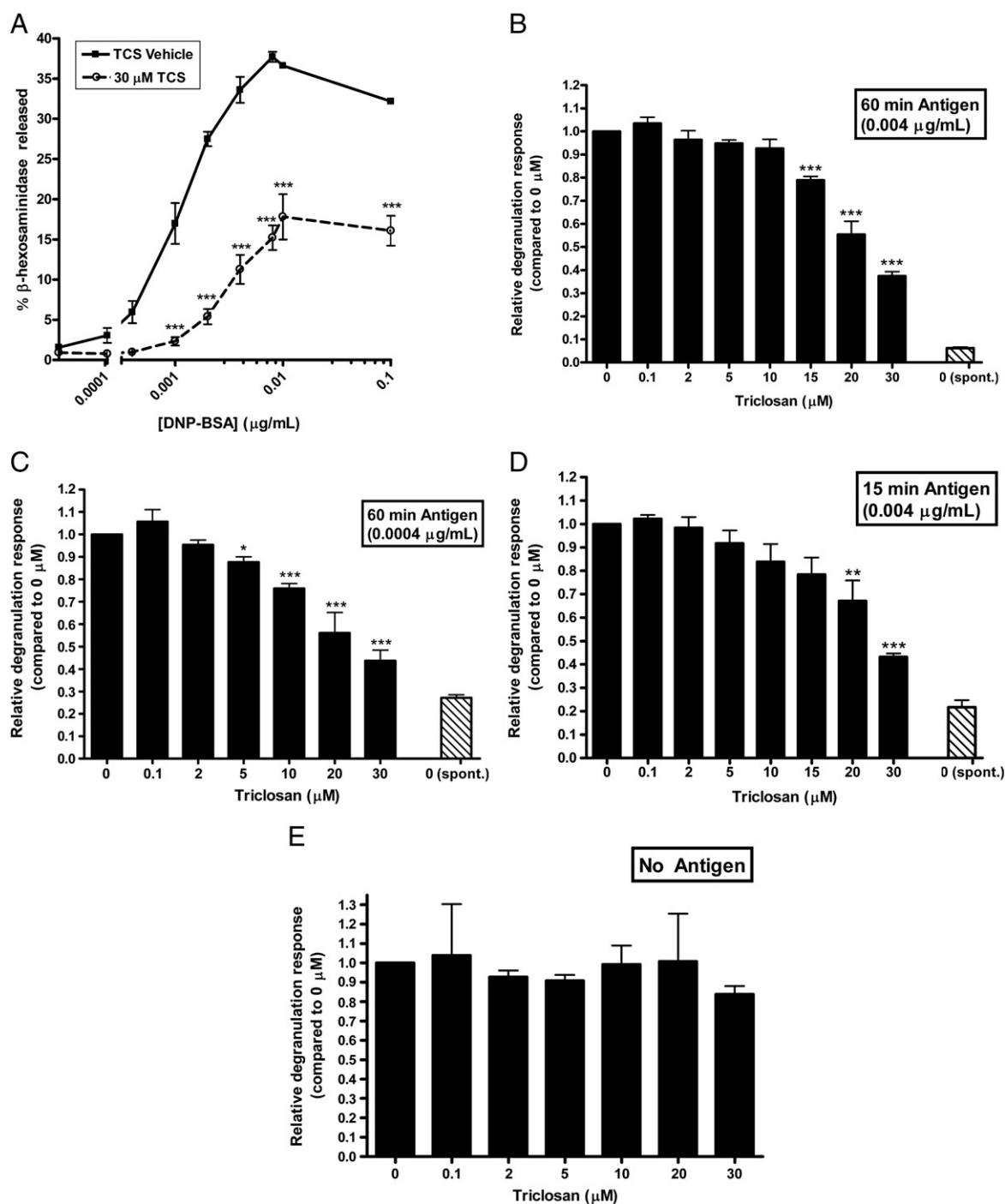


Fig. 1. Dose-responsive relative degranulation response of RBL mast cells exposed to different concentrations of TCS. A representative dose-response curve for DNP-BSA antigen (Ag) containing either TCS vehicle (0.24% ethanol) only or 30 μM TCS is shown on a logarithmic scale in (A). IgE-sensitized cells were stimulated with either 0.004 $\mu\text{g mL}^{-1}$ Ag for 1 h (B), 0.0004 $\mu\text{g mL}^{-1}$ Ag for 1 h (C), or were subjected to a 15-min exposure at the 0.004 $\mu\text{g mL}^{-1}$ Ag (D). For spontaneous release, non-IgE-sensitized cells were exposed to TCS for 1 h (no antigen present) (E). A spontaneous release measurement also appears for comparison (B–D). In B–E, the normalized degranulation response is plotted against TCS exposure doses. Values represent the mean $\pm$ SEM of 3–13 independent experiments; three replicates were used in each experiment. *** $p < 0.001$; ** $p < 0.01$; * $p < 0.05$.

of interference with the enzyme–substrate reaction used to quantify β -hexosaminidase (Fig. S1A), nor due to TCS effects on background fluorescence (Fig. S1B). These data demonstrate a strong inhibition of multivalent Ag-stimulated degranulation function in RBL mast cells due to TCS co-exposure.

Next, to discover whether or not the degree of TCS inhibition is dependent upon concentration of stimulatory antigen, a low Ag dose (0.0004 $\mu\text{g mL}^{-1}$) was utilized (Fig. 1C) for 1 h. This dose elicits a degranulation response that is ~25% of the maximal response in

the absence of TCS (see above). At 5 μM TCS, statistically significant inhibition of degranulation emerged, resulting in levels that were 0.88-fold $\pm$ 0.02. Increasing concentrations of TCS further inhibited the response. At 10 μM TCS, degranulation was 0.76-fold $\pm$ 0.02; at 20 μM TCS, 0.56-fold $\pm$ 0.09; and, at 30 μM TCS, degranulation was 0.44-fold $\pm$ 0.05. For comparison, the average spontaneous 0 μM release level was 0.27-fold $\pm$ 0.01 of the degranulation response for the 0 μM TCS control. We have found here that when RBL cells are stimulated to a low level (0.0004 $\mu\text{g mL}^{-1}$) for 1 h (Fig. 1C),

significant TCS inhibition of degranulation begins at lower TCS concentrations than when a higher Ag concentration ($0.004 \mu\text{g mL}^{-1}$) (Fig. 1B) is used ($5 \mu\text{M}$ vs. $15 \mu\text{M}$, respectively). However, $20 \mu\text{M}$ TCS with low Ag (Fig. 1C) is as effective as $20 \mu\text{M}$ TCS with high Ag (Fig. 1B); the same comparison is true for $30 \mu\text{M}$. Thus, multivalent antigen concentration does not play a large role in the severity of TCS's effects on mast cells.

We next shortened the duration of antigen/TCS co-exposure from 1 h, to 15 min (Fig. 1D) in order to test if TCS's effects occur rapidly. In these experiments, RBL cells were stimulated to degranulate with $0.004 \mu\text{g mL}^{-1}$ DNP-BSA based on initial 15-min Ag dose–response data (Fig. S1C). The Ag dose response curve for 15 min reveals a shift in the dose response from 1-h data, as it is defined by a much lower “maximal” response due to incomplete release of β -hexosaminidase (as compared to 1-h data in Fig. 1B). The average absolute degranulation response elicited by $0.004 \mu\text{g mL}^{-1}$ Ag for 15 min over the course of all experiments was $7.3\% \pm 0.4\%$. Significant dampening was observed by $20 \mu\text{M}$ TCS, where levels were $0.67\text{-fold} \pm 0.04$. At $30 \mu\text{M}$ TCS, degranulation was $0.50\text{-fold} \pm 0.04$; compare to spontaneous levels ($0.22\text{-fold} \pm 0.01$ of 15-min Ag-stimulated control). Therefore, TCS was able to significantly inhibit the release of β -hexosaminidase of DNP-BSA Ag-activated mast cell degranulation by as early as 15 min. Taken together, Figs. 1B–D clearly show that TCS strongly inhibits RBL mast cell degranulation.

In contrast to the Ag-stimulated 1-h data, we did not observe significant degranulation responses to TCS for non-stimulated (no IgE or Ag) RBL cells (Fig. 1E). This lack of a TCS effect on spontaneous degranulation shows that TCS only affects degranulation when a second environmental agent, such as antigen, is present.

TCS concentrations that effectively inhibit degranulation are not cytotoxic to RBL cells

We tested whether TCS is cytotoxic to RBL cells in 1-h cytotoxicity experiments designed to simulate the conditions of the degranulation experiments, as described in Materials and methods. Fig. 2A presents cytotoxicity data from a 1-h lactate dehydrogenase (LDH) release assay (Roche Diagnostics), which measures damage to the plasma membrane. No significant increase in % LDH release was observed until $150 \mu\text{M}$ TCS, in which average release was $66\% \pm 6\%$ (SEM) of high control. As shown in Fig. S2, we confirmed that LDH absorbance was not affected by TCS.

Fig. 2B depicts data from 1-h trypan blue exclusion experiments, in which viable cells were identified by virtue of their ability to actively exclude trypan blue dye. A significant decrease in cell viability was not observed until $150 \mu\text{M}$ TCS, by which concentration, no viable cells were counted. Taken together, the data in Figs. 2A and B strongly suggest that $30 \mu\text{M}$ or lower TCS concentrations are not cytotoxic at 1 h. Using a clonogenic (colony-forming) assay on RBL cells (data not shown), we determined a 24-h LC50 value for TCS of $40\text{--}43 \mu\text{M}$ (95% confidence interval). In summary, these cytotoxicity data strongly suggest that cell death is not a mechanism by which TCS inhibits mast cell degranulation.

TCS inhibition of RBL mast cell degranulation is not specific to a single type of IgE-receptor crosslinker used on the mast cell surface

Next, we determined whether or not TCS inhibition of degranulation can be achieved when IgE receptors are crosslinked with anti-IgE IgG antibody in place of DNP-BSA antigen. Only two IgE-bound Fc ϵ R1 receptors can be crosslinked together by one anti-IgE antibody, whereas greater than two receptors can be clustered when multivalent DNP-BSA Ag is used. RBL cells were co-incubated with TCS and either a moderate or low concentration of antibody, identified from initial dose response data (Fig. S3). In the absence of TCS, the moderate antibody dose, $10 \mu\text{g mL}^{-1}$, elicited an average absolute

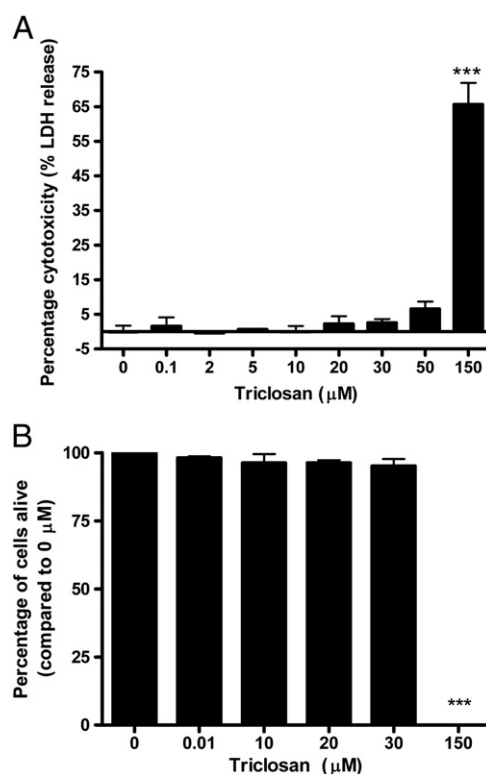


Fig. 2. Cytotoxicity of 1-h exposure to TCS assessed by Cytotoxicity Detection Kit (LDH) from Roche (A) and trypan-blue exclusion cytotoxicity assay (B). Values presented are means $\pm$ SEM of three independent experiments, except for four independent repeats in the LDH assay (for 0 μM , 10–30 μM , and 150 μM TCS samples). ***Statistically different ($p < 0.001$) from 0 μM TCS (in LDH experiment), or statistically different from 0.01 μM TCS (in trypan blue exclusion assay) in a one-way ANOVA followed by Tukey's post test.

degranulation response in our experiments of $24\% \pm 3\%$ (SEM) ($\sim 60\%$ of the maximal response found with DNP-BSA stimulation as shown in Fig. 1A), while the low antibody dose, $4 \mu\text{g mL}^{-1}$, elicited an average absolute response of $11\% \pm 1\%$ ($\sim 28\%$ of the maximal DNP-BSA response in Fig. 1A).

Fig. 3A depicts experiments performed with the moderate anti-IgE antibody dose, in which statistically significant suppression of RBL cell degranulation was obtained by $20 \mu\text{M}$ TCS, representing a response $0.61\text{-fold} \pm 0.04$ of 0 μM TCS control. Stronger inhibition was observed for cells treated for 1 h with $30 \mu\text{M}$ TCS, which produced a response that was $0.32\text{-fold} \pm 0.03$.

Fig. 3B depicts data from experiments performed with the low anti-IgE dose, which elicited an absolute response level similar to that of $0.0004 \mu\text{g mL}^{-1}$ DNP-BSA (Fig. 1C). In Fig. 3B, we show that TCS significantly inhibited low-dose antibody ($4 \mu\text{g mL}^{-1}$) crosslinking by $5 \mu\text{M}$ TCS in a manner that proceeded with clear dose responsiveness. At $5 \mu\text{M}$, the degranulation response was $0.86\text{-fold} \pm 0.03$ of 0 μM TCS control; at $10 \mu\text{M}$, $0.66\text{-fold} \pm 0.02$; at $15 \mu\text{M}$, $0.51\text{-fold} \pm 0.04$; at $20 \mu\text{M}$, $0.34\text{-fold} \pm 0.03$; and at $30 \mu\text{M}$, 0.19 ± 0.02 . Thus, TCS more potently inhibits low-dose anti-IgE-stimulated degranulation than that stimulated by the higher ($10 \mu\text{g mL}^{-1}$) antibody concentration. Also, even though the absolute level of degranulation in the absence of TCS ($\sim 10\%$ β -hexosaminidase release) is very similar to that elicited by the lower DNP-BSA dose ($0.0004 \mu\text{g mL}^{-1}$; Fig. 1C), TCS more strongly inhibited this anti-IgE-stimulated response (Fig. 3B). Overall, TCS strongly suppressed anti-IgE-activated degranulation in a dose-responsive manner for both high and low concentrations of anti-IgE IgG antibody (Figs. 3A and B). These results are similar to those found with DNP-BSA antigen (data in Figs. 1B and C), indicating that TCS's inhibition of mast cell degranulation is not specific to a single type of crosslinker used to aggregate Fc ϵ R1 IgE receptors.

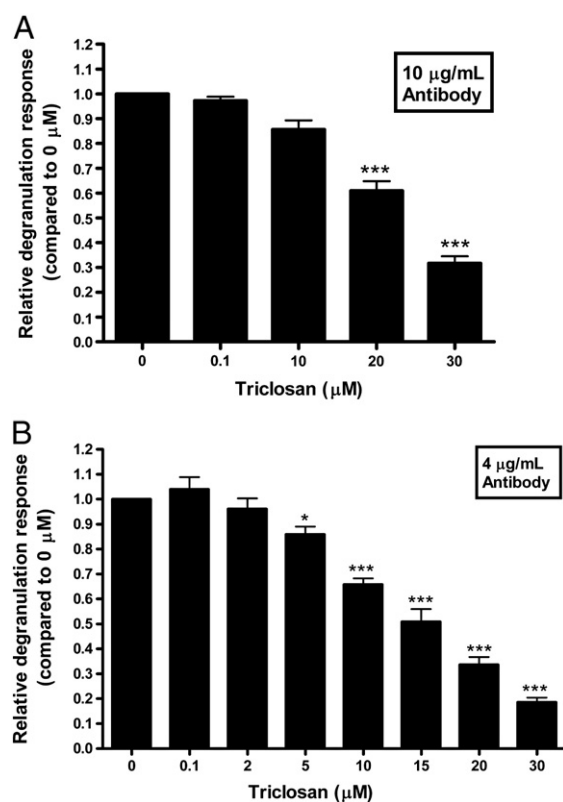


Fig. 3. Effects of TCS on RBL cell degranulation caused by IgE receptor crosslinking by anti-IgE antibody. In place of Ag, anti-IgE antibody was used to stimulate degranulation. Cells were exposed for 1 h to TCS, along with either (A) $10 \mu\text{g mL}^{-1}$ or (B) $4 \mu\text{g mL}^{-1}$ anti-IgE IgG. Values presented are means $\pm$ SEM of three independent experiments. *** $p < 0.001$ and * $p < 0.05$.

TCS inhibits degranulation most dramatically in RBL cells activated via calcium ionophore

Experiments with the Ca^{2+} ionophore A23187 were performed to investigate the mechanism(s) of TCS-induced inhibition of RBL cell degranulation. Calcium ionophore was utilized to activate the cells at the calcium influx step, thus bypassing $\text{Fc}\epsilon\text{R1}$ engagement and other upstream molecular events, while still activating a pathway that culminates in degranulation. Use of ionophore allows dissection of whether TCS's effect is up- or down-stream of calcium influx. Moderate and low ionophore concentrations were selected on the basis of preliminary dose response degranulation data (data not shown). In the absence of TCS (ethanol vehicle only present), the average absolute degranulation response due to a moderate ionophore dose (1.5×10^{-7} M) was $23 \pm 2\%$ (SEM); the average absolute response due to a low ionophore dose (0.85×10^{-7} M) was $11 \pm 2\%$. Note that the low ionophore dose elicited an average absolute degranulation response ($\sim 10\%$) that was very close to that elicited by $0.0004 \mu\text{g mL}^{-1}$ DNP-BSA (Fig. 1C) and $4 \mu\text{g mL}^{-1}$ anti-IgE antibody (Fig. 3B). In control experiments, we determined that Ca^{2+} ionophore (1.5×10^{-7} M), by itself, is not cytotoxic to RBL cells (Fig. 4A), and that, likewise, neither is ionophore *plus* TCS up to $50 \mu\text{M}$ (Fig. S4).

Fig. 4B presents data from RBL cells that have been co-incubated for 1 h with 1.5×10^{-7} M Ca^{2+} ionophore and TCS (or ethanol vehicle only). Significant decreases in degranulation were observed by $5 \mu\text{M}$ TCS (0.4-fold $\pm$ 0.2). At greater concentrations of TCS, degranulation levels were strongly depressed: 0.3-fold $\pm$ 0.2 (by $10 \mu\text{M}$ TCS), 0.2-fold $\pm$ 0.1 (by $15 \mu\text{M}$), 0.2-fold $\pm$ 0.1 (by $20 \mu\text{M}$), and 0.12-fold $\pm$ 0.05 (by $30 \mu\text{M}$) of the $0 \mu\text{M}$ TCS control response. Note that, interestingly,

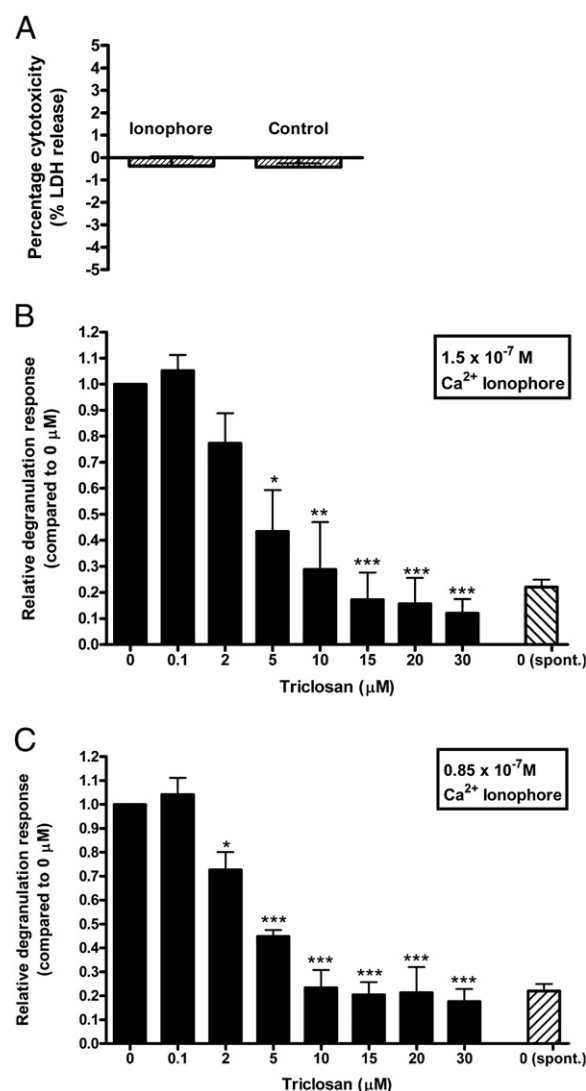


Fig. 4. Effects of TCS on calcium ionophore-stimulated RBL cell degranulation. (A) To evaluate the cytotoxicity of the ionophore, an LDH assay was performed. Percentage cytotoxicity (% LDH release) is plotted for ionophore (1.5×10^{-7} M and 4×10^{-5} v/v dimethyl sulfoxide [DMSO]) and Control (4×10^{-5} v/v DMSO). Statistical analysis was performed using a one-tailed t-test ($p < 0.05$). RBL cells were also exposed for 1 h to TCS, along with either (B) 1.5×10^{-7} M or (C) 0.85×10^{-7} M calcium ionophore A23187. A spontaneous release value is depicted for reference. Data are mean $\pm$ SEM and represent three independent experiments. *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$.

$15\text{--}30 \mu\text{M}$ TCS depressed degranulation to levels approximately equal to the spontaneous (no-ionophore) release level.

In a separate set of experiments, RBL cells were co-incubated for 1 h with 0.85×10^{-7} M Ca^{2+} ionophore and TCS (or ethanol vehicle only) (Fig. 4C). As little as $2 \mu\text{M}$ TCS resulted in statistically significant inhibition of β -hexosaminidase release (0.73-fold $\pm$ 0.06). Dramatic inhibition relative to $0 \mu\text{M}$ TCS control response was seen following this low dose ionophore: for $5 \mu\text{M}$ TCS, 0.45-fold $\pm$ 0.02; for $10 \mu\text{M}$ TCS, 0.23-fold $\pm$ 0.06; for $15 \mu\text{M}$, 0.20-fold $\pm$ 0.05; for $20 \mu\text{M}$ TCS, 0.21-fold $\pm$ 0.09; and for $30 \mu\text{M}$ TCS, 0.18-fold $\pm$ 0.05. Note that $10\text{--}30 \mu\text{M}$ TCS suppressed the release of β -hexosaminidase to levels similar to that of spontaneous release. Overall, Fig. 4 indicates that TCS's target for inhibition of RBL mast cell degranulation is not exclusively upstream of the calcium-signaling event that occurs prior to the release of allergic mediators. Also, a comparison of Fig. 4 to Figs. 1B and C, leaves open the possibility that TCS is better able to inhibit these later (post- Ca^{2+} influx) events.

Membrane ruffling effects corroborate TCS inhibition of mast cell degranulation

RBL cells not stimulated with DNP-BSA Ag (Figs. 5A–D) serve as controls to RBL cells stimulated with $0.0004 \mu\text{g mL}^{-1}$ DNP-BSA Ag (Figs. 5E–H), the same Ag concentration use in Fig. 1C. Cells were then fixed, stained with Alexa488-conjugated phalloidin to label F-actin, and imaged with an Olympus confocal microscope system. When sufficiently stimulated by antigen, RBL cells undergo membrane ruffling, defined by a dramatic rearrangement of the actin cytoskeleton. Fig. 5A presents the typical appearance of the plasma membrane of RBL cells that have not been stimulated by antigen

(under spontaneous release conditions): F-actin is located largely around the periphery of these resting cells. Addition of TCS to non-stimulated RBL cells for 1 h prior to cell fixation/labeling did not affect membrane morphology or F-actin distribution (Figs. 5B–D). For comparison, a representative image of the morphological changes due to 1-h stimulation with $0.0004 \mu\text{g mL}^{-1}$ DNP-BSA antigen is presented in Fig. 5E: FcεRI aggregation caused extensive F-actin remodeling and membrane ruffling, as expected. In Fig. 5F, co-incubation of these cells with $10 \mu\text{M}$ TCS did not substantially affect the extent of ruffling. It is apparent, however, that with 20 and $30 \mu\text{M}$ TCS, a major decrease in membrane ruffling is effected (Figs. 5G and H). Overall, these data are consistent with what was found for the

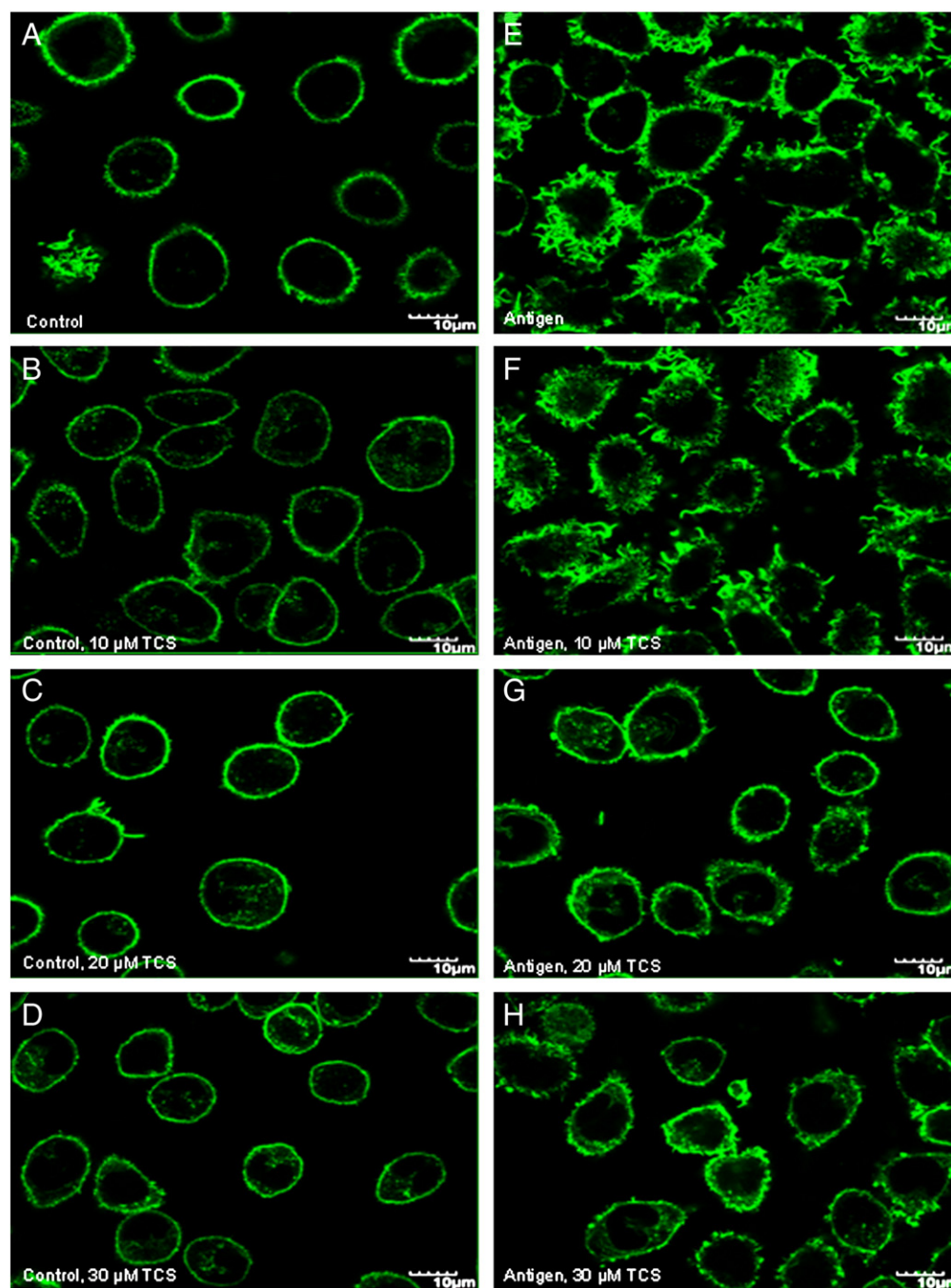


Fig. 5. Confocal fluorescence microscopy of Alexa488-phalloidin, labeling F-actin in fixed RBL cells. Membrane ruffling of IgE-sensitized RBL cells labeled with Alexa Fluor 488 conjugated phalloidin upon 1-h incubation with Tyrodes-BSA buffer (A–D) or $0.0004 \mu\text{g mL}^{-1}$ Ag (E–H) and either TCS vehicle (A and E), $10 \mu\text{M}$ TCS (B and F), $20 \mu\text{M}$ TCS (C and G), or $30 \mu\text{M}$ TCS (D and H). One representative data set from three independent experiments is shown. Images were acquired and displayed with identical settings. Scale bar, $10 \mu\text{m}$.

fluorescent microplate assays of degranulation (data in Figs. 1C and E): both degranulation (β -hexosaminidase release) and concomitant membrane ruffling are strongly inhibited by 20 and 30 μ M TCS.

Discussion

The antimicrobial agent TCS has been shown clinically to alleviate allergic skin diseases such as atopic dermatitis (Barkvold and Rolla, 1995a, 1995b; Kjaerheim et al., 1995; Sporik and Kemp, 1997; Tan et al., 2010; Wohlrab et al., 2007), but there is little known mechanistically about this process. The central finding of this study is that TCS significantly inhibits mast cell degranulation, which is involved in both physiological and pathological processes (i.e., parasitic defense and immediate hypersensitivity reactions). We determined that, in a dose-responsive manner, TCS treatment suppresses RBL mast cell degranulation stimulated by multivalent antigen, anti-IgE antibody, or Ca^{2+} ionophore and also abrogates concomitant membrane ruffling. This inhibition of mast cell function may be a mechanism by which TCS alleviates atopic dermatitis.

TCS readily undergoes dermal absorption (Black et al., 1975; Kanetoshi et al., 1992; Sandborgh-Englund et al., 2006), affording it the potential to interact with immune cells in the skin. The micromolar doses (~ 10 – 30 μ M) of TCS used in this study reflect concentrations about three orders of magnitude lower than the millimolar levels (~ 3 – 35 mM) commonly found in personal care products such as soap that are directly applied to the skin (Rodricks et al., 2010). Humans appear to predominantly excrete TCS through the urine (Bagley and Lin, 2000; Queckenberg et al., 2010), which can be used as a bio-marker for exposure (Calafat et al., 2008). Despite its short half-life, internal TCS exposure has been shown to correlate to levels found in products tested in multiple application studies (Bagley and Lin, 2000; Allmyr et al., 2008). Elsewhere, absorption of TCS from dermal spray and soap preparations has been reported to be a significant fraction of the dose administered (Lin, 2000). For example, Queckenberg et al. (2010) found $\sim 10\%$ absorption in six healthy Caucasians (gender balanced) after one 12-h dermal administration of a 2% TCS-containing cream; note that this absorption level could result in a fairly high skin concentration of TCS because a 2% TCS cream is ~ 70 mM in TCS, so 10% absorption could mean 7 mM TCS in the skin, a very high concentration relative to doses used in this current study. Also in the Queckenberg et al. (2010) study, the TCS absorbed was mostly excreted within 24 h. In our degranulation studies, we found an IgE-mediated dampened degranulation response in as few as 15 min.

Mast cell degranulation is a well-defined signaling pathway, in which early biochemical events are triggered by Fc ϵ RI aggregation at the plasma membrane. Aggregation of high-affinity IgE receptors was achieved in our studies by either multivalent antigen or anti-IgE antibody. Importantly, the type of cross-linking agent utilized did not dictate whether or not TCS could significantly dampen mast cell signaling. Thus, TCS's inhibition of mast cell degranulation is occurring somewhere in the signal transduction cascade other than at the crosslinking step at the cell surface. Other crucial molecular events that enable exocytosis of granules include the binding of inositol 1,4,5-triphosphate (IP_3) to its receptors on the endoplasmic reticulum, which results in cytosolic calcium release, followed by an increase in free cytosolic calcium (Ferris et al., 1989; Millard et al., 1989; Putney, 2009; Putney et al., 2001). From here, vesicle carriers fuse with the plasma membrane through soluble N-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) membrane fusion proteins, and allergic mediators are released from the cell (Blank et al., 2002). Calcium signaling therefore represents a much farther downstream event in the mast cell degranulation pathway than Fc ϵ RI receptor crosslinking, yet TCS was also able to significantly inhibit the release of β -hexosaminidase when early signaling events were bypassed with the use of Ca^{2+} ionophore as cell stimulant.

This indicates that the target(s) for TCS inhibition in the degranulation pathway is/are not located exclusively upstream of the calcium-signaling event and may likely be downstream of calcium influx. Additionally, we detected TCS dose-responsive suppression of membrane ruffling at TCS concentrations in line with our biochemical degranulation data, suggesting that TCS's target(s) in mast cells are common to both the signaling pathways that lead to degranulation or membrane ruffling. While others have shown that TCS inhibits inflammatory gene pathways in human cells (Barros et al., 2010), we did not necessarily expect TCS to affect the exocytotic process of degranulation.

Our results support the clinical use of TCS as an inhibitor of allergic skin disease such as eczema and provide a possible mechanism – inhibition of mast cell degranulation. It has been observed that patients with atopic dermatitis can display cutaneous hyper-reactivity resulting from exposure to otherwise innocuous environmental factors. Eczema, for example, is often associated with increased serum IgE levels, increased sensitization to allergens, and increased expression of Fc ϵ RI (Boguniewicz and Leung, 2010). Moreover, TCS and TCS-based creams are rarely sensitizing in animals or in human patients, including those with chronic skin disease such as eczema and atopic dermatitis (Jappe et al., 2003; Schena et al., 2008; Tan et al., 2010; reviewed in Bhargava and Leonard, 1996). A very low prevalence of TCS sensitization (0.7% in a study of 275 patients) was also found in high-risk chronic eczema patients sensitized to haptens such as nickel sulfate (Schena et al., 2008). The dermal toxicity of TCS in human clinical studies has been extensively investigated in both prophetic patch test studies (one 24-h skin application) and repeat-insult patch tests (two or more applications to the same locus over a longer period of time). For these tests, neither a sensitizing effect nor a photosensitizing effect was found (reviewed in Fang et al., 2010).

The immune-suppressing potential of TCS has not been exhaustively studied, but Udoji et al. (2010) recently demonstrated that concentrations of TCS up to 10 μ M could almost completely suppress the lytic function of human natural killer (NK) cells, a function needed to destroy virally infected cells and tumors. In these studies, NK cells showed significant impairment of lytic function, an effect that persisted for up to 6 days following a one-time, 1 h TCS exposure (Udoji et al., 2010). In the context of our findings of suppressed mast cell degranulation, the possibility that TCS might generally inhibit innate immune cell function warrants further investigation.

An early favorable safety profile expanded the use of TCS into an array of consumer products, but it must be mentioned that TCS is a putative endocrine disrupting chemical (Ahn et al., 2008; Foran et al., 2000; Gee et al., 2008; Helbing et al., 2011; Ishibashi et al., 2004; Kumar et al., 2009; Matsumura et al., 2005; Veldhoen et al., 2006). Mast cells have been shown to be sensitive to estrogenic chemicals (Narita et al., 2007; Zaitsu et al., 2007), possibly explaining epidemiological evidence of a strong female gender bias in allergies and asthma beginning around the time of puberty (Anto et al., 2010; Jensen-Jarolim and Untersmayr, 2008; Lee et al., 2006; McHugh et al., 2009; Melgert et al., 2007; Schatz et al., 2006; Vink et al., 2010). Work by Narita et al. (2007) demonstrated that environmental estrogens could increase degranulation in antigen-activated mast cells; however, in our studies, TCS inhibited degranulation of activated RBL cells in the presence of full-hormone medium. Nonetheless, we cannot rule out other types of endocrine disruption, such as anti-estrogen activity, as a potential mechanism. For example, in MCF-7 breast cancer cells, Gee et al. (2008) found that 10 μ M TCS inhibits estradiol-induced gene transcription using an estrogen response element (ERE)-reporter gene assay. Matsumura et al. (2005) also found that vitellogenin was reduced in TCS-exposed frogs, raising the possibility that TCS could be suppressing degranulation by inhibiting the action of estrogens in cell media. In all, determining whether or not TCS's mode of degranulation inhibition is via an endocrine disruption will lend insight into the endocrine-disrupting potential of TCS.

For the past several decades, much of what we know about human mast cell biology has been garnered from studies in rodent models. Since their isolation (Barsumian et al., 1981), the rat cell line RBL-2H3 has been a valuable resource for the study of diseases of allergic signaling in humans, especially in light of the lack of a suitable human mast cell line until very recently. Overall, the FcεRI-mediated release from RBL-2H3 shares many of the same features as the release from human basophils and rodent mast cells (Fewtrell et al., 1979; Metzger et al., 1986). RBL and mature human mast cells are both highly granulated, excitable cells that degranulate to release allergic mediators in response to appropriate stimuli.

In sum, our data support clinical evidence suggesting that TCS would be an effective topical dermatologic agent or would point to a useful drug target for treatment of allergic skin disease. Further studies are needed to elucidate the underlying mechanism(s) in RBLs, and to assess whether these same conclusions can be extended to human mast cells.

Conflict of interest statement

There are no conflicts of interest. This research was funded by a Research Starter Grant in Pharmacology/Toxicology from the PhRMA Foundation (J.A.G.), by the Maine Agricultural & Forest Experiment Station Grant Number ME08004-10 (J.A.G.), and by University of Maine startup funding (J.A.G.) (departmental; College of Natural Sciences, Forestry, and Agriculture; and the Office of the Vice President for Research at the University of Maine). R.K.P. benefited from pre-doctoral research funding from the Graduate School of Biomedical Sciences (the University of Maine), and both R.K.P. and L.M.H. were supported in part by Maine Agricultural & Forest Experiment Station research assistantships. Sponsors had no role in study design; in the collection, analysis and interpretation of data; in the writing of the report; or in the decision to submit the paper for publication.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at doi:10.1016/j.taap.2011.10.012.

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