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Abteilung Physiologie I

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Die Rolle der PI 3-kinase und Serum- und Glukokortikoid-induzierbaren Kinasen 1 und 3 in der Regulation der Mastzellfunktion

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Abbreviations

Ag	antigen
ANOVA	analysis of variance
BMMC	bone marrow mast cell
Btk	Bruton's tyrosine kinase
CRAC	calcium release activated channels
DMSO	dimethyl sulfoxide
DNP-HSA	dinitrophenyl-human serum albumin
EDTA	ethylenediaminetetraacetic acid
EGTA	ethyleneglycoltetraacetic acid
ELISA	enzyme-linked immunosorbent assay
ENaC	epithelial natrium channel
ER	endoplasmic reticulum
FcεRI	high affinity receptor for IgE
FBS	fetal bovine serum
IgE	immunoglobulin E
IL	interleukin
ITAM	immunoreceptor tyrosine-based activation motif
KCa3.1	intermediate conductance Ca^{2+}-activated K^+ channel
KO	knockout type
LPS	lipopolysaccaride
LT	leukotriene
NHE3	Na^+/H^+ exchanger
PBS	phosphate buffered saline
PDK1	phosphoinositide dependent kinase 1
PI 3-kinase	phosphoinositide 3-kinase
PIP2	phosphatidylinositol 3,4-bisphosphate
PIP3	phosphatidylinositol 3,4,5-trisphosphate
PG	prostaglandin
PKB	protein kinase B
PKC	protein kinase C
PKG	protein kinase G

PLCγ	phospholipase Cγ
PMA	phorbol myristate ester
PTK	protein tyrosine kinase
RBL	rat basophilic leukemia cells
SCF	stem cell factor
SEM	standard error of the mean
SGK1	serum- and glucocorticoid-inducible kinase 1
SGK3	serum- and glucocorticoid-inducible kinase 3
SH2	src homology-2
SOC	store-operated channel
STIM	stromal interacting molecule
TGF-β	transforming growth factor β
T_H2	T helper 2
TLR	toll-like receptor
TNF	tumor necrosis factor
TRP channels	transient receptor potential channels
TRPC channels	canonical subfamily of transient receptor potential channels
TRPM channels	melastatin subfamily of transient receptor potential channels
TRPV channels	vanilloid subfamily of transient receptor potential channels
Wt	wild-type

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1. INTRODUCTION

1.1. Allergic diseases

In the last 20-30 years, the prevalence of allergic diseases has increased significantly - a trend that shows no signs of abating. It is estimated that 600 million people worldwide experience allergic rhinitis and 300 million people worldwide have asthma. In Europe allergic diseases, such as allergic rhinitis, asthma and eczema are the most common chronic diseases and their prevalence is growing. Up to one child among three is affected, and trends indicate that by 2015, half of all Europeans may be suffering from an allergy. Some allergies may be fatal; others seriously compromise the quality of life: over 70% of allergy patients feel limited in their daily activities. There is currently no cure for allergy and asthma, which generate costs both in treatment and regular health care use (Zuberbier 264-73;Mullol et al. 327-34;Biedermann and Rocken 534-41).

Mast cells remain at the core of our understanding of allergic inflammation, they contribute to many of the initiating and subsequent events in allergic diseases and are thus one of the major targets for investigations and therapeutic interventions in allergy (Nauta et al. 354-60).

1.2. Immunobiology of mast cells

1.2.1. Origin and development

Mast cells develop from progenitor cells that in turn arise from uncommitted hematopoietic stem cells in the bone marrow. Mast cell progenitors have been described in peripheral blood and represent a distinct pool of cells separate from leukocytes or mononuclear cells. Mast cells express the receptor c-kit for stem cell factor (SCF), a specific growth factor for mast cells. SCF is regarded as a cardinal factor in mast cell biology, after binding to c-kit, it has the capacity to induce differentiation, migration and growth of mast cells. Kirshenbaum and colleagues (Kirshenbaum 497-+;Kirshenbaum et al. 2333-42) have described $CD34^{+}$, c-kit^{+}($CD117^{+}$) and $CD13^{-}$ precursors that develop into mast cells. Mast cells deprived of SCF undergo apoptosis (Okayama and Kawakami 97-115;Berent-Maoz et al. 2272-78;Moller et al. 1330-36;Biedermann 99-109).

Another mast cell growth factor – interleukin 3 (IL-3) - seems to be crucial for early mast cell proliferation in the mouse system (Okayama and Kawakami 97-115;Itakura et al. 803-11).

Mast cells undergo terminal differentiation in tissues. They are widely distributed throughout all vascularised organs and are particularly abundant (3000-25000 mast cells/mm^3) in proximity to environmentally exposed surfaces, e.g. the skin, and in the gastrointestinal and respiratory tracts. In these tissues they occur near blood vessels, epithelia, smooth muscles and nerves. These mature cells may divide further (Yong 409-24).

1.2.2. Morphology and heterogeneity

Up to 40% of the volume of mast cell is occupied by membrane-enclosed metachromatically stained basophilic secretory granules. There are 50 to 500 secretory granules in one mature mast cell. Within a given mast cell, these granules are usually of a uniform size, but there is variability from cell to cell. Mast cell granules originate from the Golgi apparatus, which is responsible for the synthesis and organization of the preformed mediators contained therein (Yong 409-24).

Tissue mast cells are highly heterogeneous with great variability in size, granule contents, cytokine production and receptor expression. Both *in vitro* experience and *in vivo* data suggest, that this heterogeneity represents an exquisite developmental sensitivity to local signals (Okayama and Kawakami 97-115). Similarly, the maintenance of mast cells within tissues is controlled by the local environment, in particular by the production of SCF by stromal cells.

In rodent tissues mast cells are classified into two distinct subtypes: connective tissue mast cells, preferentially located in skin, and mucosal mast cells, dominantly found in mucosa, such as the intestine (Kitamura, Oboki, and Ito 164-74).

1.2.3. Activation and functions

Mast cell activation

Mast cells can be activated in different ways. Stimulation of the mast cell activation, initiated either by interaction of the antigen specific antibodies or the antigen with the corresponding mast cell receptors, is referred to as immunologic activation. Alternatively, the stimulation, induced by substances such as neuropeptides, basic compounds, cytokines, and certain drugs, is called nonimmunologic activation. Both immunologic and nonimmunologic stimulations produce morphologically similar degranulation events (Fig. I). However, biochemical processes preceding the degranulation are different.

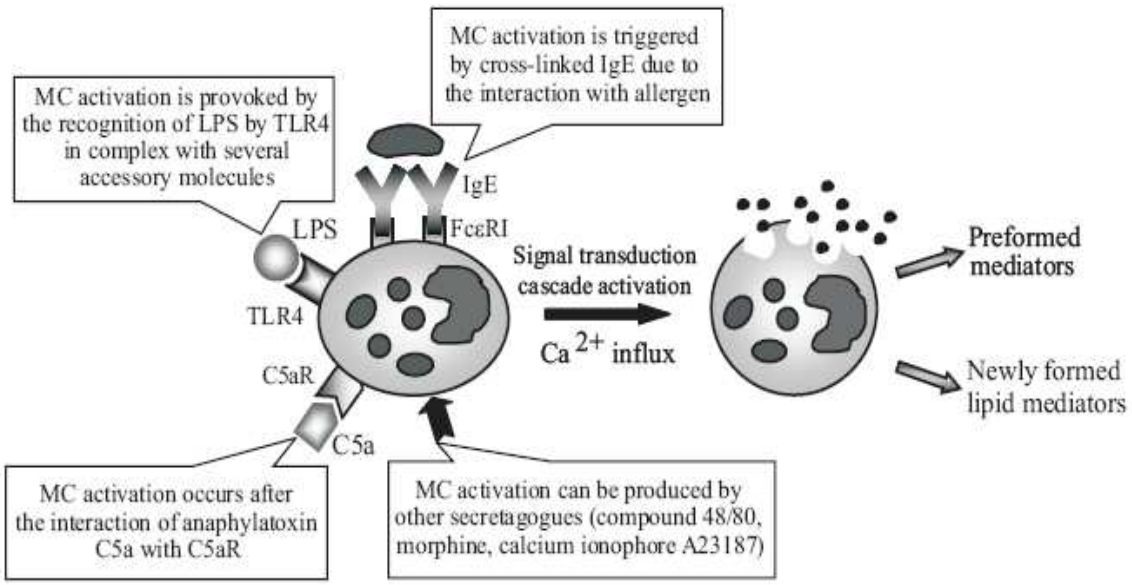

Figure I:Schematic presentation of the different ways of mast cell activation

Activation through FcεRI

The classical mast cell activation during inflammatory reaction occurs through the high affinity receptor FcεRI. FcεRI is a tetrameric complex, where an extracellular α chain binds the Fc portion of immunoglobulin E (IgE), whereas a transmembrane β chain along with disulfide-linked transmembrane γ chains participate in the signal transduction. Aggregation or so called "cross-linking" of IgE molecules bound to FcεRI on mast cells with allergen/antigen (Ag) causes mast cell degranulation. The cascade of signal transduction pathways triggers solubilization of the granule contents, granule swelling, membrane ruffling, fusion of the perigranular and plasma membranes, and, finally, leading to the exocytosis of granule content.

Degranulation of the mouse mast cells can be also triggered by aggregation of the surface-expressed IgG receptors, FcγRII and FcγRIII. These low-affinity receptors may regulate high-affinity IgE receptor-mediated activation (Yoshioka et al. 452-61;Bruhns, Fremont, and Daeron 662-69;Castells 287-92).

Other ways of mast cell activation

Complement-dependent signals are important components of the mechanisms by which mast cells are activated during infections. Mast cells express multiple receptors for the complement components (anaphylatoxins) C3a, C4a and C5a. These receptors include CD11b (CR3), CD11c (CR4), which are up-regulated during systemic mastocytosis, and C5aR. (Soruri et al.).

The TLR (Toll-like receptor) family of pattern-recognition receptors has an important role in many host defence mechanisms. Different members of the TLR family are activated by pathogen-associated or endogenous proteins. The mast cell uses selected TLRs to respond to pathogens. For example, mast cells respond to lipopolysacharide (LPS) through TLR4. In contrast, activation by peptidoglycan from gram-positive bacteria and the yeast cell-wall component zymosan are mediated through the TLR2 (Fehrenbach et al. 2087-94;Gangloff and Guenounou 115-25).

It was demonstrated that a number of cytokines (IL-1, IL-3) cause the release of histamine, SCF and macrophage inflammatory protein-1α activate the degranulation of mast cells *in vitro* and *in vivo* (Lukacs et al. 2262-68).

Dextrans and lectins appear to activate mast cells through a multipotent interaction with the cell membrane and a cross-linking of glucose receptors on the membrane. Other compounds that can directly activate mast cells include calcium ionophores, substance P (neuropeptide), compound 48/80, and drugs such as morphine, codeine and synthetic adrenocorticotropic hormone, adenosine and endothelin (Eszlari et al. 267-85;Collins et al. 843-49).

Mast cell functions

Mast cells exert their biological functions almost exclusively by humoral immune mechanisms. Nearly all mast cell functions are restricted to the release of mediators (although there are a few reports of mast-cell phagocytosis and other non-humoral functions in mice and rats). The array of mediators released by mast cells is enormous and explains how mast cells can be involved in so many different physiological (Fig. II) and pathophysiological conditions.

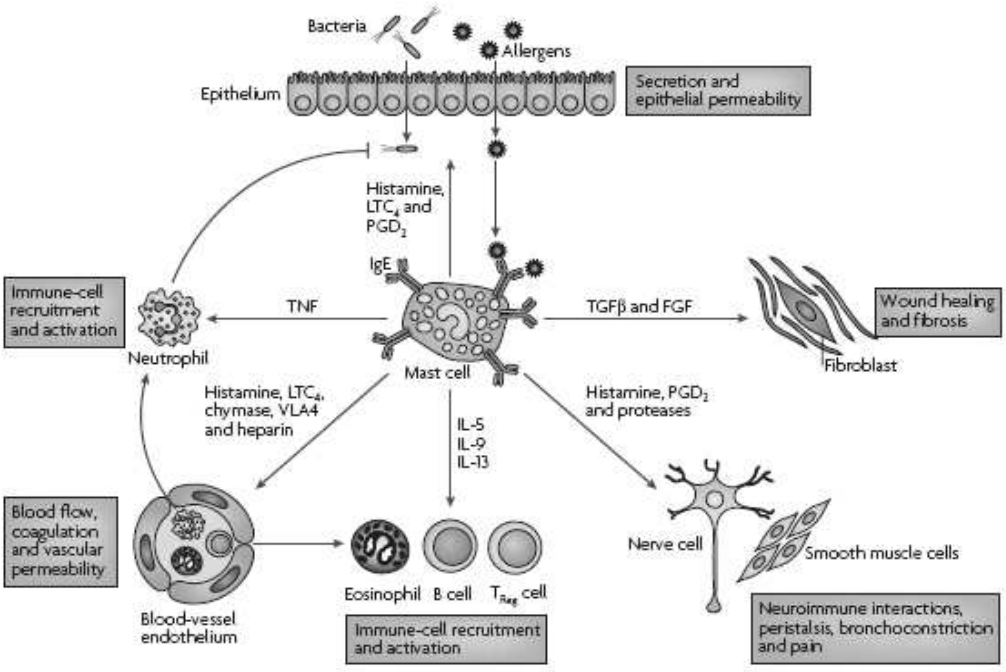

Figure II: Proposed functions of mast cells under normal conditions

Physiological mast-cell functions include the regulation of epithelial functions (secretion and epithelial permeability), smooth-muscle functions (peristalsis and bronchoconstriction), endothelial functions (blood flow, coagulation and vascular permeability), immune functions (recruitment and activation of neutrophils, eosinophils and lymphocytes), neuronal functions (neuroimmune interactions, peristalsis and pain) and other tissue functions (wound healing and fibrosis). The physiological triggers might include growth and other tissue factors, infectious agents, neuropeptides, protein antigens and physiochemical conditions, such as a change in pH or in osmolarity, FGF, fibroblast growth factor; IL, LTC_4, leukotriene C_4; PGD_2, prostaglandin D_2; TGFβ, transforming growth factor-β; TNF, tumour-necrosis factor; T_{Reg} cell, $CD4^+CD25^+$ regulatory T cell; VLA4, very late antigen 4 (Kirchhoff et al. 1591-94).

1.3. Mast cells in allergic diseases

It is well established that mast cells mediate the early phase of type I hypersensitivity reactions, as well as late-phase reactions that occur facultatively following early-phase reactions, and that are thought to cause the recurrent and chronic symptoms (Fig. III). Mast cells participate also in hypersensitivity reactions other than type I reactions, such as type IV(Nauta et al. 354-60).

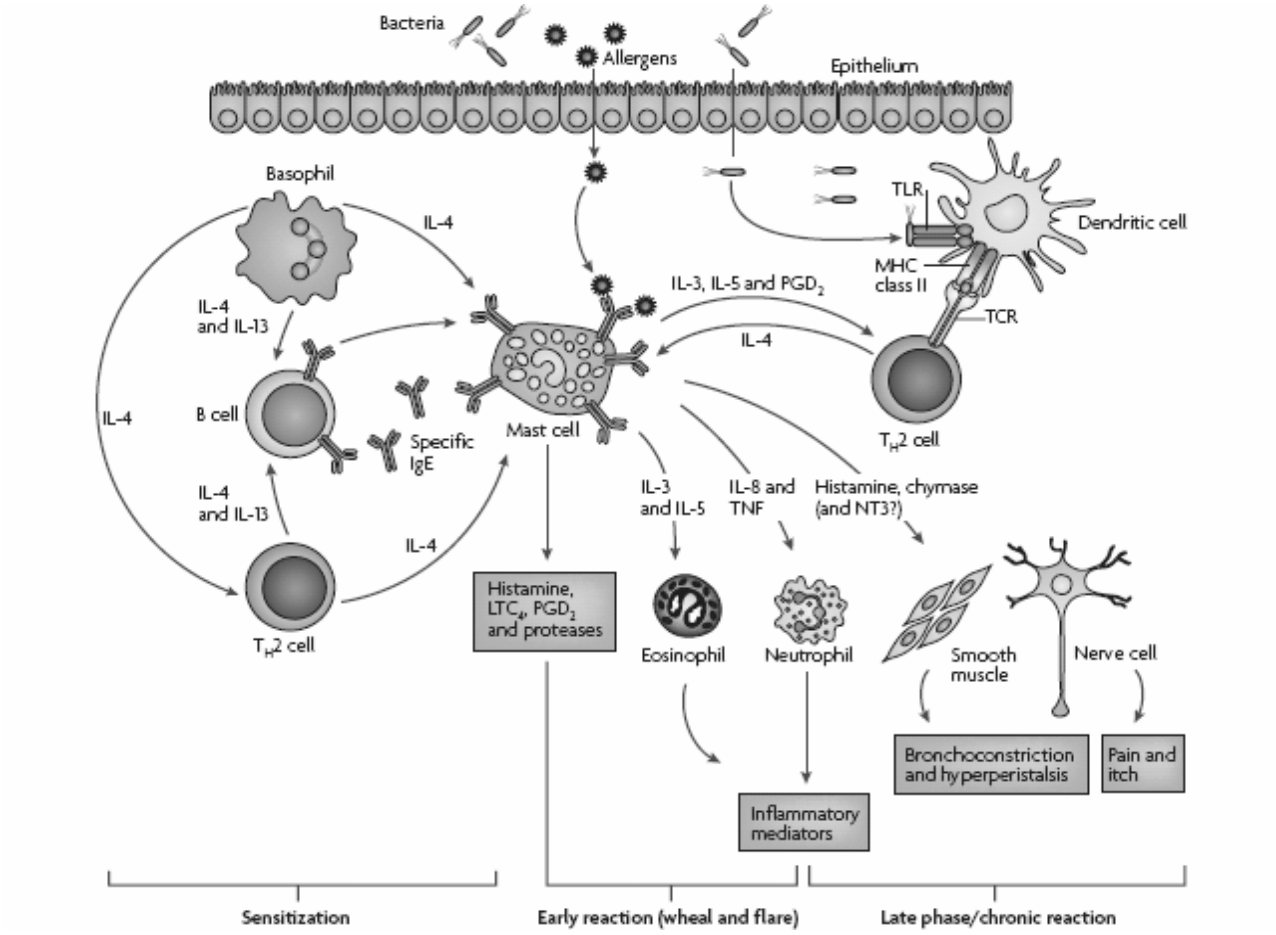

Figure III: Role of mast cells in allergic inflammation

Type I hypersensitivity is characterized by an allergic reaction that occurs immediately following contact with the antigen, referred to as the allergen (most common of them are animal products, insect sting venoms, foods, drugs, pollens etc). Mast-cell activation by IgE cross-linking with allergen requires access of allergen into the tissue and input from the adaptive immune system to be effective. Mast-cell activation needs the synthesis of specific

IgE by B cells (regulated by IL-4 and IL-13 derived from T helper 2 (T_H2) cells and basophils) and mast-cell priming by IL-4 for enhanced mediator release(Scholz, Prieschl, and Baumruker 7-18;Leung 157-67).

Within seconds to minutes after IgE cross-linking, granules in the cytoplasm of the mast cell fuse with each other and with the cell surface membrane, ejecting their contents into the extracellular milieu. The contents of the granules include histamine, proteoglycans (heparin, chondroitin sulfate), a series of neutral proteases, broadly grouped into tryptases, chymases and carboxypeptidases, and other enzymes, for example ß-hexosaminidase (so called “preformed” mediators). Histamine promotes vascular permeability; proteoglycans provide a scaffold within the granule that allows the packaging of proteases; and the neutral proteases cleave proteins from matrix and plasma in addition to activating propeptides such as the precursors for IL-1β and angiotensin II. The murine mast cell protease 6 also contributes potently to neutrophil chemotaxis. Certain subsets of mast cells store TNF within the granules as well, representing the body's only source of TNF available for immediate release(Scholz, Prieschl, and Baumruker 7-18).

Within minutes of IgE-mediated activation, mast cells begin to generate eicosanoids derived from cleavage of arachidonic acid from membrane phospholipids. Important arachidonic acid metabolites include the leukotrienes (leukotriene B4 and the cysteinyl leukotrienes), which increase vascular permeability, induce vasoconstriction and recruit leukocytes, and prostaglandins, including the neutrophil chemoattractant and vasoactive mediator prostaglandin D2.

The subsequent release of mast cell mediators induces an early reaction, consisting classically of a “wheal and flare” reaction of the skin or the mucosa.

Within hours, a later phase of mast cell activation through IgE becomes evident with the induction of new gene transcription and translation, generating a host of cytokines and chemokines. IL-3, IL-5, IL-8, TNF, neurotrophin 3 and proteases contribute to the initiation of a facultative late-phase reaction by recruiting and activating eosinophils, neutrophils and TH2 cells, and by interaction with tissue cells such as nerve cells, smooth-muscle cells, endothelial cells and the epithelium. Ongoing dysregulation of such cell types not only causes symptoms of

allergy, but also organ dysfunction, including loss of barrier function and, subsequently, increased bacterial translocation. This enables non-specific triggers to access mast cells, dendritic cells and other cells. Triggers such as bacterial products, or immunoglobulin (fragments) such as monomeric IgE and light chains might perpetuate the inflammatory process, even in the absence of allergen (Goksu and Ozdemir 577-88).

Symptoms depend on the form of allergy and allergen responsible for the occurrence of allergy, and can vary from mild to fatal. Anaphylaxis is referred to as acute systemic and very severe type I hypersensitivity allergic reaction in humans and other mammals, which involves particularly the skin, respiratory tract, cardiovascular system and gastrointestinal tract. Anaphylactic shock, the most severe type of anaphylaxis, occurs when an allergic response triggers a quick release from mast cells of large quantities of immunological mediators leading to systemic vasodilation (associated with a sudden drop in blood pressure and hypothermia in mice) and edema of bronchial mucosa (resulting in bronchoconstriction and difficulty breathing). Anaphylactic shock can lead to death in a matter of minutes if left untreated (Przybilla, Ring, and Rueff 1025-31).

1.4. Role of ion channels in mast cell function

Role of Ca^{2+} channels

IgE-dependent activation of mast cells is characterised by an influx of extracellular Ca^{2+}, which is essential for subsequent release of both preformed mediators and newly generated autacoids and cytokines.

In rodent mast cells there is evidence of Ca^{2+} influx through non-selective cation channels and store-operated channels (SOCs). The SOCs activated following depletion of intracellular Ca^{2+} stores are also known as calcium release-activated Ca^{2+} channels (CRAC). Previous data has shown a close association between Ca^{2+} influx through CRAC in rat basophilic leukemia (RBL) cells, which serve as a model of mucosal mast cells, and both degranulation and arachidonic acid metabolism with subsequent secretion of LTC4. (Chang et al. 4622-31;Liu, Groschner, and Ambudkar 93-104;Fischer et al. 1255-62)**.**

The pore-forming subunits of SOCs are presented by Orai, a recently discovered family of proteins, which consists of three closely conserved cell surface proteins (Orai1–3 or CRACM1-3) (Prakriya et al. 230-33;Feske et al. 179-85). Orai interacts with stromal interacting molecule (STIM), which acts as an endoplasmic reticulum (ER) Ca^{2+} sensor, that activates CRAC channels when Ca^{2+} levels in ER stores drop (Mignen, Thompson, and Shuttleworth 703-15;Csutora et al. 14524-31). Upon calcium store depletion, STIM proteins reorganize from their diffuse positions throughout the ER membrane to form discrete points close to the plasma membrane. Activated Orai colocalizes with STIM, concentrating calcium influx to the membrane region containing both proteins (Oh-Hora and Rao 250-58).

Researchers studying STIM1-deficient mice have found that in mast cells STIM1 is required for FcεRI-induced Ca^{2+} influx, degranulation, activation of the transcription factors NF-κB and NFAT, and IgE-dependent anaphylaxis *in vivo* (Baba et al. 81-88).

In studies exploiting STIM- and CRACM1-deficient mice it has been shown that STIM and CRACM1 are required for mast cell FcεRI-induced degranulation, synthesis of lipid mediators, cytokine release and IgE-dependent allergic responses *in vivo* (Vig et al. 89-96). Thus, it has been

conclusively demonstrated that the second stage of FcεRI-induced Ca^{2+} mobilization, which is the influx of Ca^{2+} mediated by STIM1 and CRACM1, is essential for mast cell activation *in vitro* and *in vivo* (Vig et al. 89-96;Baba et al. 81-88).

Another big group of Ca^{2+}-permeable channels is a family of transient receptor potential (TRP) channels. TRP channels are classified by their homology, including members of the canonical/TRPC subfamily, melastatin/TRPM subfamily and vanilloid/TRPV subfamily. There is an evidence that canonical transient receptor potential channels (TRPCs), which are Ca^{2+}-permeable nonselective cation channels, may associate with STIM1 and CRACM1 to enhance Ca^{2+} entry (Vig et al. 89-96;Liao et al. 2895-900).

A thermally activated TRPV2 channel has been identified in mouse BMMCs. It is expressed in the plasma membrane and since the cells exhibit a rise in cytosolic free Ca^{2+} and degranulate at > 50ºC, which is dependent on the presence of extracellular Ca^{2+}, it is appears to be biologically active (Stokes et al. 669-83;Stokes et al. 137-47).

A requirement for TRPC5 in addition to STIM1 and CRACM1 for optimal FcεRI-induced Ca^{2+} influx and degranulation has been identified in a RBL-2H3 cell line by using inhibitory RNA. The authors speculate, that TRPC5 associates with STIM1 and CRACM1 in a stoichiometric way to enhance FcεRI-induced Ca^{2+} influx and degranulation (Zhao et al. 561-68).

The rate of Ca^{2+} influx through store-operated channels is also dependent on the membrane potential, which is regulated in part by Ca^{2+}-activated nonselective cation channels such as TRPM4. Its activation results in an influx of Na^{+} that depolarizes membrane and limits the driving force for Ca^{2+} entry through CRAC channels in BMMCs**.** FcεRI-induced degranulation, release of leukotrienes and production of TNF, but not IL-6, are higher in TRPM4-deficient BMMCs, and TRPM4-deficient mice develop more severe acute (but not late-phase) inflammation during IgE-mediated passive cutaneous anaphylaxis responses. Accordingly, it has been proposed that TRPM4 acts as a 'molecular brake' on Ca^{2+} influx after FcεRI-induced activation of mast cells *in vitro* and *in vivo* (Mathar et al. 35).

Role of K^+ and Cl^- channels

Mast cells express K^+ and Cl^- channels which are likely to play an important role in mast cell activation responses through their effect on membrane potential (Bradding et al. 614-20;Duffy et al. 4261-70).

Mast cells express the intermediate conductance Ca^{2+} activated K^+ channel, $K_{Ca}3.1$ (also known as IKCa1 or SK4) (Sausbier et al. 42;Woelbing et al. S1). This channel maintains a negative membrane potential during cell activation. Following FcεRI cross-linking, mast cells open $K_{Ca}3.1$ channels. The negative cell membrane potential generated by the activity of K^+ channels enhances Ca^{2+} influx and mediator release due to both the favourable electrical driving force for Ca^{2+} entry and enhanced Ca^{2+} conductance through SOCs that mediates Ca^{2+} influx following cell activation (Duffy et al. 1653-62).

1.5. Role of PI 3-kinase in FcεRI signaling

The phosphoinositide 3-kinases (PI 3-kinases) are a ubiquitously expressed enzyme family that, through the generation of phospholipid second messengers, play a key role in the regulation of many cellular processes. PI 3-kinases participate in a broad range of signaling processes downstream of growth factors, antigens, hormones and adhesion receptors, regulating motility, proliferation, survival, glucose metabolism and cytoskeletal organization. They are classified into several distinct groups (types I-III), based on their primary structure, mode of regulation and substrate specificity. PI 3-kinases principally transduce signals through the catalytic generation of phosphatidylinositol (3,4,5)-trisphosphate, PI(3,4,5)P3, and phosphatidylinositol (3,4)-biphosphate, PI(3,4)P2, two second messengers that facilitate the recruitment of pleckstrin homology domain containing signaling proteins to the plasma membrane (Marone et al. 159-85).

Mast cell degranulation following FcεRI aggregation is generally believed to be dependent on PI 3-kinase activation. Aggregation of FcεRI leads to the activation of ß subunit-associated Lyn, a Src family protein tyrosine kinase (PTK). Activated Lyn phosphorylates tyrosine residues in the immunoreceptor tyrosine-based activation motifs (ITAMs) in the cytoplasmic regions of ß and γ subunits. Phosphorylated ß and γ ITAMs recruit Lyn and Syk, respectively. Another Src family PTK, Fyn, was also shown to associate with FcεRI and to play a complementary role, particularly by activating PI 3-kinase. These PTKs phosphorylate numerous targets and activate several signaling pathways, including the PI 3-kinase, phospholipase C (PLC) and several MAPK pathways. The activated PLCγ hydrolyzes PI(4,5)P2 to D-myo-inositol 1,4,5-trisphosphate, Ins(1,4,5)P3, and diacylglycerol, which induce the release of Ca^{2+} from intracellular stores and the activation of protein kinase C, respectively. On the other hand, stimulation of PI 3-kinase results in transient accumulation of milimolar levels of PI(3,4,5)P3 and PI(3,4)P2 (Daeron 445-65). These signaling events lead to degranulation and cytokine and chemokine production.

Although the involvement of PI 3-kinase in Ag-induced degranulation is established, a clear consensus regarding how products derived from the action of PI 3-kinase mediate the cellular responses has yet to emerge.

Data from several laboratories propose a role of PI 3-kinase in the regulation of intracellular Ca^{2+} ($[Ca^{2+}]_i$) increase in mast cells. Evidence suggests that PI 3-kinase may mediate $[Ca^{2+}]_i$ elevation via two distinct mechanisms. First, *in vitro* data indicate that PI(3,4,5)P3 stimulates Ins(1,4,5)P3 production by activating PLCγ isozymes. This PLCγ activation may be attained directly by facilitating membrane translocation or indirectly via Btk. Second, PI 3-kinase may increase $[Ca^{2+}]_i$ by facilitating Ca^{2+} mobilization across plasma membranes. This premise was prompted by data showing the inhibitory effect of PI 3-kinase inhibitors on Ag-induced Ca^{2+} response, and is in line with the notion that two distinct Ca^{2+} influx pathways (capacitative vs. non-capacitative) are operative in Ag-stimulated RBL-2H3 cells (Lee et al. 581-92;Lee et al. 581-92;Ching et al. 14814-20).

It is noteworthy that the proposed involvement of PI 3-kinase in the regulation of a non-capacitative Ca^{2+} influx pathway is reminiscent of a finding of a PI(3,4,5)P3-sensitive Ca^{2+} entry mechanism in platelets and Jurkat T-cells. Consequently, it is hypothesized that this PI(3,4,5)P3-sensitive Ca^{2+} entry is a conserved mechanism that plays a crucial role in the regulation of receptor-mediated Ca^{2+} signaling among these hematopoietic cells. This hypothesis is corroborated by data showing the involvement of this PI(3,4,5)P3-mediated Ca^{2+} influx in FcεRI-mediated Ca^{2+} response in RBL-2H3 cells (Djouder et al. 1243-50;Oka et al. 837-46;Fischer et al. 1255-62). Considering that PI 3-kinase and PLCγ are the downstream effectors of the FcεRI-mediated tyrosine kinase cascade, it is proposed that these two enzymes act in a concerted manner to initiate Ca^{2+} response to Ag stimulation. In stimulated cells, PI 3-kinase and PLCγ act on the mutual substrate PI(4,5)P2 to generate PI(3,4,5)P3 and Ins(1,4,5)P3, respectively, which activate Ca^{2+} channels at different cellular compartments to provide the elevated $[Ca^{2+}]_i$, required for optimal physiological responses (Finkelman 506-15;Bradding and Conley 979-83;Duffy et al. 4261-70).

In other cell types, PI 3-kinase-mediated signaling has been also shown to participate in the regulation of ion channels. In cells transfected with the intermediate conductance Ca^{2+}-activated K^+ channels $K_{Ca}3.1$, PI 3-kinase inhibition significantly decreased channel activity (Srivastava et al. 3630-38).

Despite the critical role of PI 3-kinase, on the one hand, and Ca- and Ca^{2+}-activated K^{+} channels, on the other hand, the influence of PI 3-kinase on the activity of these mast cell channels and thus mast cell function is not fully understood.

1.6. Serum- and glucocorticoid-inducible kinases 1 and 3

PI 3-kinase-dependent signaling involves 3-phosphoinositide (PIP3)-dependent kinase PDK1, which phosphorylates and thus activates PKB and serum- and glucocorticoid-inducible kinases 1 (SGK1) and 3 (SGK3) (Lang et al. 1151-78).

SGK1 was originally cloned in 1993 as an immediate early gene transcriptionally stimulated by serum or glucocorticoids in rat and mammary tumor cells. Transcription of SGK1 was also shown to occur rapidly in response to many agonists like mineralocorticoids (Grahammer et al. R945-R950;Richards 209-18), follicle stimulating hormone (Richards 209-18;Friedrich et al. 303-07), transforming growth factor (TGF- β) (Friedrich et al. 303-07), thrombin (BelAiba et al. 828-36), hypertonicity (Klaus et al. 1539-47), high glucose (Boini et al. 2059-66) and neuronal injury or excitotoxicity.

SGK1 belongs to the 'AGC' subfamily of serine/threonine protein kinases, which include protein kinase A (PKA) or adenosine 3', 5' monophophate (cAMP)-dependent protein kinase, PKG or guanosine 3', 5' monophosphate (cGMP)-dependent protein kinase and isoforms of PKC. SGK1 is present in the genomes of all eukaryotic organisms examined so far, including Caenorhabditis elegans, Drosphila, fish and mammals. Structure of SGK1 has been highly conserved through evolution like many other protein kinases.

Two other isoforms of SGK1 have been identified in mammals and are named as SGK2 and SGK3. The catalytic domains of SGK2 and SGK3 isoforms share 80% amino acid sequence identity with one another and with SGK1.

The human gene encoding SGK1 was found in chromosome 6q23. SGK-like gene which encodes a protein having predicted amino acid sequence identical to that of human SGK3 was found in chromosome 8q12.2.

SGK1 is expressed in humans including the pancreas, liver, heart, lung, skeletal muscle, placenta, kidney and brain, but SGK1 is not expressed in all cell types within those tissues. For example, SGK1 transcript levels are found high in acinar cells in the pancreas. High transcript levels of SGK1 are also found in the distal tubule and collecting duct of the kidney and in thick ascending

limb epithelial cells. It expresses most abundantly in liver, kidney and pancreas. As like SGK1, SGK3 mRNA is present in all human and murine tissues examined but expression is particularly high in the mouse heart and spleen and in the embryons (Lang et al. 1151-78).

SGK3 is identical to the cytokine-independent survival kinase, CISK. The "SGK-like" gene in chromosome 8q12.3 encodes a protein which predicted amino acid sequence is virtually identical to that of human SGK3. SGK3 is expressed in all tissues tested thus far (Kobayashi et al. 189-97). Unlike SGK1, SGK3 is not under transcriptional control of serum and glucocorticoids (Lang et al. 1151-78).

Neither knockout of SGK3, nor of both, SGK1 and SGK3 leads to a severe phenotype suggesting that neither SGK1 nor SGK3 are required for survival. Gene targeted mice lacking functional SGK3 have a strikingly delayed hair growth, which has been attributed to apoptosis of keratinocytes. Moreover, SGK3 deficient mice have decreased basal intestinal glucose transport and a subtle decrease of locomotion (Lang et al. 1151-78;McCormick et al. 4278-88).

To become functional, SGK1 is activated by phosphorylation through a signaling cascade including PI 3-kinase and phosphoinositide dependent kinase PDK1 and PDK2/H-motif kinase.

SGK1 and its isoforms have a considerable physiological role through the regulation of transporters and ion channels. For instance, they regulate the epithelial Na^+ channel, ENaC, the voltage-gated Na^+ channel, SCN5A, the K^+ channels ROMK1, KCNE1/KCNQ1 and Kv1.3 , the cation channel TRPV5 (Palmada et al. 175-82;Embark et al. 203-12), the Na^+/H^+ exchanger NHE3 (Sandu et al. G868-G876), the dicarboxylate transporter NaDCT, the glutamate transporters EAAT1 (Lang et al. 1151-78), EAAT3 (Rexhepaj et al. 2214-22), EAAT4 (Rajamanickam et al. 858-66;Bohmer et al. 1242-48) and EAAT5 and the Na^+/K^+-ATPase (Embark et al. 601-06;Henke et al. 370-74). The regulatory activity of SGK1 and its isoforms plays a diverse role in essential cell functions such as epithelial transport, excitability, cell proliferation and apoptosis (Lang et al. 1151-78).

However, it is not known, whether SGK1 and SGK3 participate in the regulation of mast cell ion channels and thus in the regulation of mast cell functions.

2. AIMS OF THE STUDY

The manifestations of mast-cell-driven allergic reactions are considered to be mainly a consequence of the release of pro-inflammatory mediators following Ag-induced aggregation of IgE-bound FcεRIs expressed on the mast-cell surface.

Mast cell stimulation involves activation of ion channels, such as Ca^{2+}, K^+ and Cl^- channels. Upon mast cell activation, store-operated and receptor-mediated Ca^{2+} channels allow the entry of Ca^{2+} into the cell, which triggers exocytosis and thus degranulation. The electrical driving force for Ca^{2+} entry is provided by the cell membrane potential, which is maintained by the activity of K^+ channels. Accordingly, cell membrane depolarization decreases Ca^{2+} influx and subsequent degranulation. Ca^{2+}-activated K^+ channels are critical in this scenario, as pharmacological inhibition of these channels disrupts degranulation.

Ion channel activity and function are dependent on PI 3-kinase. Signaling molecules downstream of PI 3-kinase include among others SGK1 and SGK3.

The present study has been performed to elucidate the role of PI 3-kinase in the regulation of mast cell Ca^{2+} and Ca^{2+}-activated K^+ channels, and thus in modulation of mast cell function. Also we aimed at investigating whether the PI 3-kinase-dependent ion channel regulation and function of mast cells similarly involves SGK1 and SGK3. Thus, the function of mast cells has been studied in gene-targeted mice lacking SGK1 (*sgk1*$^{-/-}$) and their wild-type littermates (*sgk1*$^{+/+}$), and SGK3 (*sgk3*$^{-/-}$) and their wild-type littermates (*sgk3*$^{+/+}$).

3. MATERIALS AND METHODS

3.1. Chemicals and reagents

Albumin, dinitrophenyl (DNP-HSA)	Sigma-Aldrich, Munich, Germany
Allophycocyanin-labelled anti-CD117	BD Pharmingen, Heidelberg, Germany
Anti-DNP IgE, clone SPE-7	Sigma-Aldrich, Munich, Germany
Apamin	Sigma-Aldrich, Munich, Germany
Clotrimazole	Sigma-Aldrich, Munich, Germany
DMSO	Sigma-Aldrich, Munich, Germany
EGTA	Sigma-Aldrich, Munich, Germany
Fetal bovine serum	Hyclone/Perbio, Bonn, Germany
Fluorescein-isothiocyanate-labelled anti-CD34	BD Pharmingen, Heidelberg, Germany
Fura-2/AM	Molecular Probes, Goettingen, Germany
Iberiotoxin	Sigma-Aldrich, Munich, Germany
Ionomycin	Calbiochem, Darmstadt, Germany
LY-294002 hydrochloride	Sigma-Aldrich, Munich, Germany
PBS	GIBCO, Carlsbad, Germany
Penicilliln/Streptomycin	Invitrogen, Karlsruhe, Germany
Phycoerythrin-labelled anti-FcεRI	eBioscience GmbH, Frankfurt, Germany
PMA	Calbiochem, Darmstadt, Germany
P-nitrophenyl N-acetyl-β-D-glucosaminide	Sigma-Aldrich, Munich, Germany
Recombinant mouse IL-3	RD systems, Wiesbaden-Nordenstadt, Germany
RPMI 1640 +L-glutamine	GIBCO, Carlsbad, Germany
SCF	Peprotech, Tebu-bio, Rocky Hill, NJ, USA
Thapsigargin	Invitrogen, Karlsruhe, Germany
TRAM-34	Sigma-Aldrich, Munich, Germany
Tris base	Sigma-Aldrich, Munich, Germany

Triton X-100	Sigma-Aldrich, Munich, Germany
Tyrode’s Salt Solution	Sigma-Aldrich, Munich, Germany
Wortmannin	Sigma-Aldrich, Munich Germany

3.2. Animals

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.

For PI 3-kinase experiments 129/SvJ, 129/SvP2 mice were used.

Generating of *sgk1*$^{-/-}$ mice and their wt littermates

A conditional targeting vector was generated from a 7-kb fragment encompassing the entire transcribed region on 12 exons. The neomycin resistance cassette was flanked by two loxP sites and inserted into intron 11. Exons 4-11, which code for the *sgk1* domain, were "floxed" by inserting a third loxP site into intron 3. Targeted R1 embryonic stem cells were transiently transfected with Cre recombinase. A clone with a recombination between the first and third loxP site (type I recombination) was injected into C57BL/6 blastocytes. Male chimeras were bred to 129/SvJ females. Heterozygous *sgk1*-deficient mice were backcrossed to 129/SvJ wild-type mice for two generations and then intercrossed to generate homozygous *sgk1*$^{-/-}$ and *sgk1*$^{+/+}$ littermates (Wulff et al. 1263-68). The animals were genotyped by PCR using standard methods. The study has been performed in 6-8 week-old female and male *sgk1*$^{+/+}$ and *sgk1*$^{-/-}$ mice.

Generating of *sgk3*$^{-/-}$ mice and their wt littermates

The targeting strategy for disruption of the SGK3 gene involved removing parts of exons 10 (which contains the ATP-binding site necessary for the catalytic activity of SGK3) and 11, deleting intron 10, and introducing an in-frame STOP codon into exon 11. Plasmid pNTK loxp (gift from Dr. S. Coughlin) was used to generate the targeting vector. Two mouse genomic fragments, containing exons 8-11 and exons 10-17, were amplified from 129X1/SvJ DNA by PCR and cloned into pCR4- TOPO and pCR-XL-TOPO (both Invitrogen, Carlsbad, California, USA) respectively and characterized by restriction enzymes. The exon 8-11-containing construct was used as a 5 template in a second round of PCR to generate a 2.6-kb exon 8-10 fragment with a

BamHI site added to the 5’ end and an MfeI site added to the 3’ end. This fragment was used as the short arm and was inserted into the BamHI/MfeI sites of the targeting vector. The 10-kb long arm fragment was generated by using the exon 10-17-containing construct as a template in a second round of PCR. A ClaI site was added to the 5’ end, and a STOP codon was added before the start of exon 11; a XhoI site was added to the 3’ end. This fragment was inserted into the ClaI/XhoI sites of the targeting vector. The targeting vector was linearized by digestion with XhoI, and electroporated into RW-4 embryonic stem cells (derived from 129X1/SvJ mice). G418- and gancyclovir-resistant clones were initially screened by PCR using oligonucleotide primers located inside and outside the targeted locus to confirm homologous recombination. Two positive clones were expanded and their genomic DNA analyzed by Southern blot analysis following digestion by MfeI. An external probe (a 2kb restriction fragment lying between exons 1 and 7) was used to verify correct targeting. The two positive clones were injected into C57BL/6 blastocysts and transferred into pseudopregnant females. Chimaeric males, identified by their agouti coat color, were mated with C57BL/6 females. To generate mice homozygous for the targeted allele, the resulting s*gk3*$^{+/-}$ males and females were interbred (McCormick et al. 4278-88).

3.3. Culture of bone marrow mast cells

Mast cells were isolated from femoral bone marrow (BMMCs) of 6-8 week-old male and female mice and cultured for 4 weeks in RPMI 1640 containing 10% FBS, 1% penicillin/streptomycin, 20 ng/ml interleukin-3 and 100 ng/ml of the c-kit ligand stem cell factor. BMMC maturation was confirmed by flow cytometry (FACS Calibur, Becton Dickinson) using the following antibodies: phycoerythrin-labelled anti-FcεRI, allophycocyanin-labelled anti-CD117 and fluorescein-isothiocyanate-labelled anti-CD34. The cells were kept in culture 4-6 weeks prior to the experiments. BMMCs were sensitized with monoclonal mouse anti-dinitrophenyl IgE (anti-DNP IgE, 5-10 µg/ml per 1 x 10^6 cells) in culture medium and challenged acutely with antigen, dinitrophenyl-human serum albumin (Ag, DNP-HSA, 50 ng/ml). In experiments with PI 3-kinase inhibition, LY-294002 (10 µM) was added to the medium 30 min before IgE.

3.4. Patch clamp

Patch clamp experiments were performed at room temperature in voltage-clamp, fast-whole-cell mode. 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). Borosilicate glass pipettes (2-4 MOhm tip resistance; GC 150 TF-10, Clark Medical Instruments, Pangbourne, UK) 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, N.Y., 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.

The pipette solution contained (in mM): 140 K-gluconate, 5 KCl, 1.2 $MgCl_2$, 2 EGTA, 1.26 $CaCl_2$ (pCa 7), 2 Na_2ATP and 10 HEPES/KOH (pH 7.2) and was used in combination with NaCl Ringer bath solution. Where indicated DNP-HSA (50 ng/ml), the channel blocker

TRAM-34 (300 nM) and/or the Ca^{2+} ionophore ionomycin (1 µM) were added to the bath solution.

For some experiments Ca^{2+}-free Ringer was used that contained 1 mM EGTA. Where indicated the Ag DNP-HSA (50 ng/ml), the channel blockers clotrimazole (1.5 µM), apamin (500 nM) and iberiotoxin (100 nM), as well as the Ca^{2+} ionophore ionomycin (1 µM) were added to the bath solution. DNP-HSA, apamin and iberiotoxin were prepared as stock solutions in water, clotrimazole in ethanol and ionomycin in dimethyl sulfoxide (DMSO). The final concentration of DMSO and ethanol were 0.01% and 0.005%, respectively.

The offset potentials between both electrodes were zeroed before sealing, and the potentials were corrected for liquid junction potentials as estimated according to Barry & Lynch (Barry and Lynch 101-17). 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 depicted as downward deflections of the original current traces.

3.5. Measurement of intracellular Ca^{2+}

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 the objective (Fluar 40×/1.30 oil, Zeiss). Emitted fluorescence intensity was recorded at 505 nm and data acquisition was performed by using specialized computer software (Metafluor, Universal Imaging, Downingtown, PA). As a measure for the increase of cytosolic Ca^{2+} activity, the slope and peak of the changes in the 340/380 nm ratio were calculated for each experiment.

BMMCs were sensitized with IgE (10 µg/ml) for 1 hour at 37°C and subsequently loaded with Fura-2/AM (2 µM) for 20 min at 37°C. Intracellular Ca^{2+} was measured prior to and following addition of DNP-HSA to IgE-sensitized BMMCs in the absence or presence of extracellular Ca^{2+}.

For PI 3-kinase experiments BMMCs were pretreated with LY-294002 (10 µM) for 30 min at 37° C or left untreated. The cells were then sensitized with IgE (10 µg/ml) for 1 hour at 37°C and subsequently loaded with Fura-2/AM (2.5 µM) for 20 min at 37°C. Intracellular Ca^{2+} was measured prior to and following addition of DNP-HSA to IgE-sensitized BMMCs in the absence or presence of extracellular Ca^{2+}.

Alternatively, changes in cytosolic Ca^{2+} were monitored in both control and LY-294002-treated BMMCs upon depletion of the intracellular Ca^{2+} stores. Experiments were carried out prior to and during exposure to nominally Ca^{2+}-free solution. In the absence of Ca^{2+} the intracellular Ca^{2+} stores were depleted by inhibition of the vesicular Ca^{2+} pump by thapsigargin (1 µM).

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 $MgSO_4$, 1 $CaCl_2$, 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, 5 EGTA, 5 glucose, pH 7.4.

3.6. Measurement of β-hexosaminidase and IL-6 release

Mature BMMCs (0.3 x 10^6 per well) were seeded in a 96-well plate in fresh medium with 10 µg/ml anti-DNP IgE antibody for 1 hour or overnight. Afterwards cells were washed in Tyrode's Salt Solution and challenged with DNP-HSA (50 ng/ml) or for positive control with 100 ng/ml phorbol myristate ester (PMA) and 1 µM ionomycin for 15 min at 37°C. 20 µl supernatant and 20 µl of 2 mM p-nitrophenyl N-acetyl-β-D-glucosaminide diluted in 0.2 M citrate buffer, pH 4.5, were mixed and incubated for 2 hours at 37°C. The reaction was terminated by the addition of 60 µl 1M Tris buffer, pH 9.0, and the absorbance was measured at 405 nm in an ELISA microplate reader.

The data are evaluated as the percent of the total release (0.1 % Triton X-100) corrected for spontaneous release.

The concentration of IL-6 released from mast cells, which were sensitized with 1 µg/ml IgE for 6 hours at 37°C and then stimulated with DNP-HAS (50 ng/ml) for 30 min, 6 hours, or 24 hours, was measured by ELISA according to the instructions of the manufacturer (BD Biosciences).

3.7. Model of passive systemic anaphylaxis and measurement of histamine serum concentrations

Mice were sensitized with 2 µg/1 g weight anti-DNP IgE by intraperitoneal application. The next day or after 5 hours, mice were challenged with either DNP-HSA (4.8 µg/1 g weight) or PBS. Body temperature was monitored before and each 1-7 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-hours light and 12-hours dark cycle (7 a.m.–7 p.m.).
Data are expressed as a change in body temperature following treatment (Δ°C).

Thirty minutes after induction of an anaphylactic reaction, blood was taken for the analysis of histamine plasma concentration. Histamine was measured by ELISA according to the instructions of the manufacturer (IBL-Hamburg).

3.8. Statistics

Data are provided as means ± SEM, *n* represents the number of independent experiments. All data were tested for significance using Student's unpaired two-tailed *t*-test, one sample *t*-test or ANOVA (Dunnets test), where applicable. Results with $p<0.05$ were considered statistically significant.

4. RESULTS

BMMC growth and maturation

BMMCs expressed CD117, CD34 and FcεRI, receptors, typically expressed by mast cells. No significant difference in receptor abundance was observed between BMMCs from SGK1 knockout mice (*sgk1*$^{-/-}$) and their wild-type littermates (*sgk1*$^{+/+}$) (Fig. 1), as well as from SGK3 knockout mice (*sgk3*$^{-/-}$) and their wild-type littermates (*sgk3*$^{+/+}$) (Fig. 3).

The forward scatter in cultured mast cells from *sgk1*$^{+/+}$ and *sgk1*$^{-/-}$ mice is shown in Fig. 2. The forward scatter was slightly but significantly smaller in *sgk1*$^{-/-}$ BMMCs than in *sgk1*$^{+/+}$ BMMCs, pointing to a smaller volume of SGK1-deficient mast cells.

No significant difference was observed in forward scatter in mast cells from *sgk3*$^{+/+}$ and *sgk3*$^{-/-}$ mice.

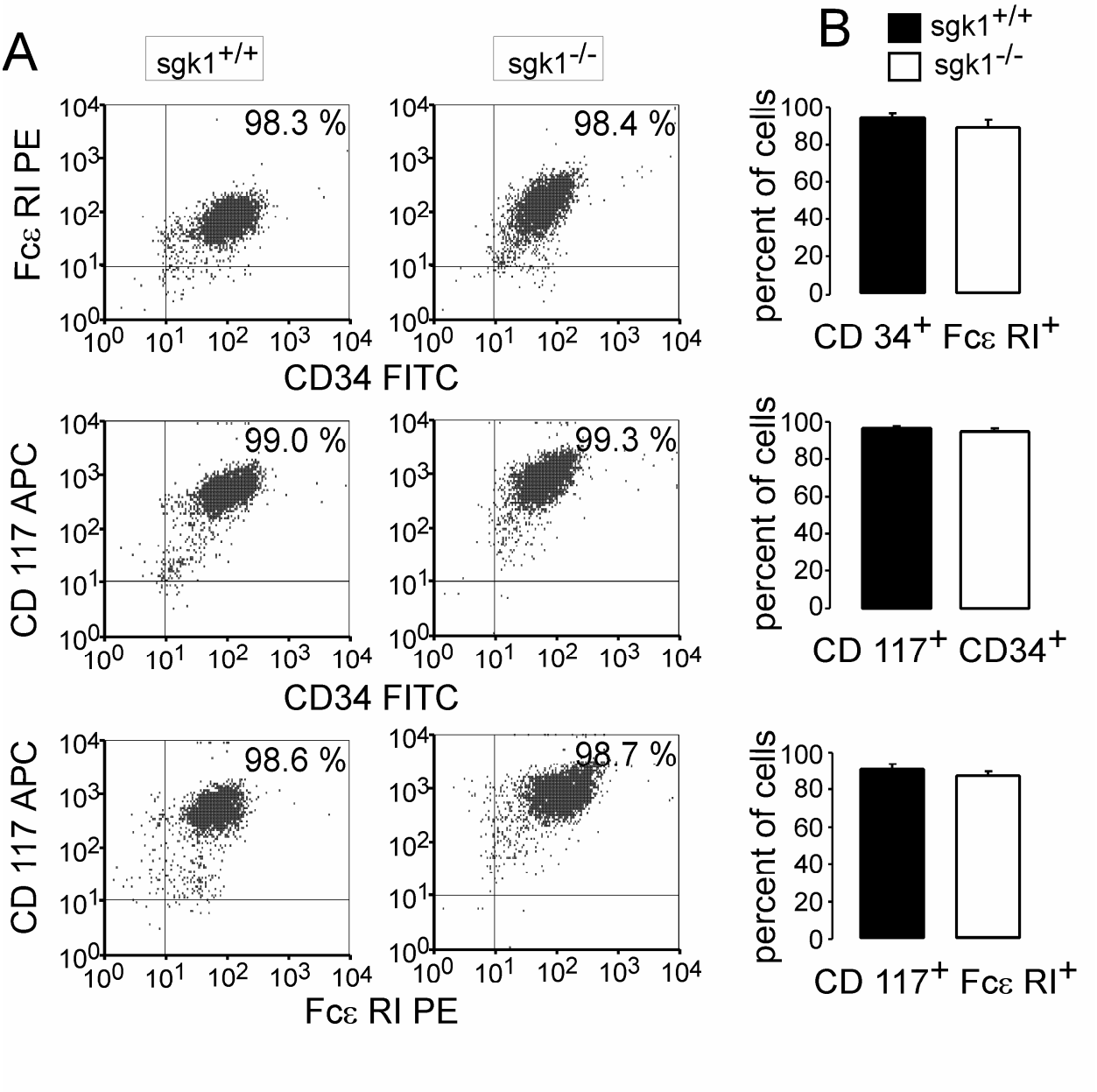

Figure 1: Maturation of BMMCs from *sgk1*$^{+/+}$ and *sgk1*$^{-/-}$ mice

A. Original dot plots of CD34-, CD117- and FcεRI-positive BMMCs from sgk1$^{+/+}$ and sgk1$^{-/-}$ mice. Numbers depict the percent of cells in the respective quadrants, acquired within the mast cell gate.

B. Percentage of mast cells in primary culture. Mean percent (±SEM; n=6 individual BMMC cultures) of *sgk1*$^{+/+}$ (closed bars) and *sgk1*$^{-/-}$ (open bars) BMMCs acquired within the mast cell gate.

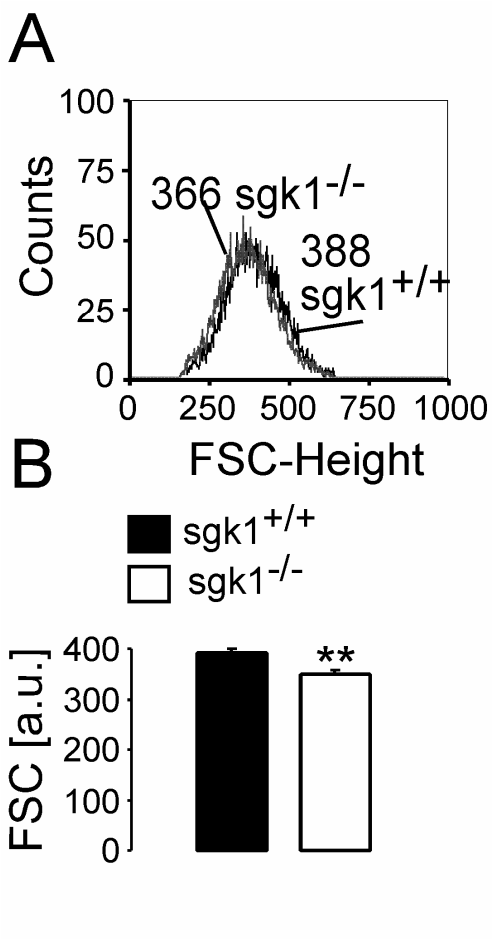

Figure 2: Forward scatter in BMMCs from *sgk1*$^{+/+}$ and *sgk1*$^{-/-}$ mice

A. Overlaid original traces illustrating the forward scatter of cultured BMMCs from *sgk1*$^{+/+}$ (black line) and *sgk1*$^{-/-}$ (grey line) mice.

B. Arithmetic means (±SEM; n=14) of forward scatter (FSC) of cultured BMMCs in arbitrary units (a.u.), from *sgk1*$^{-/-}$ mice (open bars) and *sgk1*$^{+/+}$ (closed bars). ** (p=0.004) indicates significant difference from *sgk1*$^{+/+}$, two-tailed unpaired *t*-test.

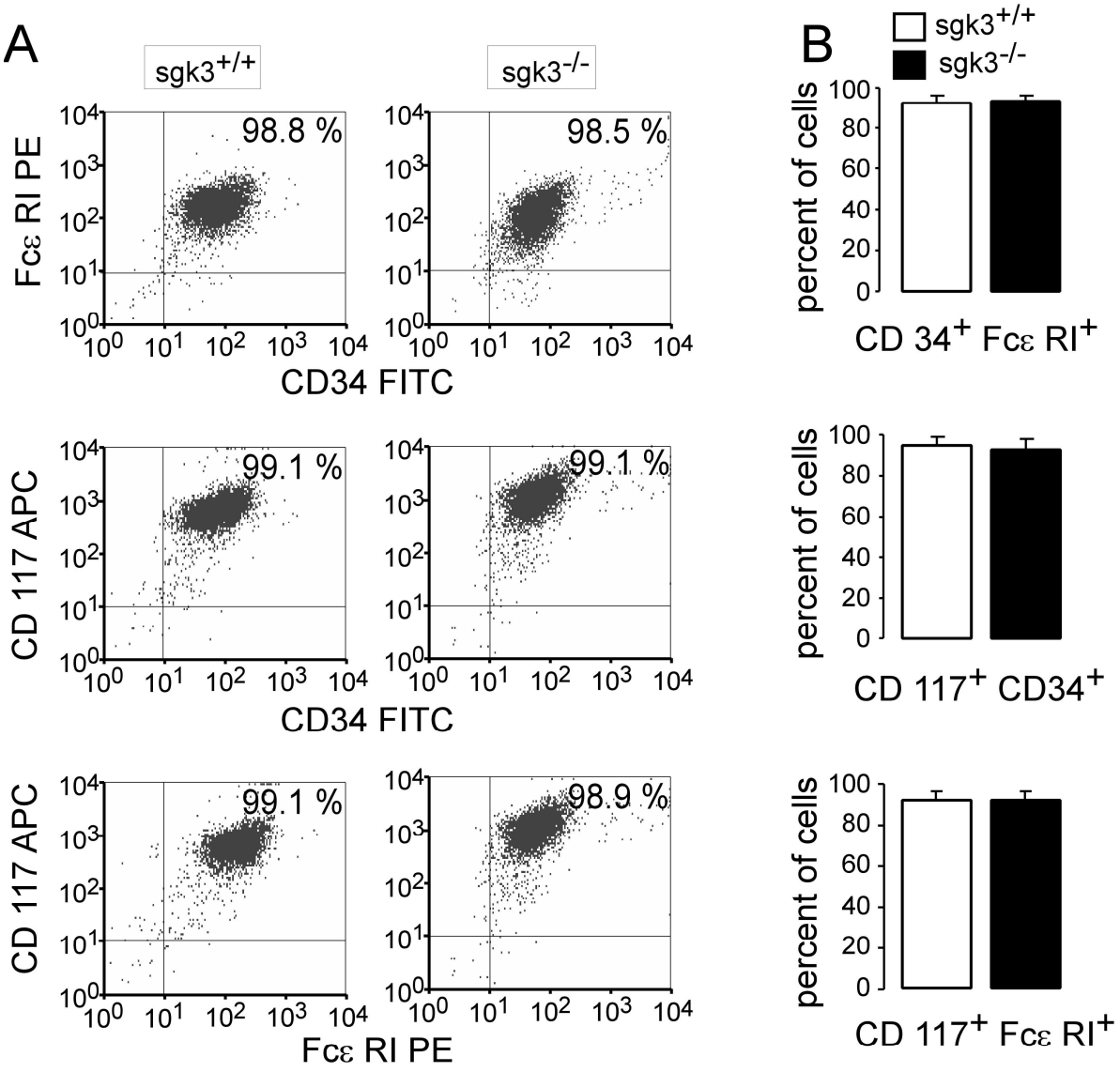

Figure 3: Maturation of BMMCs from *sgk3*$^{+/+}$ and *sgk3*$^{-/-}$ mice

A. Original dot plots of CD34-, CD117- and FcεRI-positive BMMCs from *sgk3*$^{+/+}$ and *sgk3*$^{-/-}$ mice. Numbers depict the percent of cells in the respective quadrant, acquired within the mast cell gate.

B. Frequency of mast cells in primary culture. Mean percent (±SEM; n=6 individual BMMC cultures) of *sgk3*$^{+/+}$ (open bars) and *sgk3*$^{-/-}$ (closed bars) BMMCs acquired within the mast cell gate.

4.1. Blunted Ag-induced Ca^{2+} entry in BMMCs upon PI 3-kinase inhibition

BMMCs were sensitized with IgE (10 µg/ml) for 1 hour, washed and then stimulated with Ag. According to Fura-2 fluorescence experiments exposure of control BMMCs to Ag was followed by a sharp increase in cytosolic Ca^{2+} concentration. This effect was markedly reduced when the mast cells were pretreated with PI 3-kinase inhibitor LY-294002 (10 µM, 30 min). Correspondingly, the fluorescence Δratio (peak) and the slope of the ratio (Δratio/time) upon stimulation with Ag were significantly smaller ($p < 0.001$) in BMMCs pretreated with LY-294002 than in control cells (Fig. 4B). Stimulation of 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 LY-294002-treated and control cells (Fig. 4D). Interestingly, basal free Ca^{2+} concentration was significantly lower (fluorescence ratio 1.37±0.08, n=20) in LY-294002-treated IgE-sensitized BMMCs (10 µM, 30 min) than in control cells (fluorescence ratio 1.56±0.09, n=25).

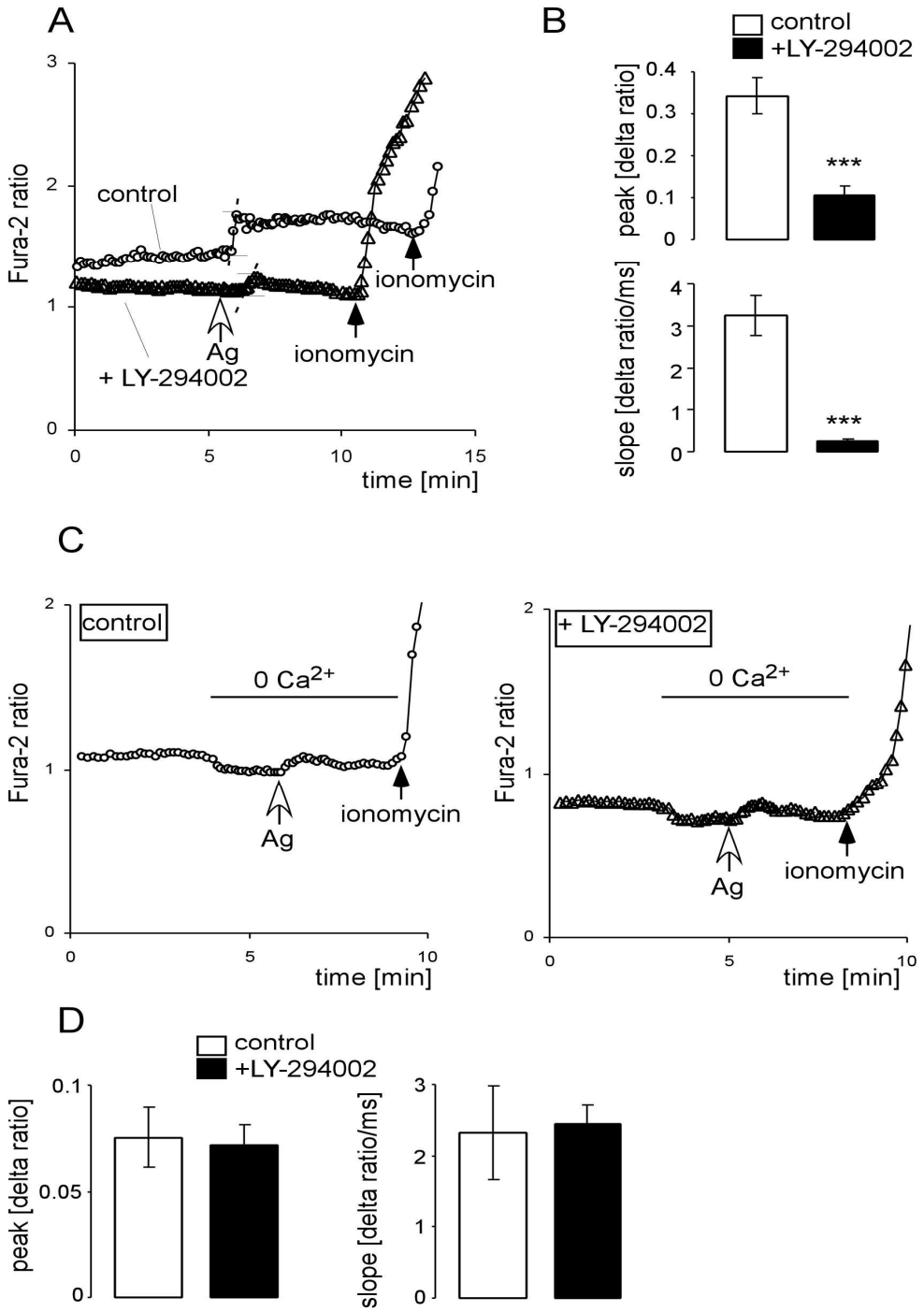

Figure 4: Ag-induced increase of intracellular Ca^{2+} is blunted by PI 3-kinase inhibitor LY-294002

A. Representative tracings showing the Fura-2 fluorescence ratios (340/380 nm) in Fura-2/AM loaded control and LY-294002 (10 μM)-treated BMMCs prior to and following acute addition of Ag. At the end of each experiment ionomycin (10 μM) was added for calibration. For quantification of the Ca^{2+} entry into BMMCs, the slope (delta ratio/ms, indicated by dash lines) and peak (delta ratio, indicated by ticks) were calculated following addition of Ag.

B. Mean (± SEM) of the peak value (left) and slope (right) of the change in Fura-2 fluorescence for control (n=25, open bars) and LY-294002-treated BMMCs (10 μM, n=20, closed bars) following addition of Ag to the bath solution. *** ($p<0.001$) indicates significant difference between both groups (two-tailed unpaired *t*-test).

C. Representative tracing showing the Fura-2 fluorescence ratio before and after addition of Ag in the absence of extracellular Ca^{2+} in control (left) and LY-294002- treated (right) BMMCs. To maintain a Ca^{2+}-free environment, EGTA (0.5 mM) was added to the Ca^{2+}- free bath solution.

D. Mean (± SEM) of the peak value (left) and slope (right) of the fluorescence ratio change for control (n=6, open bars) and LY-294002-treated (10 μM, n=5, closed bars) BMMCs upon stimulation with Ag in a Ca^{2+}-free solution.

4.2. Influx of Ca^{2+} through SOCs is PI 3-kinase-dependent

Further experiments were performed to determine, whether Ca^{2+} entry following Ca^{2+} store depletion, i.e. activation of SOCs, was similarly sensitive to PI 3-kinase inhibition. The stores were depleted by inhibition of the vesicular Ca^{2+}-ATPase by addition of thapsigargin (1 μM) in Ca^{2+}-free medium. Thapsigargin triggered a release of Ca^{2+} from intracellular stores, which was not significantly modified by LY-294002-treatment. Readdition of Ca^{2+} (1 mM) to the medium in the continued presence of thapsigargin led to an entry of Ca^{2+} through plasma membrane Ca^{2+} channels and to a subsequent increase of the fluorescence ratio (Fig. 5A). Both, peak and slope values were significantly lower in LY-294002-treated compared to control BMMCs (Fig. 5B). Thus, PI 3-kinase stimulates the influx of Ca^{2+} through the SOCs.

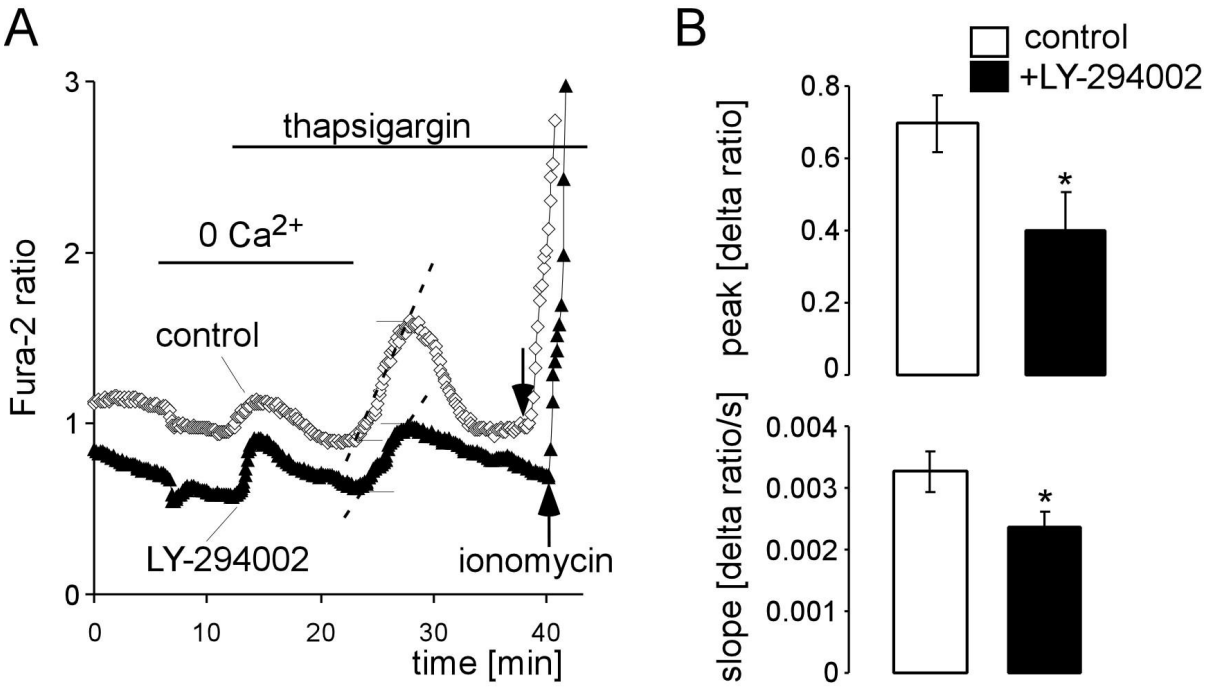

Figure 5: LY-294002 reduces the entry of extracellular Ca^{2+} through store-operated Ca^{2+} channels
A. Representative tracings showing the Fura-2 fluorescence ratio (340/380 nm) in Fura-2/AM loaded control and LY-294002 (10 μM)-treated BMMCs. Experiments were carried out prior to and during exposure to nominally Ca^{2+}-free bath solution. Where indicated, thapsigargin (1 μM) was added to the nominally Ca^{2+}-free bath solution. Upon readdition of extracellular Ca^{2+} in the presence of thapsigargin the slope (delta ratio/time, indicated by dash lines) and peak (delta ratio, indicated by ticks) of the Fura-2 fluorescence ratio change were calculated, reflecting the entry of Ca^{2+} through the SOCs. At the end of each experiment ionomycin (10 μM) was added for calibration purposes.
B. Mean (± SEM) of the peak value (upper graph) and slope (lower graph) of the change in Fura-2 fluorescence for control (n=25, open bars) and LY-294002-treated (10 μM, n=19, closed bars) BMMCs following readdition of extracellular Ca^{2+} in the presence of thapsigargin. * ($p<0.05$) indicates significant difference between both groups (two-tailed unpaired *t*-test).

4.3. Ag-induced activation of Ca^{2+}-activated K^+ channels is PI 3-kinase-dependent

Patch clamp experiments showed that the exposure of BMMCs to Ag was followed by activation of K^+ currents, which were inhibitable by the specific $K_{Ca}3.1$ blocker TRAM-34 (300 nM, Fig. 6A). Preincubation of the cells with LY-294002 (10 μM) for 30 min, prior to sensitization with IgE, resulted in a dramatic decrease of Ag-activated K^+ conductance (Fig. 6B, C).

Alternatively, Ca^{2+}-activated K^+ channel activation was induced by treatment of the BMMCs with the Ca^{2+} ionophore ionomycin (1 µM). Ionomycin-induced K^+ currents were not significantly different between LY-294002-treated and non-treated cells (Fig. 6C) suggesting that the surface expression and maximal activity of the Ca^{2+}-activated K^+ channel did not differ between the two groups. This again suggests that LY-294002 decreased primarily the Ag-stimulated Ca^{2+} signal.

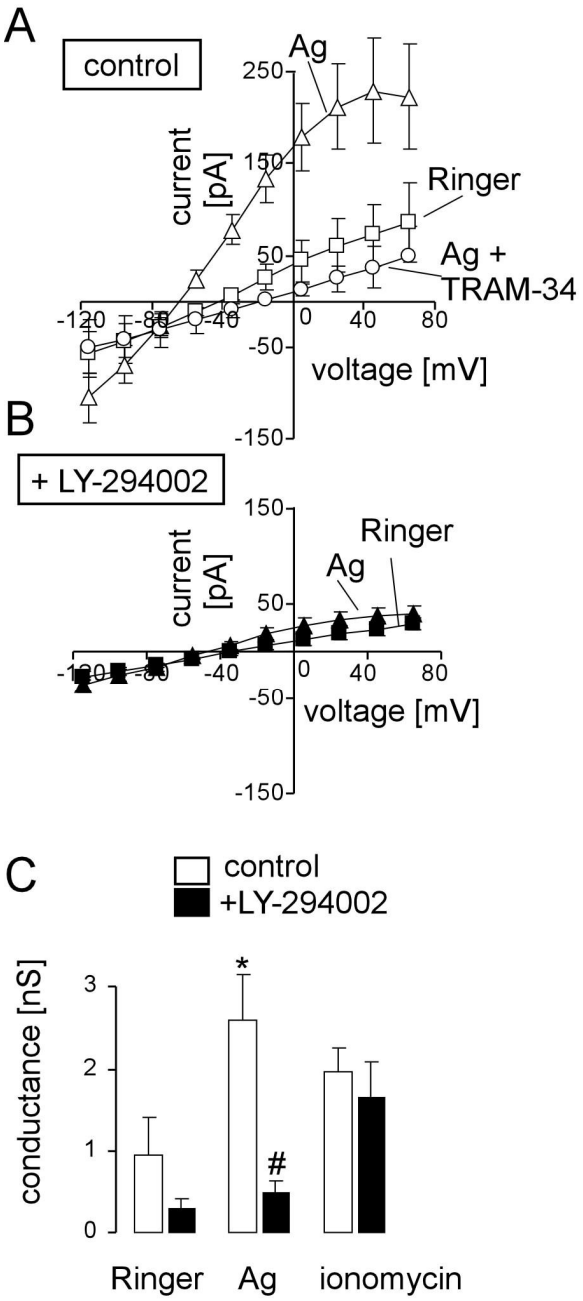

Figure 6: Ag-induced K^+ current in BMMCs is inhibited by LY-294002

A, B. Mean I-V relationships (± SEM, n=5-7) in control (A, open symbols) or LY-294002-treated (10 μM, 30 min, B, closed symbols) BMMCs prior to (Ringer, squares) or 3 min after stimulation with Ag (triangles) and then after inhibition by 300 nM TRAM-34 (A, Ag + TRAM-34, open circles).

C. Mean whole-cell conductance (± SEM, n=5-7) in control (open bars) and LY-294002-treated (closed bars) BMMCs as recorded in (A, B) prior to (Ringer) and after stimulation with either Ag or ionomycin (1 μM). Data were calculated by linear regression between -55 and +5 mV. * ($p<0.05$) indicates significant difference from control cells in Ringer; # ($p<0.05$) indicates significant difference between control and LY-294002-treated cells (ANOVA).

4.4. β-hexosaminidase release is inhibited by LY-294002

Mast cell degranulation in response to IgE-mediated stimulation, measured by β-hexosaminidase release, was significantly reduced in LY-294002-treated cells (Fig. 7), results confirming previous observations (Ali, Watson, and Osborne 103-05;Tkaczyk et al. 48474-84).

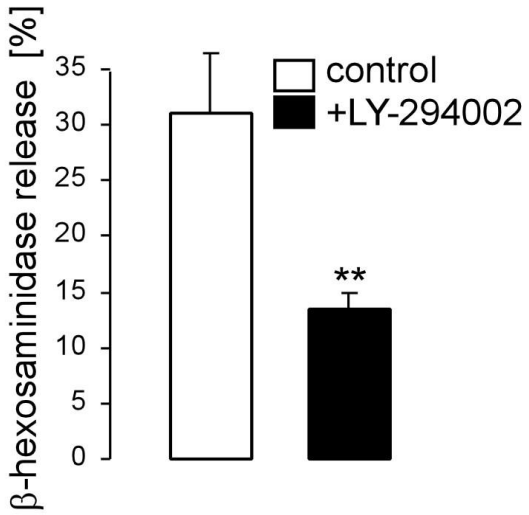

Figure 7: Degranulation of Ag-stimulated BMMCs is inhibited by LY-294002

Mean β-hexosaminidase release (± SEM, n=10 individual experiments) from control (open bar) and LY-294002-treated (closed bar) BMMCs stimulated for 15 min with Ag. 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. ** indicates significant difference between both groups ($p<0.001$; two-tailed unpaired *t*-test).

4.5. Reduced Ag-induced Ca^{2+} entry into BMMCs from *sgk1*$^{-/-}$ mice

In order to study the responses of *sgk1*$^{-/-}$ and *sgk1*$^{+/+}$ BMMCs upon stimulation with Ag via the FcεRI, the BMMCs were incubated overnight with IgE (10 µg/ml), washed and then stimulated with DNP-HSA.

According to Fura-2 fluorescence, IgE-dependent activation of BMMCs was followed by an increase of cytosolic Ca^{2+} concentration. The rate of increase was significantly larger in *sgk1*$^{+/+}$ than in *sgk1*$^{-/-}$ BMMCs (Fig. 8A, B). Accordingly, the maximal fluorescence Δratio (peak) and

the slope of the ratio (Δratio/time) upon stimulation with Ag were significantly smaller in *sgk1*$^{-/-}$ BMMCs than in *sgk1*$^{+/+}$ BMMCs (Fig. 8B). To further assess the effect of SGK1 on Ca^{2+} mobilization, the cells were stimulated with IgE and Ag in the absence of extracellular Ca^{2+} (Fig. 8C). The measured release from intracellular stores was not significantly different between the genotypes (Fig. 8D).

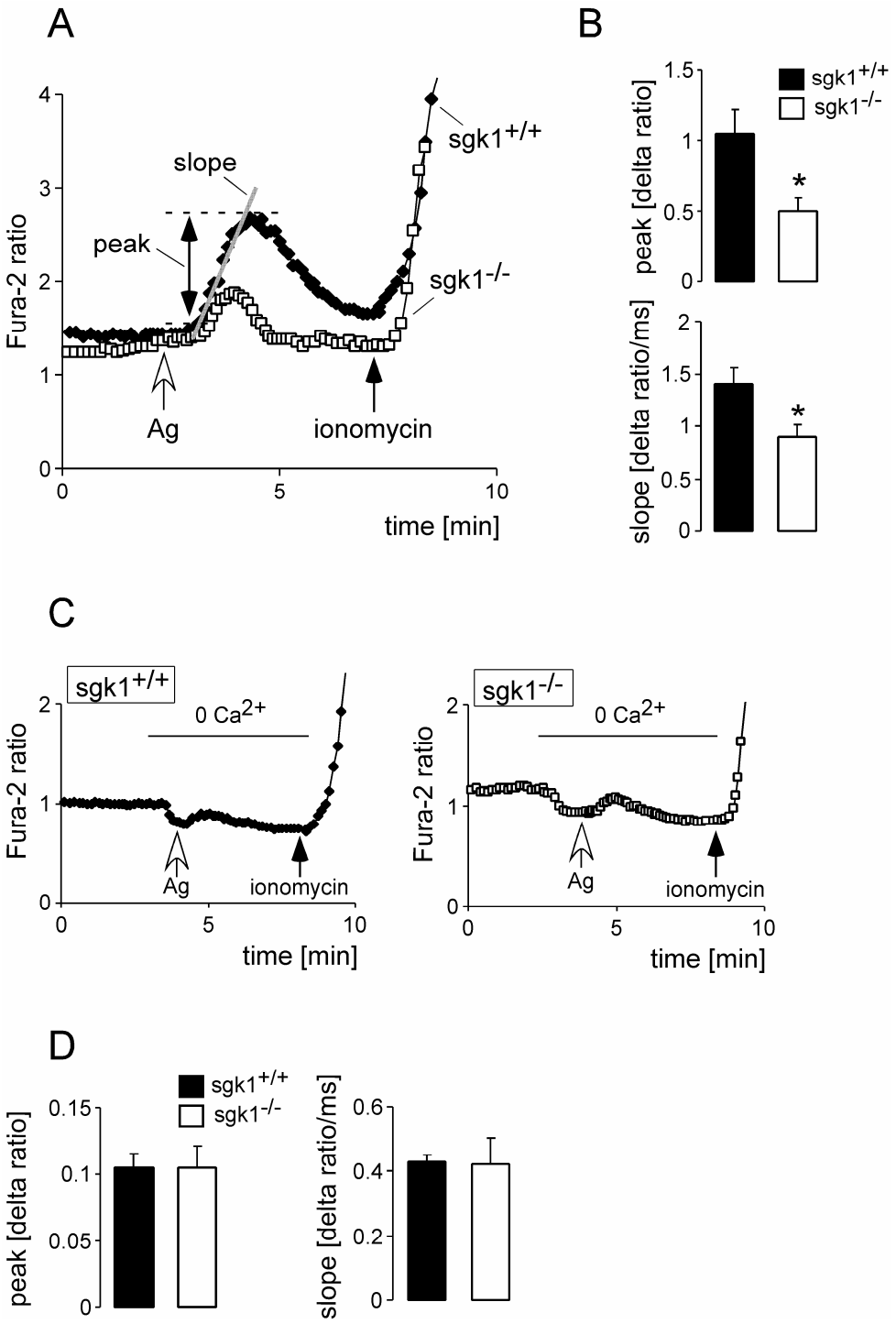

Figure 8: Ag induced Ca^{2+} entry into BMMCs from *sgk1*$^{+/+}$ and *sgk1*$^{-/-}$ mice

A. Representative tracings showing the ratio of 340/380 nm Fura-2 fluorescence in Fura-2/AM loaded BMMCs prior to and following acute addition of Ag. At the end of each experiment, ionomycin (10 µM) was added for calibration. For quantification of the Ca^{2+} entry into the BMMCs, the slope (delta ratio/ms, grey line) and peak (delta ratio, dotted lines) were calculated following addition of Ag.

B. Mean (±SEM) of the peak (upper graph) and slope (lower graph) of the fluorescence ratio change for *sgk1*$^{+/+}$ (n=8, closed bars) and *sgk1*$^{-/-}$ (n=9, open bars) BMMCs following stimulation with Ag. * ($p<0.05$) indicates significant difference between both groups (two-tailed unpaired *t*-test).
C. Representative tracings showing the Fura-2 fluorescence ratio before and after addition of Ag in the absence of extracellular Ca^{2+} in Fura-2/AM loaded BMMCs. To reach a Ca^{2+}-free environment, EGTA (0.5 mM) was added to the Ca^{2+}-free bath solution.
D. Mean (±SEM) of the peak value (left) and slope (right) of the fluorescence ratio change for *sgk1*$^{+/+}$ (n=6, closed bars) and *sgk1*$^{-/-}$ (n=4, open bars) BMMCs upon stimulation with Ag in a Ca^{2+}-free solution.

4.6. Reduced Ag-induced Ca^{2+} entry into BMMCs from *sgk3*$^{-/-}$ mice

In order to study the responses of *sgk3*$^{-/-}$ and *sgk3*$^{+/+}$ BMMCs, the cells were sensitized with IgE (10 µg/ml) for 1 hour at 37°C and subsequently loaded with Fura-2AM (2 µM) for 20 min at 37°C.

Increase of cytosolic Ca^{2+} in *sgk3*$^{-/-}$ cells stimulated with IgE and cognate Ag was impaired if compared to *sgk3*$^{+/+}$ cells (Fig. 9A, B). To further assess the effect of SGK3 on Ca^{2+} mobilization, the cells were stimulated with IgE and Ag in the absence of extracellular Ca^{2+} (Fig. 9C). Under these conditions, the measured release of Ca^{2+} from the intracellular stores was not different between *sgk3*$^{+/+}$ and *sgk3*$^{-/-}$ cells (Fig. 9D), thus the entry of extracellular Ca^{2+} was impaired in *sgk3*$^{-/-}$ BMMCs.

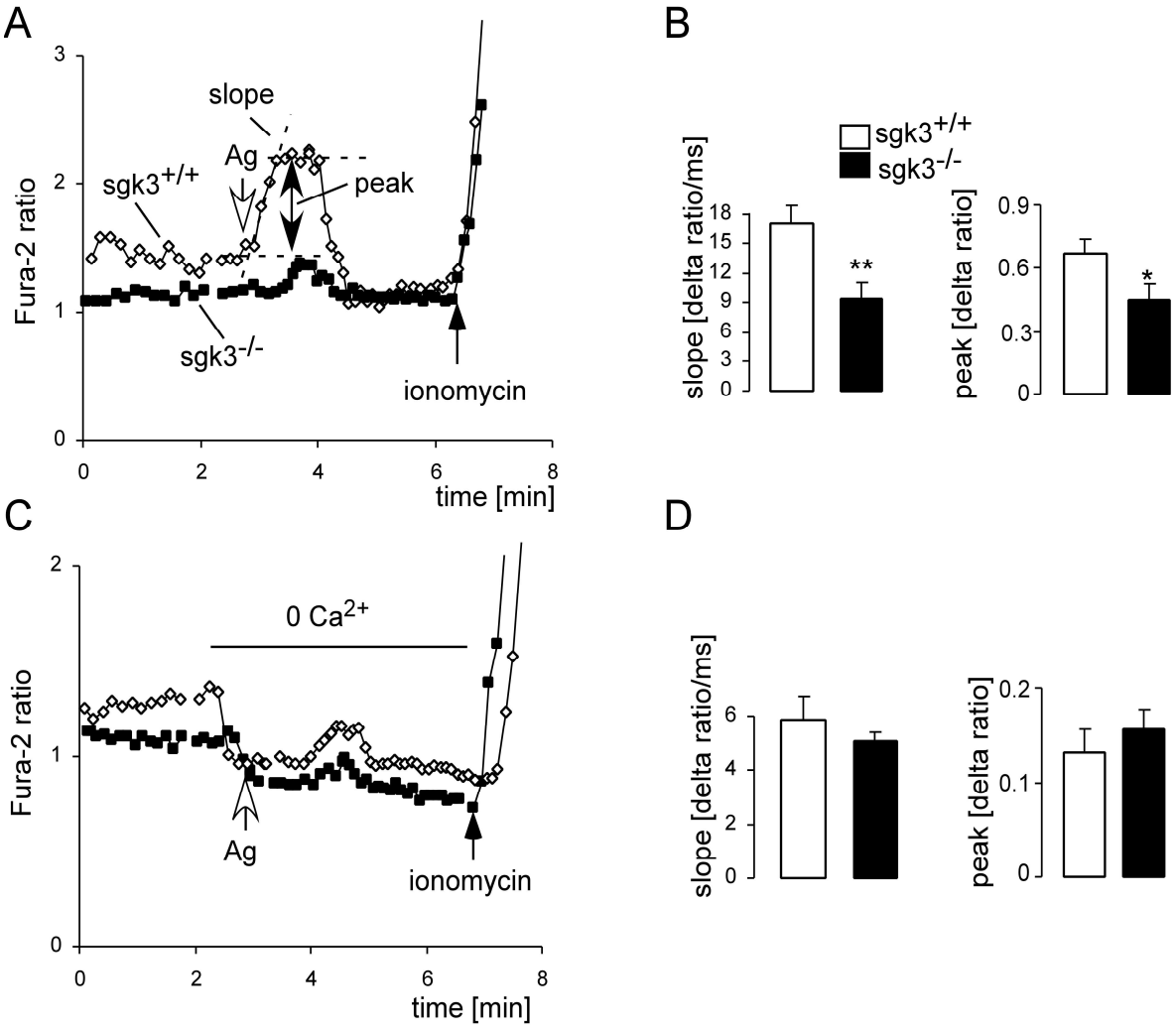

Figure 9: Ag induced Ca^{2+} entry into BMMCs from *sgk3*$^{+/+}$ and *sgk3*$^{-/-}$ mice

A. Representative original tracings showing the Fura-2 fluorescence ratio of 340 over 380 nm in Fura-2/AM loaded BMMCs from *sgk3*$^{+/+}$ and *sgk3*$^{-/-}$ mice prior to and following acute addition of Ag. At the end of each experiment, ionomycin (10 µM) was added for calibration. For quantification of the Ca^{2+} entry into the BMMCs, the slope (Δ ratio/ms) and peak (Δ ratio) were calculated following addition of Ag as indicated in the figure.

B. Mean (± SEM) of the slope (left) and peak (right) of the fluorescence ratio change for *sgk3*$^{+/+}$ (n = 9, open bars) and *sgk3*$^{-/-}$ (n = 8, closed bars) BMMCs following stimulation with Ag. * ($p<0.05$) and ** ($p<0.01$) indicate significant difference between both groups (two-tailed unpaired *t*-test).

C. Representative original tracings showing the ratio of 340/380 nm Fura-2 fluorescence in Fura-2 loaded *sgk3*$^{+/+}$ and *sgk3*$^{-/-}$ BMMCs before and after addition of Ag in the absence of extracellular Ca^{2+}. To reach a Ca^{2+}-free environment, EGTA (0.5 mM) was added to the Ca^{2+}-free bath solution.

Mean (± SEM) of the slope (left) and peak value (right) of the fluorescence ratio change for *sgk3*$^{+/+}$ (n = 5, open bars) and *sgk3*$^{-/-}$ (n = 5, closed bars) BMMCs upon stimulation with Ag in a Ca^{2+}-free solution.

4.7. Impairment of Ca^{2+}-activated K^+ currents and membrane hyperpolarization in *sgk1*$^{-/-}$ BMMCs upon IgE-dependent stimulation

In patch clamp experiments Ag stimulation of *sgk1*$^{-/-}$ and *sgk1*$^{+/+}$ BMMCs led to a rapid increase of K^+-selective conductance which was dependent on the presence of extracellular Ca^{2+}, inhibited by 1.5 µM clotrimazole (Fig. 10A-C) and not sensitive to apamin (500 nM) or iberiotoxin (100 nM, data not shown). The same current could be induced by Ca^{2+} ionophore ionomycin (1 µM, Fig. 10F). This current was thus carried by Ca^{2+}-activated K^+ channels, which have recently been identified as $K_{Ca}3.1$ (Shumilina et al. 8040-47) and are known to be activated following FcεRI cross-linking in human mast cells (Duffy et al. 4261-70;Duffy et al. 1006-08). The current induced by Ag in *sgk1*$^{-/-}$ BMMCs was significantly smaller than in wild-type cells (Fig. 10D, E), whereas treatment of the cells with ionomycin led to activation of the K^+ channels to a similar extent in *sgk1*$^{+/+}$ and *sgk1*$^{-/-}$ BMMCs (Fig. 10F, G). Thus, Ca^{2+}-activated K^+ channels are similarly expressed and are similarly sensitive to activation by Ca^{2+} in *sgk1*$^{+/+}$ and *sgk1*$^{-/-}$ BMMCs. Accordingly, the blunted activation by Ag results from decreased Ca^{2+} entry into *sgk1*$^{-/-}$BMMCs.

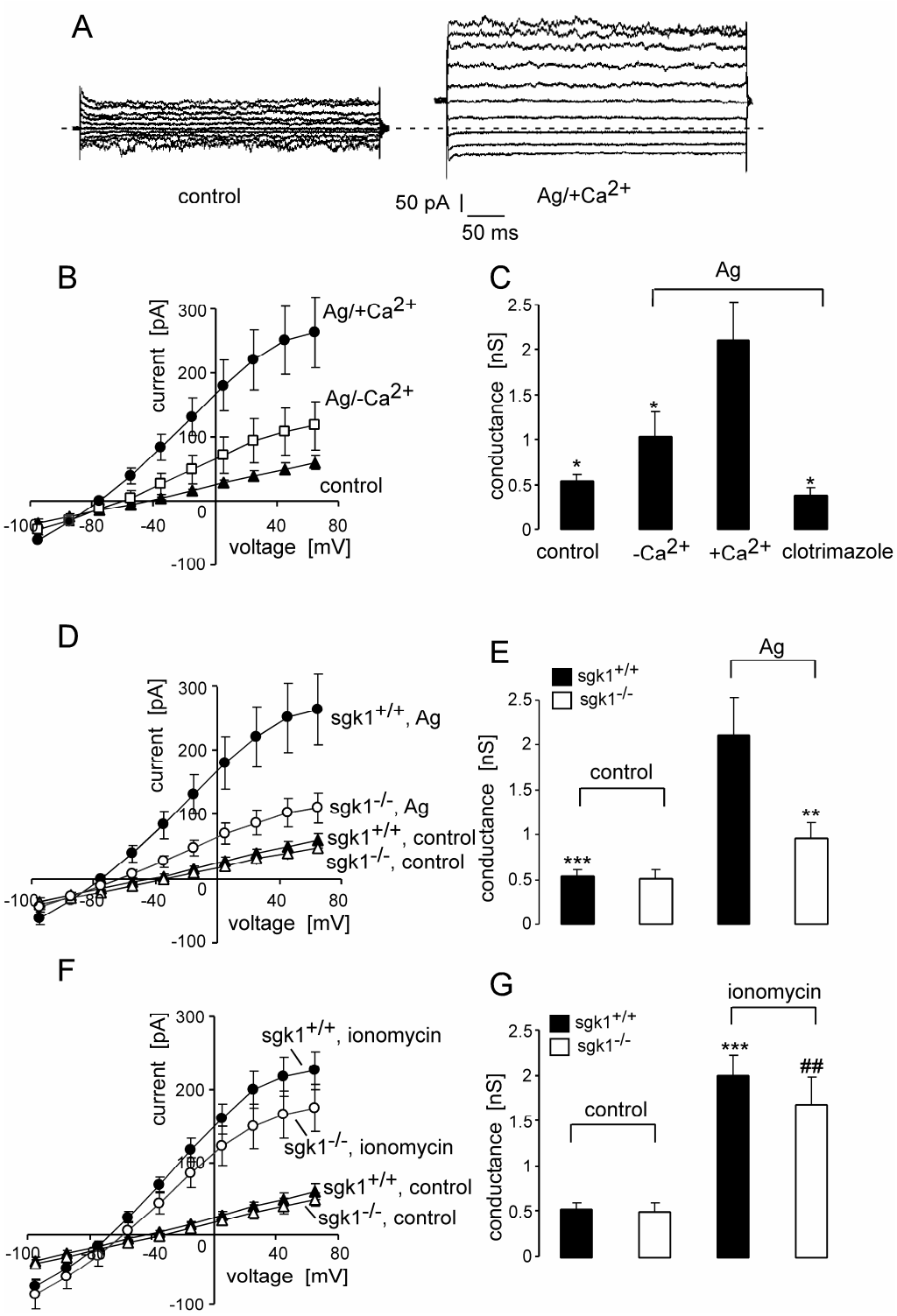

Figure 10: Ca^{2+}-activated K^+ current in *sgk1*$^{+/+}$ and *sgk1*$^{-/-}$ BMMCs upon IgE-Ag-induced stimulation

A. Representative whole-cell currents from *sgk1*$^{+/+}$ BMMCs elicited by 400 ms pulses ranging from -115 to +65 mV in 20 mV increments from a holding potential of -35 mV. Currents were recorded in

standard NaCl bath solution prior to (control) and 2 min after stimulation with Ag. The dotted line indicates the zero current value.

B. Mean I-V relationships (±SEM, n=5-11) in *sgk1*$^{+/+}$ BMMCs prior to (control, closed triangles) or 3 min after stimulation with Ag added either to Ca^{2+}-free NaCl (Ag/-Ca^{2+}, open squares) or 1 mM Ca^{2+}-containing standard NaCl bath solution (Ag/+Ca^{2+}, closed circles).

C. Mean whole-cell conductance in paired experiments (±SEM, n=5) as recorded in (B) prior to or 3 min after stimulation with Ag added either to Ca^{2+}-free NaCl or 1 mM Ca^{2+}-containing standard NaCl bath solution and after inhibition with 1.5 µM clotrimazole. Data were calculated by linear regression between -95 and +45 mV. * indicates significant difference from respective value following addition of the Ag to Ca^{2+}-containing NaCl bath ($p<0.05$; ANOVA).

D. Mean I-V relationships (±SEM, n=11-13) of currents in *sgk1*$^{+/+}$ (closed symbols) and *sgk1*$^{-/-}$ (open symbols) BMMCs prior to (control, triangles) or 3 min after (Ag, circles) stimulation with Ag (50 ng/ml) added to standard NaCl bath solution.

E. Mean whole-cell conductance (±SEM) of *sgk1*$^{+/+}$ (closed bars) and *sgk1*$^{-/-}$ (open bars), as recorded in (D) prior to and after stimulation with Ag. Data were calculated by linear regression between -95 and +45 mV. ** (***) indicate significant difference from Ag-stimulated *sgk1*$^{+/+}$ cells, (*** $p<0.001$, ** $p<0.01$; ANOVA).

F. Mean I-V relationships (±SEM, n=9-12) of currents in *sgk1*$^{+/+}$ (closed symbols) and *sgk1*$^{-/-}$ (open symbols) BMMCs prior to (control, triangles) or 2 min after (ionomycin, circles) stimulation with ionomycin (1 µM) added to standard NaCl bath solution.

G. Mean whole-cell conductance (±SEM, n=9-12) of *sgk1*$^{+/+}$ (closed bars) and *sgk1*$^{-/-}$ (open bars) BMMCs, as recorded in (F) prior to and after stimulation with ionomycin. Data were calculated by linear regression between -95 and +45 mV. *** ($p<0.001$) indicates significant difference from control *sgk1*$^{+/+}$ cells, $^{\#\#}$ ($p<0.01$) indicates significant difference from control *sgk1*$^{-/-}$ cells, ANOVA.

4.8. Impairment of Ca^{2+}-activated K^+ currents and membrane hyperpolarization in *sgk3*$^{-/-}$ BMMCs upon IgE-dependent stimulation

The K^+ currents of *sgk3*$^{-/-}$ and *sgk3*$^{+/+}$ BMMCs upon receptor stimulation were measured in patch-clamp experiments (Fig. 11). Addition of Ag to the bath solution resulted in a rapid increase of a K^+-selective conductance in *sgk3*$^{+/+}$ cells and to a significantly lesser extent in *sgk3*$^{-/-}$ cells (Fig. 11). However, when the cells were stimulated with a Ca^{2+} ionophore ionomycin, no difference in measured K^+ currents was detected between the genotypes (Fig. 11C). This suggests that the surface expression and maximal activity of the Ca^{2+}-activated K^+

channel did not differ between the two groups and SGK3 deficiency primarily decreased the Ag-stimulated Ca^{2+} entry.

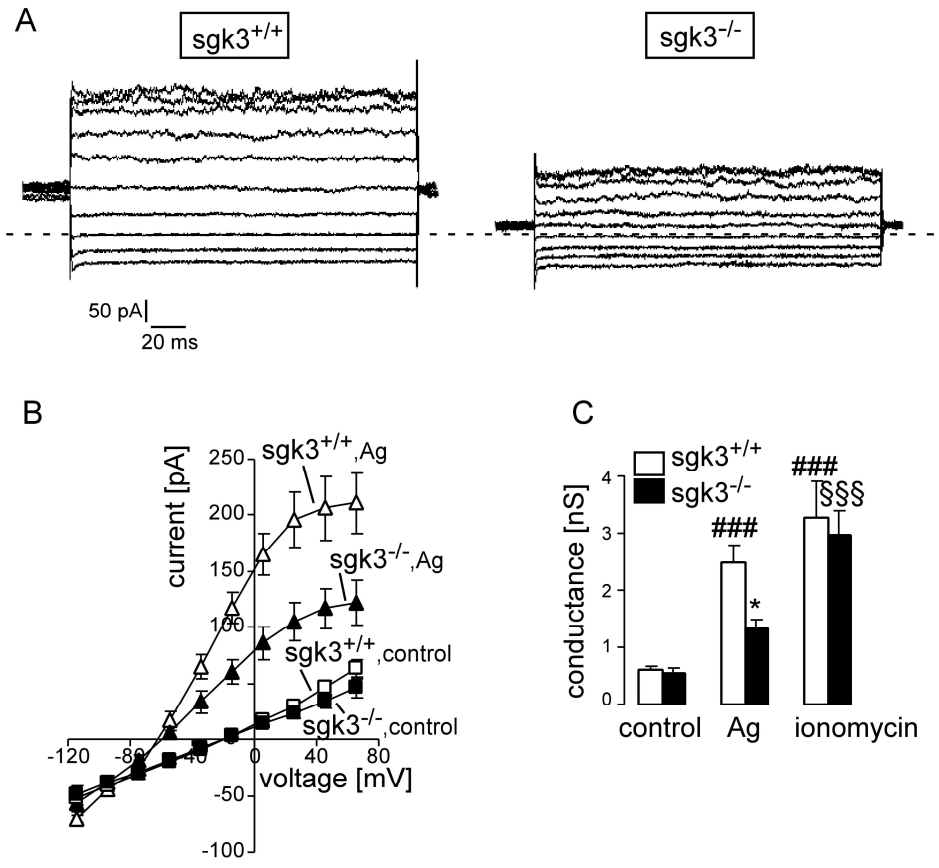

Figure 11: Ag-induced K^+ currents are reduced in *sgk3*$^{-/-}$ BMMCs

A. Representative whole-cell currents from *sgk3*$^{+/+}$ (left) and *sgk3*$^{-/-}$ (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 standard NaCl bath solution 3 min after stimulation with Ag. The dotted line indicates the zero current value.

B. Mean I-V relationships (± SEM, n=7) in *sgk3*$^{+/+}$ (open symbols) and *sgk3*$^{-/-}$ (closed symbols) BMMCs prior to (control, squares) and 3 min after stimulation with Ag (Ag, 50 ng/ml, triangles).

C. Mean whole-cell conductance (± SEM) of *sgk3*$^{+/+}$ (open bars) and *sgk3*$^{-/-}$ (closed bars) BMMCs as recorded in (B) prior to (control) and after stimulation with either Ag or ionomycin (1 µM). Data were calculated by linear regression between -55 and +5 mV. * ($P<0.05$) indicate significant difference between *sgk3*$^{+/+}$ and *sgk3*$^{-/-}$ cells; ### ($P<0.001$) indicate significant difference from *sgk3*$^{+/+}$ cells under control conditions and §§§ ($P<0.001$) indicate significant difference from *sgk3*$^{-/-}$ cells under control conditions (ANOVA).

Decreased Ca^{2+} entry in *sgk*$^{+/+-}$ and *sgk3*$^{-/-}$ BMMCs could result in decreased Ag-induced mediator release. To determine whether SGK1 and SGK3 deficiency influences mast cell degranulation, the release ß-hexosaminidase was measured in *sgk1*$^{-/-}$, *sgk3*$^{-/-}$ and respective wild-type cells.

4.9. Altered degranulation ability of Ag-stimulated *sgk1*$^{-/-}$ BMMCs

We also tested the ability of *sgk1*$^{+/+}$ and *sgk1*$^{-/-}$ BMMCs to undergo degranulation upon stimulation with Ag via the FcεRI receptor. BMMCs release β-hexosaminidase, an enzyme stored in mast cell granules. Without stimulation, the release was 8.5 ± 1.5% (n=5) in *sgk1*$^{+/+}$ BMMCs and 7.9±1.4% (n=5) in *sgk1*$^{-/-}$ BMMCs, values not significantly different between genotypes. Activation by Ag (50 ng/ml) or by ionomycin (1 µM) and PMA (100 ng/ml) stimulated degranulation in both genotypes. The effect was, however significantly smaller in *sgk1*$^{-/-}$ BMMCs than in *sgk1*$^{+/+}$ BMMCs (Fig. 12). The early Ag-induced release of IL-6 (30 min of stimulation with Ag) was also significantly blunted in *sgk1*$^{-/-}$ cells (Fig. 12B, left). However, no difference between the two genotypes was observed when the cells were stimulated with Ag for a longer time (6 or 24 hours; Fig. 12B, right). These data confirm that SGK1 is a critical component of the signaling cascade leading to release of mast cell mediators.

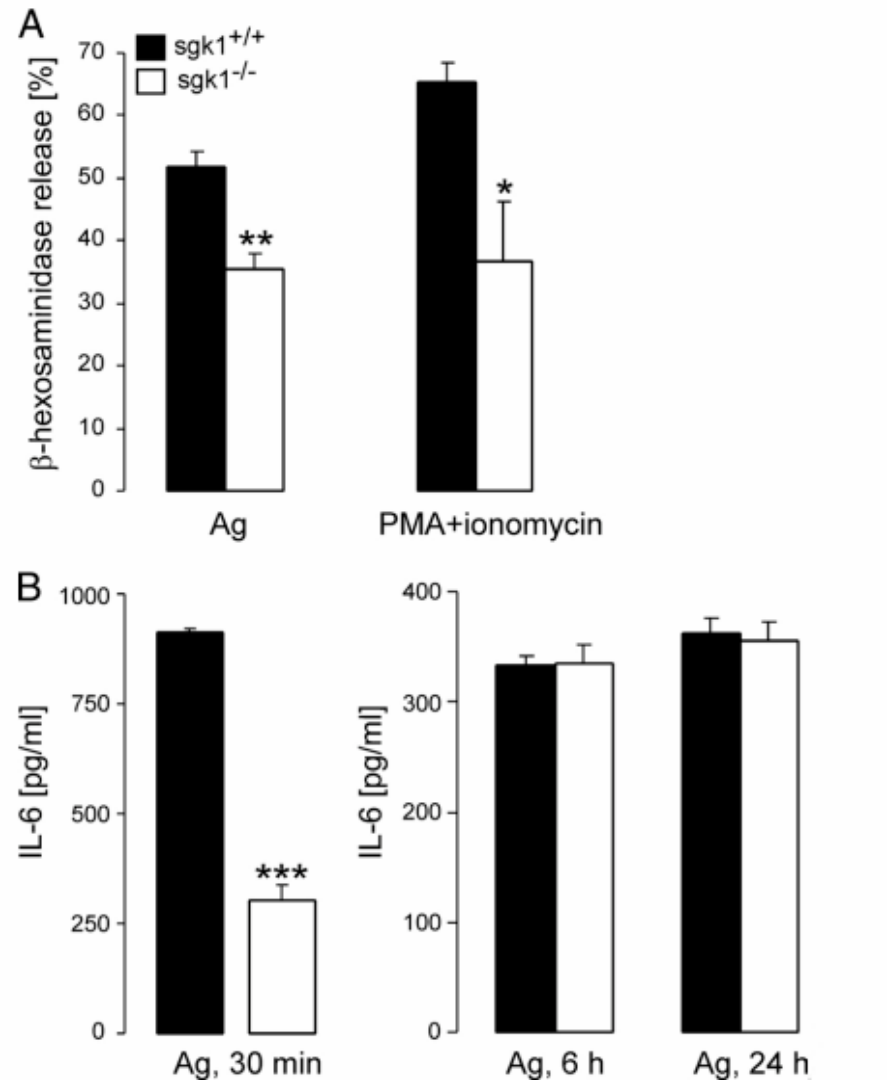

Figure 12: β-Hexosaminidase and IL-6 release from *sgk1*$^{-/-}$ and *sgk1*$^{+/+}$ BMMCs

A. Mean β-hexosaminidase release (±SEM; n=5 individual experiments) from cultured *sgk1*$^{-/-}$ BMMCs and their wild-type littermates stimulated for 15 min with 50 ng/ml Ag or 100 ng/ml PMA and 1 µM ionomycin. The stimulated β -hexosaminidase release in each experiment was corrected for the spontaneous release. ** ($p<0.01$) and * ($p < 0.05$), significant difference between genotypes, two-tailed unpaired t test.

B. Mean IL-6 release (±SEM; n=3 individual experiments) from cultured *sgk1*$^{-/-}$ BMMCs and their wild-type littermates sensitized for 6 hours with IgE and then stimulated either for 30 min with Ag (50 ng/ml, left) or 6 hours, or 24 hours (right). Each experiment was performed in duplicate; *** ($p<0.001$), two-tailed un- paired t test.

4.10. Altered degranulation ability of Ag-stimulated *sgk3*$^{-/-}$ BMMCs

To determine whether SGK3 deficiency influences mast cell degranulation, the release of β-hexosaminidase was measured in *sgk3*$^{+/+}$ and *sgk3*$^{-/-}$ cells. As shown in Fig. 13, β-hexosaminidase release was significantly reduced in *sgk3*$^{-/-}$ BMMCs.

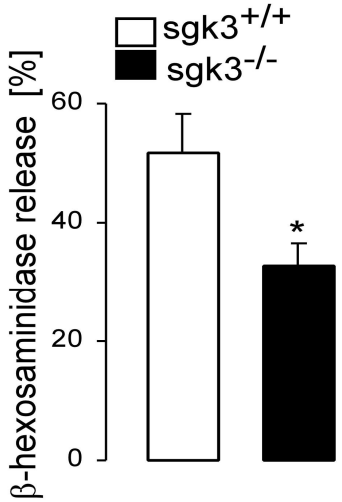

Figure 13: Degranulation of Ag-stimulated *sgk3*$^{-/-}$ and *sgk3*$^{+/+}$ BMMCs

Arithmetic means (± SEM, n=5 individual experiments) of β-hexosaminidase release from cultured *sgk3*$^{-/-}$ BMMCs (closed bars) and their wild-type littermates *sgk3*$^{+/+}$ (open bars) stimulated for 15 minutes with. 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. * indicates significant difference between genotypes ($p<0.05$; two-tailed unpaired *t*-test).

4.11. Impaired acute response to anaphylaxis and decreased histamine serum concentrations in *sgk1*$^{-/-}$ mice

Further studies have been performed to explore whether the impaired stimulation of Ca^{2+} entry into *sgk1*$^{-/-}$ BMMCs affects their *in vivo* function. To this end, a model for the passive systemic anaphylaxis has been tested in both *sgk1*$^{+/+}$ and *sgk1*$^{-/-}$ mice. The mice were sensitized with anti-DNP IgE intraperitoneally. After overnight rest, they received Ag or saline as a control by intraperitoneal injection and body temperature was monitored over time. The measured drop in

body temperature following Ag treatment was reduced in *sgk1*$^{-/-}$ mice, thus pointing to an impairment of *sgk1*$^{-/-}$-deficient mast cell function *in vivo* (Fig. 14A, B).

Serum histamine level measured 30 min after induction of the anaphylactic reaction was significantly lower in *sgk1*$^{-/-}$ mice (Fig. 14C).

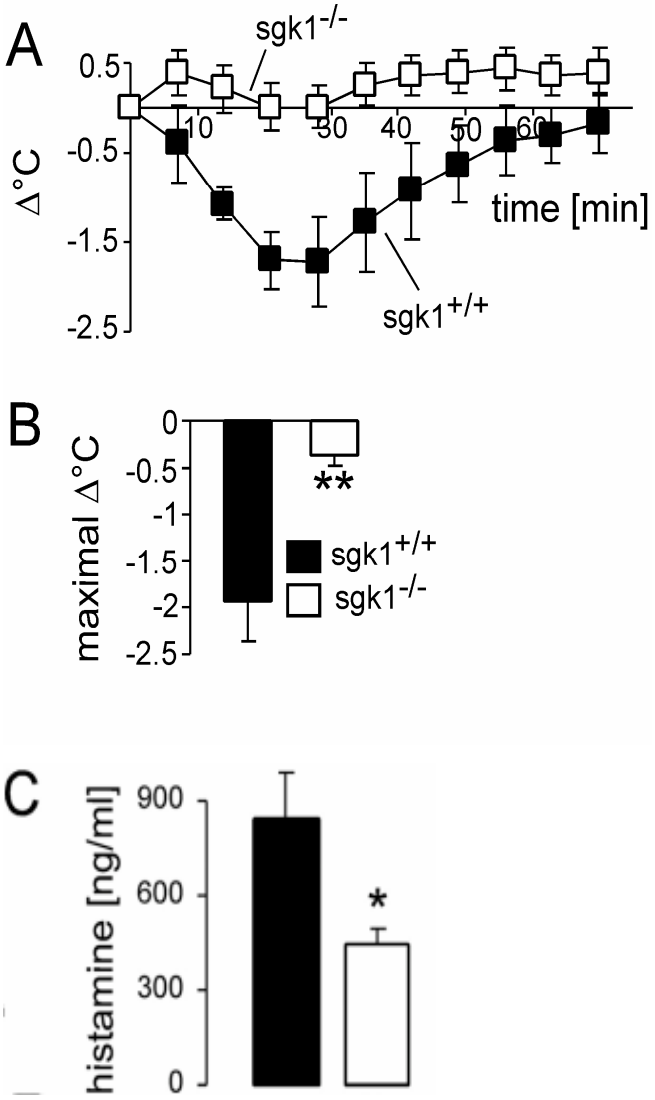

Figure 14: Systemic anaphylactic reaction in *sgk1*$^{-/-}$ and *sgk1*$^{+/+}$ mice

A. Arithmetic means (±SEM) of changes in body temperature (Δ°C) of *sgk1*$^{-/-}$ mice (n=8, open squares) and their wild-type littermates *sgk1*$^{+/+}$ (n=7, closed squares) following induction of anaphylaxis. Mice were given intraperitoneal anti-DNP IgE (2µg/g body weight) and challenged with 4,8 µg/g body weight DNP-HSA after overnight rest.

B. Arithmetic means (± SEM) of maximal changes in body temperature (Δ°C) of *sgk1*$^{-/-}$ mice (n=8, open bars) and their wild-type littermates *sgk1*$^{+/+}$ (n=7, closed bars) following induction of anaphylaxis. ** indicates significant difference between genotypes (p=0.002; two-tailed unpaired *t*-test).
C. Serum histamine level 30 min after induction of anaphylaxis in *sgk1*$^{-/-}$ mice (open bars) and their wild-type littermates *sgk1*$^{+/+}$ (closed bars). Data represent the results obtained from 3 mice and from each mouse 3 individual probes were analyzed. * indicates significant difference between genotypes.

4.12. Impaired acute response to anaphylaxis in *sgk3*$^{-/-}$ mice

To evaluate whether the observed alterations in *sgk3*$^{-/-}$ BMMCs were also relevant for mast cell function *in vivo*, we triggered passive systemic anaphylaxis in s*gk3*$^{+/+}$ and *sgk3*$^{-/-}$ mice. The mice were sensitized with anti-DNP IgE intraperitoneally. After 5 hours rest, mice received Ag or saline as a control by intraperitoneal injection and body temperature was monitored over time. As shown in Fig. 15, the decrease in body temperature following Ag treatment was dramatically decreased in *sgk3*$^{-/-}$ mice, an observation indeed pointing to severe impairment of mast cell function *in vivo*.

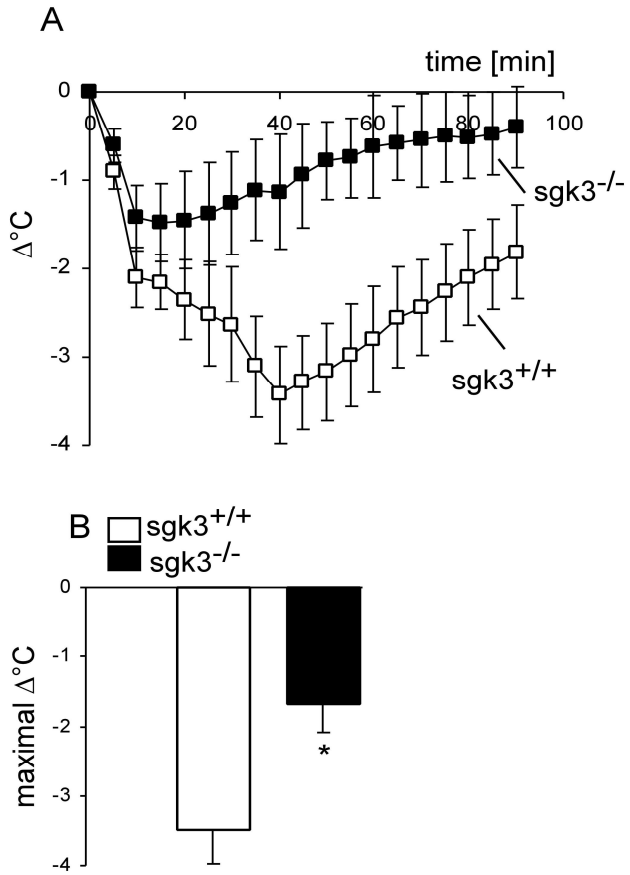

Figure 15: Systemic anaphylactic reaction in *sgk3*$^{+/+}$ and *sgk3*$^{-/-}$ mice

A. Arithmetic means (± SEM) of changes in body temperature (°C) of *sgk3*$^{-/-}$ mice (n=5, closed squares) and their wild-type littermates *sgk3*$^{+/+}$ (n=6, open squares) following induction of anaphylaxis. Mice were given intraperitoneally anti-DNP IgE (2µg/g body weight) and challenged with 4,8µg/g body weight DNP-HSA after 5 hours.

B. Arithmetic means (± SEM) of maximal changes in body temperature (°C) of *sgk3*$^{-/-}$ mice (n=5, open bars) and their wild-type littermates *sgk3*$^{+/+}$ (n=6, closed bars) following induction of anaphylaxis. * indicates significant difference between genotypes ($p<0.05$; two-tailed unpaired *t*-test).

5. DISCUSSION

The present study reveals a role of PI 3-kinase in the regulation of ion channel activity and thus the function of mast cells. Our data proved the importance of PI 3-kinase by the facts, that, first, Ca^{2+} signaling in response to FcεRI cross-linking was dramatically reduced upon inhibition of PI 3-kinase by LY294002. Second, the capacitative Ca^{2+} entry following store depletion, reflecting the activity of SOCs, was also sensitive to PI 3-kinase inhibition. Third, inhibition of PI 3-kinase abrogated IgE-mediated stimulation of Ca^{2+}-activated K^+ channels.

In the RBL-2H3 mast cell line two distinct Ca^{2+} influx pathways, capacitative and receptor-mediated, have been demonstrated. The latter, non-capacitative Ca^{2+} influx mechanism, was shown to be PIP_3- sensitive (Ching et al. 14814-20). In our experiments, capacitative Ca^{2+} entry was also dependent on the activity of PI 3-kinase, though to a less extent than IgE-dependent Ca^{2+} response. The capacitative Ca^{2+} entry could be affected indirectly via the activity of Ca^{2+}-activated K^+ channels. Mast cell Ca^{2+}-activated K^+ channels are important amplifiers of Ca^{2+} entry since their activation hyperpolarizes the cell membrane and thus provides the electrical driving force for Ca^{2+} influx through SOCs (Parekh and Penner 901-30). Recently $K_{Ca}3.1$ channel has been shown to be critically important for the regulation of Ca^{2+} entry and mast cell degranulation in mouse BMMCs (Shumilina et al.).

The present results show that ionomycin-dependent activation of mast cell Ca^{2+}-activated K^+ channels is not dependent on PI 3-kinase, which means that neither the number of functional K^+ channels, nor their maximal activity is reduced upon PI 3-kinase inhibition.

In theory, the PIP_3-sensitive Ca^{2+} influx (that is independent of the filling state of internal Ca^{2+} stores) may precede capacitative Ca^{2+} entry. This Ca^{2+} influx together with the PI 3-kinase-independent Ca^{2+} release from the internal stores then leads to the activation of Ca^{2+}-activated K^+ channels, with subsequent hyperpolarization of the cell membrane. Capacitative Ca^{2+} entry through SOCs would be enhanced by this hyperpolarization. The observed reduced IgE-mediated activation of Ca^{2+}-activated K^+ channels in the presence of the PI 3-kinase inhibitor LY-294002 could thus result from an impaired influx of Ca^{2+} through PIP_3-sensitive channels. A decreased activation of K^+ channels, in turn, is expected to impair the entry of Ca^{2+} through SOCs. However, assessment of the initial capacitative Ca^{2+} entry (through SOCs) in the present

study was performed under conditions, where cytosolic Ca^{2+} activity was rather decreased and thus K^+ channel activity presumably not stimulated. Thus, PI 3-kinase most probably directly regulates SOC surface expression or activity.

A recent study performed on fetal liver-derived mast cells from STIM-1-deficient mice has shown that STIM-1, a sensor of Ca^{2+} in the ER and an activator of SOCs, is an essential positive regulator of mast cell activation (Baba et al. 81-88). SOC influx was suggested to be a key mechanism of Ca^{2+} entry active in these cells, since the sustained increase in intracellular free Ca^{2+} concentration after FcεRI stimulation was almost completely abrogated in *Stim1*$^{-/-}$ mast cells (Baba et al. 81-88). However, the residual Ca^{2+} influx measured in *Stim1*$^{-/-}$ cells could represent a SOC-independent pathway. SOC-independent, PIP_3-sensitive Ca^{2+} influx mechanism has been demonstrated in platelets, Jurkat T cells (Johnson et al. 831-37) and RBL-2H3 mast cells (Ching et al. 14814-20). The molecular identity of PIP_3-sensitive Ca^{2+} entry system has not been identified. There is evidence that TRPC channels could represent this mechanism, since PIP_3 activates TRPC6 and to a less extent TRPC3 in HEK293 cells stably expressing these channel isoforms (Tseng et al. 11701-08); 2004). Moreover, PI 3-kinase further promotes the activity of TRPV2 channels (Penna et al. 495-507), which were shown to be expressed in bone marrow derived mast cells (Stokes et al. 137-47).

Ion channels expressed in RBL-2H3 cells are, however, clearly distinct from those expressed in mouse BMMCs. At rest, RBL-2H3 cells exhibit a strongly inwardly-rectifying K^+ conductance carried by Kir2.1 channel (Wischmeyer, Doring, and Karschin 115-20;Wischmeyer, Doring, and Karschin 34063-68) which is absent in mouse BMMCs. On the other hand, Ca^{2+}-activated K^+ channels activated following FcεRI cross-linking in mouse BMMCs, have never been demonstrated electrophysiologically in RBL-2H3 cells.

The present observations do not rule out a direct action of PI 3-kinase signaling on Ca^{2+}-activated K^+ channels. In activated primary human CD4 T cells, activation of $K_{Ca}3.1$ was shown to be dependent on phosphatidylinositol-3 phosphate (PI(3)P) and inhibited by LY-294002 and wortmannin (Srivastava et al. 3630-38;Srivastava et al. 146-54;Srivastava et al. 665-75). Nucleoside diphosphate kinase B, a mammalian hisitidine kinase, was shown to function downstream of PI(3)P to activate $K_{Ca}3.1$ (Srivastava et al. 3630-38;Srivastava et al. 146-54;Srivastava et al. 665-75).

PI 3-kinase activates PDK1, which in turn activates the three SGK and the three protein kinase B/Akt isoforms (Lang et al. 1151-78).

We found distinct functional differences of mast cells in gene-targeted mice lacking SGK1(*sgk1*$^{-/-}$) and their wild-type littermates (*sgk1*$^{+/+}$), as well as in mice lacking SGK3 (*sgk3*$^{-/-}$) and their wild-type littermates (*sgk3*$^{+/+}$). Deficiency of SGK1 and SGK3 blunts Ca^{2+} entry, the currents through Ca^{2+}-activated K^+ channels and degranulation. Most importantly, anaphylactic reaction was strongly impaired in *sgk1*$^{-/-}$ and *sgk3*$^{-/-}$ mice.

It has been shown, that SGK1 increases the cell membrane abundance and activity of the Ca^{2+} channel TRPV5 (Embark et al. 203-12). It is conceivable that SGK1 has a similar stimulating effect on TRPV2.

SGK1/SGK3-dependent Ca^{2+} entry affects the activation of the Ca^{2+}-activated K^+ channels following Ag exposure. The K^+ channel activation following ionomycin is, however, similar in *sgk1*$^{-/-}$, *sgk3*$^{-/-}$ and the correspondent wild-type BMMCs. Thus, SGK1 and SGK3 blunt the Ag-induced activation of those channels largely by compromising Ca^{2+} entry with only little or no direct effect on those K^+ channels.

In other systems, SGK1 has been shown to activate a wide variety of different K^+ channels (Baltaev et al. 26-33;Embark et al. 601-06;Embark et al. 203-12;Gamper et al. 625-34;Henke et al. 194-99;Palmada et al. 629-34;Ullrich et al. 1090-99;Wärntges et al. 617-24;Yoo et al. 23066-75;Yun et al. 2823-30).

The present study does not rule out Ca^{2+}-independent effects of SGK1 on K^+-channels and other functions of mast cells. SGK1 may not only participate in the basic functions of mast cells, but as well in their regulation by hormones and mediators. SGK1 is genomically upregulated by glucocorticoids (Firestone, Giampaolo, and O'Keeffe 1-12), mineralocorticoids (Chen et al. 2514-19;Naray-Fejes-Toth et al. 16973-78;Shigaev et al. F613-F619), 1,25-dihydroxyvitamin D_3 ($1,25(OH)_2D_3$) (Akutsu et al. 1127-39), cell shrinkage (Waldegger et al. 4440-45), gonadotropins (Alliston et al. 1934-49;Alliston et al. 385-95;Gonzalez-Robayna et al. 1283-300;Richards et al. 223-54), and TGFß (Lang et al. 8157-62;Waldegger et al. 1081-88). The kinase is activated by

IGF1 and insulin through PI 3-kinase and PDK1 (Alessi et al. 6541-51;Alessi and Cohen 55-62;Divecha, Banfic, and Irvine 3207-14;Gamper et al. 625-34;Kobayashi and Cohen 319-28;Kotani et al. 2313-21;Park et al. 3024-33). Glucocorticoids rather suppress mast cell function (Andrade, Hiragun, and Beaven 7254-62;Collado-Escobar, Cunha-Melo, and Beaven 244-50;Fushimi et al. 134-42;Krishnaswamy et al. 167-76;Matsuda et al. 1357-63;Mazingue, Dessaint, and Capron 65-77;Robin et al. 2719-26;Wershil et al. 1391-98), an effect which, according to the present observations, cannot be mediated by upregulation of SGK1.

It has been shown on *Xenopus* oocyte system, that SGK3 stimulate the activity of further ion channels including the epithelial Na^+ channel ENaC (Friedrich et al. 693-96;Palmada et al. G143-G150), the renal and cochlear Cl^- channel complex ClC-Ka/barttin (Embark et al. 1918-25) , the cell volume regulated Cl^- channel ClC2 (Palmada et al. 1001-06), the cardiac voltage-gated Na^+ channel SCN5A (Boehmer et al. 1079-84), the cardiac K^+ channels KCNE1/KCNQ1 (Embark et al. 601-06) and HERG(Maier et al. 177-86), the voltage-gated K^+ channels Kv1.3 (Gamper et al. 625-34;Henke et al. 194-99;Warntges et al. 617-24), Kv1.5 (Ullrich et al. 1090-99) and Kv4.3 (Baltaev et al. 26-33).

SGK3 stimulates the activity of the Na^+-glucose cotransporter SGLT1 (Dieter et al. 862-70) the glutamine transporter SN1 (Boehmer et al. 156-62), the glutamate transporters EAAT1 (Boehmer et al. 1181-88), EAAT2 (Boehmer et al. 911-21), EAAT3 (Schniepp et al. 1442-49) and EAAT5 (Boehmer et al. 738-42), the dicarboxylate cotransporter NaDC-1 (Boehmer et al. 998-1003), the creatine transporter CreaT (SLC6A8) (Shojaiefard, Christie, and Lang 742-46) and the Na^+/K^+-ATPase activity (Henke et al. 370-74).

Basal intestinal glucose transport is decreased in SGK3 knockout mice (Sandu et al. 437-44). Similarly, intestinal transport is impaired in the SGK1/SGK3 double knockout mouse. The impaired intestinal nutrient uptake may contribute to the delayed growth of both SGK3 knockout mice (McCormick et al. 4278-88) and SGK1/SGK3 double knockout mice (Grahammer et al. R945-R950).

SGK3 has been shown *in vitro* to confer cell survival (Liu, Yang, and Songyang 1233-36;Xu et al. 699-705), an effect which may be related to the effect of SGK3 on Kv1.3 channel activity. In human embryonic kidney cells and Jurkat lymphocytes Kv1.3 is involved in the regulation

of cell proliferation (Gamper et al. 625-34;Lang et al. 147-57) and apoptosis (Gulbins et al. 7661-66;Szabo et al. 20465-69;Lang et al. 147-57). The antiapoptotic effect has further been attributed in part to phosphorylation of transcription factors (Brunet et al. 952-65;Liu, Yang, and Songyang 1233-36;Xu et al. 699-705). Moreover, SGK3 has been shown to phosphorylate and thus inactivate Bad (Liu, Yang, and Songyang 1233-36;Xu et al. 699-705). Phosphorylated Bad binds to the chaperone 14-3-3 and is thus prevented from travelling to the mitochondria, where it triggers apoptosis (Lizcano, Morrice, and Cohen 547-57).

As was expected, knockout of PDK1 is not compatible with the survival. Reduced expression of PDK1 in PDK1 hypomorphic mice results in remarkably decreased size of the animals. The size of *sgk1*$^{-/-}$ mice is, however, normal, indicating that SGK1 could be replaced by the other five PDK1-dependent SGK and protein kinase B/Akt isoforms. One of those kinases is apparently SGK3, given that growth is delayed in SGK3 knockout mice. Along those lines, PDK1-dependent transport regulation is not abrogated in *sgk1*$^{-/-}$ mice under control conditions. Apparently, SGK1 is not vital for housekeeping functions but is critically important under defined stress conditions. Thus, at least in theory, pharmacological inhibition of SGK1 may counteract allergic reactions without severe side effects.

In conclusion, the present observation confirms the role of PI 3-kinase, SGK1 and SGK3 in the regulation of mast cell function. Our results define a critical role for PI 3-kinase in regulating mast cell Ca^{2+} entry, Ca^{2+}-dependent K^+ channel activation and degranulation upon IgE-mediated stimulation. SGK1 and SGK3 are also important for the regulation of Ca^{2+} entry, Ca^{2+}- activated K^+ channel activation, and degranulation of mast cells upon IgE-Ag-dependent stimulation. IgE-dependent systemic anaphylactic response is markedly attenuated in mice lacking functional SGK1 or SGK3. Thus, the present observations disclose new regulators of mast cell function, which could aid the development of novel therapies for the treatment of allergic diseases and other mast cell-mediated diseases by means of mast cell ion channel modulation.

6. SUMMARY

Mast cells play a central role in IgE-dependent allergic reactions including allergic rhinitis, asthma, anaphylactic shock and delayed hypersensitivity reactions.

The mechanism of mediator secretion from mast cells in disease includes modulation of ion channel activity. Cross-linking of the mast cell IgE-receptor (FcεRI) by antigen leads to stimulation of Ca^{2+} entry with subsequent mast cell degranulation and release of inflammatory mediators. Ca^{2+} further activates Ca^{2+}-activated K^+ channels, which in turn provide the electrical driving force for Ca^{2+} entry. The phosphoinositol-3 (PI3)-kinase pathway plays a pivotal role in the stimulation of mast cells. Previous studies have shown, that PI 3-kinase is required for mast cell activation and degranulation. Kinases activated through the PI 3-kinase pathway include serum- and glucocorticoid-inducible kinases 1 (SGK1) and 3 (SGK3). The present study has been performed to elucidate whether PI 3-kinase participates in the regulation of mast cell Ca^{2+} and Ca^{2+}-activated K^+ channels, and whether PI 3-kinase-dependent modulation of those channels is involved in the regulation of mast cell function. Also we explored the role of SGK1 and SGK3 in mast cell function.

To this aim the whole-cell patch clamp experiments and Fura-2 fluorescence measurements for determination of cytosolic Ca^{2+} concentration were performed in LY-294002-treated and untreated mouse bone marrow-derived mast cells (BMMCs). In response to FcεRI cross-linking, Ca^{2+} entry but not Ca^{2+} release from the intracellular stores was dramatically reduced upon inhibition of PI 3-kinase by LY294002. Ca^{2+} entry following readdition of Ca^{2+} after Ca^{2+}-store depletion with thapsigargin was also decreased by LY-294002, pointing to inhibition of store-operated channels (SOCs). Moreover, inhibition of PI 3-kinase abrogated IgE-mediated, but not ionomycin-induced stimulation of Ca^{2+}-activated K^+ channels.

To explore the role of SGK1 and SGK3, mast cells have been isolated from bone marrow of SGK1 and SGK3 knockout mice (*sgk1*$^{-/-}$, *sgk3*$^{-/-}$) and their wild-type littermates (*sgk1*$^{+/+}$, *sgk3*$^{+/+}$). Forward scatter, as determined by FACS analysis, was significantly smaller in *sgk1*$^{-/-}$ than in *sgk1*$^{+/+}$ BMMCs, pointing to a decrease in cell volume. There was no difference in forward scatter between *sgk3*$^{-/-}$ and *sgk3*$^{+/+}$ BMMCs. Upon Ag stimulation via the FcεRI receptor, Ca^{2+} entry but not Ca^{2+} release from intracellular stores was markedly impaired in *sgk1*$^{-/-}$and *sgk3*$^{-/-}$

BMMCs. The currents through Ca^{2+}-activated K^+ channels induced by Ag were significantly higher in *sgk1*$^{+/+}$ and *sgk3*$^{+/+}$ BMMCs than in *sgk1*$^{-/-}$ and *sgk3*$^{-/-}$ cells, respectively. Treatment of the cells with Ca^{2+} ionophore ionomycin (1 µM) led to similar activation of the K^+ channels in all tested genotypes, indicating that the Ca^{2+}-activated K^+ channels are similarly expressed and sensitive to activation by Ca^{2+} in *sgk1*$^{-/-}$ and *sgk3*$^{-/-}$ BMMCs as in their wt littermates, and that blunted stimulation of Ca^{2+}-activated K^+ channels was secondary to decreased Ca^{2+} entry. Compared to wild-type mice, IgE-Ag-induced degranulation of *sgk1*$^{-/-}$ and *sgk3*$^{-/-}$ BMMCs was significantly blunted. The decrease in body temperature following Ag treatment, which reflects anaphylactic reaction, was dramatically reduced in *sgk1*$^{-/-}$ and *sgk3*$^{-/-}$ mice, pointing to impaired mast cell function *in vivo*.

Thus, our observations disclose PI 3-kinase-dependent regulation of Ca^{2+}- and Ca^{2+}-activated K^+ channels, which in turn participate in triggering mast cell degranulation. SGK1 and SGK3 are critically important for the regulation of Ca^{2+} entry, Ca^{2+}-activated K^+ channel activation and degranulation of mast cells upon IgE-Ag-dependent stimulation

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8. PUBLICATIONS

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