

Blunted IgE-Mediated Activation of Mast Cells in Mice Lacking the Ca^{2+} -Activated K^+ Channel $\text{K}_{\text{Ca}3.1}$ ¹

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Mast cell stimulation by Ag is followed by the opening of Ca^{2+} -activated K^+ channels, which participate in the orchestration of mast cell degranulation. The present study has been performed to explore the involvement of the Ca^{2+} -activated K^+ channel $\text{K}_{\text{Ca}3.1}$ in mast cell function. To this end mast cells have been isolated and cultured from the bone marrow (bone marrow-derived mast cells (BMMCs)) of $\text{K}_{\text{Ca}3.1}$ knockout mice ($\text{K}_{\text{Ca}3.1}^{-/-}$) and their wild-type littermates ($\text{K}_{\text{Ca}3.1}^{+/+}$). Mast cell number as well as in vitro BMMC growth and CD117, CD34, and FcεRI expression were similar in both genotypes, but regulatory cell volume decrease was impaired in $\text{K}_{\text{Ca}3.1}^{-/-}$ BMMCs. Treatment of the cells with Ag, endothelin-1, or the Ca^{2+} ionophore ionomycin was followed by stimulation of Ca^{2+} -activated K^+ channels and cell membrane hyperpolarization in $\text{K}_{\text{Ca}3.1}^{+/+}$, but not in $\text{K}_{\text{Ca}3.1}^{-/-}$ BMMCs. Upon Ag stimulation, Ca^{2+} entry but not Ca^{2+} release from intracellular stores was markedly impaired in $\text{K}_{\text{Ca}3.1}^{-/-}$ BMMCs. Similarly, Ca^{2+} entry upon endothelin-1 stimulation was significantly reduced in $\text{K}_{\text{Ca}3.1}^{-/-}$ cells. Ag-induced release of β-hexosaminidase, an indicator of mast cell degranulation, was significantly smaller in $\text{K}_{\text{Ca}3.1}^{-/-}$ BMMCs compared with $\text{K}_{\text{Ca}3.1}^{+/+}$ BMMCs. Moreover, histamine release upon stimulation of BMMCs with endothelin-1 was reduced in $\text{K}_{\text{Ca}3.1}^{-/-}$ cells. The in vivo Ag-induced decline in body temperature revealed that IgE-dependent anaphylaxis was again significantly (by ~50%) blunted in $\text{K}_{\text{Ca}3.1}^{-/-}$ mice. In conclusion, $\text{K}_{\text{Ca}3.1}$ is required for Ca^{2+} -activated K^+ channel activity and Ca^{2+} -dependent processes such as endothelin-1- or Ag-induced degranulation of mast cells, and may thus play a critical role in anaphylactic reactions. *The Journal of Immunology*, 2008, 180: 8040–8047.

Allergic reactions of the immediate type (1, 2), including allergic rhinitis (3), asthma (4), and anaphylaxis, are mediated by the cross-linking of IgE receptors (FcεRI) on mast cells. In addition, mast cells contribute to the development of T cell-mediated delayed-type hypersensitivity reactions such as contact hypersensitivity or experimental allergic encephalitis, a murine model of multiple sclerosis (5–7). Upon activation, mast cells release a variety of factors such as cytokines and proteases and thus regulate the function of inflammatory cells such as neutrophils and T cells (5, 8–16). Moreover, it is well-understood that mast cells, similar to dendritic cells, can serve as important mediators between innate and adaptive immunity (17).

Mast cells can secrete histamine and immunoreactive leukotriene C_4 in response to nonimmunological stimuli, such as endothelin-1, a potent vasoconstrictor (18, 19). Mast cell activation, upon

both FcεRI aggregation and endothelin-1 stimulation, involves an increase in intracellular Ca^{2+} concentration, which is partially accomplished by Ca^{2+} release from intracellular stores (20–25). The increase of cytosolic Ca^{2+} also results from activation of store-operated Ca^{2+} channels (SOCs)⁵ in the plasma membrane, which are important for the release of inflammatory mediators (26–33).

The electrical driving force for Ca^{2+} entry is provided by the cell membrane potential, which is maintained by the activity of K^+ channels. Activation of K^+ channels, with subsequent hyperpolarization of the cell membrane, enhances mast cell degranulation, because cell membrane Ca^{2+} influx is greater at negative membrane potentials (34). Conversely, depolarization of the cell membrane by activation of the cation channel TRPM4 decreases Ca^{2+} influx and subsequent degranulation (35). Furthermore, decreasing the chemical driving force for K^+ exit by increasing extracellular K^+ concentration (36) or inhibiting K^+ channels with Ba^{2+} and quinidine (37) inhibits mast cell degranulation.

K^+ channels expressed in mast cells include Ca^{2+} -activated K^+ channels, which have been implicated in the regulation of mast cell exocytosis (30). However, the nonselective $\text{K}_{\text{Ca}3.1}$ blocker charybdotoxin only modestly inhibited histamine release from human lung mast cells (38). In contrast, the $\text{K}_{\text{Ca}3.1}$ opener 1-EBIO enhanced IgE-dependent Ca^{2+} influx and degranulation in response to a submaximal stimulus (38). $\text{K}_{\text{Ca}3.1}$ has not been demonstrated in rodent mast cells by electrophysiological means. However, in the RBL-2H3 rat mast cell line charybdotoxin and the

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⁵ Abbreviations used in this paper: SOC, store-operated Ca^{2+} channel; BMMC, bone marrow-derived mast cell; HSA, human serum albumin; RVD, regulatory cell volume decrease; TRAM-34, 1-[(2-chlorophenyl)diphenylmethyl]-1H-pyrazole.

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nonselective blocker cetiedil did attenuate degranulation and activation-dependent hyperpolarization of the plasma membrane (38, 39). However, another $K_{Ca3.1}$ blocker clotrimazole had no effect in these cells at pharmacologically relevant concentrations (39, 40). The precise role that $K_{Ca3.1}$ plays in mast cell mediator release is therefore unclear, although its presence does appear to be important for human lung mast cell migration (41). Moreover, the pharmacological tools are not necessarily specific for $K_{Ca3.1}$, because clotrimazole and TRAM-34 (1-[(2-chlorophenyl)diphenylmethyl]-1H-pyrazole) were reported to also inhibit nonselective cation channels (42, 43).

The present study has therefore been performed to define the contribution of the Ca^{2+} -activated K^+ channel $K_{Ca3.1}$ to mast cell function, both in vitro and in vivo. To this end, we isolated mast cells from gene-targeted mice lacking $K_{Ca3.1}$ ($K_{Ca3.1}^{-/-}$) (44) and their wild-type littermates ($K_{Ca3.1}^{+/+}$). Our findings show that loss of $K_{Ca3.1}$ channel expression significantly impairs mast cell volume regulation, IgE- and endothelin-1-induced mast cell Ca^{2+} influx and degranulation, as well as the anaphylactic response. $K_{Ca3.1}$ channels are, therefore, a critical regulator of mast cell function.

Materials and Methods

Mice

All animal experiments were conducted according to the guidelines of the American Physiological Society, as well as the German law for the welfare of animals, and were approved by local authorities. $K_{Ca3.1}$ -deficient mice ($K_{Ca3.1}^{-/-}$) were generated as described (44) and maintained at the animal facility of the Pharmaceutical Institute (Department of Pharmacology and Toxicology, University of Tübingen, Tübingen, Germany). Briefly, targeted deletion of $K_{Ca3.1}$ in mice was performed using the Cre/loxP system yielding L1/+ clones. Correctly targeted L1/+ clones were injected into C57BL/6 blastocysts and the resulting chimeras were mated with 129Sv mice to obtain germline transmission. Heterozygous offspring were intercrossed with C57BL/6 mice to produce wild-type ($K_{Ca3.1}^{+/+}$) and $K_{Ca3.1}^{-/-}$ mice on a hybrid 129Sv/C57BL/6 background (always F_2). Genotyping was performed by PCR using three primers amplifying either the wild-type (264 bp) or the knockout (507 bp) allele (for details, see Ref. 44). Homozygous $K_{Ca3.1}^{-/-}$ mice are viable, fertile, and do not show reduced life expectancy. Either litter- or age-matched wild-type ($K_{Ca3.1}^{+/+}$) and $K_{Ca3.1}^{-/-}$ mice of either sex (with hybrid 129Sv/C57BL/6 background, always F_2 generation) were randomly assigned to the experimental procedures with respect to German legislation on animal protection.

Culture of bone marrow-derived mast cells (BMMCs)

Mast cells were isolated from femoral bone marrow of 6- to 8-wk-old naive $K_{Ca3.1}^{+/+}$ and $K_{Ca3.1}^{-/-}$ mice and cultured for 4 wk in RPMI 1640 (Invitrogen Life Technologies) containing 10% FCS, 1% penicillin/streptomycin, 20 ng/ml IL-3 (R&D Systems), and 100 ng/ml of the *c-kit* ligand stem cell factor (PeproTech). Mast cell numbers were determined by a trypan blue dye exclusion assay in 0.1% trypan blue. Viable (trypan blue-negative) cells were counted every 3–5 days and growth curves were drawn using the cultured time as abscissa and number of living cells as ordinate. BMMC maturation was confirmed by flow cytometry (FACSCalibur; BD Biosciences) using the following specific fluorescent-labeled Abs: PE-labeled anti-FcεRI (eBioscience), allophycocyanin-labeled anti-CD117 (BD Pharmingen), and FITC-labeled anti-CD34 (BD Pharmingen). Cells were kept in culture 4–6 wk before the experiments. For experiments, BMMCs were sensitized for 1 h with monoclonal mouse anti-DNP mouse IgE (anti-DNP IgE, 5–10 μ g/ml per 1×10^6 cells, clone SPE-7; Sigma-Aldrich) in culture medium and challenged with DNP-human serum albumin (DNP-HSA; 5–100 ng/ml; Sigma-Aldrich). Alternatively, BMMCs were stimulated by endothelin-1 (100 nM; PeptaNova).

Patch clamp

Patch-clamp experiments have been performed at room temperature in voltage-clamp, fast whole-cell mode (45). 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: 145 mM NaCl, 5 mM KCl, 2 mM $MgCl_2$, 1 mM $CaCl_2$, 10 mM glucose, 10 mM HEPES/NaOH (pH 7.4). Borosilicate glass pipettes (2- to 4-megohm (Mohm) tip resistance; GC 150 TF-10; Clark Medical Instruments) manufactured by a microprocessor-driven DMZ puller (Zeitz) were used in combination with a

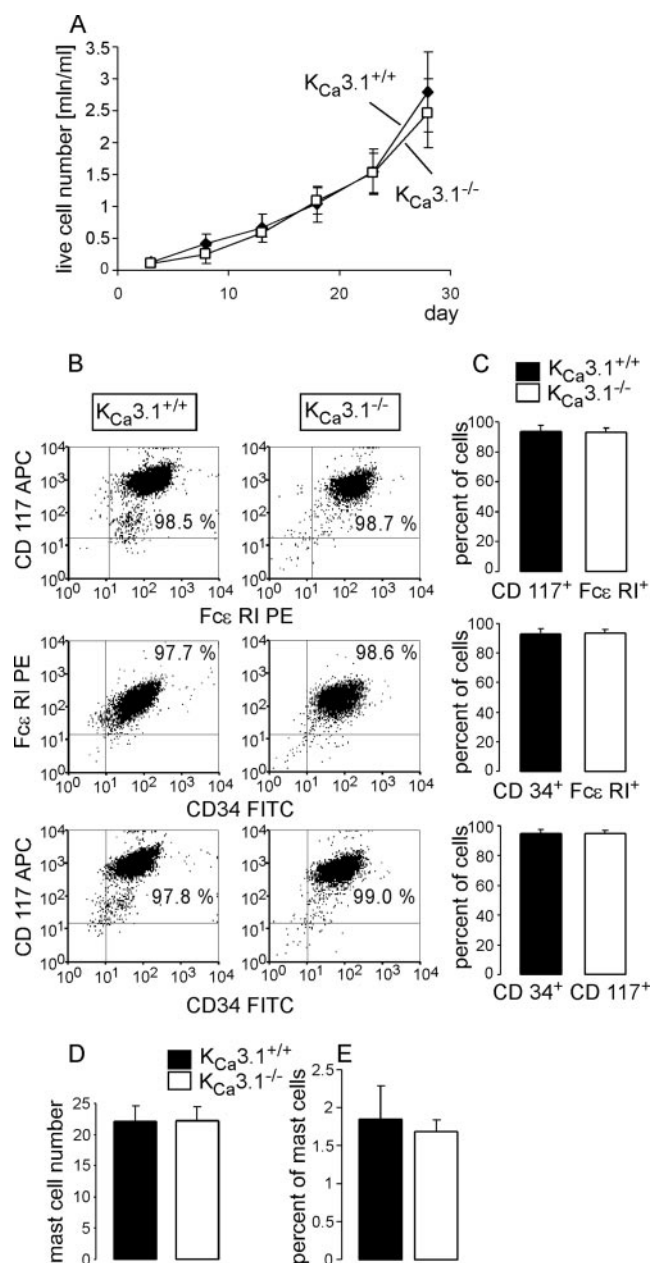
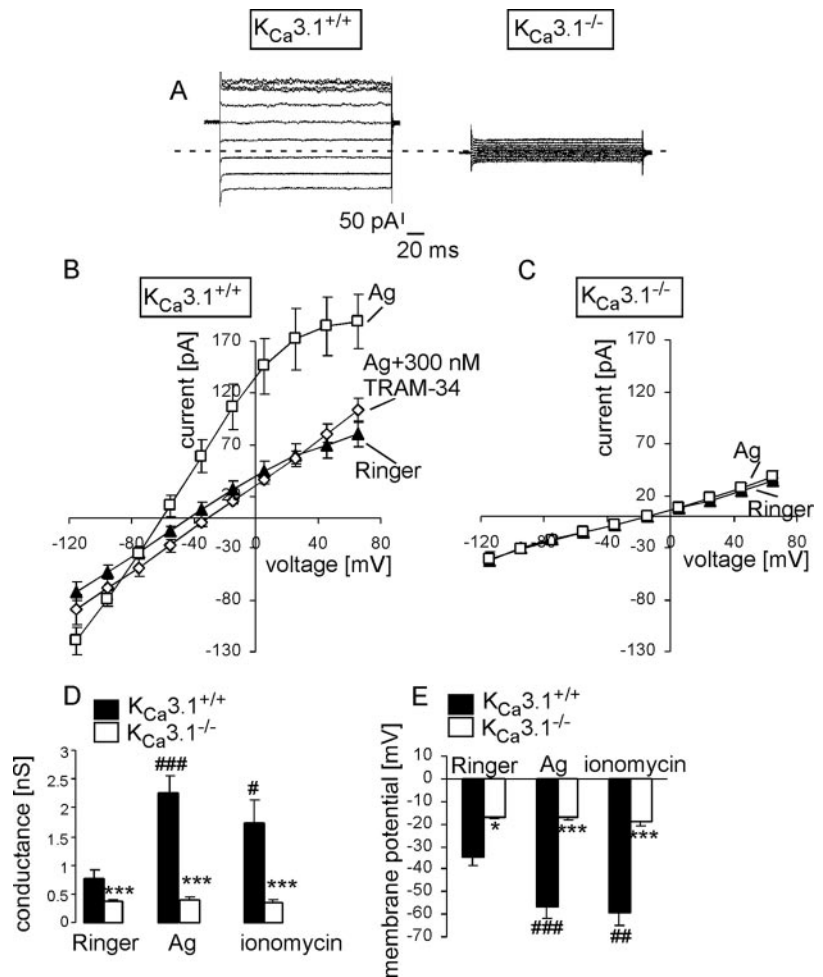


FIGURE 1. Mast cell number and in vitro BMMC growth and maturation. **A**, Time course of $K_{Ca3.1}^{+/+}$ and $K_{Ca3.1}^{-/-}$ BMMC culture growth. **B**, Original dot plots of CD117-, CD34- and FcεRI-positive BMMCs from $K_{Ca3.1}^{+/+}$ and $K_{Ca3.1}^{-/-}$ mice. Numbers depict the percent of cells in the respective quadrant, acquired within the mast cell gate. **C**, Amount of BMMCs in primary culture. Mean percent ($\pm$ SEM, $n = 3$ –4 individual BMMC cultures) of $K_{Ca3.1}^{+/+}$ (■) and $K_{Ca3.1}^{-/-}$ (□) BMMCs acquired within the mast cell gate. **D**, Density of mast cells ($\pm$ SEM) in skin, analyzed by staining of ear sections with toluidine blue. Numbers depict toluidine-positive cells in 18 view fields from three $K_{Ca3.1}^{+/+}$ and three $K_{Ca3.1}^{-/-}$ mice. **E**, Percent of mast cells ($\pm$ SEM) among leukocytes in the peritoneum of three $K_{Ca3.1}^{+/+}$ and three $K_{Ca3.1}^{-/-}$ mice.

MS314 electrical micromanipulator (MW; Märzhäuser). The currents were recorded by an EPC-9 amplifier (HEKA) using Pulse software (HEKA) and an ITC-16 Interface (Instrutech). 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 and 3 kHz low-pass filtered.

The pipette solution contained: 140 mM K-gluconate, 5 mM KCl, 1.2 mM $MgCl_2$, 2 mM EGTA, 1.26 mM $CaCl_2$ (pCa 7), 2 mM Na_2ATP , and 10 mM HEPES/KOH (pH 7.2), and was used in combination with NaCl

FIGURE 2. Lack of hyperpolarizing Ca^{2+} -activated K^+ currents upon Ag-induced activation in $K_{Ca3.1}^{-/-}$ BMMCs. **A**, Representative whole-cell currents from $K_{Ca3.1}^{+/+}$ (left) and $K_{Ca3.1}^{-/-}$ (right) BMMCs elicited by 200-ms pulses ranging from -115 to $+65$ mV in 20-mV increments from a holding potential of -35 mV. Currents were recorded in a standard NaCl bath solution 2 min after stimulation with Ag (50 ng/ml). The dotted line indicates the zero current value. **B**, Mean I-V relationships ($\pm$ SEM, $n = 8-13$) in $K_{Ca3.1}^{+/+}$ BMMCs before (Ringer, $\blacktriangle$) or 3 min after stimulation with Ag (Ag, 50 ng/ml, $\square$) and then after inhibition by 300 nM TRAM-34 (Ag + 300 nM TRAM-34, $\diamond$). **C**, Mean I-V relationships ($\pm$ SEM, $n = 12-14$) in $K_{Ca3.1}^{-/-}$ BMMCs before (Ringer, $\blacktriangle$) or 3 min after stimulation with Ag (Ag, 50 ng/ml, $\square$). **D**, Mean whole-cell conductance ($\pm$ SEM) of $K_{Ca3.1}^{+/+}$ ($\blacksquare$) and $K_{Ca3.1}^{-/-}$ ($\square$) BMMCs as recorded in **A**, before (Ringer) and after stimulation with either Ag (Ag, 50 ng/ml) or ionomycin ($1 \mu M$). Data were calculated by linear regression between -55 and $+5$ mV. ***, ($p < 0.001$) indicates significant difference between $K_{Ca3.1}^{+/+}$ and $K_{Ca3.1}^{-/-}$ cells (ANOVA); # ($p < 0.05$) and ### ($p < 0.001$) indicate significant difference from $K_{Ca3.1}^{+/+}$ cells in Ringer alone (ANOVA). **E**, Mean membrane potential ($\pm$ SEM, $n = 5-11$) in $K_{Ca3.1}^{+/+}$ ($\blacksquare$) and $K_{Ca3.1}^{-/-}$ ($\square$) BMMCs before (Ringer) and after stimulation with either Ag (Ag, 50 ng/ml) or ionomycin ($1 \mu M$). * ($p < 0.05$) and *** ($p < 0.001$) indicate significant difference between $K_{Ca3.1}^{+/+}$ and $K_{Ca3.1}^{-/-}$ cells; ## ($p < 0.01$) and ### ($p < 0.001$) indicate significant difference from $K_{Ca3.1}^{+/+}$ cells in Ringer alone (ANOVA).



Ringer bath solution. Where indicated, the Ag DNP-HSA (50 ng/ml), endothelin-1 (100 nM), the channel blocker TRAM-34 (300 nM; Sigma-Aldrich), and/or the Ca^{2+} ionophore ionomycin ($1 \mu M$; Sigma-Aldrich) were 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 (46). 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 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 are depicted as downward deflections of the original current traces.

Intracellular calcium measurements

Intracellular Ca^{2+} measurements were performed as described (47) using fura-2AM. Briefly, BMMCs were loaded with fura-2AM ($2 \mu M$; Molecular Probes) for 20 min at $37^\circ C$. The acetomethyl ester of fura-2 is an uncharged nonfluorescent molecule easily permeating the cell membrane. Once the dye is inside the cell, the lipophilic groups are cleaved by esterases. This results in the charged fluorescent form of the dye that is trapped within the cell. Additionally, the hydrolysis of the esterified groups is essential for binding of the target ion Ca^{2+} . The cells were continuously superfused to remove leaked-out extracellular fura-2.

Fluorescence measurements were conducted with an inverted phase-contrast microscope (Axiovert 100; Zeiss). Cells were excited alternatively at 340 and 380 nm and the light was deflected by a dichroic mirror into either the objective (Fluar $\times 40/1.30$ oil; Zeiss) or a camera (Proxitronic) mounted on the microscope. Emitted fluorescence intensity was recorded at 510 nm and data acquisition was performed by using Metafluor computer software (Universal Imaging). To determine the fluorescence intensity in single cells, the regions of interest were determined as circuits closely surrounding individual cells.

After a control period to wash the cells and remove any nongathered dye, intracellular Ca^{2+} was measured before and following addition of DNP-HSA (50 ng/ml) to IgE-sensitized BMMCs, in the absence or presence of extracellular Ca^{2+} . Alternatively, Ca^{2+} was measured upon stimulation of BMMCs with endothelin-1 (100 nM). As a measure of the increase of cytosolic Ca^{2+} , the slope and peak of the changes in the 340/380-nm ratio were calculated for each experiment.

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

Measurements of cell volume

Regulatory cell volume decrease was estimated from fura-2AM fluorescence at the intracellular Ca^{2+} concentration-insensitive isosbestic point (48). Briefly, the BMMCs were loaded with $2 \mu M$ fura-2AM for 20 min at $37^\circ C$ and then excited at 357 nm. Fluorescence emission was recorded at 510 nm. To measure cell volume-regulatory behavior, the initial average fluorescence of the fura-2AM-loaded cells was obtained in standard Ringer (containing: 60 mM NaCl, 5 mM KCl, 1 mM $CaCl_2$, 1.2 mM $MgSO_4$, 2 mM Na_2HPO_4 , 32 mM HEPES, 5 mM glucose (pH 7.4), 290 milliosmole (mosm/L) for 2–3 min and was standardized to 1.00 (f_0). Subsequently, the osmolarity was reduced to 160 mosm/L and changes in the fluorescence intensity (f) according to changes in cell volume were recorded. For data interpretation, the measured fluorescence was compared with the initial fluorescence intensity (f/f_0).

Measurement of degranulation and cytokine production

Mature bone marrow mast cells were seeded on 96-well plates in fresh medium with anti-DNP IgE for 1 h. Afterward, cells were washed in

Tyrod's salt solution (Sigma-Aldrich) and challenged with DNP-HSA (50 ng/ml), or for positive control with 100 ng/ml PMA ester (Sigma-Aldrich) and 1 μ M ionomycin (Sigma-Aldrich), for 15 min. A total of 20 μ l of supernatant and 20 μ l of 2 mM 4-nitrophenyl *N*-acetyl- β -D-glucosaminide (Sigma-Aldrich) diluted in 0.2 M citrate buffer (pH 4.5) was added to each well of the 96-well plate and color was developed for 2 h at 37°C. The reaction was terminated with 1 M Tris buffer (pH 9.0) and the absorbance was measured at 405 nm in an ELISA microplate reader (Sunrise remote; TECAN). The data were corrected for spontaneous release and are expressed as the percent of total release (0.1% Triton X-100). Histamine and IL-6 release from mast cells was measured by ELISA according to the instructions of the manufacturer (IBL and BD Biosciences/BD Pharmingen, respectively).

Peritoneal lavage

Anesthetized mice were euthanized by cervical dislocation and the abdominal skin was cleansed with 70% ethanol. A total of 4 ml of sterile 0.9% NaCl 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 200 $\times$ *g* for 5 min and the cell pellets were resuspended in RBC lysis buffer for 1 min, recentrifuged, and then the cell pellet was resuspended in PBS. Slides were prepared as "thick blood films" and fixed with 4% paraformaldehyde. After staining with toluidine blue, at least 600 cells/slide were counted. Mast cell numbers are expressed as normalized to the corresponding leukocyte counts.

Determination of mast cell numbers in the ear

Anesthetized mice were euthanized by cervical dislocation and the skin was cleansed with 70% ethanol. Ears were cut off at the base, fixed in 4% paraformaldehyde overnight and finally embedded in paraffin. Three- to 4- μ m-thick tissue sections of the midsections of the ears were prepared, deparaffinized, and stained with toluidine blue. Mast cell numbers of at least three slices per ear were determined using a Zeiss Axiovert 200 microscope with a LD Achroplan $\times$ 40 lens in bright field mode.

Peripheral systemic anaphylaxis/Ag-induced anaphylaxis

Mice were sensitized with 50 μ g/250 μ l of anti-DNP IgE by i.p. application. The next day, mice were challenged with either DNP-HSA (120 μ g/200 μ l) or PBS. Body temperature was monitored before and each 7 min after Ag challenge with a BAT-10 type T thermocouple thermometer and a RET-3 rectal probe for mice (Physitemp Instruments; distributed by World Precision Instruments) using a Duo18 data recording system (World Precision Instruments) during the midportion of the light phase of the light cycle. Mice were placed with the tail raised and the Vaseline-covered probe was inserted 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 a.m. to 7 p.m.). Data are expressed as a change in body temperature following treatment (Δ °C).

Statistics

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

Results

BMMC growth and maturation in *K_{Ca}3.1^{-/-}* mice

To investigate the functional role of *K_{Ca}3.1* in mast cells, cells were derived from the bone marrow (BMMCs) of *K_{Ca}3.1^{+/+}* and *K_{Ca}3.1^{-/-}* mice; growth (Fig. 1A) and expression of the mast cell surface markers CD117, CD34, and Fc ϵ RI (Fig. 1, B and C) were determined. No significant difference in the cell growth or the abundance of any of the three markers for mast cell maturation was observed between BMMCs of the two genotypes (Fig. 1, A and C). Thus, targeted deletion of *K_{Ca}3.1* in mice did not influence the growth and maturation of BMMCs.

Normal mast cell numbers in *K_{Ca}3.1^{-/-}* mice

The density of mast cells in skin, analyzed by staining of ear sections with toluidine blue, was comparable in *K_{Ca}3.1^{+/+}* and

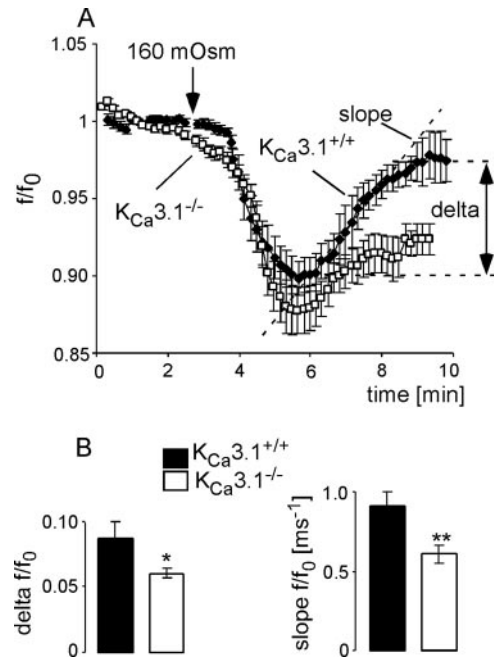


FIGURE 3. Impaired RVD of *K_{Ca}3.1^{-/-}* BMMCs upon acute hypo-osmotic challenge. *A*, BMMCs labeled with fura-2 and bathed in 290 mosm medium were imaged and then perfused with 160 mosm at the point indicated by the arrow. The curve represents relative fluorescence as a measure of cell volume changes. Note that a volume increase (cell swelling) is indicated by a decrease of fluorescence. *B*, Mean ($\pm$ SEM) of the Δ (left) and slope (right) of the RVD in *K_{Ca}3.1^{+/+}* (*n* = 10, ■) and *K_{Ca}3.1^{-/-}* (*n* = 12, □) BMMCs. * (*p* < 0.05) and ** (*p* < 0.01) indicate significant difference between genotypes (two-tailed unpaired *t* test).

K_{Ca}3.1^{-/-} mice (Fig. 1D). Similarly, the number of mast cells in the peritoneum was not different between genotypes (Fig. 1E).

Lack of Ca^{2+} -activated K^{+} currents and membrane hyperpolarization in *K_{Ca}3.1^{-/-}* BMMCs upon IgE-dependent stimulation

To analyze the currents upon stimulation of mast cells, BMMCs were incubated with IgE (5 μ g/ml) directed against the hapten DNP for 1 h, washed and then stimulated with Ag, DNP coupled to the carrier protein HSA (DNP-HSA, 50 ng/ml). According to patch-clamp experiments, the exposure of BMMCs to Ag was followed by a sharp increase of K^{+} -selective conductance in *K_{Ca}3.1^{+/+}* but not in *K_{Ca}3.1^{-/-}* BMMCs (Fig. 2, A–D). The K^{+} current was inhibited by TRAM-34, but not by apamin or iberiotoxin (Fig. 2B and data not shown). Similarly, treatment of the cells with ionomycin led to activation of the K^{+} channels in *K_{Ca}3.1^{+/+}* BMMCs but not in *K_{Ca}3.1^{-/-}* BMMCs (Fig. 2D). Thus, *K_{Ca}3.1* accounts for Ca^{2+} -activated K^{+} channel activity in BMMCs.

The membrane potential of BMMCs was measured using the current-clamp mode of the patch-clamp technique. Before application of DNP-HSA to sensitized BMMCs, the membrane potential was more depolarized (-17.0 ± 0.6 mV, *n* = 7) in *K_{Ca}3.1^{-/-}* than in *K_{Ca}3.1^{+/+}* (-35.6 ± 5.3 mV, *n* = 10) BMMCs (Fig. 2E). Applying TRAM-34 (300 nM) under resting conditions led to a pronounced depolarization (-15.4 ± 1.9 mV, *n* = 5) in *K_{Ca}3.1^{+/+}* cells, demonstrating dependence of the resting cell membrane potential on *K_{Ca}3.1*. Stimulation with 50 ng/ml DNP-HSA, was followed by a hyperpolarization to -56.9 ± 4.6 mV (*n* = 10) in *K_{Ca}3.1^{+/+}* cells, but did not significantly alter the membrane potential of *K_{Ca}3.1^{-/-}* BMMCs (-17.4 ± 1.1 mV, *n* = 7). The Ca^{2+}

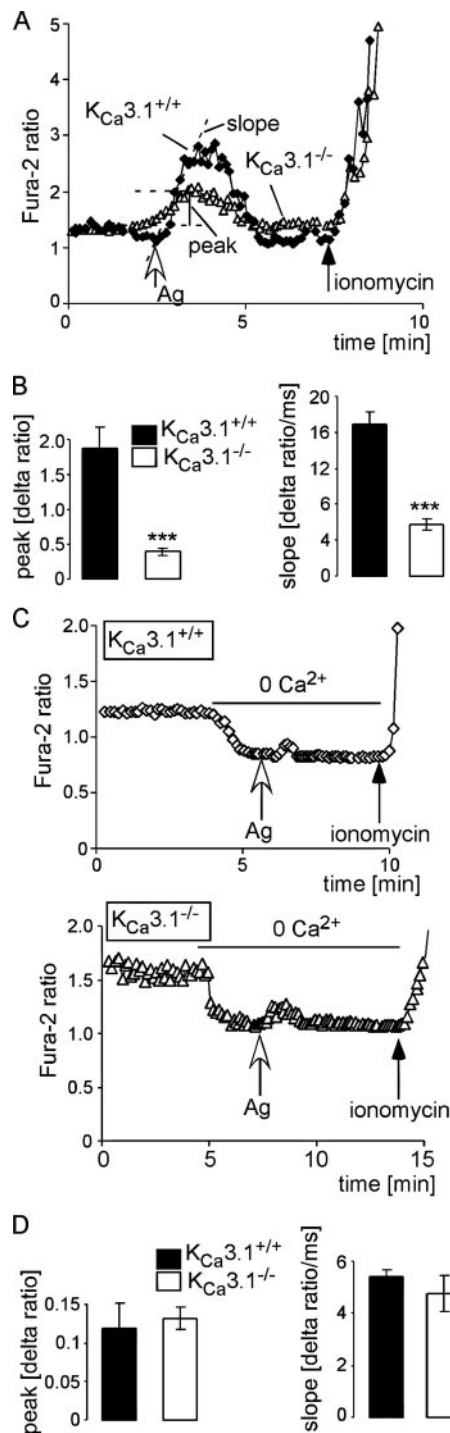


FIGURE 4. Decreased Ca^{2+} entry upon Ag-IgE stimulation in $K_{Ca}3.1^{-/-}$ BMMCs. **A**, Representative original tracings showing the ratio of 340/380 nm fura-2 fluorescence in fura-2-loaded $K_{Ca}3.1^{+/+}$ and $K_{Ca}3.1^{-/-}$ BMMCs before and after addition of Ag (Ag, 50 ng/ml). At the end of the experiments, the cells were exposed to 10 μ M ionomycin for calibration. The Ca^{2+} entry was quantified by the peak value and slope of the ratio change upon addition of Ag. **B**, Mean ($\pm$ SEM) of the peak (left) and slope (right) of the fluorescence ratio change for $K_{Ca}3.1^{+/+}$ ($n = 13$, ■) and $K_{Ca}3.1^{-/-}$ ($n = 20$, □) BMMCs upon stimulation with Ag. *** ($p < 0.001$) indicates significant difference between genotypes (two-tailed unpaired t test). **C**, Representative original tracings showing the fura-2 fluorescence ratio before and after addition of Ag (Ag, 50 ng/ml) in the absence of extracellular Ca^{2+} in a $K_{Ca}3.1^{+/+}$ (above) and a $K_{Ca}3.1^{-/-}$ (below) BMMCs. To reach a Ca^{2+} -free environment, EGTA (0.5 mM) was added to the Ca^{2+} -free bath solution. **D**, Mean ($\pm$ SEM) of the peak value (left) and slope (right) of the fluorescence ratio change for $K_{Ca}3.1^{+/+}$ ($n = 4$, ■) and $K_{Ca}3.1^{-/-}$ ($n = 4$, □) BMMCs upon stimulation with Ag.

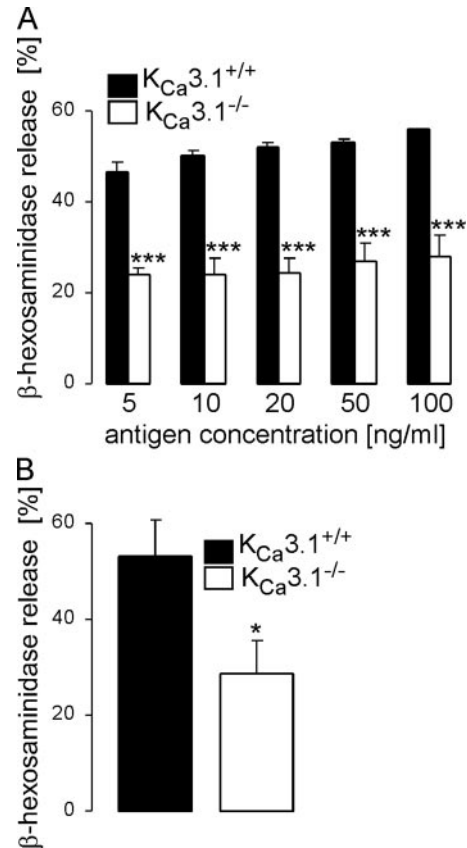


FIGURE 5. Reduced degranulation of $K_{Ca}3.1^{-/-}$ BMMCs. **A**, Mean ($\pm$ SEM, $n = 6$ individual experiments) of β -hexosaminidase release from cultured $K_{Ca}3.1^{-/-}$ BMMCs (□) and their wild-type littermates $K_{Ca}3.1^{+/+}$ (■) stimulated for 15 min with the indicated concentrations of DNP-HSA (in nanograms per milliliter). Release in the supernatant was calculated as a percentage of total cellular (0.1% Triton X-100) β -hexosaminidase. The stimulated β -hexosaminidase release in each experiment was corrected for the spontaneous release. The spontaneous release was ~6.3 and 7.2% for $K_{Ca}3.1^{+/+}$ and $K_{Ca}3.1^{-/-}$ BMMCs, respectively. *** ($p < 0.001$) indicates significant difference between genotypes (two-tailed unpaired t test). **B**, Mean ($\pm$ SEM, $n = 5$ individual experiments) of β -hexosaminidase release from cultured $K_{Ca}3.1^{-/-}$ BMMCs (□) and their wild-type littermates $K_{Ca}3.1^{+/+}$ (■) stimulated for 15 min with 100 ng/ml PMA and 1 μ M ionomycin. * ($p < 0.05$) indicates significant difference between genotypes (two-tailed unpaired t test).

ionophore ionomycin (1 μ M) similarly hyperpolarized $K_{Ca}3.1^{+/+}$ cells to -59.0 ± 5.9 mV ($n = 5$), but had no significant effect on the membrane potential of $K_{Ca}3.1^{-/-}$ BMMCs (-18.8 ± 1.8 mV, $n = 5$). In $K_{Ca}3.1^{+/+}$ cells, inhibition of DNP-HSA-induced current with TRAM-34 reversed the hyper polarization (-24.4 ± 2.6 mV, $n = 12$). Thus, $K_{Ca}3.1$ plays an important role in maintaining the resting membrane potential and in hyperpolarization upon Fc ϵ RI stimulation.

Impaired regulatory cell volume decrease in $K_{Ca}3.1^{-/-}$ BMMCs

Osmotic swelling of most cells is followed by regulatory cell volume decrease (RVD), which depends on K^+ channel activity in a wide variety of cells (49). RVD was indeed impaired in $K_{Ca}3.1^{-/-}$ BMMCs (Fig. 3A). The slope and Δ of the recovery was decreased in $K_{Ca}3.1^{-/-}$ as compared with $K_{Ca}3.1^{+/+}$ BMMCs (Fig. 3B).

Reduced Ca^{2+} entry in Ag-stimulated $K_{Ca}3.1^{-/-}$ BMMCs

According to fura-2 fluorescence experiments, exposure of IgE-sensitized mast cells to Ag led to a sharp increase in cytosolic

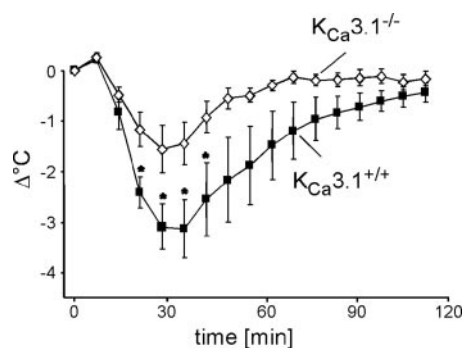


FIGURE 6. Reduced systemic anaphylactic reaction in $K_{Ca3.1}^{-/-}$ mice. Means ($\pm$ SEM, $n = 7$ mice of each genotype) of changes in body temperature ($\Delta^{\circ}\text{C}$) of $K_{Ca3.1}^{-/-}$ mice ($\diamond$) and their wild-type littermates $K_{Ca3.1}^{+/+}$ ($\blacksquare$) following induction of anaphylaxis. Mice were given i.p. anti-DNP IgE (50 μg) and challenged with 120 μg of DNP-HSA after overnight rest. * ($p < 0.05$) indicates significant difference between genotypes (two-tailed unpaired t test).

Ca^{2+} concentration in $K_{Ca3.1}^{+/+}$ BMMCs. The Ca^{2+} increase following Ag stimulation was markedly reduced in $K_{Ca3.1}^{-/-}$ BMMCs (Fig. 4A). Correspondingly, the maximal fluorescence

Δratio (peak) and the slope of the ratio ($\Delta\text{ratio}/\text{time}$) upon stimulation with Ag were significantly smaller in $K_{Ca3.1}^{-/-}$ than in $K_{Ca3.1}^{+/+}$ BMMCs (Fig. 4B). Exposure of BMMCs to a Ca^{2+} -free solution was followed by a decline of cytosolic Ca^{2+} concentration reflecting extrusion of Ca^{2+} by transport systems such as $\text{Na}^{+}/\text{Ca}^{2+}$ exchanger (Fig. 4C). Stimulation of the BMMCs with Ag in Ca^{2+} -free solution allowed an estimate of Ca^{2+} release from intracellular stores (Fig. 4C). This release was not significantly different between the genotypes (Fig. 4D).

Diminished IgE-induced degranulation in $K_{Ca3.1}^{-/-}$ BMMCs

The critical role of $K_{Ca3.1}$ in SOC-mediated Ca^{2+} influx into BMMCs suggests an important contribution of this K^{+} channel type for mast cell function, especially for Ca^{2+} -dependent processes, such as degranulation. To test this hypothesis, we analyzed the ability of $K_{Ca3.1}^{+/+}$ and $K_{Ca3.1}^{-/-}$ BMMCs to undergo degranulation in response to IgE-induced stimulation. Upon stimulation, BMMCs release β -hexosaminidase, an enzyme stored in mast cell granules (50). The granule content determined by total β -hexosaminidase levels after cell lysis with Triton X-100 was indistinguishable between the two genotypes ($p = 0.80$, t test).

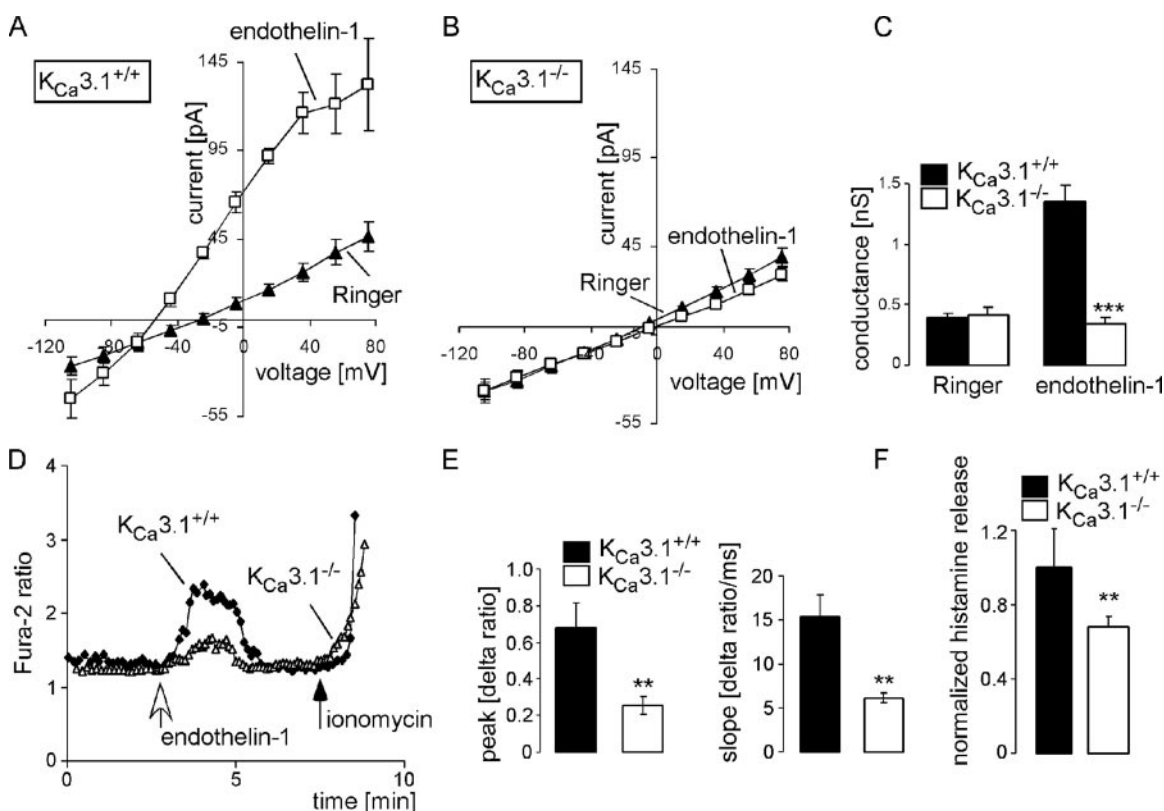


FIGURE 7. Lack of Ca^{2+} -activated K^{+} currents, reduced Ca^{2+} entry, and histamine release of endothelin-1-stimulated $K_{Ca3.1}^{-/-}$ BMMCs. **A**, Mean I-V relationships ($\pm$ SEM, $n = 4$) in $K_{Ca3.1}^{+/+}$ BMMCs before (Ringer, $\blacktriangle$) or 3 min after stimulation with endothelin-1 (100 nM, $\square$). **B**, Mean I-V relationships ($\pm$ SEM, $n = 5$) in $K_{Ca3.1}^{-/-}$ BMMCs before (Ringer, $\blacktriangle$) or 3 min after stimulation with endothelin-1 (100 nM, $\square$). **C**, Mean whole-cell conductance ($\pm$ SEM, $n = 4-5$) of $K_{Ca3.1}^{+/+}$ ($\blacksquare$) and $K_{Ca3.1}^{-/-}$ ($\square$) as recorded in **A** and **B**, before (Ringer) and after stimulation with endothelin-1 (100 nM). Data were calculated by linear regression between -55 and $+5$ mV. *** ($p < 0.001$) indicates significant difference between $K_{Ca3.1}^{+/+}$ and $K_{Ca3.1}^{-/-}$ cells (ANOVA). **D**, Representative original tracings showing the ratio of 340/380 nm fura-2 fluorescence in fura-2-loaded $K_{Ca3.1}^{+/+}$ and $K_{Ca3.1}^{-/-}$ BMMCs before and after addition of endothelin-1 (100 nM). At the end of the experiments, the cells were exposed to 10 μM ionomycin for calibration. The Ca^{2+} entry was quantified by the peak value and slope of the ratio change upon addition of endothelin-1. **E**, Mean ($\pm$ SEM) of the peak (left) and slope (right) of the fluorescence ratio change for $K_{Ca3.1}^{+/+}$ ($n = 10$, $\blacksquare$) and $K_{Ca3.1}^{-/-}$ ($n = 10$, $\square$) BMMCs upon stimulation with endothelin-1. ** ($p < 0.01$) indicates significant difference between genotypes (two-tailed unpaired t test). **F**, Mean normalized histamine release ($\pm$ SEM, $n = 6$ individual experiments) from $K_{Ca3.1}^{-/-}$ BMMCs stimulated for 30 min with 100 nM endothelin-1. The stimulated histamine release of $K_{Ca3.1}^{-/-}$ BMMCs in each experiment was first corrected for the spontaneous release and then normalized to the corresponding value of $K_{Ca3.1}^{+/+}$ cells ($\blacksquare$). The SE bar of $K_{Ca3.1}^{+/+}$ ($\blacksquare$) indicates the normalized scatter of the absolute values between the individual experiments. ** indicates significant difference from 1 ($p < 0.001$; one sample t test).

However, IgE-dependent release of β -hexosaminidase was significantly smaller in $K_{Ca3.1}^{-/-}$ as compared with $K_{Ca3.1}^{+/+}$ BMMCs, upon stimulation with Ag in a wide range of concentrations (5–100 ng/ml; Fig. 5A). Similarly, $K_{Ca3.1}^{-/-}$ BMMCs released less β -hexosaminidase when stimulated with 1 μ M ionomycin and 100 ng/ml PMA (Fig. 5B). The production of IL-6 was not significantly blunted in $K_{Ca3.1}^{-/-}$ BMMCs, though there was a downward trend (2172.34 ± 138.36 pg/ml, $n = 3$ in $K_{Ca3.1}^{+/+}$ and 1655.98 ± 24.08 pg/ml, $n = 3$ in $K_{Ca3.1}^{-/-}$ BMMCs after 24 h following Fc ϵ RI stimulation). Thus, $K_{Ca3.1}$ is indeed a decisive regulator of mast cell degranulation.

Reduced acute response to anaphylaxis in $K_{Ca3.1}^{-/-}$ mice

To evaluate whether the observed alterations in $K_{Ca3.1}^{-/-}$ BMMCs were also relevant for mast cell function in vivo, we triggered passive systemic anaphylaxis in mice. $K_{Ca3.1}^{+/+}$ and $K_{Ca3.1}^{-/-}$ mice were sensitized with anti-DNP IgE i.p. After overnight rest, mice received DNP-HSA Ag or saline as a control by i.p. injection and body temperature was monitored over time. As shown in Fig. 6, the decrease in body temperature following Ag treatment was markedly attenuated in $K_{Ca3.1}^{-/-}$ mice, an observation pointing to an impaired in vivo function of $K_{Ca3.1}$ -deficient mast cells.

Lack of Ca^{2+} -activated K^+ currents, decreased Ca^{2+} entry, and histamine release in $K_{Ca3.1}^{-/-}$ BMMCs upon endothelin-1 stimulation

BMMCs express endothelin A receptors, which are associated with Ca^{2+} -dependent release of histamine and leukotriene C_4 (18, 19). Additional experiments were thus performed to test whether $K_{Ca3.1}$ is similarly involved in the response to endothelin-1. Exposure of BMMCs to endothelin-1 (100 nM) was indeed followed by an increase of K^+ -selective conductance and hyperpolarization in $K_{Ca3.1}^{+/+}$, but not in $K_{Ca3.1}^{-/-}$ BMMCs (Fig. 7, A–C). The Ca^{2+} entry upon endothelin-1 stimulation was significantly reduced in $K_{Ca3.1}^{-/-}$ BMMCs (Fig. 7, D and E). Moreover, $K_{Ca3.1}^{-/-}$ cells secreted significantly less histamine as compared with $K_{Ca3.1}^{+/+}$ BMMCs upon endothelin-1 stimulation (Fig. 7F). Thus, $K_{Ca3.1}$ is similarly involved in the effects of endothelin-1 on Ca^{2+} -dependent mast cell responses.

Discussion

The present study discloses a key role for $K_{Ca3.1}$ in IgE-dependent mast cell degranulation. Targeted deletion of $K_{Ca3.1}$ in mice resulted in mast cell hyporesponsiveness and, as a consequence, in marked attenuation of the IgE-dependent systemic anaphylactic response. The presence of $K_{Ca3.1}$ in mast cells has been demonstrated previously in human lung mast cells by patch-clamp electrophysiology and by the presence of $K_{Ca3.1}$ mRNA in these cells (29, 30, 38). $K_{Ca3.1}$ opening is not required for, but potentiates, mast cell secretion (29, 30, 38) and plays a vital role in cell migration (41). The effect of drugs able to block $K_{Ca3.1}$ also suggested that the channel was present in the RBL-2H3 mast cell line (39, 40). The present study demonstrates for the first time that mouse BMMCs indeed express $K_{Ca3.1}$, and that BMMCs derived from $K_{Ca3.1}$ -deficient mice completely lack Ca^{2+} -activated K^+ channel activity. We show that loss of $K_{Ca3.1}$ in mouse BMMCs markedly attenuates their IgE-dependent degranulation both in vitro and in vivo.

Our findings confirm previous work that $K_{Ca3.1}$ plays a critical role in the regulation of mast cell membrane potential, and thus influences the influx of extracellular Ca^{2+} . The initial increase in cytoplasmic $[Ca^{2+}]$ following Fc ϵ RI stimulation results in the activation of $K_{Ca3.1}$ channels, which hyperpolarize the cell mem-

brane and thus provide the electrical driving force for Ca^{2+} influx through store-operated Ca^{2+} channels. Moreover, hyperpolarization increases the open probability of the Ca^{2+} channels, which are inwardly rectifying (34). Recently, it was shown that the Ca^{2+} -activated nonselective cation channel TRPM4 in mast cells acts as a negative regulator of Ca^{2+} influx following Fc ϵ RI stimulation, because the entry of cations depolarizes the membrane and limits the electrical driving force for Ca^{2+} entry through SOCs (35) and/or nonspecific cation channels permeable to divalent cations (51–53). Thus, the Ca^{2+} -activated $K_{Ca3.1}$ channel provides positive feedback for the Ca^{2+} signal, whereas the Ca^{2+} -activated TRPM4 acts as a molecular “brake” on Ca^{2+} influx.

However, the relevance of $K_{Ca3.1}$ for mast cell secretion was not entirely clear from previous studies. This was likely because the use of pharmacological inhibitors is fraught with difficulties due to nonspecific effects. For example, clotrimazole and TRAM-34 are reported to inhibit nonselective cation channels (42, 43). Our study unequivocally shows that the regulation of membrane potential by $K_{Ca3.1}$ plays a critical role in regulating the magnitude of Ca^{2+} influx and in turn degranulation.

IgE-dependent degranulation is not the only $K_{Ca3.1}$ -dependent process in mast cells. Stimulation of mast cells via endothelin receptors, which similarly requires Ca^{2+} entry (18, 19) was also impaired in $K_{Ca3.1}^{-/-}$ BMMCs. Thus, the channel is important in diverse Ca^{2+} -dependent processes of mast cells.

Targeted deletion of $K_{Ca3.1}$ in mice had no effect on mast cell growth and development. This is in good agreement with the data showing that blocking $K_{Ca3.1}$ was unable to inhibit human lung mast cell proliferation (41). We have demonstrated that the number of mast cells is normal in $K_{Ca3.1}^{-/-}$ mice. This observation is also important because it demonstrates that targeting $K_{Ca3.1}$ may not impair beneficial mast cell functions such as host defense, which makes this channel an even more attractive pharmacological target.

Beyond their role in the regulation of mast cell function, Ca^{2+} -activated K^+ channels serve a multitude of further functions in other cells. Accordingly, several defective functions have previously been described in $K_{Ca3.1}$ knockout mice, including elevated blood pressure (54) and impaired T lymphocyte and erythrocyte volume regulation (55). We show here that RVD of BMMCs was also dependent on $K_{Ca3.1}$. The present novel observation of deranged mast cell function adds to the hitherto known defects of $K_{Ca3.1}$ -deficient mice.

In conclusion, this is the first study to show that $K_{Ca3.1}$ is a critical regulator of mast cell degranulation, which plays an important role in allergic responses and anaphylaxis. Thus, $K_{Ca3.1}$ may represent a novel target for antiallergic therapy.

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Disclosures

The authors have no financial conflict of interest.

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