

ORIGINAL ARTICLE

A differential role for ceramide kinase in antigen/FcεRI-mediated mast cell activation and function

C. A. Hewson^{1,2}, J. R. Watson², W. L. Liu² and M. D. Fidock¹¹Research Enabling Group and ²Allergy & Respiratory Biology, Pfizer Global Research and Development, Sandwich, Kent CT13 9NJ, UK.Clinical &
Experimental
Allergy

Summary

Background Mast cells are specialized secretory cells releasing multiple inflammatory mediators when activated. Activation requires antigen/IgE cross-linking of FcεRI receptors, initiating a complex intracellular signalling cascade. Ceramide kinase (CERK) is a novel lipid kinase implicated in several inflammatory cellular signalling processes.

Objective We sought to investigate a role for CERK in FcεRI/IgE-mediated mast-cell activation.

Methods The rat and human mast cell-lines RBL-2H3 and LAD-2, respectively, were stimulated via FcεRI or with the active product of CERK [ceramide-1-phosphate (C-1P)].

Multiple end-points were measured by enzyme-linked immunosorbent assay; histamine (pre-formed early-phase mediator), prostaglandin D₂ (PGD₂ – rapidly metabolized early-phase mediator) and interleukin (IL) -13 (*de novo* transcribed late-phase mediator).

Results We demonstrated that C-1P alone induced release of histamine and PGD₂ and was additive to antigen-mediated activation. C-1P did not stimulate IL-13 by a statistically significant amount. Using a specific inhibitor of CERK, antigen-mediated release of histamine and PGD₂ was significantly inhibited. Finally, we identified that, for histamine, CERK was downstream of spleen tyrosine kinase, phosphoinositol-3 kinases and phospholipase C, but upstream of c-jun N-terminal kinase (JNK); while for PGD₂ CERK was positioned upstream of JNK, mitogen-activated protein kinase kinase and cyclooxygenase.

Conclusions and Clinical Relevance We have identified a differential role for CERK in mast-cell activation and begun to elucidate its position in the mast cell-signalling cascade, thereby suggesting a model by which CERK may be mediating its effects. This type of study is essential for complete understanding of activation pathways that may eventually be used to identify new targets for drug discovery in inflammatory diseases.

Keywords ceramide kinase, histamine, IL-13, mast cell

Submitted 29 March 2010; revised 26 November 2010; accepted 29 November 2010.

Correspondence:

Christopher A. Hewson, Allergy & Respiratory Biology, Pfizer Global R&D, Sandwich, Kent CT13 9NJ, UK.
E-mail: Chris.Hewson@pfizer.com

Introduction

Mast cells are specialized secretory cells derived from bone-marrow progenitors and are present within the tissues at the major interfaces between internal and external environments within the body. They have a critical role in allergic disease [1], and mediate this function by release of a large number of potent inflammatory factors, including pre-formed mediators such as histamine and proteases and rapidly metabolized lipid-derived factors such as prostaglandins and leukotrienes [2]. As well as these acute-phase mediators involved in immediate hypersensitivity reactions, mast cells are also important sources for generation of *de novo* transcribed

cytokines; including the key T-helper cell type 2 stimulating cytokines interleukin (IL)-4 and IL-13 [2]. Mast cells are activated through the interaction of specific antigen with surface bound IgE, which is bound itself, through the C3 domain of its Fc region, with the α-domain of the high affinity IgE receptor FcεRI [3, 4]. The antigen/IgE interaction causes aggregation of multiple FcεRI receptors, activating the Lyn protein tyrosine kinase (PTK), resulting in phosphorylation of immunoreceptor tyrosine-based activation motifs on the β and γ domains of the FcεRI receptor. This, in turn, allows the spleen tyrosine kinase (SYK) PTK to associate with, and be activated by, the γ domain [5] resulting in activation of phospholipase C (PLC) and further intracellular signalling pathways

leading to mast cell mediator release. A great deal of work has been undertaken to elucidate the specific signalling intermediates involved in mast cells activation as reviewed in Metcalfe *et al.* [6].

Ceramide kinase (CERK) is a lipid kinase first identified in 1989 as a calcium-stimulated lipid kinase that co-purified with rat brain synaptic vesicles [7]. CERK was subsequently identified in HL-60 cells as functionally distinct to the classic lipid kinase diacylglycerol kinase [8]. It was cloned and functionally characterized in 2002 [9] and identified as distinct but closely related to the sphingosine kinases [metabolizing sphingosine to sphingosine 1-phosphate (S-1P)]. The sphingosine kinases have been widely implicated in pro-inflammatory signalling and are present and important in mast cell activation [10]. In contrast, the action of CERK is the metabolism of ceramide to the active product ceramide 1-phosphate (C-1P). C-1P (and therefore, by inference, CERK activity) has been far less studied than S-1P, but is beginning to emerge as an important bioactive lipid mediator. Work by Pettus *et al.* [11] have identified a key role for CERK in arachidonic acid release mediated by both cytokine and calcium ionophores in the lung epithelial cell-line A549. The same group have also identified cytosolic phospholipase A₂ (cPLA₂) as a primary target for C-1P in terms of eicosanoid generation [12, 13]. In mast cell activation, a single study has identified a potential role for CERK in RBL-2H3 cells [14]. Over-expression of CERK was shown to enhance A23187-induced β -hexosaminidase release, although no investigation of Fc ϵ RI-mediated activation was investigated.

With the relatively limited, but intriguing examination of the role for CERK in mast-cell activation, the purpose of this study was to further investigate its role using multiple end-points of mast-cell activation. Additionally, we attempted to position the kinase in the overall signalling cascade of the mast cell. We chose to use the RBL-2H3 cell line as an IgE-reactive model for mast cells [15] alongside LAD-2 cells as a human model of mast cells [16]. We chose to examine histamine as an example of a clinically important pre-formed acute-phase mediator, prostaglandin D₂ (PGD₂) as an example of a rapidly metabolized acute-phase mediator and IL-13 as an example of a *de novo* transcribed late-phase mediator.

Materials and methods

Cell culture

The RBL-2H3 mast cell line was purchased from the American Type Culture Collection (ATCC number CRL-2256) and was cultured in MEM medium including Earle's salts and Glutamax[®] (Invitrogen, Carlsbad, CA, USA) and supplemented with 10% foetal calf serum (FCS) (Invitrogen). The cells were cultured at 37 °C and 5% CO₂ in a humidified incubator.

The LAD-2 mast cell line was licensed from the National Institute of Health, Bethesda, MD, USA and was cultured in StemPro[®] 34 complete medium (Invitrogen) supplemented with 2 mM L-glutamine (Invitrogen) and 10 μ g/500 mL recombinant stem-cell factor (Peprotech, Rocky Hill, NJ, USA). The cells were cultured at 37 °C and 5% CO₂ in a humidified incubator.

Mast cell activation

For C-1P activation, RBL-2H3 cells were seeded into 96-well plates at 1×10^5 cells per well in growth medium. Cells were cultured for 30-h before medium was replaced with RPMI-1640 medium (Sigma-Aldrich) containing 1% FCS and buffered with 0.15% sodium bicarbonate (Invitrogen). RPMI-1640 was then used throughout the experiment. Cells were activated with natural C-1P (bovine brain-derived – Sigma Aldrich) solubilized in 49 : 1 (v/v) methanol:dodecane at concentrations as indicated, or with vehicle alone. Control experiments using a related sphingolipid were performed as described for C-1P, but utilized natural ceramide in place of C-1P (bovine brain-derived – Sigma Aldrich). For Fc ϵ RI-mediated activation RBL-2H3 cells were seeded in growth medium containing 0.5 μ g/mL dinitrophenol (DNP)-specific murine IgE (clone SPE7 – Sigma-Aldrich, St. Louis, MO, USA). Cells were cultured and serum-starved as above before activation with 100 ng/mL DNP-human serum albumin (DNP-HSA) (Sigma-Aldrich). Cells were centrifuged at 300 g at the time-points indicated and supernatants were harvested and stored at –80 °C until required for analysis.

LAD-2 cells were sensitized overnight with 1.5 μ g/mL biotinylated human IgE (custom labelled – AbD Serotec, Raleigh, NC, USA) at 37 °C and 5% CO₂ in a humidified incubator, before being washed twice in degranulation buffer (10 mM HEPES, 137 mM NaCl, 2.7 mM KCl, 0.4 mM NaH₂PO₄, 5.6 mM D-glucose, 1.8 mM CaCl₂, 1.3 mM MgSO₄ and 0.025% BSA at pH 7). LAD-2 cells were re-suspended in fresh degranulation buffer and seeded into 96-well plates at 3×10^4 cells per well. LAD-2 cells were stimulated with 1 μ g/mL streptavidin (Sigma-Aldrich). Cells were centrifuged at 300 g at the time-points indicated and supernatants were harvested and stored at –80 °C until required for analysis.

For inhibition studies, cells were pre-treated for 15-min before activation with compounds and concentrations as indicated. Control cells were pre-treated with drug vehicle at the highest concentration used in the experiment. All compounds were purchased from Calbiochem (San Diego, CA, USA) except K1 which was kindly provided by Prof. Igarashi (Hokkaido University, Japan).

ELISA to evaluate histamine release

Histamine levels were quantified using a commercially available competition ELISA following manufacturer's

instructions (IBL, Hamburg, Germany). Briefly, supernatants and standards were acylated and diluted before being placed into pre-coated ELISA plates, together with peroxidase-conjugated histamine and histamine antiserum. Plates were incubated for 3-h, washed and developed with tetramethylbenzidine (TMB) solution (IBL) and absorbance measured at 450 nm. Higher absorbance indicated less histamine. Sensitivity was 0.2 ng/mL.

ELISA to evaluate prostaglandin D₂ release

PGD₂ levels were quantified using a commercially available competition ELISA following manufacturer's instructions (Cayman Chemical, Ann Arbor, MI, USA). Briefly, supernatants were treated with methoxyamine hydrochloride for 30-min at 60 °C and diluted before being placed into pre-coated ELISA plates, together with acetylcholinesterase-conjugated PGD₂-MOX and PGD₂-MOX antiserum. Plates were incubated overnight at 4 °C, washed and developed with Ellman's Reagent and absorbance measured at 412 nm. Higher absorbance indicated less PGD₂. Sensitivity was 3.1 pg/mL.

ELISA to evaluate interleukin-13 release

IL-13 levels were quantified using commercially available paired antibodies and standards, following manufacturer's instructions (Biosource, Camarillo, CA, USA). Briefly, ELISA plates were coated overnight with 1.25 µg/mL anti-rat IL-13 antibodies in phosphate-buffered saline (PBS) and blocked with 2% (w/v) bovine serum albumin (BSA) in PBS. Supernatants were then incubated for 2-h and washed off. Bound IL-13 was then detected with 0.25 µg/mL biotinylated anti-rat IL-13 antibodies in 0.1% w/v BSA in PBS. Finally, bound secondary antibody was detected with 0.3 µg/mL peroxidase-conjugated biotin in 0.1% (w/v) BSA in PBS and developed with TMB substrate buffer (R&D Systems, Minneapolis, MN, USA). Absorbance was measured at 450 nm. Sensitivity was 14 pg/mL.

Western blot to evaluate activation of ERK and JNK

Cells were stimulated with 3.125 µM C-1P. Cells were harvested using 2×SDS loading buffer (Invitrogen) and 1% (v/v) β-mercaptoethanol, heat denatured and the proteins resolved on a 10% Tris-Glycine gel (Invitrogen). Following transfer to an Immobilon-P PVDF membrane, ERK activation was analysed by immunoblotting with mouse anti-phospho-ERK or rabbit anti-ERK (1 : 1000 v/v – Cell Signaling Technology, Boston, MA, USA). JNK activation was analysed by immunoblotting with mouse anti-phospho-JNK or rabbit anti-JNK (1 : 1000 v/v – Cell Signaling Technology). Detection used anti-mouse IRDye® (1 : 8000 v/v Rockland Immunochemicals, Gilbertsville, PA, USA) or anti-rabbit Alexafluore (1 : 2000 v/v

Invitrogen) conjugated secondary antibodies and visualized using an Odyssey Infrared Detection System (LI-COR Biosciences, Lincoln, NE, USA).

Statistical analysis

Data are presented as mean ± the standard error of the mean. All data were analysed using one-way analysis of variance (ANOVA) and Bonferroni's multiple comparison *post hoc* test. Data were accepted as significantly different when $P < 0.05$.

Results

The active product of CERK, C-1P, is able to stimulate acute-phase activation of RBL-2H3 cells

The lipid kinase CERK has previously been implicated in mast cell degranulation using over-expression of the kinase and measurement of β-hexosaminidase release [14]. However, no work has been undertaken to establish its role in release of the more clinically relevant pre-formed mediator histamine, or other facets of mast cell mediator release such as the rapidly metabolized acute-phase mediator PGD₂ and the late-phase mediator IL-13. To investigate its role in release of these three mediators, we initially chose to stimulate RBL-2H3 cells with the active product of CERK, natural length C-1P, as has been previously used in other systems [12, 13].

Stimulation of RBL-2H3 cells with 3.125 µM C-1P produced a relatively late release of histamine (Fig. 1a). No release of histamine was observed over control/vehicle until 4-h post-stimulation, when a significant increase in histamine was observed [86 ng/mL compared with 30 ng/mL in vehicle-treated cells ($P < 0.001$) and 39 ng/mL in control cells]. This release of histamine was further increased at 6-h post-stimulation [144 ng/mL histamine compared with 56 ng/mL in vehicle cells ($P < 0.001$) and 60 ng/mL in control cells].

C-1P stimulation of RBL-2H3 cells also produced a relatively late release of PGD₂ (Fig. 1b). As for histamine, no induction of PGD₂ release was observed 1-h post-stimulation (standard time-point for antigen-mediated PGD₂ release). A small induction of PGD₂ release was observed 2-h post-stimulation, although this was not statistically significant until 4-h post-stimulation [768 pg/mL compared with 17 pg/mL in vehicle-treated cells ($P < 0.001$) and 71 pg/mL in control cells]. This was further increased by 6-h [1031 pg/mL PGD₂ compared with 222 pg/mL in vehicle cells ($P < 0.001$) and 86 pg/mL in control cells].

In contrast to both histamine and PGD₂ release, C-1P stimulation did not produce a statistically significant induction of IL-13 production, even when the time-course was extended to 24-h post-stimulation (Fig. 1c).

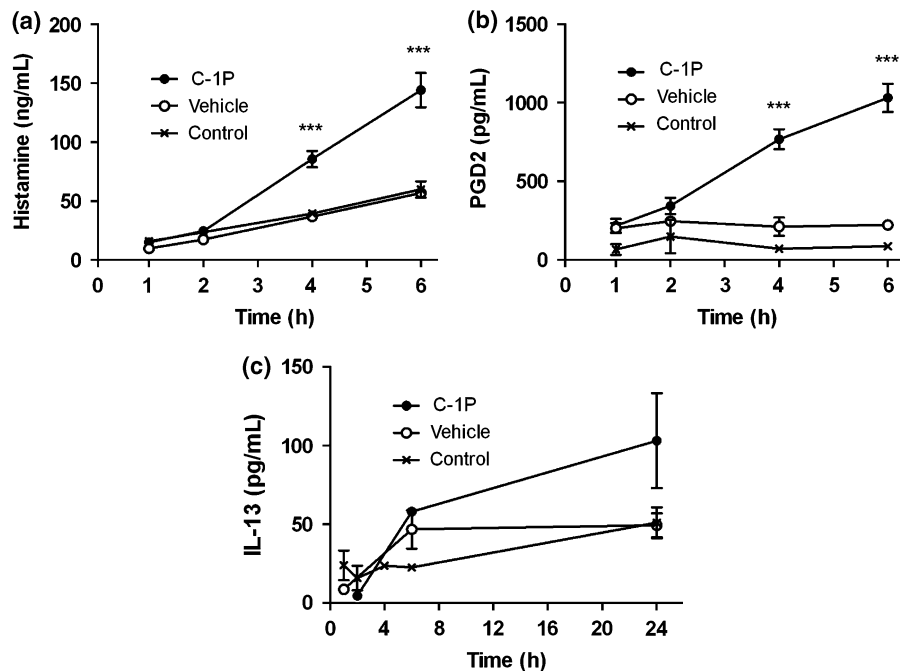


Fig. 1. C-1P stimulates a time-dependent increase in histamine, prostaglandin D₂ (PGD₂) and IL-13. RBL-2H3 cells were stimulated with 3.125 μ M C-1P or vehicle at the same concentration. Supernatants were harvested at 1-, 2-, 4- and 6-h post-stimulation and the amount of (a) histamine and (b) PGD₂ release was quantified by ELISA. (c) For quantification of IL-13 release, an additional 24-h post-stimulation supernatant was harvested and analysed by ELISA. ***Data significantly different to cells treated with C-1P vehicle at $P < 0.001$ ($n = 3$ in duplicate).

With the identification that C-1P mediated a specific time-dependent release of histamine and PGD₂ we decided to further validate the role of CERK in activation of mast cells and performed dose-response studies 6-h post-stimulation.

RBL-2H3 cells were therefore stimulated with a variety of doses of C-1P and supernatants were harvested 6-h post-stimulation. Comparisons were then made between C-1P stimulated cells and un-stimulated control cells.

C-1P stimulation produced a dose-dependent release of histamine (Fig. 2a). 1.563 μ M C-1P produced a statistically significant release of histamine compared with control cells [155 ng/mL compared with 99 ng/mL ($P < 0.05$)]. This increased to a maximal plateau with 6.25 and 12.5 μ M C-1P [277 ng/mL ($P < 0.001$) and 274 ng/mL ($P < 0.001$), respectively]. No discernible stimulation of histamine release was observed in cells treated with drug-vehicle at an equivalent concentration as the 12.5 μ M C-1P condition.

As seen for histamine, C-1P also produced a dose-responsive increase in PGD₂ release (Fig. 2b). In these experiments, a statistically significant increase in PGD₂ was observed using 3.125 μ M C-1P [2223 pg/mL ($P < 0.01$) compared with 414 pg/mL]. This induction was increased to 4756 pg/mL ($P < 0.001$) with 12.5 μ M C-1P. No stimulation was observed in the control drug-vehicle cells.

To address the possibility of metabolism of exogenously applied C-1P back to ceramide, and stimulation of histamine and PGD₂ through non-C-1P specific sphingolipid action, we applied bovine brain-derived natural ceramide

solubilized in 98 : 2 (v/v) methanol:dodecane to RBL-2H3 cells alongside C-1P at the same doses as Figs 2a and b. Only 12.5 μ M ceramide significantly stimulated histamine release over control cells ($P < 0.05$), and induced significantly less histamine than C-1P at 12.5, 6.25 and 3.125 μ M [$P < 0.001$, $P < 0.001$ and $P < 0.05$, respectively (data not shown)]. The same was demonstrated for ceramide-induced PGD₂ release (data not shown).

C-1P enhances antigen-mediated activation of RBL-2H3 cells

With the demonstration that C-1P stimulated release of histamine and PGD₂ in a time- and dose-dependent manner, we decided to examine the ability of C-1P to increase antigen-mediated activation. In addition, we chose to examine increases in IL-13 production to establish whether CERK was involved in antigen-mediated IL-13 production. We and others have previously optimized antigen-mediated stimulation of histamine and PGD₂ at 1-h post-stimulation (data not shown; [17]). The observation that C-1P took longer meant that we were keen to establish an effect of C-1P in a more physiologically relevant time-frame. Cells were therefore pre-treated for 1-h with a variety of doses of C-1P before stimulation with DNP-HSA acting as antigen and histamine and PGD₂ assayed 1-h later.

C-1P produced a dose-responsive additive increase in antigen-mediated histamine release (Fig. 3a). All doses of

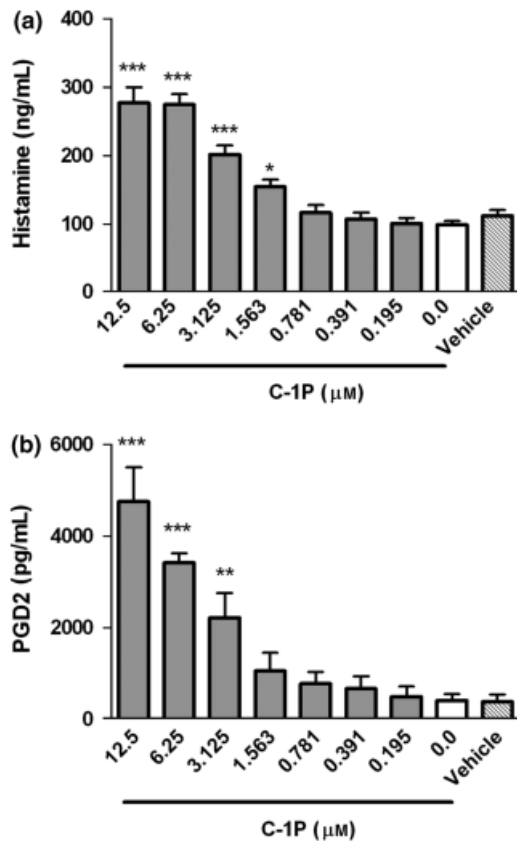


Fig. 2. C-1P stimulates a dose-responsive release of histamine and prostaglandin D₂ (PGD₂). RBL-2H3 cells were stimulated with a variety of doses of C-1P, or C-1P vehicle at the top dose, and supernatants harvested 6-h post-stimulation. (a) Histamine and (b) PGD₂ release were quantified by ELISA. *, **, ***Data significantly different to cells treated with C-1P vehicle at $P < 0.05$, 0.01 and $P < 0.001$ respectively ($n = 3$ in duplicate).

C-1P increased histamine release over antigen-stimulated alone cells, but the induction was not statistically significant until 0.781 μM [172 ng/mL compared with 110 ng/mL ($P < 0.01$)]. This reached maximum with 6.25 μM C-1P [203 ng/mL ($P < 0.001$)]. While no induction of histamine was observed with 6.25 μM C-1P at 2-h (Fig. 1a), when the cells were primed with IgE antibody for these experiments a small induction was observed with 12.5 and 6.25 μM C-1P alone. However, lower doses did not produce an induction in conjunction with antigen.

As for histamine, C-1P produced an additive increase in PGD₂ release (Fig. 3b). In this case, 1.563 μM C-1P was required to produce a statistically significant increase in PGD₂ release over antigen-alone cells [8712 pg/mL compared with 2245 pg/mL ($P < 0.01$)]. Again a maximum response was observed with 6.25 μM C-1P [699 pg/mL ($P < 0.001$)]. As seen for histamine, when the cells were primed with IgE for these experiments, C-1P at 12.5 and 6.5 μM produced a small increase in PGD₂ release that had not been observed previously (Fig. 1b).

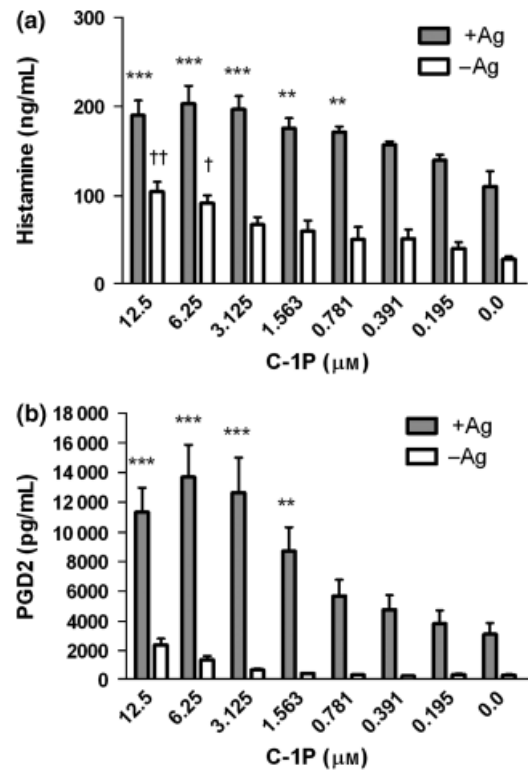


Fig. 3. C-1P synergises with FcεRI/antigen activation to enhance release of histamine and prostaglandin D₂ (PGD₂). RBL-2H3 cells were sensitized with IgE and stimulated with a variety of doses of C-1P or C-1P vehicle for 1-h before some were further activated with 2 ng/mL dinitrophenol-human serum albumin (DNP-HSA) (+Ag). Controls (-Ag) were treated with medium alone. Supernatants were harvested after 1-h and analysed for histamine (a) and PGD₂ release (b) by ELISA. **, ***Data significantly different to antigen-alone stimulated cells (+Ag, 0.0 μM C-1P) at $P < 0.01$ and < 0.001 , respectively. † and †† indicate data significantly different to unstimulated cells (-Ag, 0.0 μM C-1P) at $P < 0.05$ and < 0.01 respectively ($n = 3$ in duplicate).

Finally, we examined the response of antigen-mediated IL-13 release to the addition of C-1P. IL-13 was assessed 6-h post-stimulation as had been previously shown to be optimum for this end-point (data not shown). While small increases were observed, the induction was not significant (data not shown).

Pharmacological inhibition of CERK abrogates antigen-mediated release of PGD₂ and histamine from rat and human mast cells

With the demonstration that the active product of CERK (C-1P) causes release of acute-phase mediators from mast cells, we next sought to confirm the role of the CERK using N-acetyl sphingosine/C₂-ceramide (NAS) as an inhibitor of CERK activity at high concentrations [10, 14] alongside a specific pharmacological inhibitor of CERK - K1 [18, 19].

A variety of doses of NAS or K1 were used to pre-treat RBL-2H3 for 15-min before antigen stimulation. Supernatants

were harvested 1-h post-stimulation and histamine and PGD₂ levels quantified by ELISA.

NAS produced a clear and dose-dependent inhibition of both histamine and PGD₂ release (Fig. 4a). For histamine, the calculated IC₅₀ was 31 μM $\pm$ 7.1, with PGD₂ inhibition slightly less potent with an IC₅₀ of 40 μM $\pm$ 8.5. K1 produced a dose-dependent partial inhibition of histamine release, with 30 and 10 μM K1 significantly inhibiting its release [58% and 30%, respectively ($P < 0.001$ and 0.05, respectively)] (Fig. 4b). K1 produced a more effective dose-dependent inhibition of PGD₂ release, with 30 and 10 μM significantly inhibited the release of PGD₂ by 98% and 75%, respectively ($P < 0.001$) (Fig. 4c).

With the clear demonstration of a role for CERK in rat mast-cell activation, we next sought to confirm a role for CERK in human mast cells, using the recently described LAD-2 cell-line. Various doses of K1 were used to pre-treat these cells 15-min before the stimulation and histamine and PGD₂ release was quantified 1-h post-stimulation. In LAD-2 cells, K1 significantly inhibited histamine release only at 100 μM [87% ($P < 0.001$)] (Fig. 5a). However, against PGD₂ 100, 30, 10 and 3 μM K1 significantly inhibited its release [100%, 99%, 63% ($P < 0.001$) and 21% ($P < 0.05$) respectively] in an analogous manner as in RBL-2H3 cells (Fig. 5b).

CERK is positioned downstream of PLC, but upstream of JNK/MEK/COX in the signalling cascade of antigen-activated RBL-2H3 cells

With the demonstration that CERK has an important role in antigen-mediated release of histamine and PGD₂, we were then keen to position the kinase in the overall Fc ϵ RI signalling cascade. RBL-2H3 cells were therefore pre-treated with IC₅₀ concentrations of compounds previously identified to inhibit antigen-mediated histamine and PGD₂ release (data not shown; see Table 1 for details) and then stimulated with 3.125 μM C-1P. Supernatants were harvested 6-h post-stimulation and the amount of histamine and PGD₂ released was assessed by ELISA.

It was observed that inhibitors of the more upstream kinases (SYK inhibitor of SYK kinase, wortmannin inhibition of PI3K and U73122 inhibition of PLC) had no statistically significant effect on histamine release (Fig. 6a). However, when cells were pre-treated with SP600125 or AS601245 to inhibit JNK activity, a statistically significant inhibition of C-1P induction of histamine release was observed [57% inhibition ($P < 0.001$) and 59% ($P < 0.001$), respectively].

For C-1P-mediated PGD₂ release, we observed a similar situation as for histamine (Fig. 6b). Inhibition of SYK,

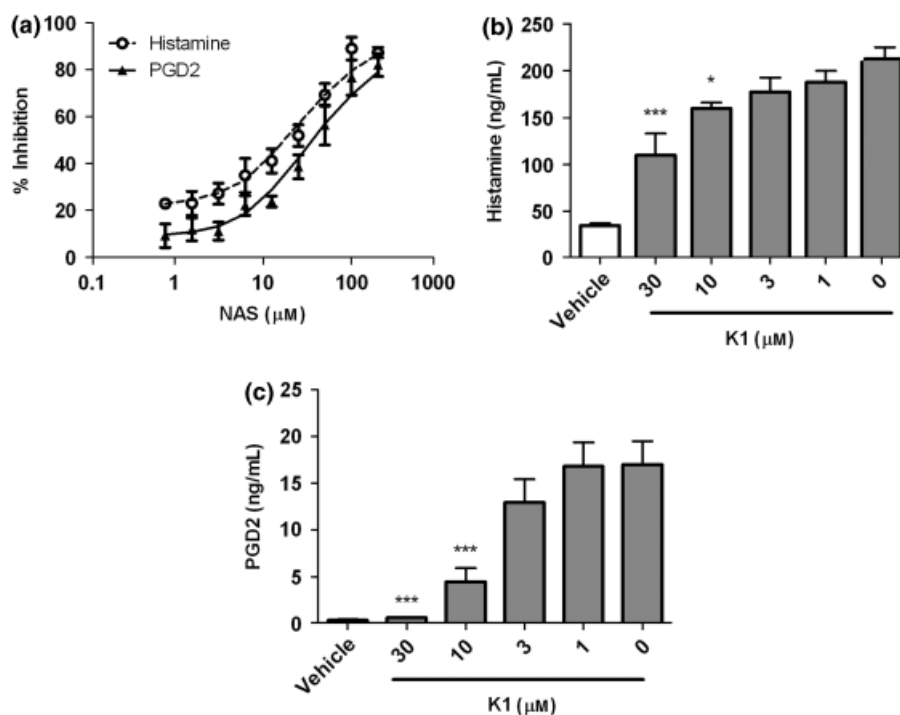


Fig. 4. N-acetyl sphingosine/C₂-ceramide (NAS) and the ceramide kinase (CERK)-specific inhibitor K1 prevents antigen-mediated histamine and prostaglandin D₂ (PGD₂) release in a rat mast-cell line. (a) Sensitized cells were pre-treated NAS for 15-min before stimulation with 100 ng/mL Dinitrophenol-human serum albumin (DNP-HSA). Supernatants were harvested 1-h post-stimulation and the amounts of histamine and PGD₂ quantified by ELISA. Percentage inhibition of release was calculated for each mediator compared with DNP-HSA stimulated, untreated cells (144 ng/mL histamine and 5776 pg/mL PGD₂) and IC₅₀ values were calculated for both end-points ($n = 3$ in duplicate). Sensitized RBL-2H3 cells were pre-treated with the CERK inhibitor K1 and then stimulated with 100 ng/mL DNP-HSA. After 1-h stimulation (a) histamine release and (c) PGD₂ production was assessed by ELISA ($n = 4$ in duplicate). *, ***, Data significantly different to stimulated cells at $P < 0.05$, 0.01 and 0.001, respectively.

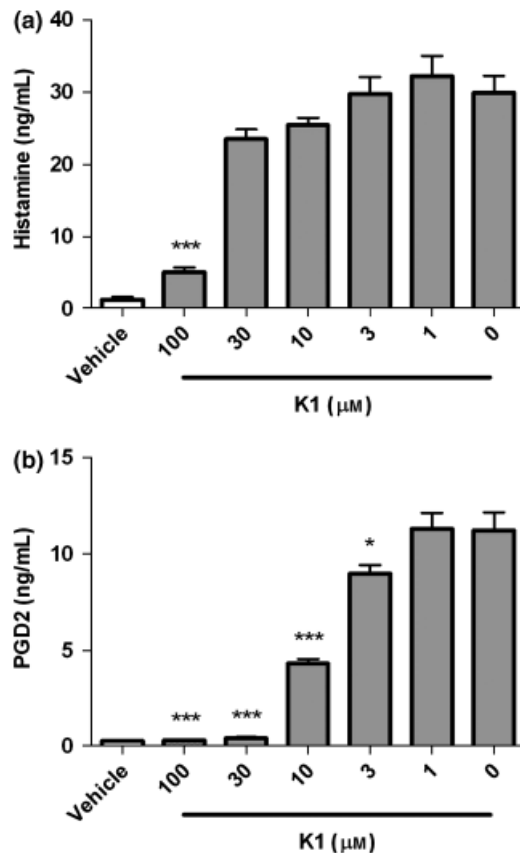


Fig. 5. The ceramide kinase (CERK)-specific inhibitor K1 prevents antigen-mediated histamine and prostaglandin D₂ (PGD₂) release in a human mast-cell line. Sensitized LAD-2 cells were pre-treated with the CERK inhibitor before stimulation with 1 μg/mL streptavidin. After 1-h stimulation (a) histamine release and (b) PGD₂ production was assessed by ELISA ($n = 3$ in duplicate). *, ***Data significantly different to stimulated cells at $P < 0.05$ and 0.001 , respectively.

PI3K and PLC failed to inhibit C-1P induction of PGD₂ release, indicating that CERK activation likely occurs downstream of these kinases. When cells were pre-treated with SP600125 or AS601245, we saw a significant inhibition of PGD₂ production [65% inhibition ($P < 0.001$) and 57% inhibition ($P < 0.001$), respectively], confirming the position of CERK as upstream of JNK activation. In addition, when MEK was inhibited with PD98059 or U0126, or COX was inhibited using indomethacin or ibuprofen we observed a significant inhibition of C-1P-mediated PGD₂ release [60% inhibition ($P < 0.001$) and 42% inhibition ($P < 0.001$), respectively for MEK, and 59% inhibition ($P < 0.001$) and 54% inhibition ($P < 0.001$), respectively for COX] demonstrating that CERK activation is also upstream of MEK and COX.

Confirmation that CERK activity was upstream of JNK and MEK/ERK was assessed by Western blot. When cells were stimulated with 3.125 μM C-1P a time-dependent induction of ERK phosphorylation was observed at 1 and 2 h post-stimulation (Fig. 6c) and at 2 and 4 h post-stimulation for JNK phosphorylation (Fig. 6d).

Discussion

Using the active natural product of the kinase (C-1P) and a variety of biochemical and molecular methodologies we demonstrated that CERK was essential for FcεRI/antigen-mediated mast cell degranulation and release of pre-formed mediators exemplified here by histamine. In addition to demonstrating a novel physiological role for CERK in mast cell degranulation, we identified further novel roles for CERK in production and release of rapidly metabolized lipid-derived factors and *de novo* synthesized

Table 1. IC₅₀ values for compound inhibition of antigen-mediated mast cell secretion

Compound	Target	IC ₅₀		
		Histamine	PGD ₂	IL-13
SYK inhibitor	SYK	414 ± 35.8 nM	144 ± 40.0 nM	490 ± 60.0 nM
Wortmannin	PI-3K	16.1 ± 4.74 nM	27.3 ± 4.05 nM	9.8 ± 1.13 nM
LY294002	PI-3K	5.6 ± 2.19 μM	11.1 ± 2.84 μM	7.3 ± 1.40 μM
U73122	PLC	3.00 ± 0.334 μM	2.46 ± 0.244 μM	5.70 ± 1.230 μM
SP600125	JNK1/2/3	6.50 ± 2.440 μM	0.93 ± 0.099 μM	4.60 ± 0.219 μM
AS601245	JNK1/2/3	9.00 ± 1.47 μM	0.97 ± 0.21 μM	8.00 ± 1.91 μM
PD98059	MEK	> 100 μM	360 ± 66.0 nM	> 100 μM
U0126	MEK1/2	> 100 μM	273 ± 45.6 nM	> 100 μM
Indomethacin	COX	> 100 μM	14 ± 1.05 nM	> 100 μM
Ibuprofen	COX1	> 100 μM	38 ± 5.8 nM	> 100 μM
BAY11-7082	NFκB	> 100 μM	> 100 μM	11.1 ± 1.31 μM
Dexamethasone	Glucocorticoid	> 100 μM	> 100 μM	7.9 ± 0.68 nM

IC₅₀ values are indicated for each compound and end-point measured ± standard deviation. > 100 μM indicates no inhibitory effect was observed up to and including 100 μM. In addition, the p38 inhibitors SB203580 and SB202190 were tested at up to 100 μM, but had no inhibitory effect on any end-point measured.

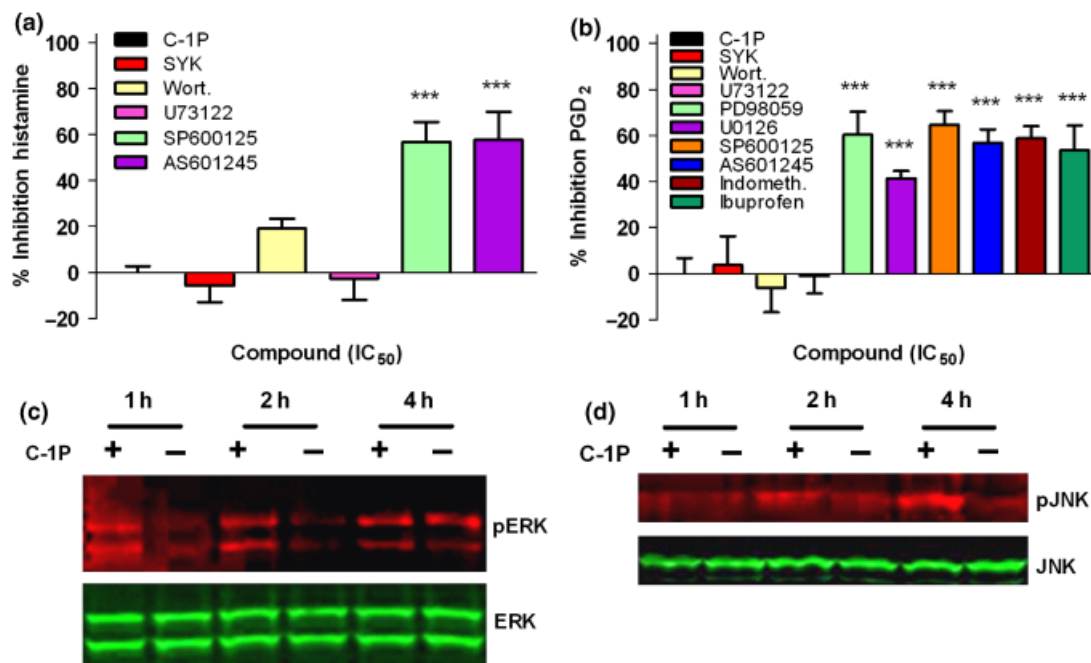


Fig. 6. Ceramide kinase (CERK) is positioned downstream of SYK, PI3K and PLC, but upstream of c-jun N-terminal kinase (JNK), MEK and COX in activated mast cells. RBL-2H3 cells were pre-treated with compounds to inhibit SYK (SYK inhibitor), PI-3K (Wortmannin), PLC (U73122), JNK (SP600125, AS601245), MEK (PD98059, U0126), COX (indomethacin, ibuprofen) at IC₅₀ concentrations identified in Fig. 1 and Table 1, before stimulation with 3.125 μ M C-1P. Supernatants were harvested 6-h post-stimulation and histamine (a) and prostaglandin D₂ (PGD₂) (b) levels quantified by ELISA. ***Data significantly different to C-1P stimulated cells at $P < 0.001$ ($n = 3$ in duplicate). Additionally, RBL-2H3 cells were stimulated with 3.125 μ M C-1P. Cells were harvested at 1, 2 and 4-h post-stimulation and the activation states of (c) ERK and (d) JNK assessed by phospho-specific Western blot. Images are representative of three separate experiments.

late-phase mediators, using PGD₂ and IL-13 as examples. In addition, we identified a differential role for CERK when comparing the three different end-points. Using the same techniques as for histamine, we identified that CERK also played an important role in PGD₂ generation. For IL-13 production, the requirement for CERK was less apparent, with no statistically significant increases obtained with C-1P stimulation of RBL-2H3 cells. Finally, we identified the likely position of CERK as downstream of SYK, phosphoinositide 3-kinase (PI-3K) and PLC, but upstream of JNK for histamine and also upstream of JNK, MEK/ERK and COX for PGD₂.

A single study has sought to examine the role of CERK in mast cell degranulation by calcium ionophores using over-expression of CERK [14]. In addition to potential off-target and cytotoxic effects, a recent review also highlighted the possibility that over-expression of CERK may alter the levels of S-1P via inter-conversion, rather than demonstrating a CERK/C-1P specific effect [20]. With these caveats in mind, we used exogenously applied C-1P to control levels within the cell to protect against cytotoxicity, exogenously applied ceramide to control for inter-conversion/metabolism issues and sought to confirm the requirement of CERK using a specific pharmacological inhibitor. In addition, other studies have uncovered release of biogenic amines without general

degranulation [21] and have highlighted differential inhibitory effects on β -hexosaminidase compared with histamine release [22, 23]. It was therefore important that we demonstrated that the previous work was not specific to β -hexosaminidase, and CERK activation is relevant to important mediators of inflammation. Using these techniques, we were able to improve the understanding of CERK in allergic disease by activating mast cells through their physiological mechanism (Fc ϵ RI aggregation following IgE recognition of antigen) and by studying histamine release as a clinically relevant marker of disease mechanism. Additionally, we were keen to examine the translation from the commonly used rat RBL-2H3 mast-cell model to a human cell. To this end we employed the recently described LAD-2 human mast-cell model to establish a comparable role for the CERK in antigen-mediated activation of human mast-cells.

With the numerous caveats around stimulation with C-1P and the specificity of NAS, we were keen to establish evidence using a more specific inhibitor of CERK. K1 has been demonstrated to act as specific inhibitor of CERK and has been demonstrated to cause inhibition of β -hexosaminidase release from rodent mast-cells [18, 19]. We confirmed the role of CERK in antigen-mediated histamine release, with a comparable level of inhibition as previously observed for β -hexosaminidase release and

further identified a role for CERK in human mast-cell degranulation using the LAD-2 cell line. Additionally, we established the novel role for CERK in the production and release of PGD_2 from mast cells – in this case identifying a more potent and efficacious level of inhibition than had been identified previously for β -hexosaminidase and histamine release. Again, we were able to confirm the effects observed in a rat cell line with those observed using human cells.

No work to date has attempted to position CERK in a signalling cascade. While it is generally understood that CERK is a calcium-dependent kinase [7] and acts on cPLA_2 in a number of cell-types [12, 13], no studies have gone further and none have investigated this in mast cells. Here, we describe an initial attempt at positioning CERK in the signalling cascade following $\text{Fc}\epsilon\text{RI}$ activation. Using standard compounds, we sought to examine which compounds also inhibited action of C-1P, reasoning that these compounds would identify downstream signalling intermediates. Those that did not inhibit C-1P activation would be upstream. We identified that SYK, PI3K and PLC inhibition had no effect on C-1P-mediated PGD_2 or histamine release. In contrast, inhibition of JNK did inhibit histamine and PGD_2 release, along with MEK and COX inhibition of PGD_2 release. We then confirmed that C-1P stimulation caused activation of both ERK and JNK by Western blot. Thus, we have demonstrated that, in mast cells, CERK activation lies upstream of JNK, MEK and COX activation and likely lies downstream of PLC (Fig. 7). How this fits with previous studies identifying cPLA_2 as a target remains unclear. While it has been reported that C-1P interacts directly with cPLA_2 in respiratory epithelial cells and macrophages [12, 13], we have demonstrated that JNK and MEK activation are also required downstream of CERK in mast cell production of PGD_2 . However, it has been shown that both JNK and MEK/ERK induce phosphorylation of cPLA_2 leading to PGE_2 production in bronchial epithelial cells [24] and MEK/ERK activate cPLA_2 in murine mast cells [25]. We therefore hypothesize that C-1P may have dual roles in direct activation of cPLA_2 as well as indirect activation via MAPKs. Alternatively, CERK may have a mast cell-specific mode of action with the indirect activation of cPLA_2 through activation MEK and JNK only (Fig. 7).

In summary, we have identified that the novel lipid kinase CERK is an important mediator of cell signalling events occurring during $\text{Fc}\epsilon\text{RI}$ -mediated mast cell activation. We have confirmed previous work examining a role

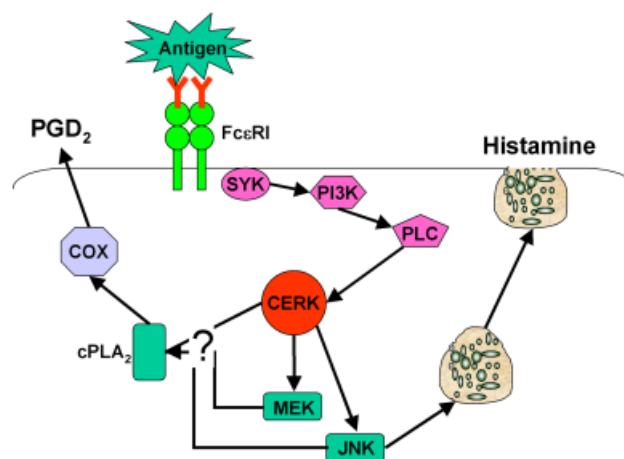


Fig. 7. A model for the involvement of ceramide kinase (CERK) in antigen-mediated activation of mast cells. $\text{Fc}\epsilon\text{RI}$ aggregation initiates a signalling cascade including activation of SYK, PI3K and PLC leading to the activation of CERK. CERK metabolizes ceramide to the active C-1P which then activates c-jun N-terminal kinase (JNK) pathways leading to histamine release. Additionally, C-1P also activates MEK pathways that are required for prostaglandin D_2 (PGD_2) production. With other workers identifying direct interactions between C-1P and cPLA_2 leading to arachidonic release as a substrate for COX [12, 13], we hypothesize a dual role for C-1P activating cPLA_2 directly in addition to an indirect activation of cPLA_2 via JNK and MEK, thereby causing PGD_2 release from mast cells.

in degranulation, and extended this study by looking at a more disease-specific marker of degranulation and identification of the role CERK plays in $\text{Fc}\epsilon\text{RI}$ -mediated degranulation. In addition, we have identified a completely novel role in PGD_2 release and demonstrated no significant role in IL-13 production. We therefore also identified a novel differential importance for CERK depending on which end-point is studied; with CERK being vital for histamine and PGD_2 production, but of little importance for IL-13 release. Finally, we have positioned CERK as downstream of SYK, PI3K and PLC, but upstream of JNK, MEK and COX activation. Studies such as this provide key information on important pathologically relevant cell signalling cascades that will eventually lead to novel pharmacological intervention to prevent and control allergic disease.

Acknowledgements

Conflicts of Interest: All authors are employed and the work funded by Pfizer Ltd.

References

- Galli SJ, Maurer M, Lantz CS. Mast cells as sentinels of innate immunity. *Curr Opin Immunol* 1999; 11:53–9.
- Church MK, Shute JK, Sampson AP. Mast cell-derived mediators. In: Adkinson NF, Yunginger JW, Busse WW, Bochner BS, Holgate ST, Simons FER., eds. *Middleton's allergy: principles and practice*, 6th Edn. Philadelphia: Mosby, 2003; 186–209.
- Weetall M, Shopes B, Holowka D, Baird B. Mapping the site of interaction between murine IgE and its high affinity

- receptor with chimeric Ig. *J Immunol* 1990; **145**:3849–54.
- 4 Nissim A, Eshhar Z. The human mast cell receptor binding site maps to the third constant domain of immunoglobulin E. *Mol Immunol* 1992; **29**:1065–72.
- 5 Jouvin M, Adamczewski M, Numerof R, Letourneur O, Valle A, Kinet J. Differential control of the tyrosine kinases Lyn and Syk by the two signaling chains of the high affinity immunoglobulin E receptor. *J Biol Chem* 1994; **269**:5918–25.
- 6 Metcalfe DD, Baram D, Mekori YA. Mast cells. *Physiol Rev* 1997; **77**:1033–79.
- 7 Bajjalieh S, Martin T, Floor E. Synaptic vesicle ceramide kinase A calcium-stimulated lipid kinase that co-purifies with brain synaptic vesicles. *J Biol Chem* 1989; **264**:14354–60.
- 8 Kolesnick R, Hemer M. Characterization of a ceramide kinase activity from human leukemia (HL-60) cells. Separation from diacylglycerol kinase activity. *J Biol Chem* 1990; **265**:18803–8.
- 9 Sugiura M, Kono K, Liu H *et al*. Ceramide kinase, a novel lipid kinase. Molecular cloning and functional characterization. *J Biol Chem* 2002; **277**:23294–300.
- 10 Baumruker T, Bornancin F, Billich A. The role of sphingosine and ceramide kinases in inflammatory responses. *Immunol Lett* 2005; **96**:175–85.
- 11 Pettus BJ, Bielawska A, Spiegel S, Roddy P, Hannun YA, Chalfant CE. Ceramide kinase mediates cytokine- and calcium ionophore-induced arachidonic acid release. *J Biol Chem* 2003; **278**:38206–13.
- 12 Pettus BJ, Bielawska A, Subramanian P *et al*. Ceramide 1-phosphate is a direct activator of cytosolic phospholipase A2. *J Biol Chem* 2004; **279**:11320–6.
- 13 Subramanian P, Stahelin RV, Szulc Z, Bielawska A, Cho W, Chalfant CE. Ceramide 1-phosphate acts as a positive allosteric activator of group IVA cytosolic phospholipase A2{alpha} and enhances the interaction of the enzyme with phosphatidylcholine. *J Biol Chem* 2005; **280**:17601–7.
- 14 Mitsutake S, Kim TJ, Inagaki Y, Kato M, Yamashita T, Igarashi Y. Ceramide kinase is a mediator of calcium-dependent degranulation in mast cells. *J Biol Chem* 2004; **279**:17570–7.
- 15 Oliver JM, Seagrave J, Stump RF, Pfeiffer JR, Deanin GG. Signal transduction and cellular response in RBL-2H3 mast cells. *Prog Allergy* 1988; **42**:185–245.
- 16 Kirshenbaum AS, Akin C, Wu Y *et al*. Characterization of novel stem cell factor responsive human mast cell lines LAD 1 and 2 established from a patient with mast cell sarcoma/leukemia; activation following aggregation of FcepsilonRI or Fc gammaRI. *Leuk Res* 2003; **27**:677–82.
- 17 Mishra NC, Rir-sima-ah J, Boyd RT *et al*. Nicotine inhibits Fc{varepsilon}RI-induced cysteinyl leukotrienes and cytokine production without affecting mast cell degranulation through {alpha}7/{alpha}9/{alpha}10-nicotinic receptors. *J Immunol* 2010; **185**:588–96.
- 18 Kim J-W, Inagaki Y, Mitsutake S *et al*. Suppression of mast cell degranulation by a novel ceramide kinase inhibitor, the F-12509A olefin isomer K1. *Biochim Biophys Acta (BBA) – Mol Cell Biol Lipids* 2005; **1738**:82–90.
- 19 Kumada H, Mitsutake S, Inagaki Y *et al*. Kinetics of the ceramide kinase inhibitor k1, a suppressor of mast-cell activation. *Biosci Biotechnol Biochem* 2007; **71**:2581–4.
- 20 Chalfant CE, Spiegel S. Sphingosine 1-phosphate and ceramide 1-phosphate: expanding roles in cell signaling. *J Cell Sci* 2005; **118**:4605–12.
- 21 Theoharides TC, Bondy PK, Tsakalos ND, Askenase PW. Differential release of serotonin and histamine from mast cells. *Nature* 1982; **297**:229–31.
- 22 Alexandrakis M, Singh L, Boucher W, Letourneau R, Theofilopoulos P, Theoharides TC. Differential effect of flavonoids on inhibition of secretion and accumulation of secretory granules in rat basophilic leukemia cells. *Int J Immunopharmacol* 1999; **21**:379–90.
- 23 Trnovsky J, Letourneau R, Haggag E, Boucher W, Theoharides TC. Quercetin-induced expression of rat mast cell protease II and accumulation of secretory granules in rat basophilic leukemia cells. *Biochem Pharmacol* 1993; **46**:2315–26.
- 24 Mizumura K, Hashimoto S, Maruoka S *et al*. Role of mitogen-activated protein kinases in influenza virus induction of prostaglandin E2 from arachidonic acid in bronchial epithelial cells. *Clin Exp Allergy* 2003; **33**:1244–51.
- 25 Fonteh AN, Atsumi G-I, LaPorte T, Chilton FH. Secretory phospholipase A2 receptor-mediated activation of cytosolic phospholipase A2 in murine bone marrow-derived mast cells. *J Immunol* 2000; **165**:2773–82.