

Role of acid sphingomyelinase in the regulation of mast cell function

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Summary

Background Degranulation of mast cells is stimulated by store-operated Ca^{2+} -entry (SOCE). In other cell types, Ca^{2+} -entry is modified by ceramide. Exogenously added ceramide has been shown to trigger mast cell apoptosis. Effects of endogenously produced ceramide in mast cells remained, however, elusive. Ceramide may be produced from sphingomyelin by acid sphingomyelinase (Asm).

Objective This study explored the impact of Asm on mast cell functions.

Methods Mast cells were isolated from bone marrow (BMMCs) or peritoneal lavage of gene-targeted mice lacking Asm ($asm^{-/-}$) and their wild-type littermates ($asm^{+/+}$). BMMC maturation and apoptosis-associated annexin V binding were determined by flow cytometry. Asm activity was assessed enzymatically, cytosolic Ca^{2+} activity ($[\text{Ca}^{2+}]_i$) utilizing Fura-2 fluorescence, current across the cell membrane by whole-cell patch clamp, degranulation from hexosaminidase-release and migration utilizing a transwell chamber. *In vivo* anaphylaxis was derived from decrease in body temperature.

Results Peritoneal mast cell number, BMMC phenotype, spontaneous BMMC apoptosis as well as BMMC CD117, CD34 and FcεRI expression were similar in both genotypes. In $asm^{+/+}$ BMMCs, stimulation with antigen resulted in a fast ~2.5-fold increase in Asm activity. Release of Ca^{2+} from internal stores and hence several Ca^{2+} -dependent functions were strongly impaired in $asm^{-/-}$ BMMCs. Thus, antigen-induced increase in $[\text{Ca}^{2+}]_i$ in IgE-sensitized cells, antigen- but not ionomycin-induced currents through Ca^{2+} -activated K^+ -channels ($\text{K}_{\text{Ca}3.1}$), IgE/antigen-triggered β-hexosaminidase release, and antigen-induced migration were all lower in $asm^{-/-}$ BMMCs than in $asm^{+/+}$ BMMCs. Pharmacological inhibition of Asm by amitriptyline (500 nM, 3 h) in $asm^{+/+}$ BMMCs similarly decreased antigen-induced increase in $[\text{Ca}^{2+}]_i$, $\text{K}_{\text{Ca}3.1}$ currents, β-hexosaminidase release and migration. The decrease in body temperature upon the induction of systemic anaphylaxis was significantly less pronounced in $asm^{-/-}$ mice than in $asm^{+/+}$ mice, an observation pointing to *in vivo* significance of Asm.

Conclusions and Clinical Relevance Asm is a novel, powerful regulator of mast cell function and thus a potential target in the treatment of allergic reactions.

Keywords acid sphingomyelinase, allergy, anaphylaxis, Ca^{2+} channels, degranulation, K^+ channels, $\text{K}_{\text{Ca}3.1}$

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Introduction

Mast cells are key players in several IgE-dependent allergic reactions [1, 2], such as allergic rhinitis [3], asthma [4, 5], anaphylactic shock and delayed hypersensitivity reactions [6–9]. Upon activation, mast

cells release several cytokines regulating the function of other inflammatory cells, such as neutrophils and T cells [8, 10–15].

Mast cell degranulation is stimulated by the increase in cytosolic Ca^{2+} concentrations due to Ca^{2+} release from intracellular stores followed by the activation of store-operated Ca^{2+} entry (SOCE) [16–24]. Furthermore, Ca^{2+} can enter the cell via non-selective cation channels such as TRPC6 [25]. Ca^{2+} entry and thus

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degranulation is further influenced by K⁺ channels [17, 26–28] and Cl[−] channels [20, 21], which set the negative membrane potential. Those ion channels are novel promising targets for mast cell inhibition in allergic disorders [29].

In other cell types, Ca²⁺ entry [30] and exocytosis [31] are modified by acid sphingomyelinase (Asm), the product of *Smpd1* gene, an enzyme implicated in a wide variety of clinical disorders including lung inflammation, fibrosis and infection [32], cystic fibrosis [33], cardiovascular disease [34, 35], Wilson's disease [36], multiple sclerosis [37], major depression [38], Parkinson's disease [39], Alzheimer's disease [38, 40, 41] and diabetes [42–44].

Sphingomyelin metabolites, ceramide and sphingosine, have been proposed as intracellular mediators of apoptotic signals [45–54]. In mast cells, exogenously added Asm has been shown to trigger apoptosis [55, 56]. Exogenously added C2 ceramide and sphingosine have been shown to inhibit mediator release from murine bone marrow-derived cultured mast cells (BMMCs) [55]. Expression and enzymatic activity of endogenous Asm as well as ceramide formation are increased in BMMCs deficient in IL-15, a potent anti-apoptotic cytokine [56]. The role of Asm in responses other than apoptosis has not been well studied in mast cells, and the functional role of endogenous Asm or ceramide in mast cells remained largely elusive.

This study explored the impact of acid sphingomyelinase on Ca²⁺ concentration, degranulation and migration of mast cells. To this end, mast cells have been isolated from bone marrow of acid sphingomyelinase-deficient mice (*asm*^{−/−}) and from their wild-type littermates (*asm*^{+/+}).

Materials and methods

Mice

All animal experiments were conducted according to the German law for the welfare of animals and were approved by the local authorities. Experiments were performed in gene-targeted mice lacking functional acid sphingomyelinase (*asm*^{−/−}) and their wild-type littermates (*asm*^{+/+}). The mice have been generously provided by R. Kolesnick [57].

Culture of bone marrow-derived mast cells

Mast cells were isolated from femoral and tibial bone marrow of 6- to 8-week-old-naïve *asm*^{+/+} and *asm*^{−/−} mice and cultured for 4 weeks in RPMI 1640 (Gibco, Darmstadt, Germany) containing 10% FCS (PAA), 1% penicillin/streptomycin (PAA), 20 ng/mL IL-3 (Immunotools, Friesoythe, Germany) and 100 ng/mL of the c-kit

ligand stem cell factor (Immunotools). BMMC maturation was confirmed by flow cytometry (FACSCalibur; BD Biosciences, Heidelberg, Germany) using the following specific fluorescent-labelled antibodies: PE-labelled anti-FcεRI (eBioscience, Frankfurt, Germany), allophycocyanin-labelled anti-CD117 (BD Pharmingen, Heidelberg, Germany) and FITC-labelled anti-CD34 (BD Pharmingen). Cells were kept in culture 4–6 weeks before the experiments. For experiments, BMMCs were sensitized for 1 h with monoclonal mouse anti-DNP mouse IgE (anti-DNP IgE, 5–10 µg/mL per 1 × 10⁶ cells, clone SPE-7; Sigma-Aldrich, Seelze, Germany) in culture medium and challenged with DNP-human serum albumin (DNP-HSA; 2,4-Dinitrophenyl hapten conjugated to human serum albumin, 50 ng/mL; Sigma-Aldrich).

Transmission electron microscopy

Mast cells were fixed with pre-warmed Karnovsky's fixative for 10 min at 37°C and stored at 4°C. Cell pellets were embedded in 3.5% agarose at 37°C and coagulated at room temperature. Embedded cell pellets were fixed in Karnovsky's solution. Post-fixation was based on 1.0% osmium tetroxide containing 1.5% K-ferrocyanide in aqua bidest for 2 h. Following standard methods, blocks were embedded in glycidic ether and cut using an ultra microtome (Ultracut, Reichert, Vienna, Austria). Ultra-thin sections (30 nm) were mounted on copper grids and analysed using a Zeiss LIBRA 120 transmission electron microscope (Carl Zeiss, Oberkochen, Germany) operating at 120 kV.

Peritoneal lavage

Anaesthetized mice were euthanized by cervical dislocation, and the abdominal skin was cleansed with 70% ethanol. Sterile 0.9% NaCl (4 mL) was then instilled into the peritoneum. The abdomen was massaged gently for 1 min and then opened with sterile scissors. Recovered peritoneal lavage fluid was centrifuged at 2000 g for 5 min, and the cell pellets were resuspended in red blood cell lysis buffer for 1 min and recentrifuged, and then, the cell pellet was resuspended in phosphate-buffered saline (PBS). Slides were prepared as 'thick blood films' and fixed with 4% paraformaldehyde. After staining with toluidine blue, at least 10 view fields/slides were counted.

Phosphatidylserine translocation

Apoptotic cell membrane scrambling was evidenced from annexin V binding to phosphatidylserine (PS) at the cell surface. To this end, the percentage of PS-translocating cells was evaluated by staining with fluorescein isothiocyanate (FITC)-conjugated annexin V.

In brief, 1×10^6 cells were harvested and washed twice with PBS and then resuspended in annexin V binding buffer (BD Bioscience). The cells were incubated with FITC-annexin V for 15 min at room temperature in the dark and then analysed by flow cytometry.

Asm activity assay

The activity of the Asm was measured as previously described [58]. Briefly, 5×10^5 cells were incubated with IgE (10 μ g/mL, 1 h) or with IgE (10 μ g/mL, 1 h) followed by antigen (DNP-HSA, 50 ng/mL, 5 min) or left untreated (control), frozen in liquid nitrogen and kept at -80°C . The cells were then lysed in 200 μ L of ice-cold buffer containing 50 mM sodium acetate (pH 5.0), 0.1% NP40. After 10 min lysis on ice, the lysates were sonicated 3 times for 10 s each using a tip sonicator at low energy. The lysates were incubated with 0.02 μ Ci of [^{14}C]sphingomyelin for 30 min at 37°C . The substrate was dried before use and resuspended in 50 mM sodium acetate (pH 5.0), 0.1% NP40, followed by 10-min bath sonication to promote the formation of micelles. The reaction was stopped by the addition of 1 mL of $\text{CHCl}_3:\text{CH}_3\text{OH}$ (v/v). Phases were separated by 5-min centrifugation at 20 000 g, and an aliquot of the aqueous phase was applied for liquid scintillation counting. Hydrolysis of [^{14}C]sphingomyelin by sphingomyelinase results in the release of [^{14}C]choline chloride into the aqueous phase, whereas ceramide and unreacted [^{14}C]sphingomyelin remain in the organic phase. Therefore, the release of [^{14}C]choline chloride (nmol/g/h) serves to determine the activity of the Asm.

Patch clamp

Patch clamp experiments have been performed at room temperature in voltage-clamp, fast-whole-cell mode [59]. BMMCs were continuously superfused by a flow system inserted into the dish. The bath was grounded via a bridge filled with NaCl Ringer solution, containing (in mM) 145 NaCl, 5 KCl, 2 MgCl_2 , 1 CaCl_2 , 10 glucose, 10 HEPES/NaOH (pH 7.4, 300 mOsm). Borosilicate glass pipettes (2–4 M Ω tip resistance; GC 150 TF-10, Harvard Apparatus, March-Hugstetten, Germany), manufactured by a microprocessor-driven DMZ puller (Zeitz, Augsburg, Germany), were used in combination with a MS314 electrical micromanipulator (MW, Märzhäuser, Wetzlar, Germany). The currents were recorded by an EPC-9 amplifier (HEKA, Lambrecht, Germany) using Pulse software (HEKA) and an ITC-16 Interface (Instrutech, Port Washington, NY, USA). Whole-cell currents were determined as 10 successive 200-ms square pulses from a -35 mV holding potential to potentials between -115 mV and $+65$ mV. The currents were recorded with an acquisition frequency of 10 kHz and 3 kHz low-pass filtered [60, 61].

The pipette solution contained (in mM) 140 K-glucuronate, 5 KCl, 1.2 MgCl_2 , 2 EGTA, 1.26 CaCl_2 (pCa 7), 2 Na_2ATP and 10 HEPES/KOH (pH 7.2, 280 mOsm), and was used in combination with NaCl Ringer bath solution. Where indicated, the antigen dinitrophenyl-HSA (DNP-HSA, 50 ng/mL, Sigma-Aldrich) was added to the bath solution.

The offset potentials between both electrodes were zeroed before sealing. The potentials were corrected for liquid junction potentials as estimated according to Barry and Lynch [62]. The original whole-cell current traces are depicted without further filtering, and currents of the individual voltage square pulses are superimposed. The applied voltages refer to the cytoplasmic face of the cell membrane with respect to the extracellular space. The inward currents, defined as flow of positive charge from the extracellular to the cytoplasmic membrane face, are negative currents and depicted as downward deflections of the original current traces.

Intracellular calcium measurements

Intracellular Ca^{2+} measurements were performed as described [60, 63]. Briefly, BMMCs were sensitized with IgE (10 μ g/mL) for 1 h at 37°C and subsequently loaded with Fura-2/AM (2 μ M; Molecular Probes, Goettingen, Germany) for 20 min at 37°C . Fluorescence measurements were carried out with an inverted phase-contrast microscope (Axiovert 100; Zeiss, Oberkochen, Germany). Cells were excited alternatively at 340 and 380 nm, and the light was deflected by a dichroic mirror into either the objective (Fluar 40 $\times$ /1.30 oil; Zeiss) or a camera. Emitted fluorescence intensity was recorded at 505 nm, and data acquisition was performed using specialized computer software (Metafluor; Universal Imaging, Downingtown, PA, USA). The corresponding ratios (F_{340}/F_{380}) were used to obtain intracellular Ca^{2+} concentrations. The following equation was used: $[\text{Ca}^{2+}]_{\text{free}} = K_d \times ((R - R_{\text{min}})/(R_{\text{max}} - R)) \times S_f$ (K_d = dissociation constant of Fura-2; R = ratio of emission intensity, exciting at 340 nm, to emission intensity, exciting at 380 nm; R_{min} = ratio at zero free Ca^{2+} ; R_{max} = ratio at saturating Ca^{2+} ; S_f = instrumental constant). As a measure for the increase in cytosolic Ca^{2+} activity, the slope and peak of the changes in the intracellular Ca^{2+} concentration were calculated for each experiment. Intracellular Ca^{2+} was measured prior to and following addition of DNP-HSA (50 ng/mL) to IgE-sensitized BMMCs in the absence or presence of extracellular Ca^{2+} . Alternatively, changes in cytosolic Ca^{2+} were monitored upon depletion of the intracellular Ca^{2+} stores. Experiments were carried out prior to and during exposure of the cells to Ca^{2+} -free solution. In the absence of Ca^{2+} , the intracellular Ca^{2+} stores were depleted by the inhibition of the vesicular Ca^{2+}

pump by thapsigargin (1 μM ; Molecular Probes). Re-addition of Ca^{2+} allowed assessing the store-operated Ca^{2+} entry.

For intracellular calibration purposes, ionomycin (10 μM) was applied at the end of each experiment. Experiments were performed with Ringer solution containing (in mM) 125 NaCl, 5 KCl, 1.2 MgSO_4 , 2 CaCl_2 , 2 Na_2HPO_4 , 32 HEPES, 5 glucose, pH 7.4. To reach nominally Ca^{2+} -free conditions, experiments were performed using Ca^{2+} -free Ringer solution containing (in mM) 125 NaCl, 5 KCl, 1.2 MgSO_4 , 2 Na_2HPO_4 , 32 HEPES, 0.5 EGTA, 5 glucose, pH 7.4.

Measurement of degranulation

Mature bone marrow mast cells were seeded on 96-well plates in fresh medium with anti-DNP IgE antibody (5 $\mu\text{g}/\text{mL}$) for 1 h. Afterwards, cells were washed in PBS (Gibco) containing 1 mM CaCl_2 and challenged with DNP-HSA (50 ng/mL). 20 μL of supernatant and 20 μL of 2 mM 4-nitrophenyl N-acetyl- β -D-glucosaminide (Sigma-Aldrich), diluted in 0.2 M citrate buffer, pH 4.5, were added to each well of the 96-well plate, and colour was developed for 2 h at 37°C. The reaction was terminated with 1M TRIS buffer, pH 9.0, and the absorbance was measured at 405 nm in an ELISA microplate reader [64]. The data are expressed as the percentage of the total release (Triton X-100 0.1%) and are corrected for spontaneous release.

Migration

For migration assays, transwell inserts (Corning #3421) were used with a pore diameter size of 8 μm . The transwells were placed in a 24-well cell culture plate containing cell culture medium (750 μL). The upper chambers were filled with 400 μL cell culture medium containing 5×10^4 cells/well. The chamber was placed in a 5% CO_2 37°C incubator for 4 h. In the following, the non-migrated cells were gently removed by cotton-tipped swab and PBS wash. The transwells were moved to 4% PFA and incubated overnight in 4°C. Membranes were removed by scalpel, placed on slides and stained with DAPI. The migrated cells bound to the membrane were then counted.

Passive systemic anaphylaxis/antigen-induced anaphylaxis

Mice were sensitized with 30 $\mu\text{g}/250 \mu\text{L}$ anti-DNP IgE by intraperitoneal application. Five hours later, mice were challenged with either DNP-HSA (100 $\mu\text{g}/200 \mu\text{L}$) or PBS. Body temperature was monitored before and every min after antigen challenge with 8-Channel USB Thermometer (Tübingen, Germany) during the

mid-portion of the light phase of the light cycle. Mice were placed with the tail raised, and the vaseline-covered probe was inserted at a standardized distance of 2 cm until a stable temperature reading was obtained. Baseline temperature was measured after habituating mice to rectal probe insertion. Ambient room temperature was 23°C, and the animals were exposed to a 12 h light and 12 h dark cycle (7 am to 7 pm). Data are expressed as a change in body temperature following the treatment ($\Delta^\circ\text{C}$).

Statistics

Data are provided as means $\pm$ SEM, n represents the number of animals/independent experiments. All data were tested for significance using Student's unpaired two-tailed t -test or ANOVA (Dunnett's test), where applicable. $P < 0.05$ was considered to indicate statistical significance.

Results

Phenotype and number of mast cells derived from $asm^{+/+}$ and $asm^{-/-}$ mice as well as stimulation of Asm activity by antigen

To explore the impact of acid sphingomyelinase (Asm) on mast cell function, mast cells were isolated from bone marrow (BMMCs) of gene-targeted mice lacking Asm ($asm^{-/-}$) and their wild-type littermates ($asm^{+/+}$). As illustrated in Figs 1 a and b, $asm^{+/+}$ and $asm^{-/-}$ BMMCs expressed CD117, CD34 and Fc ϵ RI, that is, receptors typically expressed by mast cells, to a similar extent. The number of mast cells in the peritoneum was not different between genotypes (Fig. 1c, $P = 0.43$). Electron microscopy analysis revealed that granules of $asm^{-/-}$ BMMCs were not different from $asm^{+/+}$ BMMCs (Fig. 1d). Moreover, the rate of spontaneous apoptosis, as assessed by annexin V-positive BMMCs, was not different between $asm^{+/+}$ and $asm^{-/-}$ genotypes (Fig. 1e, $P = 0.97$). In $asm^{+/+}$ BMMCs, stimulation with IgE (1 μM , 1 h) and then with cognate antigen (DNP-HSA, 2,4-Dinitrophenyl hapten conjugated to human serum albumin, 50 ng/mL, 5 min) resulted in a ~ 2.5 -fold stimulation of Asm activity (Fig. 1f).

Antigen-induced release of Ca^{2+} from internal stores with or without genetic or pharmacological inhibition of Asm

Fura-2 fluorescence was employed to elucidate whether Asm influences cytosolic Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$). In unstimulated BMMCs, $[\text{Ca}^{2+}]_i$ was not significantly different ($P = 0.42$) between $asm^{+/+}$ BMMCs ($84.38 \pm 2.54 \text{ nM}$, $n = 134$) and $asm^{-/-}$ BMMCs ($87.51 \pm$

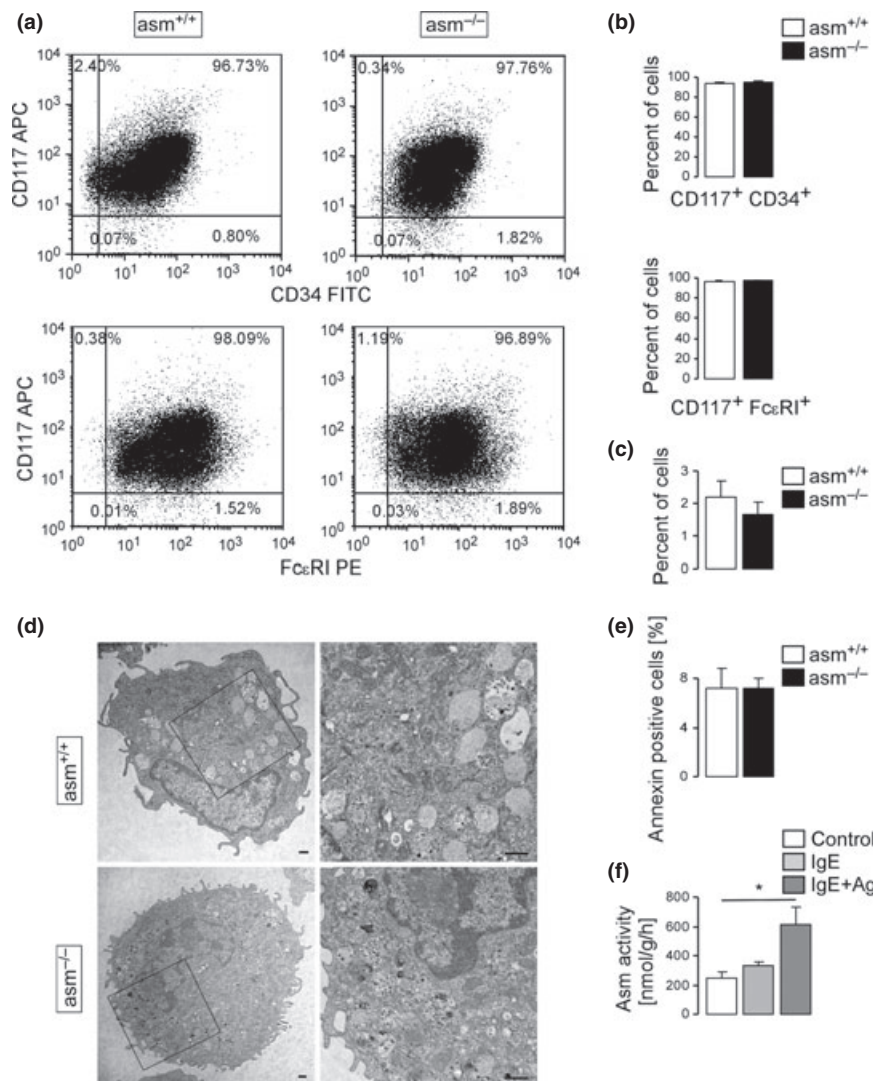


Fig. 1. Phenotype and number of mast cells derived from *asm*^{+/+} and *asm*^{-/-} mice. Stimulation of Asm activity by antigen. (a) Original dot plots of CD117-, CD34- and FcεRI-positive bone marrow-derived mast cells (BMMCs) from *asm*^{+/+} and *asm*^{-/-} mice. Numbers depict the percentage of cells in the respective quadrant. (b) Percentage of mast cells in primary culture. Mean percentage (± SEM; *n* = 6 individual BMMC cultures) of *asm*^{+/+} (open bars) and *asm*^{-/-} (closed bars) BMMCs. (c) Percentage of toluidine blue-positive mast cells (± SEM) among leukocytes in the peritoneum from 4 *asm*^{+/+} (open bars) and 4 *asm*^{-/-} (closed bars) mice. (d) Transmission electron microscopy of BMMCs from *asm*^{+/+} and *asm*^{-/-} mice. Bars: 1 μm; N = Nucleus. (e) Arithmetic means (*n* = 5 individual BMMC cultures) of the percentage of *asm*^{+/+} (open bars) and *asm*^{-/-} (closed bars) BMMCs with annexin V binding. (f) Enzymatic activity of Asm (± SEM, *n* = 6–7) in *asm*^{+/+} BMMCs treated with either IgE (10 μg 1 h, light grey bar) or IgE (10 μg 1 h) followed by antigen (Ag, 50 ng/mL, 5 min, dark grey bar) or left untreated. * (*P* < 0.05), ANOVA.

2.97 nM, *n* = 128). Stimulation with IgE (1 μM, 1 h) and then with antigen (DNP-HSA, 50 ng/mL) resulted in a sharp increase in [Ca²⁺]_i in both genotypes, an effect, however, significantly more pronounced in *asm*^{+/+} BMMCs than in *asm*^{-/-} BMMCs (Figs 2a and b). Also, when lower concentrations of IgE (300 ng/mL, 3 h) and antigen (1 ng/mL) were used, *asm*^{-/-} BMMCs responded with significantly lower and less steep [Ca²⁺]_i increase (Figs 2c and d). The pharmacological inhibitor of Asm, amitriptyline (500 nM, 3 h), similarly led to reduced antigen-dependent increase in [Ca²⁺]_i in *asm*^{+/+} cells (Figs 2e and f). To explore, whether Asm

influences intracellular Ca²⁺ release or the entry of extracellular Ca²⁺, BMMCs were sensitized with IgE and challenged with antigen in the absence of extracellular Ca²⁺ (Figs 3a and b). As a result, in the nominal absence of extracellular Ca²⁺, the exposure to IgE and antigen was followed by a transient increase in intracellular Ca²⁺, which was significantly higher in *asm*^{+/+} compared with *asm*^{-/-} BMMCs (Fig. 3b). Pharmacological inhibition of Asm by amitriptyline (500 nM, 3 h) similarly impaired antigen-induced release of Ca²⁺ (Figs 3c and d). To confirm that Asm up-regulates intracellular Ca²⁺ release, depletion of Ca²⁺ stores was

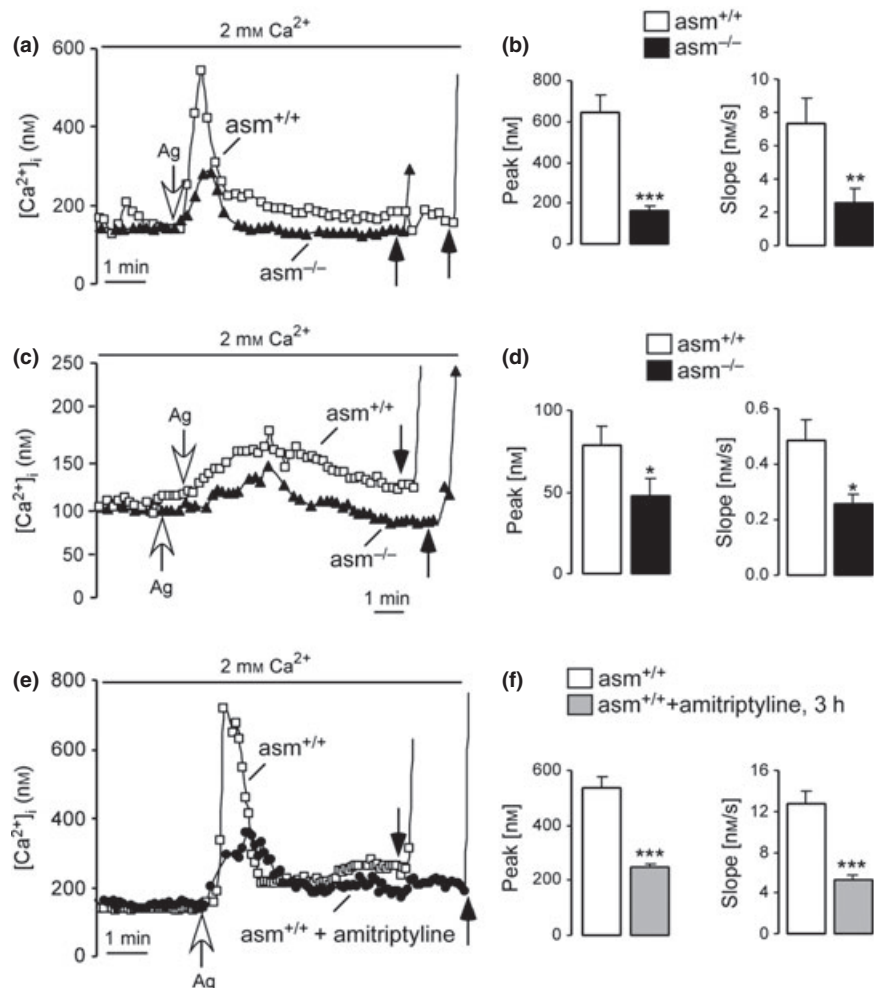


Fig. 2. Antigen-induced Ca²⁺ increase in BMMCs from *asm*^{+/+} and *asm*^{-/-} mice and amitriptyline-treated *asm*^{+/+} BMMCs. (a) Representative original tracings showing intracellular Ca²⁺ concentrations in Fura-2/AM loaded IgE (10 µg/mL, 1 h)-sensitized BMMCs from *asm*^{+/+} and *asm*^{-/-} mice prior to and following addition of antigen (Ag, 50 ng/mL, white arrow) in the presence of extracellular Ca²⁺. At the end of each experiment, ionomycin (10 µM, black arrow) was added. For quantification of the Ca²⁺ entry into the BMMCs, the peak (nm) and the slope (nm/s) were calculated following the addition of Ag. (b) Arithmetic means (± SEM) of the peak (left) and slope (right) of the change in intracellular Ca²⁺ concentrations for *asm*^{+/+} (*n* = 9, open bars) and *asm*^{-/-} (*n* = 13, closed bars) BMMCs upon stimulation with antigen (50 ng/mL) as in (a). ** (*P* < 0.01), *** (*P* < 0.001), two-tailed unpaired *t*-test. (c) Representative original tracings showing intracellular Ca²⁺ concentrations in IgE (300 ng/mL, 3 h)-sensitized *asm*^{+/+} and *asm*^{-/-} BMMCs before and after the addition of low concentration of antigen (Ag, 1 ng/mL, white arrow) in the presence of extracellular Ca²⁺. (d) Arithmetic means (± SEM) of the peak (left) and slope (right) of the change in intracellular Ca²⁺ concentrations for *asm*^{+/+} (*n* = 16, open bars) and *asm*^{-/-} (*n* = 5, closed bars) BMMCs upon stimulation with antigen (1 ng/mL) as in (c). * (*P* < 0.05), two-tailed unpaired *t*-test. (e) Representative original tracings showing intracellular Ca²⁺ concentrations in IgE (10 µg/mL, 1 h)-sensitized *asm*^{+/+} BMMCs untreated and treated with amitriptyline (500 nM, 3 h) before and after the addition of antigen (Ag, 50 ng/mL, white arrow) in the presence of extracellular Ca²⁺. (f) Arithmetic means (± SEM) of the peak (left) and slope (right) of the change in intracellular Ca²⁺ concentrations for untreated *asm*^{+/+} (*n* = 31, open bars) and *asm*^{+/+} BMMCs treated with amitriptyline (*n* = 19, grey bars) upon stimulation with antigen (50 ng/mL) as in (e). *** (*P* < 0.001), two-tailed unpaired *t*-test.

induced by inhibition of the Ca²⁺-ATPase SERCA by thapsigargin and Ca²⁺ was readed (Fig. 3e). While the Ca²⁺-release was strongly impaired in *asm*^{-/-} BMMCs, the entry of extracellular Ca²⁺ through store-operated Ca²⁺ (SOC) channels was not affected by Asm deficiency (Fig. 3f). Thus, Asm is a positive regulator of the release of intracellular Ca²⁺.

Currents through Ca²⁺-activated K⁺ channels K_{Ca}3.1 with or without genetic or pharmacological inhibition of Asm

Further experiments explored whether lack of Asm influenced Ca²⁺-activated K⁺ channels K_{Ca}3.1 in mast cells. The K⁺ currents of BMMCs upon stimulation with IgE/antigen or ionomycin were measured by whole-cell patch clamp (Fig. 4). In both genotypes, addition of

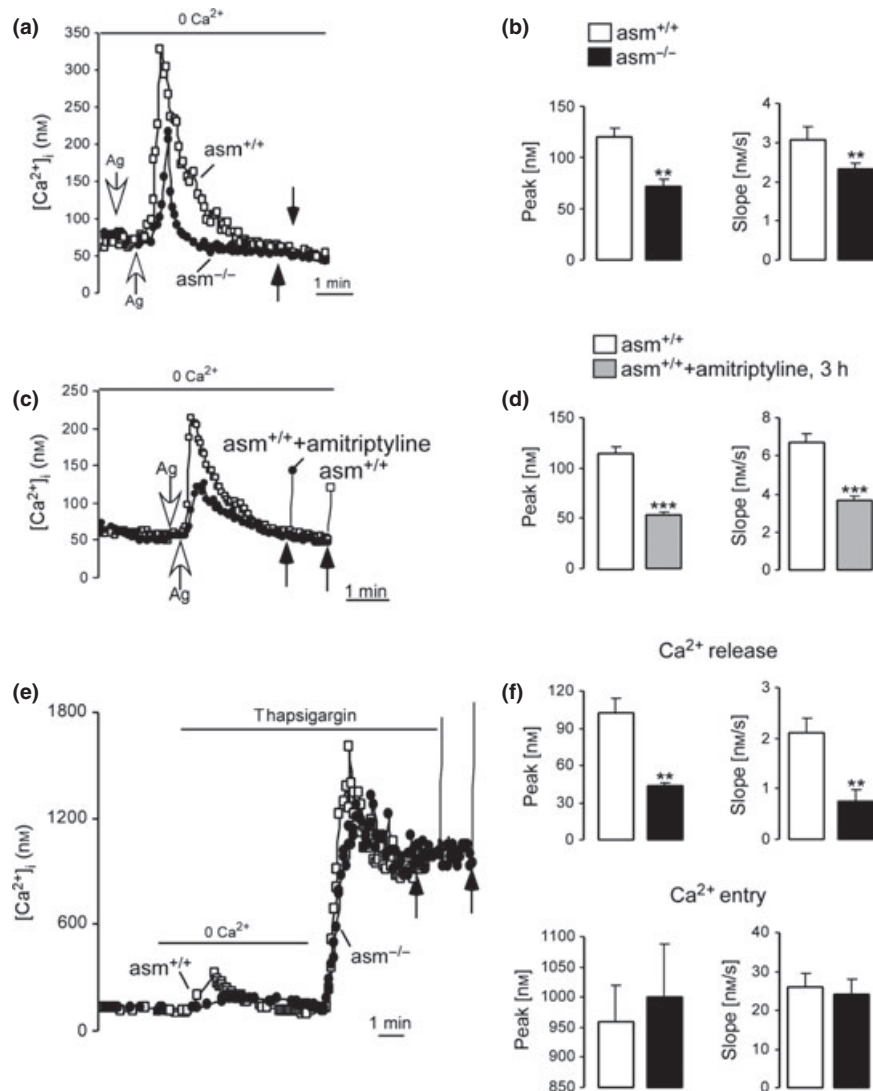
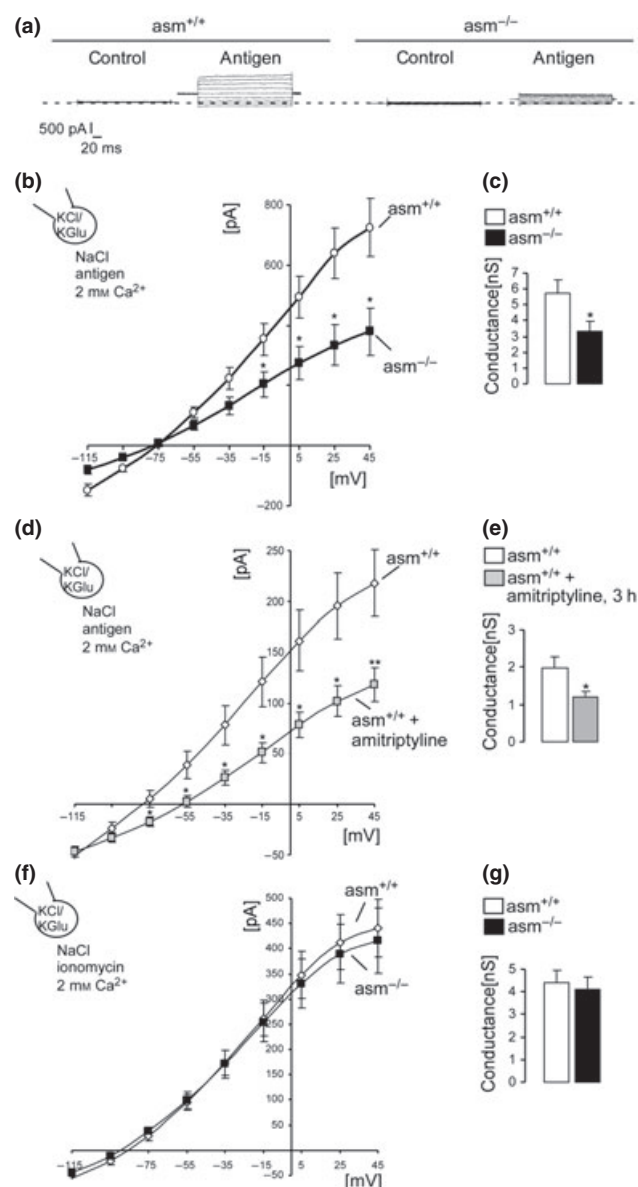


Fig. 3. Antigen-induced Ca²⁺ release in BMMCs from *asm*^{+/+} and *asm*^{-/-} mice. (a) Representative original tracings showing intracellular Ca²⁺ concentrations in IgE (10 µg/mL, 1 h)-sensitized BMMCs from *asm*^{+/+} and *asm*^{-/-} mice prior to and following addition of antigen (Ag, 50 ng/mL, white arrow) in the absence of extracellular Ca²⁺. To reach a Ca²⁺-free environment, EGTA (0.5 mM) was added to the Ca²⁺-free bath solution. (b) Arithmetic means (± SEM) of the peak (left) and slope (right) of the change in intracellular Ca²⁺ concentrations for *asm*^{+/+} (*n* = 18, open bars) and *asm*^{-/-} (*n* = 32, closed bars) BMMCs following stimulation with antigen (50 ng/mL) as in (a). ** (*P* < 0.01), two-tailed unpaired *t*-test. (c) Representative original tracings showing intracellular Ca²⁺ concentrations in IgE (10 µg/mL, 1 h)-sensitized *asm*^{+/+} BMMCs untreated and treated with amitriptyline (500 nM, 3 h) before and after the addition of antigen (Ag, 50 ng/mL, white arrow) in the absence of extracellular Ca²⁺. (d) Arithmetic means (± SEM) of the peak (left) and slope (right) of the change in intracellular Ca²⁺ concentrations for untreated *asm*^{+/+} (*n* = 41, open bars) and *asm*^{+/+} BMMCs treated with amitriptyline (*n* = 40, grey bars) upon stimulation with antigen (50 ng/mL) as in (c). *** (*P* < 0.001), two-tailed unpaired *t*-test. (e) Representative original tracings showing intracellular Ca²⁺ concentrations in *asm*^{+/+} and *asm*^{-/-} BMMCs upon store depletion by thapsigargin (1 µM) added first in Ca²⁺-free solution. Readdition of extracellular Ca²⁺ in the presence of thapsigargin reflects the entry of Ca²⁺ through the store-operated Ca²⁺ channels. (f) Arithmetic means (± SEM) of the peak (left) and slope (right) of the change in intracellular Ca²⁺ concentrations for *asm*^{+/+} (*n* = 12, open bars) and *asm*^{-/-} (*n* = 10, closed bars) BMMCs upon addition of thapsigargin in Ca²⁺-free solution (Ca²⁺ release) and upon readdition of Ca²⁺ in the continuous presence of thapsigargin (Ca²⁺ entry). ** (*P* < 0.01), two-tailed unpaired *t*-test.

antigen to the bath solution resulted in a rapid increase of a K⁺-selective conductance (Figs 4a–c). The current amplitude was increased following antigen application and reached its maximum in about 3 min. The maximal amplitude was significantly smaller in *asm*^{-/-} than in *asm*^{+/+} cells (Figs 4a–c). Moreover, in *asm*^{+/+} cells, incu-

bation with amitriptyline (500 nM, 3 h) led to a lower activation of K⁺ conductance upon antigen (Figs 4d and e). In contrast, following stimulation with the Ca²⁺ ionophore ionomycin, the K⁺ currents were not significantly different between the genotypes (Figs 4f and g).



Degranulation with or without genetic or pharmacological inhibition of Asm

At least in theory, the blunted Ca^{2+} entry in $asm^{-/-}$ BMMCs could result in decreased antigen-induced mediator release. To determine whether lack of Asm modifies mast cell degranulation, the release of β -hexosaminidase was measured in $asm^{+/+}$ and $asm^{-/-}$ cells. As illustrated in Fig. 5, β -hexosaminidase release was significantly reduced in $asm^{-/-}$ BMMCs and following incubation of $asm^{+/+}$ cells with amitriptyline (500 nM).

Migration with or without genetic or pharmacological inhibition of Asm

As Ca^{2+} -sensitive cellular functions include cell migration, additional experiments were performed to

Fig. 4. K^+ currents in $asm^{+/+}$, $asm^{-/-}$ and amytriptyline-treated $asm^{+/+}$ BMMCs. (a) Representative whole-cell currents from $asm^{+/+}$ (left) and $asm^{-/-}$ (right) IgE (10 $\mu\text{g/mL}$, 1 h)-sensitized BMMCs elicited by 200 ms pulses ranging from -115 to $+45$ mV in 20 mV increments from a holding potential of -35 mV. Currents were recorded in standard NaCl bath solution before (control) and after stimulation with antigen (50 ng/mL). The dotted line indicates the zero current value. (b) Mean I-V relationships ($\pm$ SEM) in $asm^{+/+}$ (open symbols, $n = 29$) and $asm^{-/-}$ (closed symbols, $n = 24$) BMMCs after stimulation with antigen. $*P < 0.05$, two-tailed unpaired t -test. (c) Arithmetic means ($\pm$ SEM, $n = 24$ –29) of whole-cell conductance of $asm^{+/+}$ (open bars) and $asm^{-/-}$ (closed bars) BMMCs as recorded in (b) after stimulation with antigen. Data were calculated by linear regression between -75 and $+5$ mV. $*P < 0.05$, two-tailed unpaired t -test. (d) Mean I-V relationships ($\pm$ SEM) in IgE (10 $\mu\text{g/mL}$, 1 h)-sensitized $asm^{+/+}$ BMMCs untreated (open symbols, $n = 14$) or treated with amitriptyline (500 nM, 3 h, grey symbols, $n = 15$) after stimulation with antigen. $*P < 0.05$, $**P < 0.01$, two-tailed unpaired t -test. (e) Arithmetic means ($\pm$ SEM, $n = 14$ –15) of whole-cell conductance of $asm^{+/+}$ BMMCs untreated (open bars) or treated with amitriptyline (grey bars) as recorded in (d) after stimulation with antigen. Data were calculated by linear regression between -75 and $+5$ mV. (f) Mean I-V relationships ($\pm$ SEM) in $asm^{+/+}$ (open symbols, $n = 36$) and $asm^{-/-}$ (closed symbols, $n = 36$) BMMCs after stimulation with ionomycin (1 μM). (g) Arithmetic means ($\pm$ SEM, $n = 36$) of whole-cell conductance of $asm^{+/+}$ (open bars) and $asm^{-/-}$ (closed bars) BMMCs as recorded in (f) after stimulation with ionomycin. Data were calculated by linear regression between -75 and $+5$ mV.

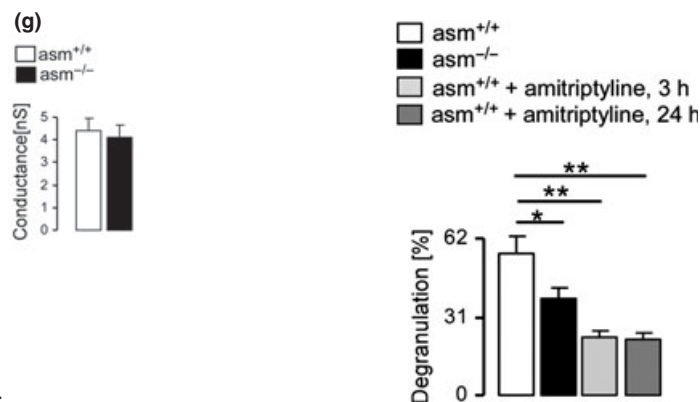


Fig. 5. Degranulation of antigen-stimulated $asm^{-/-}$ and $asm^{+/+}$ BMMCs, effect of amitriptyline. Arithmetic means ($\pm$ SEM, $n = 5$ –7 individual experiments) of β -hexosaminidase release from IgE (10 $\mu\text{g/mL}$, 1 h)-sensitized and antigen (50 ng/mL, 15 min)-stimulated BMMCs from $asm^{-/-}$ (closed bar) and $asm^{+/+}$ mice. $asm^{+/+}$ BMMCs were either untreated (open bar) or treated with amitriptyline (500 nM) for 3 h (light grey bar) or 24 h (dark grey bar). Release in the supernatant was calculated as % of total cellular (0.1% Triton X-100) β -hexosaminidase. The stimulated β -hexosaminidase release in each experiment was corrected for the spontaneous release. $*P < 0.05$, $**P < 0.01$, ANOVA.

elucidate whether Asm deficiency impacts on mast cell migration. As illustrated in Fig. 6, migration towards low antigen concentrations (1 ng/mL) was significantly less pronounced in $asm^{-/-}$ BMMCs. To further test the role of acid sphingomyelinase, migration was assessed

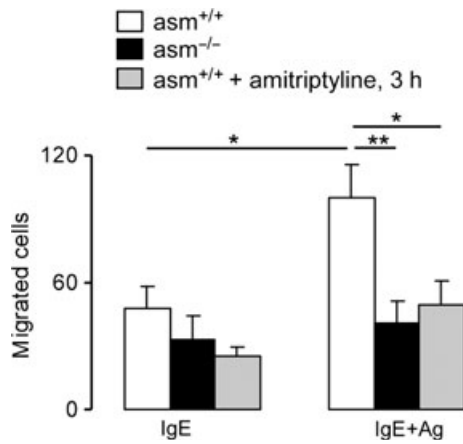


Fig. 6. Migration of *asm*^{+/+} and *asm*^{-/-} BMMCs, effect of amitriptyline. Arithmetic means $\pm$ SEM ($n = 8$) of migrating *asm*^{+/+} BMMCs, untreated (open bars) or treated with amitriptyline (500 nM, 3 h, grey bars) and untreated *asm*^{-/-} (closed bars) BMMCs. BMMCs were either stimulated with IgE alone (300 ng/mL, 3 h) or with IgE (300 ng/mL, 3 h) and then with antigen (1 ng/mL, 3 h). * ($P < 0.05$), ** ($P < 0.01$), ANOVA.

in the presence of amitriptyline (500 nM, 3 h). As shown in Fig. 6, amitriptyline indeed inhibited the migration of *asm*^{+/+} BMMCs.

Systemic anaphylactic reaction in *asm*^{+/+} and *asm*^{-/-} mice

To define whether Asm-sensitive Ca^{2+} entry and degranulation affect mast cell function *in vivo*, passive systemic anaphylaxis was tested in *asm*^{+/+} and *asm*^{-/-} mice (Fig. 7). The mice were sensitized with anti-DNP IgE intraperitoneally, and after overnight rest, they received DNP-HSA antigen or saline as a control by intraperitoneal injection. In the following, body temperature was monitored. As a result, the decline in body temperature following antigen treatment was significantly blunted in *asm*^{-/-} mice (Fig. 7).

Discussion

The present study reveals a novel function of acid sphingomyelinase (Asm), that is, regulation of mast cell Ca^{2+} concentration, Ca^{2+} -activated K^+ ($\text{K}_{\text{Ca}3.1}$) channel activity, degranulation, migration and finally, *in vivo* sensitivity to anaphylaxis. Ca^{2+} release, activity of $\text{K}_{\text{Ca}3.1}$ channels, mast cell degranulation, migration and anaphylactic reaction are all decreased in BMMCs from Asm knockout mice (*asm*^{-/-}) as compared to BMMCs from their wild-type littermates (*asm*^{+/+}). Soluble ceramide analogues or bacterial sphingomyelinase has been previously shown to induce apoptotic death of murine BMMCs even in the presence of the growth and survival factors IL-3 and/or stem cell factor [55]. The present

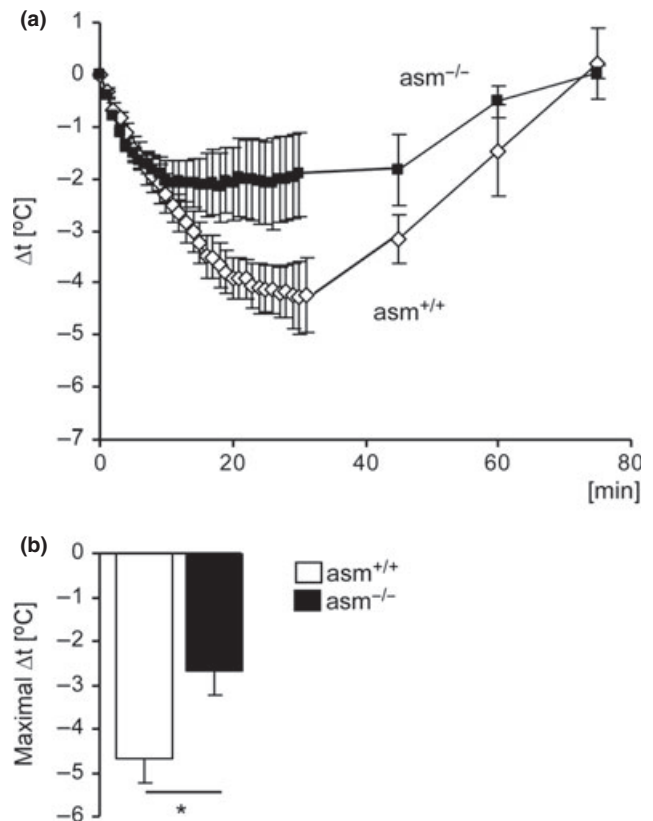


Fig. 7. Systemic anaphylactic reaction in *asm*^{+/+} and *asm*^{-/-} mice. (a) Changes in body temperature ($\Delta^{\circ}\text{C}$) of *asm*^{-/-} mice ($n = 5$, closed squares) and their wild-type littermates *asm*^{+/+} ($n = 7$, open squares) following induction of anaphylaxis ($\pm$ SEM). Mice were sensitized with anti-DNP IgE (2 $\mu\text{g/g}$ body weight) by i.p. application and challenged with DNP-HSA (4.8 $\mu\text{g/g}$ body weight, i.p.) after overnight rest. (b) Arithmetic means ($\pm$ SEM) of maximal changes in body temperature ($\Delta^{\circ}\text{C}$) of *asm*^{-/-} mice ($n = 5$, closed bars) and their wild-type littermates *asm*^{+/+} ($n = 7$, open bars) following induction of anaphylaxis. * ($P < 0.05$), two-tailed unpaired *t*-test.

study reports that in contrast to exogenously added Asm, endogenously expressed Asm up-regulates mast cell responsiveness to IgE-dependent stimulation.

According to the earlier studies [17–24, 29, 65], mast cell degranulation is governed by the release of Ca^{2+} from internal stores followed by the Ca^{2+} entry through Ca^{2+} release-activated Ca^{2+} channels (I_{CRAC}). Short-chain ceramide analogues added exogenously to RBL-2H3 mast cells have been shown to inhibit I_{CRAC} [66]. However, long-chain ceramides that are structurally closer to natural ceramides or sphingomyelinase treatment failed to significantly alter I_{CRAC} in RBL-2H3 cells [66]. Moreover, the effect of C2 ceramide on I_{CRAC} closely resembles the effect of sphingosine, a metabolite into which C2 ceramide can be converted. In the present study, the entry of Ca^{2+} upon store depletion induced by thapsigargin was not affected by Asm deficiency. However, the release of internal Ca^{2+} from the stores was strongly impaired in *asm*^{-/-} mast cells, and

consequently, the antigen-induced increase in $[Ca^{2+}]_i$ was blunted in those cells. It has recently been shown that depletion of intracellular Ca^{2+} inhibits Asm translocation to the outer leaflet of the cell membrane and formation of ceramide-enriched platform [49]. On the other hand, impaired Ca^{2+} release in $asm^{-/-}$ mast cells and rapid and strong activation of Asm upon FcεRI cross-linking demonstrate that Asm can also act upstream of the Ca^{2+} release, suggesting an existence of a positive feedback mechanism between Ca^{2+} signalling and Asm activity.

Conversion of ceramide to sphingosine (by the action of ceramidase) may be followed by phosphorylation of sphingosine (by sphingosine kinase) to yield S1P (sphingosine-1-phosphate), another bioactive metabolite essential for mast cell chemotaxis and degranulation [67]. S1P has been shown to induce Ca^{2+} release from internal stores [67], and thus, a positive effect of Asm on Ca^{2+} release in the present study may result from S1P formation downstream from Asm-generated ceramide. On the other hand, ceramide could be phosphorylated by ceramide kinase (CERK) to yield ceramide-1-phosphate, which plays an important role in mast cell activation [68]. Another mechanism could be partitioning of the FcεRI to lipid domains [69], the formation of which might depend on Asm-generated ceramide. Translocation and clustering of FcεRI in the lipid rafts are required for the activation of early signalling events initiated by FcεRI cross-linkage [69].

Entry of Ca^{2+} depends on the potential difference across the cell membrane [70]. Thus, Ca^{2+} entry is enhanced following the activation of Ca^{2+} -activated K^+ channels $K_{Ca3.1}$, which provide a positive feedback regulation of I_{CRAC} [17, 26–28, 60]. Blunted increase in $[Ca^{2+}]_i$ upon antigen stimulation in $asm^{-/-}$ mast cells presumably accounts for the impaired antigen-induced activation of $K_{Ca3.1}$ channels, as activation of $K_{Ca3.1}$ channels upon ionomycin was similar in $asm^{+/+}$ and $asm^{-/-}$ cells.

Antigen-induced mast cell migration has been shown to critically depend on $[Ca^{2+}]_i$ [71]. When intracellular or extracellular Ca^{2+} is sequestered or the membrane potential is depolarized (and thus the driving force for Ca^{2+} influx is reduced), mast cells do not migrate [71]. As Asm activity is required for both $[Ca^{2+}]_i$ increase

and $K_{Ca3.1}$ channel activation, the complete abrogation of antigen-induced migration upon genetic or pharmacological inhibition of Asm corresponds well to those previous studies. The mechanism downstream of $[Ca^{2+}]_i$ leading to mast cell migration is currently incompletely understood, but involves the actin cytoskeleton dynamics, as a rise in $[Ca^{2+}]_i$ leads to actin depolymerization [72–74].

As Asm activity influences Ca^{2+} release, activity of Ca^{2+} -activated K^+ channels, mast cell degranulation, migration and anaphylactic reaction, activation of Asm is expected to enhance the sensitivity of mast cells to activators. Acid sphingomyelinase is up-regulated or activated by a number of inflammatory mediators such as platelet-activating factor [34], amyloid [48, 75] and plasminogen activator inhibitor 1 [76]. Accordingly, Asm plays a decisive role in inflammation [32, 34, 35] and diabetes [42–44].

In conclusion, Asm up-regulates in mast cells antigen-stimulated Ca^{2+} release, subsequent activation of Ca^{2+} -activated K^+ channels, degranulation and migration. The increase in Ca^{2+} release eventually leads to enhanced sensitivity to anaphylaxis.

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Conflict of interest

The authors of this manuscript reported no financial interests or potential conflict of interests.

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