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Diacylglycerol kinase γ regulates antigen-induced mast cell degranulation by mediating Ca^{2+} influxes

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ABSTRACT

Diacylglycerol (DAG) is an important lipid that acts as a signaling messenger during mast cell degranulation after allergen cross-linking of immunoglobulin (Ig) E-bound FcεRI receptors. In this study, we determined the role of diacylglycerol kinase (DGK), which negatively regulates DAG-dependent signaling by converting DAG to phosphatidic acid (PA), in the regulation of mast cell degranulation. Treating RBL (rat basophilic leukemia)-2H3 mast cells with a type I DGK inhibitor significantly reduced antigen-induced degranulation and PA production. Among type I DGK isoforms, we observed that DGKα and DGKγ mRNAs were expressed in RBL-2H3 mast cells using reverse transcription polymerase chain reaction. DGKγ knockdown, but not DGKα, by isoform-specific short hairpin RNAs reduced mast cell degranulation and Ca^{2+} influxes from the extracellular environment. These results suggest that DGKγ regulates mast cell degranulation after FcεRI cross-linking through mobilization of intracellular Ca^{2+} through Ca^{2+} influxes.

1. Introduction

Mast cells are major effector cells in allergic responses by secreting numerous inflammatory mediators [1]. The signaling pathways involved in degranulation have been revealed using RBL(rat basophilic leukemia)-2H3 mast cells, a model of basophils and mast cells [2–4]. Antigen-driven cross-linking of high affinity immunoglobulin (Ig)E receptors (FcεRI) initiates a signaling cascade through activation of the Src family of protein kinases and tyrosine kinases [5,6]. These kinases phosphorylate and activate numerous downstream signaling molecules, including the linker for activation of T-cells (LAT; [7]). Phosphorylated LAT recruits several signaling molecules that contain Src homology 2 domains, such as the adaptor protein Grb2 and phospholipase Cγ (PLCγ; [8]).

Activated PLCγ hydrolyzes phosphatidylinositol-4,5-bisphosphate, which results in the production of inositol-1,4,5-triphosphate (IP_3) and diacylglycerol (DAG). DAG activates protein kinase C (PKC), and IP_3 mediates the release of intracellular Ca^{2+} from the endoplasmic reticulum (ER), which is required to open Ca^{2+} channels in the plasma membrane. Increases in intracellular Ca^{2+} levels and PKC activation are required for mast cell degranulation [9]. In addition to PKC, several DAG targets, such as Ras guanyl nucleotide-releasing proteins (RasGRP) and the canonical transient receptor potential (TRPC) channel protein, are required for antigen-induced degranulation [10,11]. These reports indicate that DAG plays important roles in mast cell function.

PLCγ depletion results in impaired antigen-induced degranulation and cytokine production [12], and DAG and its analogs induce degranulation in the presence of a Ca^{2+} ionophore [13]. Both DAG and phosphatidic acid (PA) are important lipid messengers for regulating degranulation. For example, phosphatidylinositol-4-phosphate 5-kinase [PI(4)P5K] and sphingosine kinase (Sphk), which are regulated by PA, are also important regulators of mast cell responses [14,15]. Thus, both DAG and PA levels in activated mast cells must be precisely controlled.

Diacylglycerol kinase (DGK) regulates the balance between DAG and PA levels in many cell types because DGK catalyzes DAG hydrolysis to generate PA. Molecular cloning has identified 10 mammalian DGK isoforms that are classified into five subfamilies [16,17]. Thus, DGK should play an important role in signal transduction during degranulation. Because both DGK substrates and products contribute to mast cell function, DGK may also be involved in mast cell degranulation. DGKζ knockout (KO) reduces

Abbreviations: DAG, diacylglycerol; DGK, diacylglycerol kinase; DNP, dinitrophenol; PA, phosphatidic acid; PKC, protein kinase C; PLC, phospholipase C; RBL, rat basophilic leukemia; Sphk, sphingosine kinase.

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PA production by approximately 50% compared with WT, but only DGKζ has been reported as a positive regulator of degranulation [18]. This partial suppression of PA production prompted us to explore the possible involvement of other DGK isoforms in mast cell degranulation.

Therefore, we determined the expression levels of DGK isoforms in RBL-2H3 mast cells. We also examined the effects of a type I DGK inhibitor (R59022) and short haipin (sh) RNAs that targeted type I DGKs on degranulation and Ca^{2+} mobilization.

2. Materials and methods

2.1. RBL-2H3 cells and those cells that stably express DGK shRNA

RBL-2H3 cells were grown in RPMI 1640 medium (Nacalai Tesque) supplemented with 10% FBS (Sigma–Aldrich), 100 U/ml of penicillin, and 100 μg/ml of streptomycin (GIBCO) in a 37 °C humidified atmosphere with 5% CO_2. pSuper-gfp/neo based plasmids that expressed shRNA, targeting DGKα and DGKγ, were transfected using an Amaxa Nucleofector System (Amaxa/Lonza), according to the manufacturer's instructions. Geneticin (0.5 mg/ml) was added to the medium at 24 h after transfection. After culture for more than 24 h, transfected cells were transferred to 96-well plates at a density of 1 cell/well for cloning. Positive clones were identified by fluorescence that was detected using confocal microscopy.

2.2. Plasmid construction

For RNA interference experiments, double-stranded oligonucleotides were subcloned into a short hairpin RNA (shRNA) expression vector pSuper.gfp/neo (OligoEngine) that contained the H1 small nuclear RNA promoter at its *Bgl*II/*Hind*III sites. Target sequences used for knockdown were: DGKα target, 5′-GGAGCAGATTGTGTTGGAA-3′ and 5′-GCGATGTGCTGAAGGTCTT-3′; DGKγ target, 5′-GGATGACGTTTCACCGCAA-3′ and 5′-GCTCTACCAAGAAAGGAAA-3′; and luciferase (control) target, 5′-AACATAAAGAAAGGCCCGG-3′.

2.3. Degranulation assay

RBL-2H3 cells (5.0×10^5/ml) were incubated overnight. For antigen stimulation, cell cultures were incubated with 0.5 μg/ml anti-mouse monoclonal dinitrophenyl (DNP)-IgE (500 ng/ml; Sigma-Aldrich). These cells were subsequently washed twice and treated with DNP-BSA for 15 min at 37 °C. Supernatants were transferred to 96-well plates and incubated with β-hexosaminidase substrate (1 mM *p*-nitrophenyl-*N*-acetyl-D-glucosaminide) at 37 °C for 1 h. To stop the reaction, 0.2 M Tris was added, and the absorbance was measured at 405 nm.

2.4. PA production assay

PA measurements were made as previously described [19]. In brief, cells were stimulated with DNP-BSA, after which lipids were extracted from cells using the Bligh–Dyer lipid extraction method [20]. Lipids were dried under a nitrogen stream and subsequently suspended in 2% Triton X-100. Lipids were incubated with lipoprotein lipase generated from *Burkholderia sp.* (Sigma–Aldrich), followed by incubation with glycerol-3-phosphate oxidase (from *Aerococcus viridans*; Sigma–Aldrich), horseradish peroxidase (Sigma–Aldrich), and Amplex red (Invitrogen). A standard curve was generated for each experiment using egg-PA (Avanti Polar Lipids). Protein contents were measured using a BCA protein assay kit (Thermo Fisher Scientific, Inc., Rockford, IL, USA) and used for normalization. Finally, PA amounts in stimulated cells were normalized to those of unstimulated cells for each experiment.

2.5. RT-PCR

DGK isoform mRNA expressions were determined by reverse transcription polymerase chain reaction (RT-PCR). Total RNA was isolated from RBL-2H3cells using TRIzol Reagent (Invitrogen). Total RNA (200 ng) was reverse-transcribed into cDNA using a ThermoScript RT-PCR System (Invitrogen) using a standard protocol and subsequently amplified using Ex Taq (TaKaRa). The specific primers used were: DGKα forward, 5′-GATGAGATTGGGTACCTGG and reverse 3′-ACTGCCATCCCGGTCC; DGKβ forward, 5′-CCAGCAAATACG TGCTTCCC and reverse 3′-TTGATGCAGGAGGGCGG; DGKγ forward, 5′-TGGACAAAGGGGGGCCTA and reverse 3′-GGGCATGACCTCTAG GTAC; DGKδ forward, 5′-GGCATGCTGACCAAACAGAAC and reverse 3′-CATCCTCGTCCTCAATGATATCC; DGKε forward, 5′-ATGGTGTAG TCTGCAGCGTTCG and reverse 3′-CACCTGGACGGGATTTAACAGCA; DGKι forward, 5′-GCAGGAGGGAAAATGTAAGCA and reverse 3′-GCAAAGGCTTCATGAGAGGA; DGKη forward, 5′-TGCCTCCATAG CTGGGAG and reverse 3′-CTGCGTAGCTTGGGATGTTC; DGKθ forward, 5′-ACGATTATGACACGTATCACCACC and reverse 3′-GGCGAA ACTGATTTTTCCTCATGG; and DGKζ forward, 5′-TCCTTGGCCGCA TTGCAG and reverse 3′-TCCTGGGCCCTGTCGAT.

2.6. Western blot analysis for endogenous DGKs translocation

Endogenous DGKs translocation to cell membranes was determined by Western blotting. Cells were cultured in 60-mm dishes (3×10^6 cells in 3 ml/dish) and incubated overnight with 0.5 μg/ml of DNP-specific IgE at 37 °C. For control dishes, after a washing step, cells were stimulated with either 10 ng/ml of antigen, DNP-BSA, or 1 μm ionomycin for 0, 1, 3, and 5 min in glucose-Pipes buffer (119 mm NaCl, 5 mm KCl, 0.4 mm $MgCl_2$, 1 mm $CaCl_2$, 5.6 mm glucose, 1 mg/ml BSA, 25 mm Pipes/NaOH; pH 7.4). After washing with ice-cold glucose-Pipes buffer, cells were scraped off and sonicated in extraction buffer (20 mm Tris–HCl, pH 7.5, 5 mm EDTA, 10 mm EGTA, 1 mm dithiothreitol, 50 μg/ml of PMSF, and 5 μg/ml of Leupeptin). The homogenate was centrifuged at 180,000*g* for 20 min at 4 °C, after which the pellet was resuspended with sonication in 1 ml of extraction buffer and centrifuged at 180,000*g* for 20 min at 4 °C. The membrane fraction was solubilized in 100 μl of extraction buffer, containing 0.1% Triton X-100. Protein concentrations were determined by a Bradford assay. The homogenate was diluted in SDS sample buffer and boiled at 95 °C for 5 min. Membrane proteins were separated by SDS–PAGE and blotted onto PVDF membranes. Western blotting was performed according to the manufacturer's, protocol and immunoreactivity was detected using ECL detection reagents.

2.7. Intracellular Ca^{2+}measurement

Cytosolic Ca^{2+} levels were measured using a fluorescent indicator. RBL-2H3 cells (1.8×10^3 cells/well) were placed in a 96-well CC3 black plate (Nunc) for 1 day before measurements. Cells were loaded with 2.5 μM Fura 2/AM and 1% Pluronic 100 (both from Dojindo) for 20 min in Siraganian buffer (119 mM NaCl, 5 mM KCl, 0.4 mM $MgCl_2$, 5.6 mM glucose, 1 mM $CaCl_2$, 0.1% BSA, and 25 mM 1,4-piperazinediethanesulfonic acid, pH 7.4). Subsequently, cells were washed and treated with 50 nM DNP-BSA. Fura2 fluorescence intensity was monitored using a Mithras LB940 microplate reader (Berthold Technologies, Germany). Emission was measured at 535 ± 20 nm after excitation at 340 ± 10 nm and 380 ± 10 nm.

3. Results

3.1. DGK inhibitor suppresses antigen-induced degranulation and PA production

We examined whether DGK was involved in antigen-induced mast cell degranulation using a type I DGK inhibitor, R59022 [21]. Antigen-induced degranulation was inhibited by this DGK inhibitor in a dose-dependent manner (Fig. 1A), which suggested that type I DGK isoforms were involved in antigen-induced degranulation. To confirm the involvement of type I DGK in degranulation, we determined the effects of R59022 on PA production after antigen stimulation. Adding DNP-BSA to IgE-sensitized RBL-2H3 cells resulted in robust PA accumulation beginning at 30 s after stimulation. This increase in PA was inhibited by R59022 treatment (Fig. 1B). This partial decrease in PA was likely due to other DGK isoforms. These results indicated that type I DGK isoforms were partially involved in antigen-induced mast cell degranulation and PA production.

3.2. Expression and translocation of DGK isoforms in RBL-2H3 mast cells

To determine the DGK isoforms expressed in RBL-2H3 cells, we used RT-PCR using cDNA from RBL-2H3 cells. Among the 9 isoforms tested, DGKα, γ, δ, ε, ζ, and θ were expressed by these cells (Fig. 2A). Because both DGKα and γ are classified as type I DGK, we focused on these two isoforms.

To examine the DGK isoforms activated during degranulation, we monitored the translocation of endogenous DGKα and γ to cell membranes after antigen cross-linking by fractionation because DGK translocation is used as an index of DGK activation [22]. The DGKα level in the membrane fraction did not change compared with unstimulated cells, whereas antigen cross-linking induced DGKγ recruitment to the membrane fraction (Fig. 2B). This suggested that among type I DGK isoforms, DGKγ was involved in degranulation after antigen cross-linking on RBL-2H3 cells.

3.3. Impaired degranulation after DGKγ knockdown

To confirm that type I DGKγ, but not DGKα, affected degranulation after antigen cross-linking, we used short hairpin RNAs (shRNAs) for DGKα and DGKγ. After establishing stable knockdown (KD) cell lines for DGKα and DGKγ using shDGKα and [20] shDGKγ, respectively, we confirmed that these shRNAs resulted in >50% reductions in DGKα or DGKγ expression levels in each cell line without affecting other isoform level by immunoblotting analysis (Fig. 3A). shRNA for luciferase, used as a negative control, had little effect on either isoform levels (Fig. 3A). DGKγ-KD cells had significantly reduced PA levels up to 1 min after FcεRI stimulation (Fig. 3B). In contrast, DGKα-KD cells produced slightly lower amounts of PA than control cells, although this difference was not significant (Fig. 3B). Corresponding to the amount of PA produced, there was reduced antigen-induced degranulation due to DGKγ-KD, but not due to DGKα-KD (Fig. 3C). These results showed that DGKγ was specifically involved in regulating antigen-induced mast cell PA production and degranulation.

3.4. DGKγ knockdown cells have defective Ca^{2+} influxes

To understand the mechanisms by which DGKγ KD inhibited antigen-induced mast cell degranulation, we examined signaling

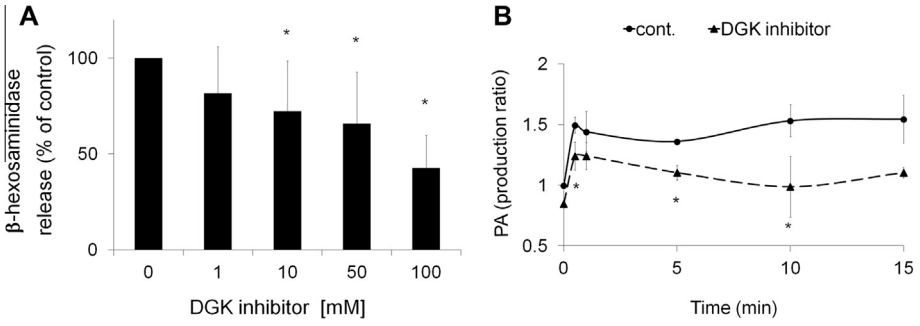

Fig. 1. DGK inhibitor effects on mast cell PA production and degranulation. (A) Total PA levels in cell lysates were determined using the enzymatic assay described in Section 2. Lysates were prepared from RBL-2H3 cells that were either untreated or pre-treated with the indicated concentrations of the DGK inhibitor R59022 for 10 min and subsequently stimulated with 50 ng/ml of DNP-BSA. PA values in stimulated cells were normalized to those of unstimulated cells for each experiment and reported as production ratios. (B) RBL-2H3 cells were incubated for 15 min with different concentrations of R59022 and subsequently stimulated with 50 ng/ml of DNP-BSA for 15 min. β-Hexosaminidase release was expressed as a percentage of control. Experiments in A and B were conducted in triplicate, and error bars indicate ± SD (*$p < 0.05$).

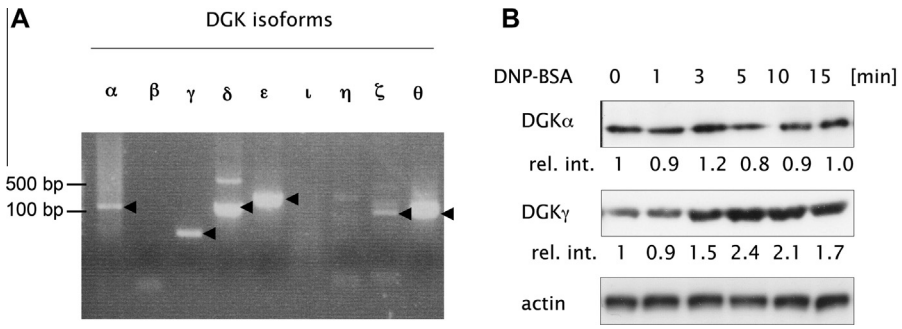

Fig. 2. Expression and translocation of DGK isoforms in RBL-2H3 cells. DGK isoform expression levels in RBL-2H3 cells were determined by RT-PCR. (B) Translocation of DGKs from the cytoplasm to subcellular membranes was analyzed by immunoblotting after DNP-BSA treatment for the indicated times. Quantitation of the corresponding protein levels was determined using densitometry and shown as relative intensity levels (rel. int.). Equal amounts of protein were determined based on the amounts of actin.

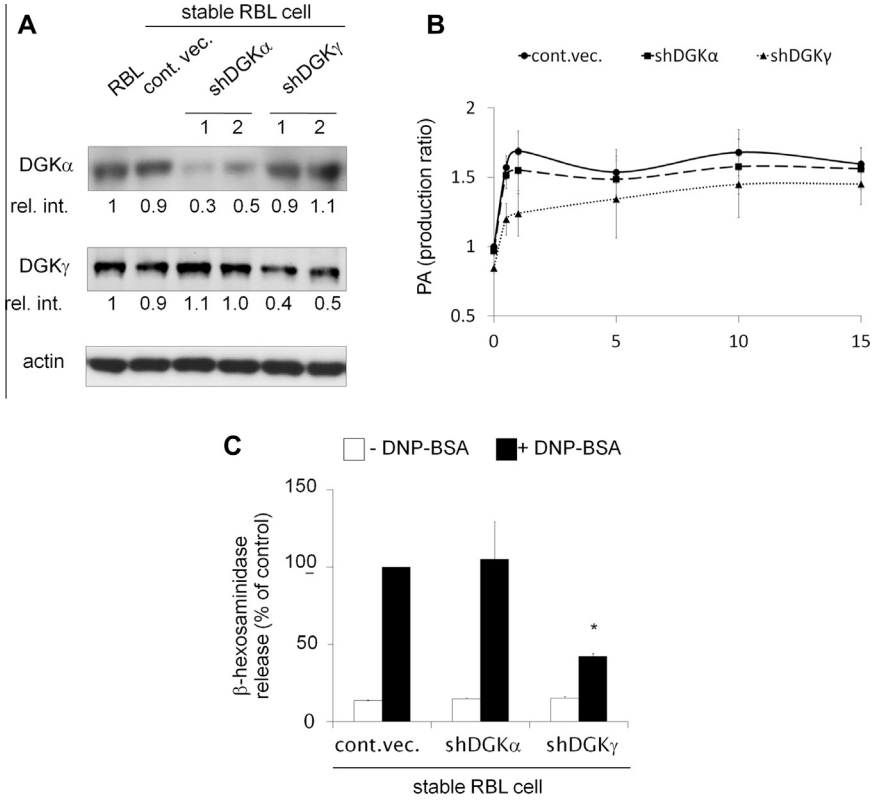

Fig. 3. DGK knockdown effects on RBL-2H3 mast cell degranulation. (A) Efficiency of small hairpin RNA (shRNA) for knocking down the expression of DGKα and γ isoforms in RBL-2H3 cells. Quantitation of the corresponding proteins was determined using densitometry and shown as relative intensity levels (rel. int.). Equal amounts of protein were determined on the basis of the amounts of actin. (B, C) DGK KD effects on mast cell PA production (B) and degranulation (C) (*$p < 0.05$).

events downstream of FcεRI. PLCγ activation is crucial for degranulation. Therefore, phosphorylation of this enzyme was monitored as an indicator of its activation after stimulation by DNP-BSA using an anti-phospho-PLCγ antibody. PLCγ phosphorylation was not obviously affected by DGKγ-KD (Fig. 4A), which suggested that DGKγ was not essential for PLCγ tyrosine phosphorylation.

Next, we investigated whether DGKγ-KD affected FcεRI-induced Ca^{2+} responses because Ca^{2+} mobilization in response to IgE receptor-mediated signaling is a key process in several aspects of mast cell function, including degranulation [23]. DGKγ-KD cells had a significantly reduced Ca^{2+} increase after antigen stimulation compared with control cells in the presence of extracellular Ca^{2+} (Fig. 4B). DGK inhibitor I treatment also suppressed the increase in antigen-induced Ca^{2+} levels (Supplementary Fig. S1). These results implied that DGKγ was important for Ca^{2+} mobilization during degranulation.

An amplified antigen-stimulated Ca^{2+} response includes Ca^{2+} release from internal stores and a Ca^{2+} influx from the extracellular medium. Ca^{2+} release from ER stores has been implicated in directly activating the Ca^{2+} influx pathway [24]. However, because PLCγ phosphorylation was normal, we hypothesized that DGKγ-KD affected intracellular Ca^{2+} mobilization independently of PLCγ activity. FcεRI-dependent release of Ca^{2+} from intracellular stores, which was measured by removing Ca^{2+} from the extracellular medium, was comparable among the different stably transfected cell lines (Fig. 4C). However, replenishing Ca^{2+} in the Ca^{2+}-free medium after 250 s of FcεRI stimulation resulted in an incomplete increase of Ca^{2+} in DGKγ-KD cells, but not in DGKα-KD cells. This indicated that DGKγ contributed to Ca^{2} influxes from the extracellular medium, which was induced by ER Ca^{2+} store depletion.

4. Discussion

Previous studies established that DAG and PA were significant lipid messengers for FcεRI-induced mast cell degranulation. Despite the significance of DGK in regulating the balance between DAG and PA production, the role of individual DGK isoforms in degranulation has not been determined, except for DGKζ [18]. DGKζ-KO bone marrow-derived mast cells (BMMCs) produced half the amounts of PA as compared with WT BMMCs during FcεRI-induced activation. This incomplete suppression of PA production prompted us to consider the possible involvement of other DGKs besides DGKζ because we observed that other DGK isoforms were expressed by RBL-2H3 mast cells (Fig. 2A). However, because other DGK isoforms were also expressed by these cells, we cannot rule out that PA derived from other DGK isoforms may have compensated for DGKγ-KD. It was also reported that FcεRI-induced Ca^{2+} responses were decreased in the absence of DGKζ. In the present study, DGKγ-KD, but not DGKα-KD abrogated FcεRI-induced PA production and Ca^{2+} influxes. Together with the results of a previous report, both DGKγ and DGKζ appeared to be involved in PA production and Ca^{2+} mobilization during mast cell activation.

We observed DGK isoform-specific roles in the signal transduction that leads to degranulation. For example, PLCγ phosphorylation was decreased in DGKζ-KO BMMCs, whereas this was not observed in DGKγ-KD cells. This difference suggests that DGKζ-derived PA regulates PLCγ activity and results in Ca^{2+} mobilization from the ER. In contrast, DGKγ controls Ca^{2+} influxes from the extracellular environment without affecting PLCγ activity and Ca^{2+} release from the ER.

We also observed differences between DGKγ and DGKα because DGKγ, but not DGKα, contributed to mast cell degranulation and

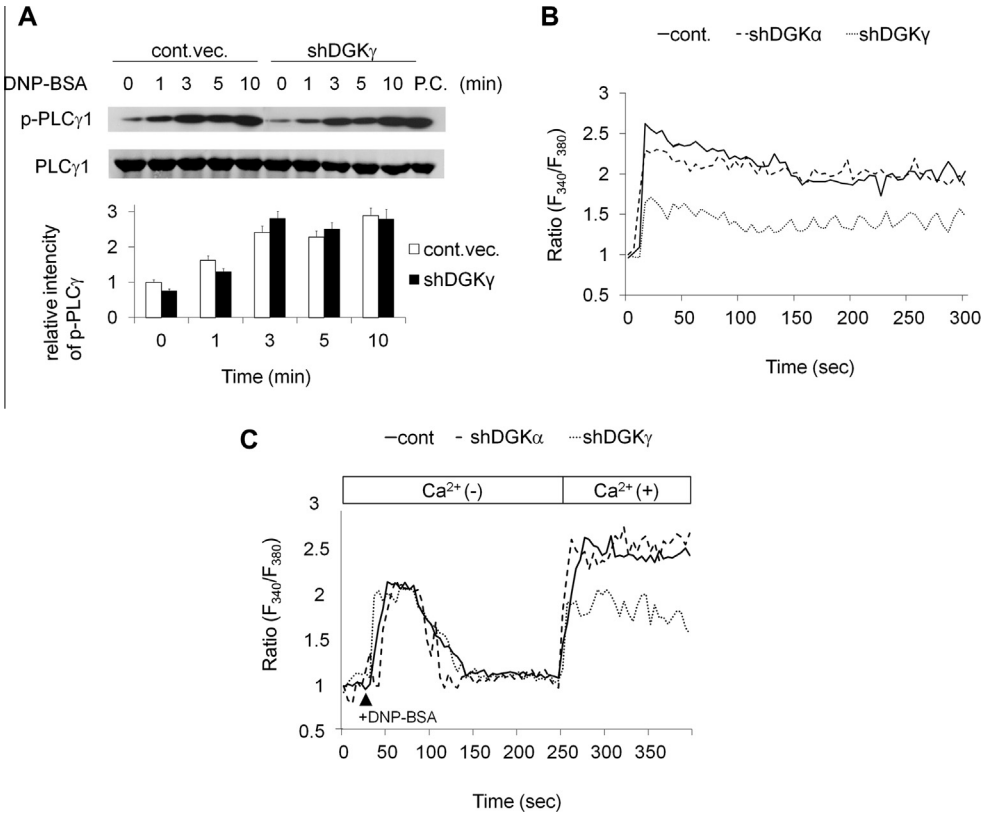

Fig. 4. DGK knockdown effects on antigen-induced PLCγ activation and Ca^{2+} mobilization in RBL-2H3 cells. (A) Normal PLCγ phosphorylation in DGKγ-KD RBL-2H3 cells after FcεRI stimulation. RBL-2H3 cell lines that stably expressed shRNA for DGKs were stimulated with 50 ng/ml of DNP-BSA. PLCγ phosphorylation was determined by Western blot using an anti-phospho-PLCγ antibody. Blots were probed with an anti-actin antibody as a loading control. (B) RBL-2H3 cell lines loaded with Fura-2 were stimulated by FcεRI to induce Ca^{2+} responses in the presence of extracellular Ca^{2+}. Ca^{2+} increase was determined with a LB940 microplate reader and shown as the emission ratio of Fura2 after excitation at 340 and 380 nm. (C) Fura-2 loaded RBL-2H3 stably expressing cell lines were stimulated with DNP-BSA in the absence of extracellular Ca^{2+}, after which fluorescent signal emission was monitored. At 25 s after stimulation, 2 mM Ca^{2+} was added to the medium.

Ca^{2+} influxes after antigen stimulation, even though both DGKα and γ are classified as type I DGKs. The distinct activities of these DGK isoforms for their diverse targets may explain this difference. For example, DGKγ, but not DGKα reportedly exhibited maximal activity without added Ca^{2+} [25], although both DGKs have Ca^{2+}-coordinating residues of two EF-hand motifs. Based on these observations, we speculated that DGKγ, but not DGKα and DGKζ can work at the upstream of Ca^{2+} influxes. Additional experiments will be necessary to fully understand the isoform-specific mechanisms for regulating degranulation.

The detailed mechanism by which DGKγ regulates Ca^{2+} influxes remains to be determined. One possible target is Orai1, which is one component of store-operated Ca^{2+} entry (SOCE) [26]. In DGKγ-KD cells, DAG, which activates PKC, accumulates concomitant with decreased PA levels. We previously reported that Orai1 was phosphorylated in serine residues by PKCβ, which resulted in negative regulation of Orai1 function [27]. Studies using Orai1 KO mice suggested that Orai1 was essential for mast cell degranulation [28]. Considering the results of these reports, DAG accumulation induced by DGKγ-KD may negatively regulate SOCE by suppressing Orai1. Our PKC activation assay showed that PKCβ activity was upregulated in unstimulated DGKγ-KD cells compared with control cells (Supplementary Fig. S2).

Another possible mechanism is a sphingosine kinase (Sphk)-induced pathway. By phosphorylating sphingosine (SPH) to generate sphingosine 1 phosphate (S1P), Sphk regulates the levels of two important bioactive lipids and affects several cellular functions [29]. Intracellular S1P formation promoted Ca^{2+}-induced calcium entry in human neutrophils [30]. In contrast, SPH has been shown to inhibit Ca^{2+} influxes [31–34]. Of particular interest, the study by [33] using RBL-2H3 cells suggested that SPH and its structural analogs were specific inhibitors of store-operated calcium release-activated Ca^{2+} currents (iCRAC). In addition, it has been shown that Sphk2-deficient mast cells had diminished SOCE and degranulation [15]. Thus, the putative mechanism by which SphK2 reduces the levels of SPH through S1P generation may exert its effect on Ca^{2+} influxes. Although the results were not statistically significant, at some time points, we observed that S1P levels after antigen treatment had decreased in DGKγ-KD cells in contrast to increased S1P levels in control cells (Supplementary Fig. S2).

In conclusion, we identified DGKγ as a novel regulator of mast cell degranulation. Generating mice deficient in this isoform may confirm the results found with RBL-2H3 cells.

Conflict of interest

The authors have no financial conflicts of interest.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2014.01.197.

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