Mast cells play important roles in both innate and adaptive immune responses. They are central effector cells in immune responses to parasites and in the pathogenesis of diseases such as asthma and allergy (1, 2). The high affinity receptor for IgE (FcεRI) is one of several cell surface receptors critical for mast cell development and function (3). FcεRI binds to IgE in the absence of antigen and subsequent cross-linking of IgE-bound FcεRI by cognate antigen induces a signaling cascade that leads to mast cell degranulation and cytokine secretion, which contribute to both chronic allergic inflammation and acute anaphylaxis. Understanding FcεRI signaling and mast cell activation is critical to devising new therapies for mast cell–mediated diseases.

Recent studies have greatly improved our understanding of FcεRI signaling. After FcεRI engagement, the Src family members Lyn and Fyn and the tyrosine kinase Syk are activated (4, 5). These molecules in turn recruit and activate other kinases such as the Tec family kinase Btk (6), phospholipid modifying enzymes including phosphatidylinositol 4,5-bisphosphate 3-kinase (PI3K) (7), the GTPase-activating molecule Vav1 (8), and adaptor molecules such as linker for activated T cells (LAT) (9), non–T cell activation linker (NTAL/LAB) (10, 11), SH2 domain containing leukocyte phosphoprotein of 76 kD (SLP-76) (12, 13), and Grb2-associated binder protein 2 (Gab2) (14). The formation of a multimolecular signaling complex coordinates activation of various downstream signaling pathways necessary for mast cell effector functions. These pathways include phospholipase Cγ (PLCγ) (15, 16), protein kinase C (PKC) isoforms (17, 18), and mitogen-activated protein kinases (MAPKs) (19). PLCγ hydrolyzes the membrane phospholipid phosphatidylinositol 4,5-bisphosphate (PIP2), leading to the generation of two important second messengers, diacylglycerol and inositol 1,4,5-trisphosphate (IP3) (20, 21). These messengers activate distinct signaling branches that ultimately lead to mast cell degranulation, cytokine production, and other effector functions.

Calcium and diacylglycerol are critical second messengers that together effect mast cell degranulation after allergen cross-linking of immunoglobulin (Ig)E-bound FcεRI. Diacylglycerol kinase (DGK)ζ is a negative regulator of diacylglycerol-dependent signaling that acts by converting diacylglycerol to phosphatidic acid. We reported previously that DGKζ−/− mice have enhanced in vivo T cell function. Here, we demonstrate that these mice have diminished in vivo mast cell function, as revealed by impaired local anaphylactic responses. Concordantly, DGKζ−/− bone marrow–derived mast cells (BMMCs) demonstrate impaired degranulation after FcεRI cross-linking, associated with diminished phospholipase Cγ activity, calcium flux, and protein kinase C–βII membrane recruitment. In contrast, Ras–Erk signals and interleukin-6 production are enhanced, both during IgE sensitization and after antigen cross-linking of FcεRI. Our data demonstrate dissociation between cytokine production and degranulation in mast cells and reveal the importance of DGK activity during IgE sensitization for proper attenuation of FcεRI signals.
second messengers, diacylglycerol (DAG) and inositol 1,4,5-trisphosphate (IP$_3$). IP$_3$ binds to its receptor in the endoplasmic reticulum and induces Ca$^{2+}$ release into the cytoplasm.

DAG recruits to the membrane and activates PKC family members and RasGRPs, which are recently identified guanine nucleotide exchange factors for Ras and Rap (20). Synergistic action of multiple downstream signals, particularly Ca$^{2+}$ and PKCs, are required to induce mast cell degranulation (18, 21, 22). Activated PKCs and MAPKs together promote transcription of many proinflammatory genes, including cytokines (22–25).

Both in vitro and in vivo evidence suggest a critical role for DAG in the regulation of mast cell function after FcεRI engagement. Treatment of mast cells with DAG analogues in the presence of a Ca$^{2+}$ ionophore can mimic FcεRI engagement and induce mast cells to degranulate and release active mediators (26, 27). Mice lacking PLC$\gamma$2, the enzyme that generates IP$_3$ and DAG, have diminished mast cell function (28, 29). Similarly, deficiency in DAG effector molecules alters mast cell function. Multiple PKCs are expressed in mast cells, and activation of both classical and novel isoforms of PKC is regulated by DAG (18, 30). Different PKCs have distinct functions in mast cells. PKC$\beta$−/− mast cells demonstrate decreased IL-6 production and degranulation in response to FcεRI engagement (22), whereas PKC$\delta$−/− mast cells respond more vigorously to suboptimal FcεRI stimulation with more sustained Ca$^{2+}$ mobilization and increased degranulation compared with WT mast cells (31). Thus, proper balance of PKC$\beta$ and PKC$\delta$ activities appears important for mast cell function.

These observations suggest that DAG levels must be tightly controlled in mast cells. One mechanism for terminating DAG signaling is by phosphorylation catalyzed by the DAG kinase (DGK) family of enzymes. Phosphorylation of DAG by DGKs converts DAG to phosphatidic acid (PA), thus preventing DAG from activating PKCs and RasGRPs (20, 32–34). Additionally, PA itself is a second messenger, thus serving as a negative control for DAG signaling is by phosphorylation catalyzed by the DAG kinase (DGK) family of enzymes. Phosphorylation of DAG by DGKs converts DAG to phosphatidic acid (PA), thus preventing DAG from activating PKCs and RasGRPs (20, 32–34).

Therefore, through conversion of DAG into PA, DGK enzymes could regulate many aspects of inositol lipid metabolism and mast cell activation upon FcεRI engagement.

We recently described mice deficient in DGK$\zeta$ and demonstrated that T cells from these animals are hypersensitive to TCR stimulation. DGK$\zeta$−/− mouse T cells mount enhanced antiviral immune responses, indicating that DGK$\zeta$ is an important in vivo negative regulator of TCR signaling and T cell activation (38, 39). We show here that DGK$\zeta$ also regulates immune receptor signaling in mast cells. To our surprise, in vivo mast cell function is impaired in DGK$\zeta$−/− mice as indicated by diminished local anaphylactic responses. To explore the mechanism underlying this finding, we have studied DGK$\zeta$−/− bone marrow–derived mast cells (BMMCs). We demonstrate that FcεRI-induced degranulation of DGK$\zeta$−/− BMMCs is diminished, correlating with impaired PLC$\gamma$ activation and PKC$\beta$II membrane translocation. Similar to what we have observed after TCR stimulation, however, FcεRI-induced activation of the Ras–Erk signaling pathway is enhanced and DGK$\zeta$−/− BMMCs produce increased IL-6 during IgE sensitization and after antigen cross-linking of the FcεRI. Moreover, mast cell survival after growth factor withdrawal is greatly increased by DGK$\zeta$ deficiency, correlating with maintained phosphorylation of Akt. These findings indicate that DGK$\zeta$ functions to maintain mast cell responsiveness to antigen stimulation during passive sensitization with IgE and demonstrate separation of cytokine production and degranulation after FcεRI stimulation of mast cells.

**RESULTS**

**Mast cells develop in DGK$\zeta$-deficient mice**

We previously reported that DGK$\zeta$ plays an important role in T cell activation (38, 39). DGK$\zeta$ is also expressed in mast cells and, as in T cells, the 115-kD isoform is prominent (Fig. 1 A). There are also smaller species reactive with the anti-DGK$\zeta$ antibody, which may be products of protein degradation, as mast cells are rich in granules containing proteases. Alternatively, these might represent products of alternate transcripts. Importantly, the three major species reactive with the DGK$\zeta$ antibody are all absent in BMMCs from targeted mice (Fig. 1 A). Mast cell development is unaffected by DGK$\zeta$ deficiency for the following reasons: the numbers of mast cells in ear, skin, stomach, and spleen are similar in WT and DGK$\zeta$−/− mice (Fig. 1 B and not depicted); electron microscopy did not reveal any differences in cell morphology or number of intracellular granules (not depicted); and KO mast cells express similar levels of c-Kit and FcεRI on their surfaces (Fig. 1 C). Additionally, DGK$\zeta$ deficiency does not result in compensatory up-regulation of transcripts for related DGK isoforms (Fig. S1, available at http://www.jem.org/cgi/content/full/jem.20052424/DC1).

**Decreased passive cutaneous anaphylaxis in DGK$\zeta$-deficient mice**

DGK$\zeta$−/− T cells have enhanced homeostatic proliferation and antiviral responses in vivo (39). To test the importance of DGK$\zeta$ in mast cell function, we assessed allergic responses by examining passive cutaneous anaphylaxis (PCA), an in vivo measure of FcεRI-dependent mast cell function. Unexpectedly, DGK$\zeta$−/− mice had significantly impaired localized anaphylactic responses (Fig. 2 A).

To explore why DGK$\zeta$−/− mice demonstrate impaired PCA responses, we first compared FcεRI-triggered degranulation in WT versus DGK$\zeta$−/− BMMCs by measuring β-hexosaminidase release (Fig. 2 B). FcεRI-induced degranulation was significantly diminished in DGK$\zeta$−/− BMMCs (Fig. 2 B). PMA plus ionomycin (Io) stimulation, however, resulted in similar degranulation in WT and DGK$\zeta$−/− BMMCs, indicating that the ability to release granules and total granule content is unaffected by DGK$\zeta$ deficiency.
The impairment of FceRI-induced mast cell degranulation likely contributes to the decreased PCA responses in DGKζ−/− mice.

Another consequence of FceRI engagement is the production of proinflammatory cytokines such as IL-6. IgE alone can induce a significant amount of IL-6 production in WT BMMCs (Fig. 2 C), which is consistent with previous reports (40). Cross-linking the receptor with antigen induces even more IL-6 production. Under both stimulation conditions, DGKζ−/− BMMCs produce approximately twofold more IL-6 than WT BMMCs. Therefore, DGKζ deficiency impairs FceRI-induced degranulation, but increases cytokine production after IgE sensitization and after cross-linking of the receptor with antigen.

Antigen stimulation of IgE-sensitized BMMCs

Cross-linking of IgE-bound FceRI results in activation of a signaling network that coordinates mast cell effector functions. To investigate why DGKζ−/− BMMCs have impaired degranulation but enhanced cytokine production after FceRI stimulation, we assessed how DGKζ deficiency affects antigen-induced FceRI signaling in IgE-sensitized BMMCs. We first verified that DGKζ phosphorylates DAG after antigen stimulation of BMMCs by measuring PA production. Addition of DNP-HSA to IgE-sensitized WT BMMCs results in robust PA accumulation beginning 5–10 min after stimulation (Fig. 3 A). As expected, antigen stimulation of DGKζ−/− BMMCs results in significantly less PA accumulation.

We next examined signaling pathways that have been implicated in cytokine production after FceRI ligation. Activation of the PI3K/Akt and Ras/Erk pathways is required for cytokine production (23, 25, 41). Low dose stimulation of IgE-sensitized WT BMMCs with DNP-HSA weakly activates Akt, but stimulation of DGKζ−/− BMMCs results in significantly increased Akt activation.
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(Fig. 3 B). FcεRI activation of Ras is believed to be the result of Grb2-SOS activation (42, 43), but recent reports suggest that DAG might contribute to Ras activation through allosteric activation of the Ras guanine-nucleotide exchange factor RasGRP4 (44–46). As shown in Fig. 3 C, FcεRI-induced Ras activity is enhanced in DGKζ−/− BMMCs. Consistent with the increased Ras activation, we observed enhanced and prolonged Mek1/2 and Erk1/2 phosphorylation in DGKζ−/− BMMCs compared with WT BMMCs (Fig. 3 D). The effect of DGKζ deficiency is selective, however, as FcεRI-induced activation of the MAPKs p38 and Jnk is similar in WT and DGKζ−/− BMMCs (unpublished data). These data demonstrate that DGKζ deficiency enhances FcεRI-induced activation of Akt, Ras, and Erk1/2, likely contributing to enhanced cytokine production.

Figure 3. Antigen stimulation of IgE-sensitized BMMCs. (A) DGKζ deficiency impairs PA production by BMMCs after FcεRI stimulation. BMMCs were sensitized for 4 h with IgE, labeled with 32P-orthophosphate, and either left unstimulated or stimulated with 10 ng/ml DNP-HSA for 10 min. Lipids were extracted, separated by TLC, and quantified using a phosphorimager. PA, the product of DAG phosphorylation, was identified by comigration with a standard. Data are presented as fold-induction calculated by cpm of stimulated cells divided by cpm of unstimulated cells. Means ± standard error of four experiments are shown; *, P < 0.05. (B–D) WT and DGKζ−/− BMMCs were sensitized with IgE for 4 h and left unstimulated (U) or were stimulated either with 4 ng/ml of DNP-HSA for different times or with 100 ng/ml PMA (P) for 5 min. (B) Akt phosphorylation was assessed by immunoblot analysis using an antibody specific for phosphorylated Ser473 of Akt. Total Akt is shown as a loading control. (C) Active Ras in lysates was measured by affinity precipitation using GST-Raf-RBD agarose beads followed by immunoblot analysis. (D) MEK1/2 and ERK1/2 activity was assessed by immunoblot analysis using phospho-specific antibodies. Total MEK1/2 and ERK1/2 serve as loading controls. (E) Membrane recruitment of PKCβII is diminished in DGKζ−/− BMMCs. IgE-sensitized BMMCs were left unstimulated (−) or were stimulated with 50 ng/ml DNP-HSA (DNP) or PMA (P) for 10 min. Triton-soluble membrane fractions were prepared and membrane translocation of PKC isoforms was determined by Western blot using antibodies to PKCβII, PKCδ, and calnexin (CNX) as a loading control. One representative blot is shown and data from five (PKCβII) or three (PKCδ) experiments were quantified and normalized to CNX; combined data are represented graphically.
Activation of PKC family members by DAG and Ca\(^{2+}\) coordinates mast cell degranulation after FceRI stimulation (22, 47). As expected, antigen cross-linking induces membrane recruitment of PKCβII and PKCδ in WT BMMCs (Fig. 3 E). However, we find that membrane recruitment of PKCβII is clearly diminished in DGKζ\(^{-/-}\) BMMCs, whereas PKCδ recruitment is preserved. Unstimulated IgE-sensitized DGKζ\(^{-/-}\) BMMCs also demonstrate diminished levels of membrane-associated PKCβII but normal PKCδ levels. Moreover, treatment with the DAG analogue PMA, which is not a DGK substrate and should be unaffected by DGK deficiency, results in impaired movement of PKCβII to the membrane in DGKζ\(^{-/-}\) BMMCs but normal movement of PKCδ (Fig. 3 E, representative blot and graphical summary of replicates). The block in PKCβII recruitment was not the result of diminished protein levels, however, as total levels of PKCβII and PKCδ in whole cell lysates were not decreased in DGKζ\(^{-/-}\) BMMCs as compared with WT BMMCs (Fig. S2, available at http://www.jem.org/cgi/content/full/jem.20052424/DC1). These data provide a likely mechanism for the impaired degranulation we observe in DGKζ\(^{-/-}\) BMMCs, as FceRI cross-linking results in less recruitment of a positive regulator (PKCβII) of mast cell degranulation, but appropriate recruitment of a negative regulator of mast cell degranulation (PKCδ). The data also suggest that DGKζ is essential for FceRI activation in addition to its effect on DAG levels, as PMA and PMA/Io (unpublished data) fail to restore PKCβII recruitment in DGKζ\(^{-/-}\) BMMCs.

**Calcium response and PLCγ activity in DGKζ\(^{-/-}\) BMMCs**

The diminished membrane recruitment of the Ca\(^{2+}\)-dependent PKCβII along with the preserved movement of the Ca\(^{2+}\)-independent PKCδ led us to question whether DGKζ deficiency affects FceRI-induced Ca\(^{2+}\) flux. Compared with WT BMMCs, DGKζ\(^{-/-}\) BMMCs have a significantly diminished Ca\(^{2+}\) response after FceRI stimulation by antigen (Fig. 4 A). Consistent with the decreased Ca\(^{2+}\) response, phosphorylation of PLCγ1 and PLCγ2 after stimulation of the FceRI was decreased in DGKζ\(^{-/-}\) BMMCs (Fig. 4 B). As tyrosine phosphorylation of PLCγ does not always correlate with its enzymatic activity (48), we analyzed PLC activity by assessing metabolism of the

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**Figure 4.** Decreased Ca\(^{2+}\) responses and PLCγ activity in DGKζ\(^{-/-}\) mast cells. WT and DGKζ\(^{-/-}\) BMMCs were sensitized for 4 h with IgE in cytokine-free media. (A) Decreased Ca\(^{2+}\) flux in DGKζ\(^{-/-}\) mast cells after FceRI stimulation. WT and DGKζ\(^{-/-}\) BMMCs were loaded with Indo-1 and stimulated with 100 ng/ml DNP-HSA to induce Ca\(^{2+}\) responses. Ca\(^{2+}\) flux was determined by flow cytometry based on the change of FL5/FL4 ratio. Data shown are representative of four experiments. (B) Decreased PLC activity in DGKζ\(^{-/-}\) BMMCs after FceRI stimulation. BMMCs were stimulated with different concentrations of DNP-HSA for 2 min. PLCγ phosphorylation was determined by Western blot with anti–phospho-PLCγ1 and anti–phospho-PLCγ2 antibodies. The blots were probed with an anti-actin antibody as a loading control. (C) Decreased PIP\(_2\) hydrolysis in DGKζ\(^{-/-}\) BMMCs. Lipids were analyzed as in Fig. 3. PIP\(_2\) was identified by comigration with a standard. Data are means ± standard error of four different experiments.

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**Figure 5.** IgE stimulation in the absence of antigen cross-linking results in enhanced signaling in DGKζ\(^{-/-}\) BMMCs. BMMCs were exposed to 100 ng/ml IgE for 30 min. Lipids were extracted and PA production was quantified as in Fig. 3. Means ± standard error of five experiments are shown. *, P < 0.05. (B) Enhanced Ras/ERK activation in DGKζ\(^{-/-}\) BMMCs. BMMCs were stimulated with 1 μg/ml IgE for various amounts of time or with 100 ng/ml PMA for 5 min. Active Ras and ERK1/2 phosphorylation were assessed as in Fig. 3. For the Ras blot, a space is included before the PMA lanes to designate that these samples were run on a separate gel.

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PLC substrate PIP_2 and production of IP_3. After receptor cross-linking in WT BMMCs, PIP_2 levels quickly decrease (Fig. 4 C), and IP_3 levels increase (Fig. 4 D) as a result of PLC activity. Upon stimulation of DGKζ_−/−_BMMCs, we do not observe a decrease but rather a slight increase in PIP_2 levels (Fig. 4 C), and IP_3 generation is impaired (Fig. 4 D). These data provide compelling evidence that DGKζ deficiency impairs activation of PLCγ after antigen stimulation of IgE-sensitized mast cells.

**IgE signaling and function in the absence of antigen**

IgE sensitization of DGKζ_−/−_BMMCs leads to increased cytokine production and impaired PKCβII recruitment to the membrane, so we therefore examined how IgE alone signaling and function is altered in DGKζ deficiency. IgE binding to FceRI induces a large increase in PA in WT BMMCs (Fig. 5 A). DGKζ_−/−_BMMCs, in contrast, have a significant impairment in PA accumulation during IgE sensitization. DAG-dependent signaling is also enhanced in DGKζ_−/−_BMMCs during IgE sensitization, as Ras and Erk1/2 activity is enhanced (Fig. 5 B).

IgE binding to the FceRI signals mast cell survival through up-regulation of antiapoptotic proteins (40, 49–52), and ex vivo mast cell survival and expansion requires IL-3 (53, 54). In the presence of IL-3, both WT and DGKζ_−/−_ mast cells survive and expand similarly (unpublished data). When IL-3 is withdrawn, WT BMMCs undergo apoptosis as expected (Fig. 6 A). Surprisingly, DGKζ_−/−_BMMCs have greatly enhanced survival in the absence of IL-3. IgE enhances survival in both WT and DGKζ_−/−_BMMCs, but because survival in the absence of cytokines is so much greater in the DGKζ_−/−_BMMCs, the IgE survival effect is less marked (Fig. 6, A and B).

Akt regulates cell survival and is activated by IgE binding to FceRI (40). Akt is phosphorylated in WT BMMCs grown in cytokine-replete media, and this activation decreases after withdrawal of cytokines (Fig. 6 C). DGKζ_−/−_BMMCs, in contrast, have enhanced Akt activity during growth in cytokine-replete media and maintain Akt activity after cytokine removal (Fig. 6 C). As cytokines stimulate inositol metabolism, including PIP_2 hydrolysis by PLCγ and phosphorylation by PI3K (55, 56), it is likely that DGKζ regulates signals generated through cytokine receptors in addition to its role in FceRI signaling. Future work will explore the biochemical basis of this observation.

**DISCUSSION**

We have studied mice that lack a key enzyme involved in DAG metabolism, DGKζ, to address the consequences of dysregulated DAG accumulation after immune receptor signaling. We demonstrated recently that DGKζ functions as a key negative regulator of TCR signaling and T cell activation (38, 39). We show here that DGKζ plays an important and unexpected role in regulating signal transduction from the FceRI in mast cells. DGKζ_−/−_BMMCs manifest decreased PA production, enhanced activation of the Ras-Mek-Erk signaling pathway, increased Akt phosphorylation, prolonged survival after cytokine deprivation, and enhanced production of IL-6 as compared with WT BMMCs. Surprisingly, IgE-mediated PCA responses are significantly decreased in DGKζ_−/−_ mice, concordant with impairment of receptor-induced PLCγ activity and degranulation in DGKζ_−/−_BMMCs. We demonstrate that signals generated by IgE in the absence of antigen cross-linking are enhanced in DGKζ_−/−_BMMCs. These data demonstrate that DGKζ is important for inactivation of DAG-mediated signaling pathways in mast cells and that DGKζ also plays a critical role in maintaining

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**Figure 6. Enhanced survival of DGKζ−/− BMMCs after IL-3 withdrawal.** WT and DGKζ−/− BMMCs were cultured in media without cytokines in the presence of different concentrations of IgE for 48 or 110 h. Survival of BMMCs was determined by staining with FITC-annexin V and PI and analyzed by flow cytometry. (A) Representative dot-plots. (B) Percentage of live cells (Annexin-V and PI negative) in a full time course and at a range of concentrations of IgE. Data shown are representative of three experiments. (C) Akt phosphorylation during growth in cytokine-replete media (+IL3/SCF) and after 18 h cytokine withdrawal (18 h, no cytokines) was assessed by immunoblot analysis. One representative blot is shown and data from three independent experiments were quantified, normalized to PLCγ1 signal, and represented graphically for comparison. P-AKT signal in WT cells grown in cytokine-replete media was arbitrarily set to 1.
FceRI responsiveness to antigen cross-linking. In addition, we demonstrate a novel FceRI signaling alteration in DGKζ−/− BMMCs, in that receptor engagement results in poor PLCγ activity and degranulation but augmented cytokine production.

The observation that DGKζ deficiency impairs PLCγ function after FceRI stimulation of mast cells was unexpected, as DGKζ was predicted to function downstream of PLCγ. It is possible that enhanced DAG-dependent signaling during IgE sensitization of DGKζ−/− BMMCs promotes feedback inhibition of PLCγ and Ca2+ responses upon antigen cross-linking of the FceRI. Short pretreatment of mast cells with phorbol esters results in feedback inhibition of PLCγ that is mediated by PKCα and PKCε (57–59). Prolonged administration of phorbol esters, in contrast, down-regulates PKC isoforms and potentiates PLC activity upon antigen stimulation (59). We do not see changes in PKC protein levels in DGKζ−/− BMMCs, and we report impaired PLC activity. PKC feedback inhibition might be indirect as well, as PKCβ can induce serine-phosphorylation of Btk at an inhibitory site, dampening Btk activation in mast cells (60). Btk phosphorylates and activates PLCγ after FceRI stimulation, so feedback inhibition of Btk could contribute to diminished PLCγ activity (61). Alternatively, enhanced Erk activation in DGKζ−/− BMMCs could inhibit PLCγ association with LAT, as has been observed in T cells after TCR engagement (62). However, we have not observed an obvious decrease in PLCγ2-LAT association in DGKζ−/− BMMCs after antigen cross-linking of the FceRI (unpublished data).

Alternatively, diminished PLCγ activity and PKCβII membrane recruitment might be a direct consequence of DGKζ deficiency. Although DGK activity terminates DAG signaling, it also creates PA (32, 33, 63). PA is a potent activator of PLCγ enzymatic activity in vitro (35). In addition, a recent report suggests that DGKζ, through production of PA, may increase intracellular PIP2 by activation of PI3K (37). Thus, DGKζ deficiency could lead to decreased PI3K activity, diminishing levels of the PLC substrate PIP2. Our PIP2 measurements did not reveal any differences in 32P incorporation into PIP2 in IgE-sensitized BMMCs (unpublished data), but more direct measures of PIP2 mass should be performed to ensure that steady state levels of 32P-labeled PIP2 reflect total mass. The importance of diminished PA production to the phenotype of DGKζ−/− BMMCs is particularly difficult to assess, as alternative pathways to generate PA exist. These pathways include the hydrolysis of phosphatidylecholine mediated by phospholipase D and the acylation of lyso-PA by lyso-PA acyltransferases (63). Phospholipase D–derived PA has been reported to regulate diverse cellular processes, particularly in vesicle transportation and exocytosis (64, 65). It is possible that PA generated by DGKζ performs a similar function to promote mast cell degranulation.

It is intriguing that membrane recruitment of PKCβII is diminished in unstimulated IgE-sensitized DGKζ−/− BMMCs and that PKCβII recruitment is not restored with PMA treatment. PMA is a DAG analogue and is not subject to phosphorylation by DGK, so we predict that if DGKζ acts only by regulating DAG levels, PMA treatment should restore PKCβII membrane recruitment. It is possible that DGKζ has adaptor functions in addition to its role in lipid metabolism, perhaps by participating in a complex with PKCβII or Btk directly (37, 66). Future work will explore this interesting observation.

We have been unable to directly measure an FceRI-induced change in DAG mass in WT or DGKζ−/− mast cells using standard biochemical approaches (unpublished data). The likely explanation is that the receptor-induced pool of DAG is a small fraction of the total cellular pool because DAG is important for other aspects of cell biology. We are currently developing imaging approaches to measure DAG localization using a fluorescently labeled DAG probe (67). We also will develop HPLC/MS/MS techniques to quantify particular DAG subspecies, which may be more markedly regulated than total cellular DAG (68). These approaches will allow us to measure dynamic changes in DAG localization after FceRI stimulation as well as assess in vivo substrate specificity of DGKζ.

The increased Akt phosphorylation in DGKζ−/− BMMCs stimulated through cytokine or FceRs is intriguing. It is possible that increased Akt activity is a consequence of greater PIP3 production in DGKζ−/− BMMCs, and preliminary data support this hypothesis. Decreased PA production in DGKζ−/− BMMCs might increase PI3K activity, as PA has been reported to inhibit this enzyme in vitro (69). Also, perhaps decreased PLCγ activity in DGKζ−/− BMMCs increases the local concentration of PIP2 available as substrate for PI3K (66). The interrelation among different PI metabolites is complex and understanding the exact lipid alterations will require further study.

Our current studies of DGKζ in mast cells reveal important roles for this enzyme in regulating FceRI signaling. DGKζ is not only essential for terminating DAG activity but also appears to be critical for maintaining optimal FceRI responsiveness to antigen cross-linking. A more complete understanding of the importance of DGK activity to mast cell function requires analysis of other DGK family members. Ongoing biochemical and genetic studies are addressing this question.

**MATERIALS AND METHODS**

**DGKζ-deficient mice.** DGKζ−/− mice have been described previously (39) and were housed in pathogen-free facilities at the University of Pennsylvania and Duke University. All experiments using animals were performed in accordance with regulations of the Institutional Animal Care and Use Committee of the University of Pennsylvania and Duke University.

**Microscopic analysis of mast cells.** Skin samples from the back of the trunk and the ears were collected from killed WT and DGKζ−/− mice. Tissues were fixed and mounted in paraffin according to standard histological procedures. Paraffin sections were stained with toluidine blue and mast cells in tissues were evaluated by microscopy. In each tissue, mast cells were counted in 10 contiguous high-power fields (10 × 100).
BMMCs. BM cells from mice were harvested and incubated with Isocele’s medium (Mediatech, Inc.) supplemented with 15% FBS (HyClone), 100 U/ml penicillin G, 100 U/ml streptomycin, and 292 μg/ml of 1-glutamine, 10 mM Heps (pH 7.4), 0.1 mM nonessential amino acid, 1 mM sodium pyruvate, and 50 μM 2-mercaptoethanol (IMDM-15) with IL-3–conditioned medium for 2 wk and further expanded for an additional 2–8 wk with the supplement of SCF–conditioned medium made from cell lines engineered to produce these cytokines.

Flow cytometry. BMMCs were analyzed directly or after 4-h sensitization with 1 μg/ml IgE. Cells were stained with fluorescently labeled antibodies in 5% FBS in PBS and were analyzed on a FACSCaliber (Becton Dickinson) with CELLQuest software. Antibodies used were PE-conjugated c-Kit and FITC-conjugated anti-IgE (BD Biosciences). To measure cell survival after cytokine withdrawal, cells were stimulated in IMDM-15 without cytokine at a concentration of 105 cells/ml in a 96-well plate with different concentrations of anti-DNP–IgE (0, 0.04, 0.2, 1, or 5 μg/ml; Sigma-Aldrich). After 48 or 110 h incubation, cells were stained with propidium iodide and annexin V–FITC (BD Biosciences) and were analyzed by flow cytometry.

Measurement of PA and PIP2. BMMCs were sensitized at 37°C for 1 h in 1 μg/ml IgE in IMDM-15 without IL-3 or SFC. Cells were harvested and rest in Tyrode’s buffer (10 mM Heps, pH 7.4, 130 mM NaCl, 1 mM MgCl2, 5 mM KCl, 1.4 mM CaCl2, 5.6 mM glucose, and 1 mg/ml bovine serum albumin) for 1 h at 37°C. 3P-phosphoric acid (MP Biomedicals) was added to a concentration of 0.25 mM/C/ml. Cells were left unstimulated or were stimulated with 10 ng/ml DNP-HSA. Stimulation was terminated by addition of 0.1 volume of ice-cold 2 M HCl and transfer of cells to ice for 10 min. Lipids were extracted with 3 ml of 2:1 methanol/chloroform and phases were separated by addition of 2 ml 1 N NaCl and 2 ml of chloroform. Lipid phase was washed 1 × with 3 ml of upper phase buffer (upper phase of 10:10:9 chloroform:methanol:1 M NaCl), dried under nitrogen, and separated by thin-layer chromatography using a basic solvent (9:7:2 chloroform:methanol:4 M NH4OH). PA and PIP2 were identified by comigration with standards (Sigma-Aldrich) and quantified using a phosphorimager.

Measurement of IP3. IgE-sensitized BMMCs were rested for 1 h in Tyrode’s buffer, then left unstimulated or stimulated for 20 or 2 s with 10 ng/ml DNP-HSA. Stimulations were terminated by addition of 0.2 vol 20% perchloric acid for 20 min on ice. Proteins were solubilized and supernatants were neutralized to pH 7.5 by titration of 1.5 M KOH 60 mM 10:10:9 chloroform:methanol:1 M NaCl, dried under nitrogen, and separated by thin-layer chromatography using a basic solvent (9:7:2 chloroform:methanol:4 M NH4OH). PA and PIP2 were identified by comigration with standards (Sigma-Aldrich) and quantified using a phosphorimager.

Western blot analysis. For DGK expression, BMMCs were lysed in 1% NP-40 lysis buffer (1% NP-40, 150 mM NaCl, 50 mM Tris, pH 7.4) with protease inhibitors. Proteins were resolved by SDS-PAGE, transferred to Trans-Blot Nitrocellulose membrane (Bio-Rad Laboratories), and probed with an anti-DGK antibody for DGK expression (71). To analyze Ras, Erk, Mek1, Jnk, and PLCγ1/2 activation, IgE-sensitized BMMCs were resuspended in Tyrode’s buffer. Cells were left unstimulated or stimulated with DNP-HSA for different times or PMA (100 ng/ml) for 5 min. For IgE alone activation of Ras and Erk, cells were not sensitized, but rather were stimulated with 1 μg/ml IgE or PMA. Phosphorylation of these proteins was determined by Western blot with antiphospho-specific antibodies for Erk1/2, Mek1/2, Jnk, Akt, and PLCγ1 and PLCγ2 (Cell Signal Technology). Membranes were stripped and reprobed with antibodies to Erk, Mek1, Jnk, Akt, and actin for loading controls. Activated Ras in cell lysates was determined by GST–Raf-RBD “pull-down” assay as described previously (38).

PKC membrane localization. BMMCs were sensitized for 4 h with 1 μg/ml IgE in IMDM-15 without cytokines and rested for 1 h at 37°C in Tyrode’s buffer. Cells were stimulated with 50 ng/ml DNP-HSA or 100 ng/ml PMA for 5 min, and membrane fractions were isolated as described previously (72). Protein concentration in Triton soluble fraction was quantified using a bicinchoninic acid assay (Pierce Chemical Co.) before PAGE analysis. Western blots were probed with antibodies to PKCβ (Cell Signal Technology), PKCβII (Santa Cruz Biotechnology, Inc.), and calcineurin (Stressgen Bioreagents).

Detection of IL-6 production. Measurement of IL-6 production by BMMCs was performed as described with modifications (13). BMMCs were sensitized in IMDM-15 plus IL-3. For the unstimulated condition, cells were resuspended in IMDM-10 at 2.5 × 107 cells/ml. 200 μl cells were seeded in each well of a 96-well plate in triplicate. For FcεRI stimulation, cells were washed and sensitized with 1 μg/ml anti-DNP IgE in IMDM-10 at 107 cells/ml for 4 h, pelleted, washed once with IMDM-10, and resuspended at 5 × 105 cells/ml in IMDM-10. 100 μl aliquots of cells were placed into wells of a 96-well plate, followed by addition of 100 μl of IMDM-10 with 2 μg/ml of anti-DNP–IgE, with 60 ng/ml DNP-HSA, or with 40 ng/ml PMA and 200 ng/ml ltr. Cells were incubated at 37°C with 5% CO2 for 24 h and IL-6 in supernatants was measured using a murine IL-6 ELISA kit (Pierce Chemical Co.). Data are normalized to PMA/Io control.

β-hexosaminidase release assay. Hexosaminidase release assay was performed as described previously with modification (13). BMMCs grown in IMDM-15 supplemented with IL-3 were stimulated with IgE at 37°C for 1 h to assess IgE–induced degranulation or were sensitized with 1 μg/ml IgE for 4 h and left unstimulated or stimulated with 10 ng/ml of DNP-HSA or PMA plus ltr at 37°C for 1 h. β-Hexosaminidase activities in the medium and in whole cell lysates were determined using p-nitro-phenyl-N-acetyl-β-D-glucosamide as the substrate.

Passive cutaneous anaphylaxis assay. The passive cutaneous anaphylaxis assay was performed according to published protocols (73). Mice were anesthetized by intraperitoneal injection of 300 μl of 2.5% 2,2,2-tribromoethanol in tert-amyl alcohol/PBS (1/40; Sigma-Aldrich), followed by subcutaneous injection of 25 ng IgE in 25 μl PBS in the right ear and 25 μl PBS in the left ear. 24 h later, mice were anesthetized and 200 μl antigen (100 μg DNP-HSA, 1% Evans blue in PBS) was injected intravenously via the retroorbital sinus, 30 min after the injection, mice were killed and both ears were removed. Ears were incubated in 200 μl formaldehyde at 55°C for 48 h to extract the dye. Tissue debris was removed by centrifugation and the intensities of the dye were measured by absorption at 610 nm. The data are calculated as OD610 of the IgE–injected ear minus the OD610 of the PBS-injected ear from the same mouse.

Calcium flux. BMMCs were sensitized with 1 μg/ml IgE for 4 h; resuspended at 107 cells/ml in Tyrode’s buffer containing 3 μg/ml Indo-1 (Invitrogen) and 4 mM probenecid; and incubated for 30 min at 37°C. Cells were washed twice with Tyrode’s buffer and resuspended at 2 × 106 cells/ml. 40 μl of cells was added into 460 μl of prewarmed Tyrode’s buffer to measure Ca2+ responses by flow cytometry (BD-LSR; Becton Dickinson). After collection of the baseline ratio of FL5 to FL4, 10 μl of 5 μg/ml DNP-HSA was added to stimulate cells. Calcium flux was assessed as the ratio of FL5 to FL4 fluorescence.

Online supplemental material. For measurement of DGK transcript levels, RNA was isolated from WT and DGKζ−/− BMMCs by TRIzol extraction (Invitrogen) and cDNA generated by reverse transcriptions (CLONTECH Laboratories, Inc.). Transcripts were quantified by SYBR green real-time PCR using the following primers: DGKα: 5′-GATGCGAGCCACCCCTGTACAAT-3′, 5′-GGACCCTAAGCATAAGGCACTC-3′; DGKβ: 5′-GGGACCCTCAAGGACCTTGTTG-3′, 5′-TCAGCTCCTGTGACCCCAACA; and DGKζ: 5′-TTCCCCAGGGCACTCTCA-3′, 5′-CA-GACGTGACATCTAGGAAGCA-3′. No products were amplified from
a no reverse transcriptase control sample (unpublished data). Transcripts were normalized to GAPDH signal (5′-GAGGTACGAGGCACGAGTTT-3′, 5′-GAATTGACCATGCGTGGAAT-3′) using the ΔΔCT method. For measurement of total cellular protein levels of PKCβII and PKCβ, WT and DGKζ−/− BMMCs were left unstimulated for 4 h in cytokine-free media with or without IgE (1 µg/ml). Cells were lysed in 1% NP-40 lysis buffer with protease inhibitors, proteins were resolved by SDS-PAGE, and Western blots were probed with antibodies to PKCβII or PKCβ. Blots were stripped and reprobed for actin as a loading control. Online supplemental material is available at http://www.jem.org/cgi/content/full/jem.20052424/DC1.

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