Regulation of anaphylactic responses by phosphatidylinositol phosphate kinase type I α

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The membrane phospholipid phosphatidylinositol 4, 5-bisphosphate [PI(4,5)P_2] is a critical signal transducer in eukaryotic cells. However, the physiological roles of the type I phosphatidylinositol phosphate kinases (PIPKIs) that synthesize PI(4,5)P_2 are largely unknown. Here, we show that the α isozyme of PIPKI (PIPKIα) negatively regulates mast cell functions and anaphylactic responses. In vitro, PIPKIα-deficient mast cells exhibited increased degranulation and cytokine production after Fcε receptor-I cross-linking. In vivo, *PIPKI*α$^{-/-}$ mice displayed enhanced passive cutaneous and systemic anaphylaxis. Filamentous actin was diminished in *PIPKI*α$^{-/-}$ mast cells, and enhanced degranulation observed in the absence of PIPKIα was also seen in wild-type mast cells treated with latrunculin, a pharmacological inhibitor of actin polymerization. Moreover, the association of FcεRI with lipid rafts and FcεRI-mediated activation of signaling proteins was augmented in *PIPKI*α$^{-/-}$ mast cells. Thus, PIPKIα is a negative regulator of FcεRI-mediated cellular responses and anaphylaxis, which functions by controlling the actin cytoskeleton and dynamics of FcεRI signaling. Our results indicate that the different PIPKI isoforms might be functionally specialized.

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Abbreviations used: BMMC, bone marrow–derived mast cell; IP_3, inositol 1,4,5-trisphosphate; LAT, linker for activation of T cells; PI3K, phosphoinositide 3-kinase; PIP_3, phosphatidylinositol 3, 4, 5-trisphosphate; PIPKI, type I phosphatidylinositol phosphate kinase; PIPKIα, α isozyme of PIPKI; PLC, phospholipase C; RBL, rat basophilic leukemia.

Engagement of mast cell FcεRI by IgE, followed by the aggregation of multiple IgE-bearing FcεRI molecules by polyvalent antigen, leads to cellular activation and the initiation of allergic reactions (1–3). Activated mast cells release preformed granule-associated chemical mediators, transcribe multiple cytokine genes, and secrete newly synthesized arachidonic acid metabolites and various proteins that trigger allergic inflammation. At the molecular level, the cross-linking of mast cell FcεRI molecules initiates various signaling cascades that lead to the generation of second messengers, protein phosphorylation, and protein–protein and lipid–protein interactions (4). Among the most important intracellular signaling molecules activated after FcεRI cross-linking are the phospholipid-metabolizing enzymes, which include phospholipase C (PLC; reference 5), src homology 2-containing inositol 5-phosphatase 1 (6), and the phosphoinositide 3-kinases (PI3Ks; references 7, 8).

Phosphorylated derivatives of phosphatidylinositol, collectively referred to as phosphoinositides, make up a minor proportion of membrane phospholipids, but are important intermediates in eukaryotic cellular responses (9, 10). Two major second messengers, phosphatidylinositol 3, 4, 5-trisphosphate (PIP_3)

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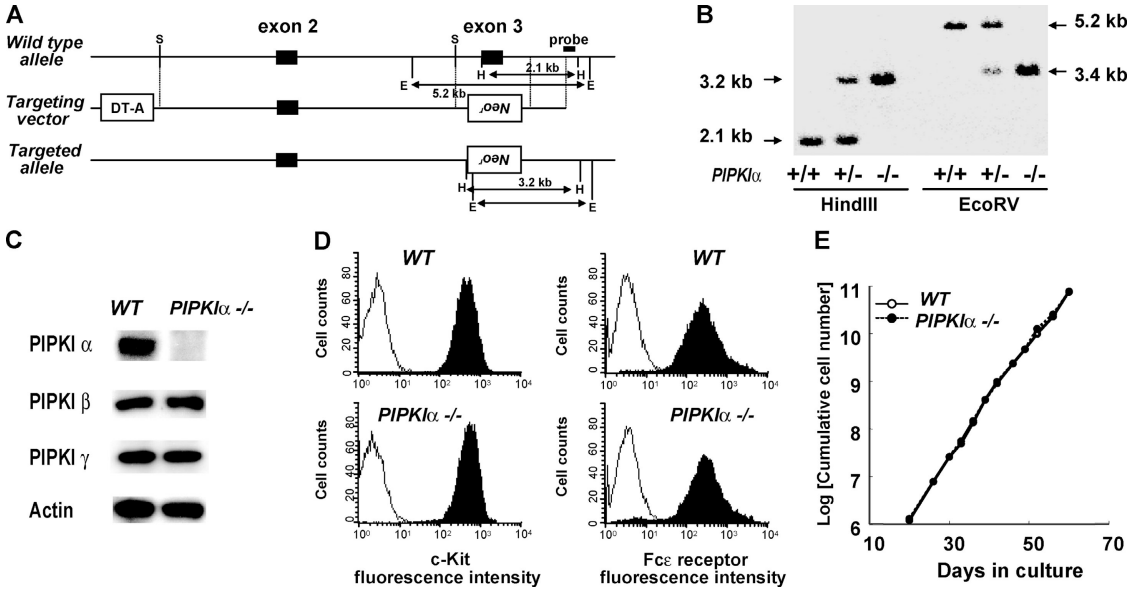

Figure 1. Gene targeting of murine *PIPKIα* and characterization of *PIPKIα*$^{-/-}$ BMMCs. (A) Partial restriction map of the genomic *PIPKIα* sequence and construction of the targeting vector bearing the neomycin resistance (*Neo*^{r}) gene. Exon 3, which encodes a portion of the kinase core domain, was replaced with a *PGK-Neo* cassette. The *PIPKIα* flanking probe used for Southern blotting and expected fragment sizes after digestion of WT and mutant genomic DNA are indicated. H, Hind III; E, EcoRV; S, SmaI; DT-A, diphtheria toxin A subunit. (B) Southern blot of genomic DNA from wild type (+/+), *PIPKIα*$^{+/-}$ (+/−), and *PIPKIα*$^{-/-}$ (−/−) E14 embryonic stem cells hybridized to the probe indicated in A. (C) Western blot of PIPKI isozyme expression in BMMCs using antibodies specifically recognizing the indicated proteins. (D) Flow cytometric analysis of the normal surface expression of c-Kit (left) and FcεRI (right) on PIPKIα$^{-/-}$ BMMCs. (E) Equivalent cumulative cell numbers of *PIPKIα*$^{+/+}$ (open circles) and *PIPKIα*$^{-/-}$ BMMCs (closed circles, dotted line) in cultures maintained for the indicated number of days. For all figures, results shown are representative of at least three independent experiments using three pairs of simultaneously established *PIPKIα*$^{+/+}$ and *PIPKIα*$^{-/-}$ BMMCs.

and inositol 1,4,5-trisphosphate (IP_3), are produced by PI3Ks and PLC, respectively. PIP_3 and IP_3 are required for intracellular signal transduction pathways activated by most cell surface receptors, including cytokine/growth factor receptors, the T cell receptor, the B cell receptor, and receptors for Ig. There is increasing evidence that the metabolism of PIP_3 and IP_3 has physiological and pathophysiological significance for immune system regulation (for reviews see references 11–15).

Phosphatidylinositol 4, 5-bisphosphate [$PI(4,5)P_2$] is a substrate shared by PI3K and PLC. Phosphorylation of $PI(4,5)P_2$ by PI3K generates PIP_3, which controls the intracellular localization and activity of several proteins containing pleckstrin homology domains (16). Conversely, hydrolysis of $PI(4,5)P_2$ by PLC produces IP_3 and DAG, second messengers necessary for the entry of calcium into cells and protein kinase C activation, respectively (13). In addition to its well-established role as a precursor of second messengers, a more direct function for $PI(4,5)P_2$ in the control of diverse cellular processes has been demonstrated recently (17–20). Based on biochemical assays and studies of permeable cell preparations, $PI(4,5)P_2$ appears to be particularly important for the regulation of cytoskeletal reorganization and intracellular membrane transport (9, 17, 18, 21–23).

The phosphatidylinositol phosphate kinases (PIPKs) are enzymes important for $PI(4,5)P_2$ synthesis. There are two types of PIPKs: type I (PIPKI) and type II (PIPKII). Three genes encode the α,β and γ PIPKI isoforms, and the γ gene further generates three splice variants (24–28); in addition, three genes encode PIPKII (29). $PI(4,5)P_2$ can be synthesized either by phosphorylation of PI(4)P by PIPKI, or by phosphorylation of PI(5)P by PIPKII. In mammalian cells, PI(4)P is at least fifty times more abundant than PI(5)P. Moreover, PIPKI (but not PIPKII) can induce dramatic changes in the actin cytoskeleton. Thus, it is generally accepted that the majority of $PI(4,5)P_2$ molecules in a mammalian cell are derived from PIPKI-mediated phosphorylation of PI(4)P. Given the broad range of potential functions of $PI(4,5)P_2$, and the fact that the steady-state level of $PI(4,5)P_2$ is much higher than that of other lipid second messengers, it is likely that some kind of functional compartmentalization of $PI(4,5)P_2$ production exists inside the cell (17, 30). Such compartmentalization could be achieved by the interplay of multiple PIPKI molecules.

In this work, we genetically inactivated the α isozyme of PIPKI (PIPKIα) in mice. PIPKIα deficiency leads to enhanced degranulation and cytokine production in response to FcεRI stimulation in cultured mast cells. Moreover, anaphylactic responses are enhanced in mice deficient for PIPKIα. From a mechanistic standpoint, our results indicate that PIPKIα is crucial for the integrity of the actin cytoskeleton and FcεRI translocation to lipid rafts. Thus, our data are the first genetic evidence for a nonredundant role of PIPKIα in vivo: the restraint of allergic reactions.

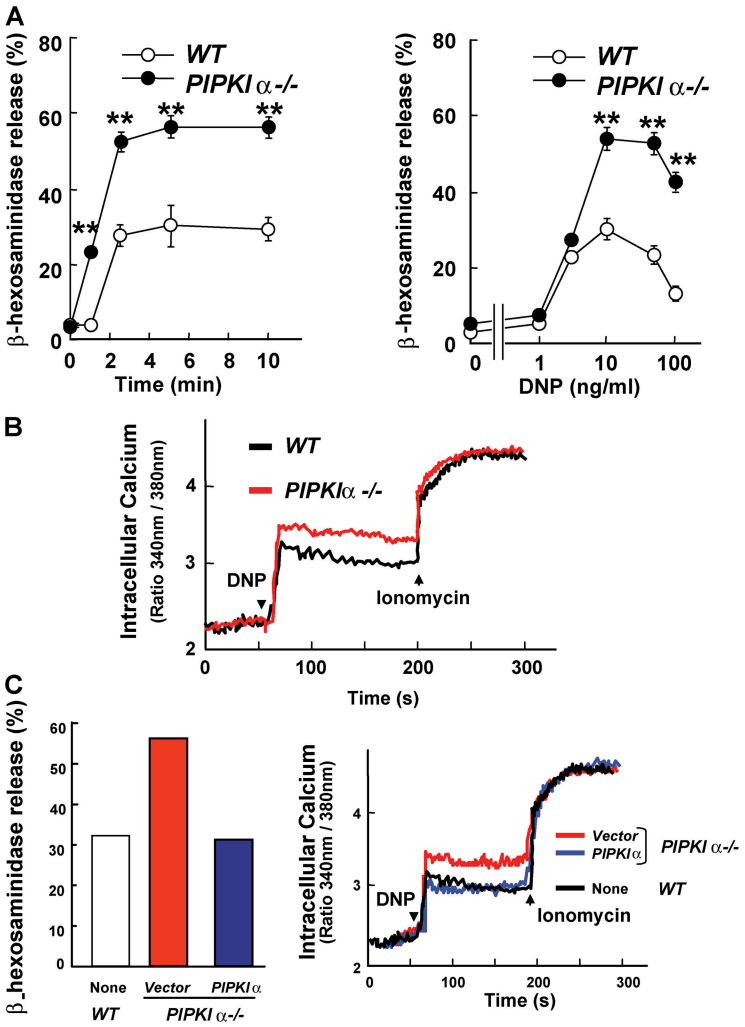

Figure 2. Enhanced FcεRI-mediated degranulation and calcium mobilization in *PIPKIα*$^{-/-}$ mast cells. (A) Increased β-hexosaminidase release. *PIPKIα*$^{+/+}$ (WT; open circles) and *PIPKIα*$^{-/-}$ (closed circles) BMMCs were preloaded with mouse anti-DNP IgE and stimulated with 50 ng/ml^{-1} DNP-HSA for the indicated times (left) or stimulated for 10 min with the indicated concentrations of DNP-HSA (right). The percentage of total cellular β-hexosaminidase that was released was taken as degranulation. Data shown are the mean ± SD of triplicate samples. **, $P < 0.01$ for *PIPKIα*$^{-/-}$ cells compared with *PIPKIα*$^{+/+}$ cells, as determined by Student's *t* test. (B) Increased calcium mobilization. IgE-sensitized, Fura-2-loaded BMMCs were stimulated with 50 ng/ml^{-1} DNP-HSA and Ca^{2+} flux was monitored by spectrofluorimetry. (C) Restoration of normal degranulation and Ca^{2+} flux after introduction of PIPKIα cDNA. Degranulation (left) and Ca^{2+} flux (right) were measured as in A and B, respectively, in WT BMMCs or *PIPKIα*$^{-/-}$ BMMCs reconstituted with either empty vector (red) or *PIPKIα* cDNA (blue).

RESULTS

Generation of *PIPKIα* knockout mice and bone marrow–derived mast cells

Murine embryonic stem cells heterozygous for a deletion mutation of the *PIPKIα* gene were generated by replacing 1.7 kb of the *PIPKIα* gene (including the region encoding the NH_2-terminal amino acids 68–106, indispensable for kinase activity) with a *PGK-Neo* cassette (Fig. 1 A). Southern blot analysis using a short arm flanking probe confirmed disruption of the gene (Fig. 1 B). No random integrations of the *PGK-Neo* cassette were detected (unpublished data). These cells were used to derive homozygous *PIPKIα*$^{-/-}$ mice, which were born at the expected Mendelian ratio, were healthy and fertile, and displayed no histological abnormalities up to 12 mo of age. There were no overt differences from the WT in lymphocyte numbers or in subpopulations present in the thymus, lymph node, spleen, and bone marrow (unpublished data).

It has been demonstrated that the PI3Ks and PLCγ, which utilize $PI(4,5)P_2$ as a substrate to generate either PIP_3 or IP_3, respectively, play critical roles in the development and effector functions of mast cells (31–34). In addition, $PI(4,5)P_2$ modulates the organization of the actin cytoskeleton, another cellular process that has been implicated in the

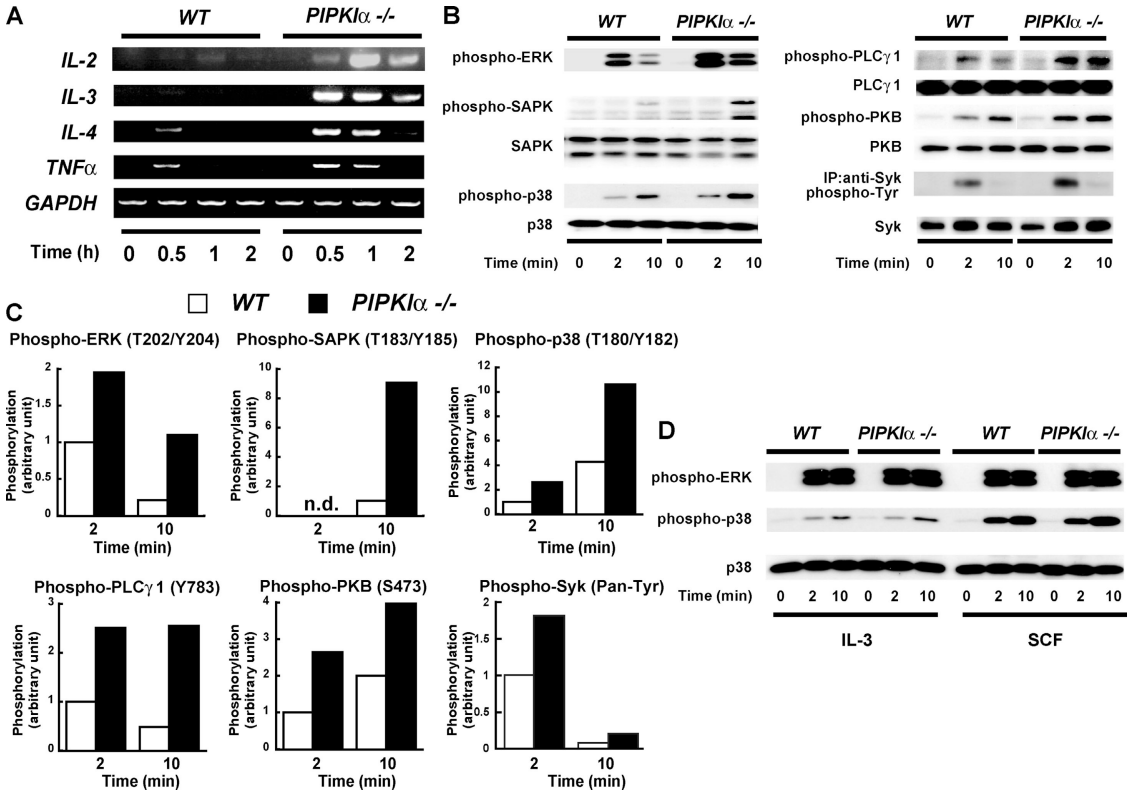

Figure 3. Augmented cytokine gene expression and FcεRI signaling in *PIPKIα*$^{-/-}$ BMMCs. (A) Increased cytokine mRNA expression. *PIPKIα*$^{+/+}$ and *PIPKIα*$^{-/-}$ BMMCs were sensitized with IgE and stimulated with DNP (50 ng/ml^{-1}) for the indicated times. Induction of mRNA expression for the indicated cytokines was detected by RT-PCR. (B) Enhanced signaling molecule phosphorylation after FcεRI cross-linking. BMMCs were sensitized with IgE and stimulated with DNP for the indicated times. For both panels, 30 μg of cell lysates were subjected to successive rounds of immunoblotting using phospho-specific and total antibodies recognizing the indicated proteins. Syk phosphorylation was monitored by immunoprecipitation of Syk followed by immunoblotting using antiphosphotyrosine antibody. One trial representative of a minimum of three experiments is shown in each case. White lines indicate that intervening lanes have been spliced out. (C) Band intensities in B were quantified using Dolphin-1 software, and relative phosphorylation levels were normalized to protein levels as described in Materials and methods. Values shown are the fold increase in the phosphorylated form of each molecule in *PIPKIα*$^{-/-}$ BMMCs compared with the value in WT cells activated for 2 min (except for SAPK, which was 10 min). (D) Normal signaling molecule phosphorylation after IL-3 or SCF stimulation. WT and *PIPKIα*$^{-/-}$ BMMCs were stimulated with either 30 ng/ml^{-1} IL-3 or 30 ng/ml^{-1} SCF and the phosphorylation of ERK and p38 was assessed as in B.

degranulation response (35, 36). To investigate the physiological role of PIPKIα in mast cells, we established IL-3–dependent bone marrow–derived mast cell (BMMC) lines from *PIPKIα*$^{+/+}$ and *PIPKIα*$^{-/-}$ littermates. BMMCs from *PIPKIα*$^{-/-}$ mice lacked PIPKIα protein, but showed normal expression of two other PIPKIs, PIPKIβ and PIPKIγ (Fig. 1 C). Both *PIPKIα*$^{+/+}$ and *PIPKIα*$^{-/-}$ BMMCs expressed similar levels of c-Kit and FcεRI, markers that are characteristic of mature BMMCs (Fig. 1 D), and *PIPKIα*$^{-/-}$ BMMCs proliferated at the WT rate (Fig. 1 E). Thus, PIPKIα expression appears to be dispensable for the cytokine-dependent emergence and differentiation of BMMCs.

Enhanced degranulation, cytokine gene expression, and FcεRI signaling in *PIPKIα*$^{-/-}$ BMMCs

To determine whether PIPKIα was required for mast cell functions, we investigated mast cell granule release by measuring the extracellular activity of β-hexosaminidase, a marker enzyme for histamine-containing granules. The total activity of β-hexosaminidase per cell did not differ between *PIPKIα*$^{+/+}$ and *PIPKIα*$^{-/-}$ BMMCs, and treatment with IgE alone did not induce granule release from BMMCs of either genotype. However, FcεRI-evoked degranulation was increased in PIPKIα-deficient mast cells compared with WT controls (Fig. 2 A). From a total of >15 experiments using five pairs of BMMC lines from littermate mice, we calculated that the degranulation at 5 min after 10 ng/ml DNP stimulation was 56.7% ± 6.2 for *PIPKIα*$^{-/-}$ BMMCs compared with 23.2% ± 3.6 for WT BMMCs. This difference was statistically significant as determined by the Mann-Whitney's U test ($P = 0.00078$) and suggested that PIPKIα is involved in the control of FcεRI signaling.

It is well known that a transient increase in intracellular calcium is essential for degranulation (34, 37). We found that

the amplitude of Ca^{2+} elevation was increased in *PIPKIα*$^{-/-}$ BMMCs compared with that in WT cells (Fig. 2 B), consistent with the enhanced degranulation observed in the mutant cells. To confirm that the enhanced degranulation truly resulted from the loss of PIPKIα expression, we reestablished PIPKIα expression in *PIPKIα*$^{-/-}$ BMMCs using retroviral infection. Degranulation and Ca^{2+} mobilization were restored to normal by infection of *PIPKIα*$^{-/-}$ BMMCs with a retrovirus containing *PIPKIα*, but not by infection with a control virus (Fig. 2 C). Together, these results demonstrate that a lack of PIPKIα expression engenders increased antigen-induced degranulation mediated by high affinity FcεRI.

Cytokines produced by mast cells are critical mediators in allergy and inflammation. Therefore, we investigated whether PIPKIα played a role in mast cell cytokine production. Quantitative RT-PCR was performed to determine the induction of various cytokine mRNA transcripts in WT and *PIPKIα*$^{-/-}$ BMMCs. FcεRI engagement induced higher levels of all cytokine mRNAs examined in *PIPKIα*$^{-/-}$ BMMCs compared with WT cells (Fig. 3 A). IL-2 and IL-3 were the molecules most markedly affected; relative quantitation revealed >50-fold increases in these mRNAs in *PIPKIα*$^{-/-}$ cells that had been stimulated for 1 h (Fig. 3 A and not depicted).

Cytokine gene expression is regulated by several signaling cascades, including those governed by mitogen-activated protein kinases (38). We stimulated *PIPKIα*$^{+/+}$ and *PIPKIα*$^{-/-}$ BMMCs with IgE plus antigen and monitored the phosphorylation (activation) of various signaling proteins. FcεRI-triggered phosphorylation of ERK1/ERK2 (T202/Y204), SAPK (T183/Y185), p38 (T180/Y182), PLCγ-1 (Y783), PKB (Ser473), and Syk was increased in *PIPKIα*$^{-/-}$ BMMCs compared with WT BMMCs (Fig. 3, B and C), indicating that multiple signaling cascades are activated in the absence of PIPKIα. Of interest, normal phosphorylation of ERK and p38 occurred in *PIPKIα*$^{-/-}$ BMMCs stimulated with either IL-3 or SCF (Fig. 3 D), and the basal phosphorylation of mitogen-activated protein kinases was not increased in *PIPKIα*$^{-/-}$ BMMCs (Fig. 3, B and D). Therefore, an absence of PIPKIα does not lead to hyperphosphorylation of these signaling molecules per se. Rather, the hyperphosphorylation of signaling molecules in *PIPKIα*$^{-/-}$ BMMCs depends on FcεRI stimulation.

Increased severity of local and systemic anaphylactic reactions in PIPKIα-deficient mice

Anaphylaxis is an extreme form of allergic reaction triggered by allergen-induced cross-linking of allergen-specific IgE present on the surface of mast cells. Mast cell degranulation is induced, which leads to the release of copious amounts of vasoactive amines and inflammatory mediators. To determine whether PIPKIα functions as a negative regulator of mast cell activation in vivo, we examined IgE-dependent anaphylactic reactions in *PIPKIα*$^{-/-}$ mice. Antigen challenge of mice that had been sensitized previously with monoclonal DNP-specific IgE antibody demonstrated that

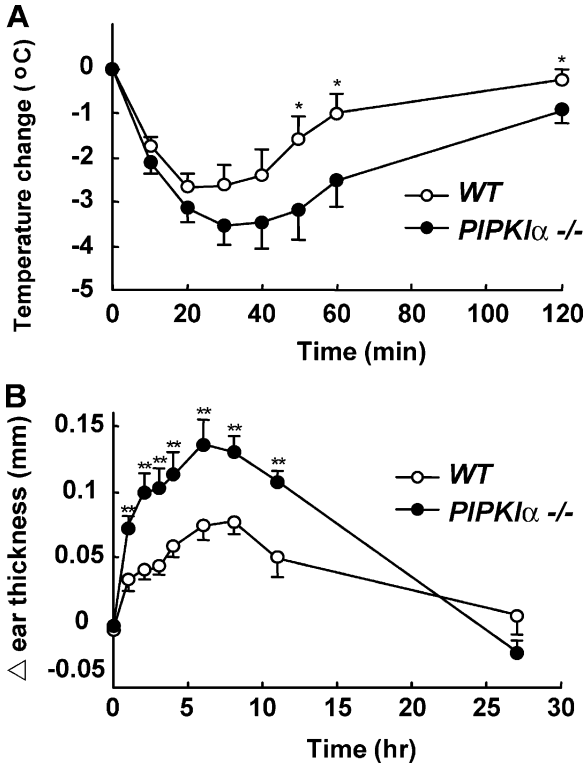

Figure 4. Enhanced anaphylactic responses in *PIPKIα*$^{-/-}$ mice. (A) Systemic anaphylaxis. WT and *PIPKIα*$^{-/-}$ mice ($n = 7$ mice per genotype) received 5 μg anti-DNP IgE i.v., followed by stimulation with 1 mg DNP-HSA per mouse. The systemic anaphylactic response was monitored by measuring rectal temperature at the indicated times after antigen injection. (B) Passive cutaneous anaphylaxis. WT and *PIPKIα*$^{-/-}$ mice ($n = 9$ per genotype) received 100 μg anti-TNP IgE i.v. After 24 h, mice were epicutaneously challenged with 10 μl 1% picryl chloride on the right ears, and with 10 μl 1% oxazolone on the left ears. Net ear swelling (thickness of the right ear minus that of the left ear) was measured with a caliper at the indicated times. Data are expressed as mean ± SD. *, $P < 0.05$ and **, $P < 0.01$ for *PIPKIα*$^{-/-}$ mice compared with *PIPKIα*$^{+/+}$ mice as determined by Student's unpaired *t* test.

PIPKIα$^{-/-}$ mice exhibited a greater degree of systemic anaphylaxis than WT mice, as assessed by core temperature changes (Fig. 4 A). An increase in passive cutaneous anaphylaxis was also observed in the mutants (Fig. 4 B). Mast cell numbers were identical in the ear skin of WT (12 ± 0.9 per field; $n = 8$) and *PIPKIα*$^{-/-}$ (12 ± 1.0 per field; $n = 8$) mice, so that the augmented anaphylaxis in *PIPKIα*$^{-/-}$ mice was most likely due to mast cell hyperreactivity to antigens. These data imply that a major physiological role of PIPKIα is to prevent inappropriate mast cell degranulation and cytokine production and, thus, anaphylaxis.

Decreased PI(4,5)P_2 levels and atypical actin cytoskeleton in PIPKIα-deficient mast cells

In view of the in vitro evidence that PIPKIα produces PI(4,5)P_2, the precursor of IP_3 and PIP_3, the finding of in-

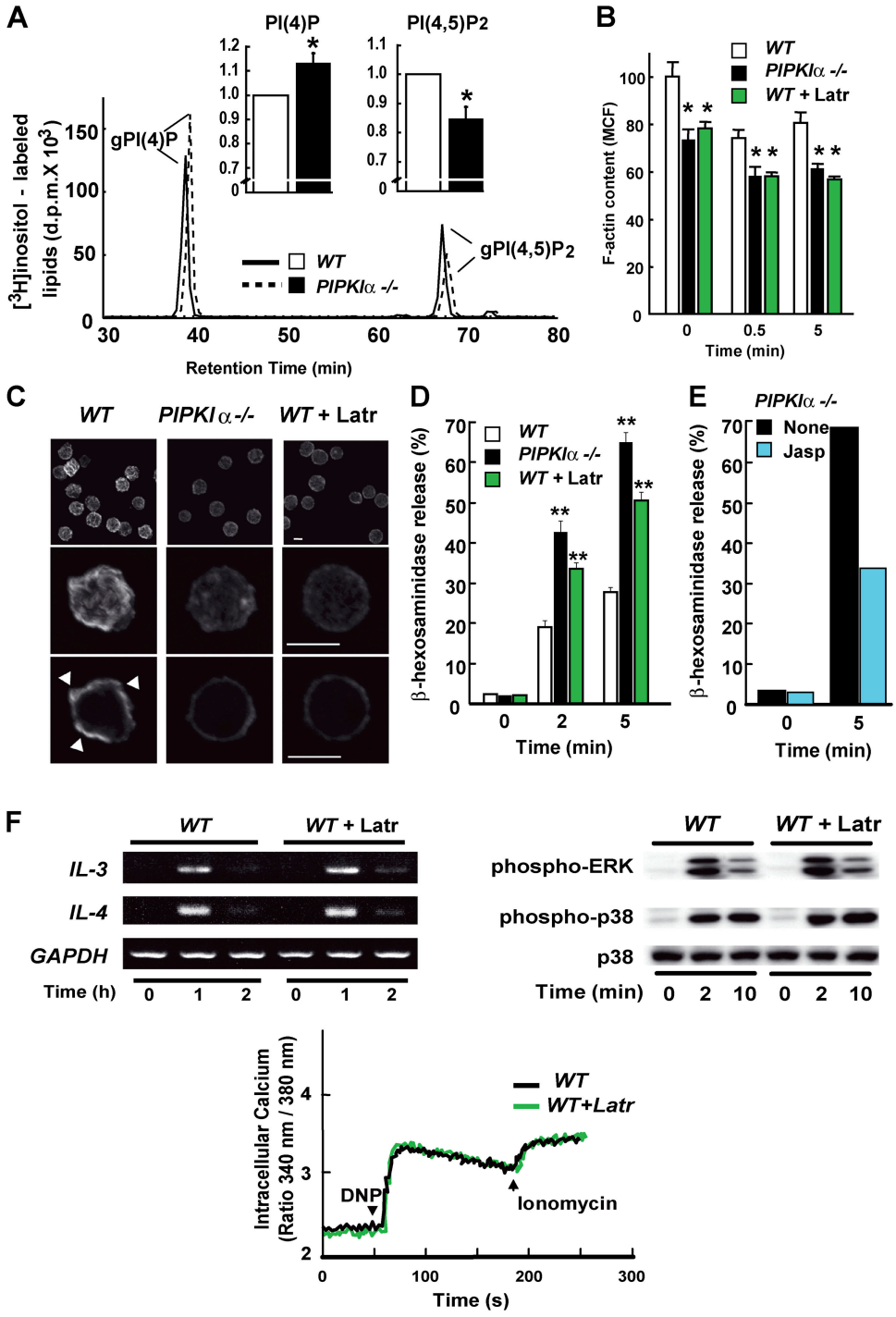

Figure 5. PI(4,5)P_2 levels and actin cytoskeleton in *PIPKIα*$^{-/-}$ BMMCs. (A) Altered phospholipids. HPLC analysis of phospholipids prepared from WT and *PIPKIα*$^{-/-}$ BMMCs that were metabolically labeled with [^{3}H]inositol for 48 h. The chromatographic tracings shown are one result representative of five independent trials. The decrease in PI(4,5)P_2 (0.84-fold) and increase in PI(4)P (1.13-fold) in *PIPKIα*$^{-/-}$ BMMCs were statistically significant (insets). *, $P < 0.05$ for *PIPKIα*$^{-/-}$ cells compared with untreated WT cells. (B) Decreased F-actin content as determined by flow cytometry. IgE-sensitized *PIPKIα*$^{-/-}$ BMMCs and IgE-sensitized WT BMMCs, which were either left untreated or pretreated with 0.5 μM latrunculin (Latr) for 15 min, were stimulated with 50 ng/ml^{-1} DNP for the indicated times. F-actin was stained with Alexa 488–labeled phalloidin and analyzed by flow cytometry. The mean channel fluorescence (MCF) in untreated WT BMMCs was arbitrarily assigned a value of 100. Data shown are the mean percentage of the control value ± SD of triplicate samples. *, $P < 0.05$ for *PIPKIα*$^{-/-}$ cells or latrunculin-treated WT cells compared with untreated WT cells at the indicated times. (C) Decreased F-actin content as determined by confocal fluorescence microscopy. F-actin in the cells examined in B was visualized using Alexa 488–labeled phalloidin. Confocal images were collected every 1 μm, and summation images (top and middle rows) or single

creased mast cell activation and allergic reactions in the absence of PIPKIα was an unexpected result. Therefore, we determined the relative contribution of PIPKIα to overall $PI(4,5)P_2$ production. The intracellular $PI(4,5)P_2$ content was assessed in BMMCs that had been metabolically labeled with [^{3}H]inositol for 48 h to achieve a stable equilibrium. Lipid extraction followed by HPLC analysis revealed a statistically significant decrease in $PI(4,5)P_2$ in BMMCs lacking PIPKIα (Fig. 5 A). A concomitant increase in PI(4)P, the substrate of PIPKIα, was observed in *PIPKIα*$^{-/-}$ BMMCs. These results provide the first genetic evidence that PIPKIα acts as a functional PI(4)P 5-kinase in vivo, and are in agreement with a recent paper describing a partial decrease in $PI(4,5)P_2$ levels in HeLa cells treated with small interference RNA for PIPKIα (39). We conclude that the loss of PIPKIα can be compensated for by other PIPKs in the context of bulk $PI(4,5)P_2$ synthesis, but that a specific $PI(4,5)P_2$ pool may exist that is exclusively maintained by PIPKIα.

To gain insights into the functional characteristics of the $PI(4,5)P_2$ compartment controlled by PIPKIα, we analyzed the production of the $PI(4,5)P_2$–derived second messenger IP_3. At 1 min after FcεRI stimulation, IP_3 production was enhanced in *PIPKIα*$^{-/-}$ BMMCs (2.8 ± 0.37 pmol/10^6) compared with WT cells (2.1 ± 0.17 pmol/10^6; $P = 0.13$; $n = 8$). In addition, the observation that PKB activation was increased in *PIPKIα*$^{-/-}$ BMMCs (Fig. 3 B) suggests that PIP_3 formation is also enhanced in the absence of PIPKIα. Thus, somewhat surprisingly, the production of the second messengers IP_3 and PIP_3 is increased, rather than decreased, in the absence of PIPKIα.

In sharp contrast, the absence of PIPKIα had a clearly suppressive effect on the filamentous actin cytoskeleton (F-actin) in BMMCs. F-actin in *PIPKIα*$^{-/-}$ BMMCs was consistently decreased to 70–80% of the WT level (Fig. 5 B). We used confocal fluorescence microscopy to examine the morphology and distribution of F-actin in *PIPKIα*$^{-/-}$ BMMCs. A dramatic reduction in F-actin staining was found in *PIPKIα*$^{-/-}$ BMMCs in comparison with WT cells (Fig. 5 C). Notably, the pattern of F-actin distribution and the cell shape of *PIPKIα*$^{-/-}$ BMMCs were similar to those of WT BMMCs treated with latrunculin, a potent inhibitor of actin polymerization (40). Cortical actin filaments were decreased in both cases. Together, these results indicate that PIPKIα is dispensable for IP_3 and PIP_3 production, but is a critical regulator of actin cytoskeletal reorganization.

A role for F-actin in the down-regulation of mast cell degranulation, but not cytokine expression

The peripheral actin cytoskeleton is essential for many biological processes involving changes to the plasma membrane architecture (41, 42). In the rat basophilic leukemia (RBL) cell line, it has been demonstrated that FcεRI-triggered actin polymerization plays a negative regulatory role in degranulation, cytokine production, and Ca^{2+} elevation as well as in FcεRI signaling (35, 43). To test whether F-actin was involved in BMMC responses, we first examined the effect of latrunculin on degranulation. WT BMMCs pretreated with latrunculin showed enhanced degranulation in response to FcεRI engagement (Fig. 5 D). Moreover, jasplakinolide, a cell-permeable stabilizer of actin filaments, suppressed degranulation in BMMCs upon FcεRI cross-linking (Fig. 5 E). Latrunculin and jasplakinolide also had parallel effects on BMMC degranulation induced by ionomycin (Fig. S1, available at http://www.jem.org/cgi/content/full/jem.20041891/DC1). These results suggest that polymerized actin can restrain degranulation, and that the augmented degranulation observed in *PIPKIα*$^{-/-}$ BMMCs can be attributed to their decreased polymerized actin content.

Next, we examined the possible involvement of the actin cytoskeleton in FcεRI-mediated cytokine production and protein phosphorylation. Contrary to its striking effect on degranulation, treatment of WT BMMCs with latrunculin before FcεRI cross-linking did not enhance cytokine induction or phosphorylation of signaling proteins, and did not lead to elevated intracellular calcium (Fig. 5 F). These observations may account for the fact that latrunculin only partially mimics the effect of PIPKIα disruption on BMMC degranulation. Our findings concur with a previous paper that demonstrated normal activation of ERK and p38 despite increased actin polymerization in BMMCs lacking Wiskott-Aldrich syndrome protein-interacting protein (44). Thus, contrary to the situation in RBL cells, alterations to the actin cytoskeleton in BMMCs do not necessarily result in anomalies to all FcεRI-evoked cellular responses. Rather, our data show that PIPKIα-mediated suppression of FcεRI-mediated cellular responses in mast cells appears to operate via at least two different mechanisms, only one of which depends on regulation of actin polymerization.

Increased localization of FcεRI in lipid rafts in *PIPKIα*$^{-/-}$ BMMCs

Although most FcεRI-mediated responses were potentiated in PIPKIα-deficient BMMCs, those induced by IL-3 and

images from the center of representative cells (bottom row) are presented. Bar, 10 μm. The WT cells exhibited a more jagged circumferential F-actin structure compared with *PIPKIα*$^{-/-}$ cells (arrowheads). (D) Increased degranulation induced by latrunculin. IgE-sensitized *PIPKIα*$^{-/-}$ BMMCs, and IgE-sensitized WT BMMCs that were either left untreated or pretreated with latrunculin (Latr) for 15 min were stimulated with 50 ng/ml^{-1} DNP for the indicated times. Degranulation was measured as in Fig. 2 A. **, $P < 0.01$ for latrunculin-treated WT cells or *PIPKIα*$^{-/-}$ cells compared with untreated WT cells at 5 min after cross-linking ($n = 4$). (E) Suppression of degranulation induced by jasplakinolide. IgE-sensitized *PIPKIα*$^{-/-}$ BMMCs that were either left untreated or pretreated with 1 μM jasplakinolide (Jasp) for 15 min were stimulated with DNP for 5 min. Degranulation was assessed as for in D. (F) Normal cytokine mRNA expression, signaling molecule phosphorylation, and Ca^{2+} mobilization in the presence of latrunculin. IgE-sensitized WT BMMCs that were either left untreated or pretreated with latrunculin for 15 min were stimulated with DNP for the indicated times. Cytokine gene expression, protein phosphorylation, and Ca^{2+} mobilization were determined as in Fig. 3, A and B, and Fig. 2 B, respectively.

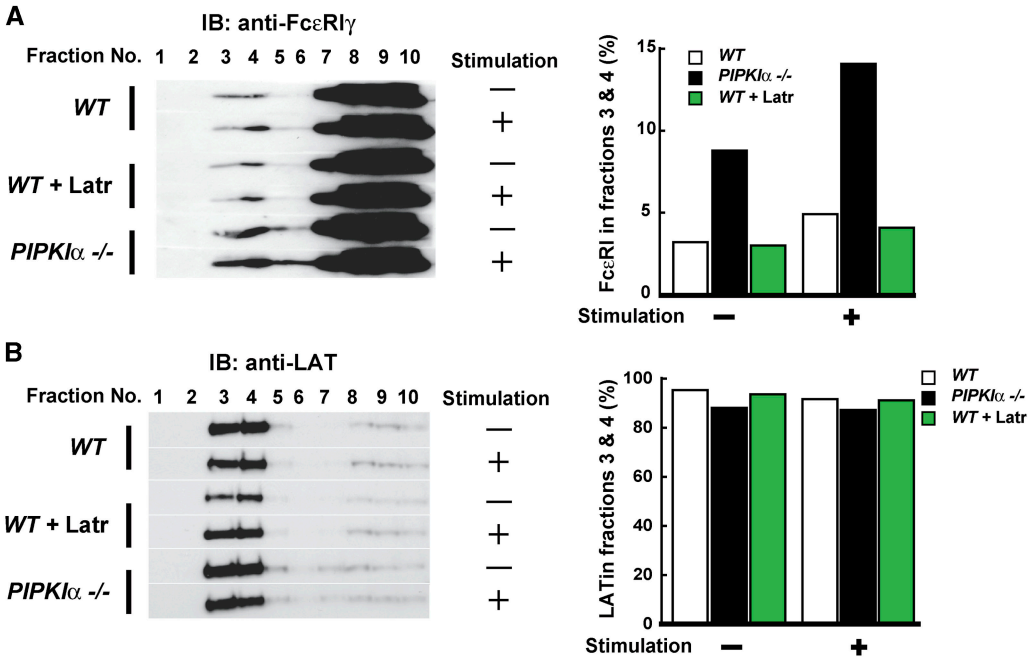

Figure 6. Regulation of FcεRI localization to lipid rafts by PIPKIα. (A) Enhanced localization of FcεRIγ to lipid rafts. BMMCs sensitized with anti-DNP IgE were either left untreated or treated with latrunculin for 15 min. Cells were incubated with or without DNP (stimulation) for 2 min, lysed in 0.5% Triton X-100 buffer, and subjected to sucrose gradient ultracentrifugation to purify lipid rafts. (left) Fractions were separated by SDS-PAGE, transferred to PVDF membranes, and immunoblotted (IB) with anti-FcεRI γ-chain antibody. Fractions 3 and 4 contain the lipid rafts. (right) The distribution of FcεRIγ in fractions 3 and 4 was quantitated by densitometric analysis of the immunoblot. (B) Normal distribution of LAT. Fractions from A were immunoblotted with anti-LAT antibody as for in A (right) and LAT distribution was densitometrically quantitated as in A (left).

SCF treatment were similar in magnitude to those of WT BMMCs. Therefore, we investigated whether an absence of PIPKIα could lead to a change in the dynamics of FcεRI distribution on the plasma membrane. Lipid rafts are defined as plasma membrane microdomains enriched with glycosphingolipids and cholesterol. These structures are now generally recognized as "signaling platforms" (45, 46). It has been shown that, after cross-linking, FcεRI molecules are recruited to the rafts where they undergo tyrosine-phosphorylation as the initiation step of several downstream signaling cascades. Indeed, disruption of lipid rafts with methyl-β-cyclodextrin inhibited FcεRI signaling in BMMCs, as exemplified by a decrease in ERK and p38 phosphorylation (Fig. S2, available at http://www.jem.org/cgi/content/full/jem.20041891/DC1).

Due to their low density, lipid rafts can be separated from nonraft plasma membrane components by ultracentrifugation of detergent-lysed cells in sucrose density gradients. As shown in Fig. 6 A, engagement of FcεRI induced translocation of at least the FcεRIγ chain component of FcεRI to the lipid raft fractions (fractions 3 and 4) of both WT and *PIPKIα*$^{-/-}$ BMMCs. Importantly, the presence of FcεRIγ in the raft fractions was significantly enhanced in *PIPKIα*$^{-/-}$ BMMCs even before aggregation. Immunoblotting for the lipid raft marker linker for activation of T cells (LAT) demonstrated that the partitioning of LAT between the lipid rafts and nonlipid raft regions of the membrane was not altered in the absence of PIPKIα (Fig. 6 B). It should also be noted that the amounts of FcεRIγ in the lipid rafts were not affected by latrunculin, suggesting that the actin cytoskeleton does not play a major role in regulating the redistribution of FcεRI. These results suggest that PIPKIα suppresses the interaction between FcεRI and the lipid rafts independently of actin cytoskeletal organization. Such a role for PIPKIα could explain its apparent ability to impede the propagation of FcεRI-mediated signaling cascades leading to BMMC cytokine production and degranulation. The precise mechanism by which PIPKIα regulates FcεRI localization is under investigation.

DISCUSSION

Mast cells play a key role in allergic reactions due to their ability to synthesize and release proinflammatory mediators and cytokines (1–3). Upon exposure to allergens, specific IgE bound to FcεRI on mast cells becomes cross-linked and intracellular signals are transduced that lead to cellular activation. These intracellular signals are tightly regulated, as spurious signals could result in unwanted, and possibly deleterious, responses. Although recent work has identified many of the proteins that positively regulate FcεRI signaling, little is known about the negative regulators of these signaling cas-

cades. In this study, we have identified a physiological role for PIPKIα as a negative regulator of FcεRI-mediated mast cell functions. BMMCs from PIPKIα-deficient mice exhibit enhanced degranulation and cytokine gene expression. As a result, loss of PIPKIα culminates in aggravated systemic and local passive anaphylaxis in vivo.

PIPKIs are lipid kinases that are critical for intracellular signaling due to their production of the versatile phospholipid $PI(4,5)P_2$. PIPKIs and $PI(4,5)P_2$ have been implicated in the regulation of the actin cytoskeleton, vesicular trafficking, cell migration, adhesion, phagocytosis, and apoptosis (9, 17, 18, 21, 22). Three genes encoding PIPKIs have been identified that show considerable homology to each other, but not to other lipid kinases. However, it has been difficult to clarify whether the multiple PIPKI isozymes have overlapping or redundant functions. The genetic examination of PIPKIα function presented in this work clearly demonstrates an essential function for a PIPKI isozyme as a modifier of FcεRI-mediated mast cell activation, and suggests that each PIPKI isozyme may play a unique physiological role. Consistent with this concept is the recent paper by Di Paolo et al., which asserts that disruption of the PIPKIγ isoform in mice leads to early postnatal lethality and synaptic defects (47). However, there must also be an overlap of PIPKI isozyme functions because PIPKIα-deficient mice are viable and fertile and display no overt histological abnormalities despite the wide tissue distribution of PIPKIα expression.

It has long been hypothesized that cortical actin filaments act as a barrier to prevent secretory granules from accessing the plasma membrane (48). This inference has been supported mainly by pharmacological evidence that inhibitors of actin polymerization potentiate degranulation. In vitro, latrunculin reportedly enhances FcεRI-mediated degranulation in both mouse BMMCs and RBL cells, although FcεRI stimulation results in a decrease in F-actin content in the former (44) and an increase in the latter (35). Thus, a major outstanding question in this field has been whether this actin-based regulation of degranulation operates physiologically. Our work shows that PIPKIα regulates actin reorganization in mast cells in a manner that is functionally important for degranulation, supporting a physiologically relevant role for the control of degranulation via the actin cytoskeleton. Moreover, our results provide insight into a potential molecular mechanism that can maintain sufficient F-actin in mast cells to suppress inappropriate degranulation. This mechanism may define the threshold for the occurrence of allergic reactions in vivo.

IP_3 production and PIP_3-dependent activation of PKB are enhanced in *PIPKIα*$^{-/-}$ BMMCs, suggesting that PIPKIα is dispensable for supplying $PI(4,5)P_2$ for the generation of second messengers. It has recently become clear that intact $PI(4,5)P_2$ (but not the products of its metabolism) can act directly as a signaling lipid (17–20). This function of $PI(4,5)P_2$ is mediated by actin regulatory proteins and components of the exocytosis/endocytosis machinery that possess $PI(4,5)P_2$ binding domains (9, 17, 18, 21–23). Given the essential role of $PI(4,5)P_2$ in actin reorganization, our data indicate that the pool of $PI(4,5)P_2$ produced specifically by PIPKIα is responsible for the maintenance of the actin cytoskeleton in mast cells. We propose that $PI(4,5)P_2$ synthesis by PIPKIα must take place in a defined membrane compartments because, despite its striking effect on F-actin content, the overall $PI(4,5)P_2$ level was only partially reduced in BMMCs lacking PIPKIα (Fig. 5). However, the putative specialized membrane compartments in which PIPKIα-mediated $PI(4,5)P_2$ synthesis occurs remain to be characterized. It should also be noted that our findings do not contradict previous studies that have unequivocally demonstrated that $PI(4,5)P_2$ interacts with and activates several proteins needed for the docking and fusion of secretory granules (49, 50). Our work suggests that, although PIPKs other than PIPKIα can supply sufficient $PI(4,5)P_2$ to allow membrane fusion, PIPKIα has an exclusive role in modulating the actin cytoskeleton and mast cell degranulation.

The mechanism underlying the augmented FcεRI signaling response and increased cytokine gene expression caused by PIPKIα deficiency appears to be distinct from that underlying the enhanced degranulation because these phenotypes could not be induced by latrunculin-mediated reduction of F-actin. As PIPKIα-deficient BMMCs responded normally to IL-3 and SCF, whereas virtually all responses mediated by FcεRI were enhanced, we speculate that PIPKIα acts at the level of FcεRI activation as well as at the level of actin cytoskeleton reorganization. Recent studies have revealed that LAT in lipid rafts plays a central role in FcεRI signaling in mast cells (51). Aggregation of FcεRI induces the inclusion of this receptor and Syk into lipid rafts. Subsequently, Syk phosphorylates LAT to create protein-binding sites that facilitate the assembly of a macromolecular complex of signaling proteins that include Grb2, Gads, SLP76, Vav, and PLCγ. Thus, the FcεRI signaling pathway becomes broadly divergent after the stage of lipid raft recruitment (36, 52, 53). In our work, we showed that the association of the FcεRIγ subunit with lipid rafts was increased in *PIPKIα*$^{-/-}$ BMMCs. Does the distribution of FcεRIγ fairly represent the distribution of FcεRI? Although the FcεRIγ chain associates with a variety of receptors, including FcγR (2), BMMCs do not degranulate in response to IgG (unpublished data). Furthermore, other receptors in which FcεRIγ participates, such as FcαR, are not expressed on BMMCs (2, 54). Therefore, we believe that the distribution of FcεRIγ truly represents the localization of functional FcεRI on the mast cell surface. To our knowledge, our investigation of PIPKIα provides the first identification of a molecule that modulates the localization of FcεRI to lipid rafts. Thus, loss of this regulatory function may account for the broad impact of PIPKIα deficiency on the multiple cellular responses elicited by FcεRI engagement (Fig. 7). It should be noted that some FcεRI-mediated responses are unaltered in the absence of

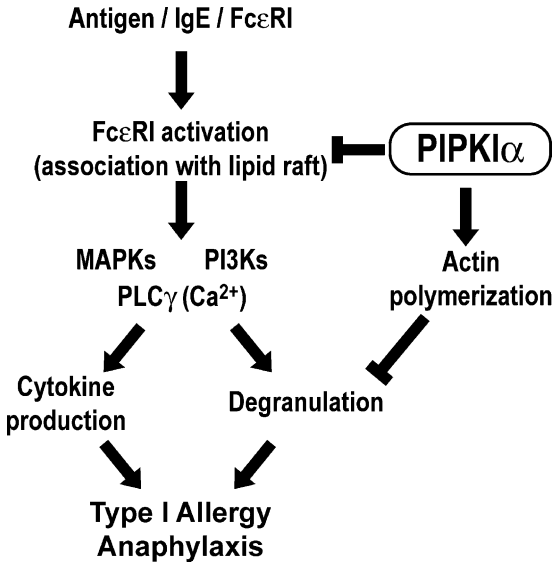

Figure 7. A model for the role of PIPKIα in mast cell activation and anaphylactic responses mediated by FcεRI. PIPKIα acts both to control FcεRI signaling after cross-linking and to promote actin polymerization, thereby inhibiting both degranulation and cytokine production. Thus, anaphylaxis is prevented.

LAT (51), indicating that signaling pathways independent of lipid rafts exist. The role of PIPKIα in such pathways remains to be defined.

Our work provides clues to the mechanisms involved in FcεRI dynamics in the plasma membrane. Because phosphoinositides (including $PI(4,5)P_2$) are reportedly enriched in lipid rafts (55), it will be interesting to analyze FcεRI distribution in mutant cells deficient for other phosphoinositide-metabolizing enzymes such as PIPKIβ and PIPKIγ. These types of studies may further establish that the $PI(4,5)P_2$ synthesized by each PIPK isozyme is functionally compartmentalized. More importantly, such investigations may clarify the precise mechanisms regulating FcεRI dynamics in the plasma membrane that influence the outcome of allergen challenge in vivo.

Our findings may have applications in the clinical arena. FcεRI engagement is known to trigger allergic reactions, and an association between human FcεRIβ chain polymorphisms and atopic phenotypes has been reported previously (56). FcεRI engagement has also been linked to the pathogenesis of parasitic diseases and autoimmune disorders (2). These associations arise because FcεRI is found on monocytes, eosinophils, platelets, Langerhans cells, and dendritic cells as well as on mast cells. Thus, the functional specialization reported here for a PIPKI isoform in the molecular attenuation of FcεRI signaling may represent a distinct mechanism underlying a subset of allergic hypersensitivities, parasite susceptibilities or autoimmune diseases. Such specialization among PIPKs could provide clinical researchers with novel therapeutic targets for these disorders.

MATERIALS AND METHODS

Generation of PIPKIα-deficient mice. Mice deficient for PIPKIα were generated using homologous recombination in embryonic stem cells as described previously (57). For further experimental details, see Supplemental Materials and methods (available at http://www.jem.org/cgi/content/full/jem.20041891/DC1). All experimental protocols were reviewed and approved by the Akita University Institutional Committee for Animal Studies.

Retroviral gene transfer. BMMC reconstitution assays using the retroviral vector pBabe-puro were performed as described previously (58). In brief, Myc epitope-tagged full-length *PIPKIα* cDNA (59) was cloned into the SalI site of pBabe-puro. This vector was transfected into a phoenix-E packaging cell line. *PIPKIα*$^{-/-}$ BMMCs were cultured in the supernatant for 1 d and selected in 2.5 mg/ml^{-1} puromycin for 3 wk before being used for experiments.

Degranulation and calcium mobilization. 2×10^6 cells ml^{-1} BMMCs were sensitized with 0.2 μg/ml^{-1} anti-DNP IgE (SPE-7) for 15 h. The cells were washed, resuspended in OPTI-MEM (GIBCO BRL) containing 0.1% BSA, and challenged with DNP-HSA (Sigma-Aldrich). The percentage of total cellular β-hexosaminidase that was released was taken as degranulation as described previously (60). For calcium mobilization, the sensitized BMMCs were incubated at room temperature for 45 min with 2 μM Fura-2-AM (Molecular Probes) in Tyrode's buffer (10 mM Hepes, pH 7.4, 112 mM NaCl, 2.7 mM KCl, 0.4 mM NaH_2PO_4, 1.6 mM $CaCl_2$, 1 mM $MgCl_2$, 2 mM glucose, and 1% BSA). FcεRI-mediated calcium mobilization was measured every 0.5 s using a spectrophotometer (Shimadzu) set for dual excitation at 340 and 380 nm and emission at 510 nm.

Immunoblotting, immunoprecipitation, and in vitro PI3K assay. BMMCs were sensitized as described before. After washing and resuspension in OPTI-MEM containing 0.1% BSA, the cells ($5–10 \times 10^6$) were stimulated with 50 ng ml^{-1} DNP-HSA. Reactions were terminated by the addition of ice-cold PBS and cell lysates were prepared in RIPA buffer (1% Triton X-100, 10 mM Tris/HCl, pH 7.4, 150 mM NaCl, 30 mM $Na_4P_2O_7$, 5 mM EDTA, 50 mM NaF, 1 mM Na_3VO_4, and a protease inhibitor cocktail from Roche Molecular Chemicals). Immunoprecipitation and immunoblotting were performed as described previously (61). The relative phosphorylation of each signaling protein was normalized to its protein level in each sample (for details, see Supplemental Materials and methods). Immune complex lipid kinase assays were performed as described previously (57).

Passive systemic and cutaneous anaphylaxis. For passive systemic anaphylaxis, mice (10–15 wk old) were sensitized for 24 h by intravenous injection of 5 μg anti-DNP IgE (SPE-7). The mice were subsequently challenged with an intravenous injection of 100 μg DNP-HSA. Body temperature was monitored using a rectal probe (Shibaura) starting from the time of antigen injection. For passive cutaneous anaphylaxis, mice (10–15 wk old) were injected intravenously with 100 μg anti-TNP IgE (62). After 24 h, ear-swelling responses were elicited by painting 10 μl 1% picryl chloride (Nakalai Tesque) in acetone on the right ears of each animal, and 10 μl 1% 4-ethoxymethylene-2-phenyl-2-oxazoline-5-one (oxazolone; Sigma-Aldrich) in acetone on the left ear. Ear thickness was measured as described previously (62).

Cellular $PI(4,5)P_2$ measurement and actin cytoskeleton assessments. BMMCs were labeled for 48 h with 10 μCi ml^{-1} [^{3}H]-myo-inositol (Amersham Biosciences) in inositol-free DMEM containing dialyzed 10% heat-inactivated FCS. Labeling was quenched and lipids were extracted as described (57). Dried lipids were deacylated and analyzed by HPLC according to Serunian et al. using a Partisphere SAX column (Whatman; reference 63). Radioactivity was assayed in 0.5-ml fractions using a liquid scintillation counter. To compare data among experiments, the raw radioactive counts determined for PI(4)P and $PI(4,5)P_2$ were normalized to the raw radioactive counts for total phosphoinositides. The normalized amounts of PI(4)P or $PI(4,5)P_2$ present in *PIPKIα*$^{+/+}$ BMMCs in each experiment were assigned a value of 1, and the relative amounts of PI(4)P or

$PI(4,5)P_2$ in *PIPKIα*$^{-/-}$ BMMCs were calculated. Significance was assessed with Student's *t* test. p-values <0.05 were considered significant. Flow cytometry and fluorescence microscopy were used to analyze the status of the actin cytoskeleton (for details, see Supplemental Materials and methods).

RT-PCR. 10^7 BMMCs sensitized with anti-DNP IgE were stimulated with 50 ng ml^{-1} DNP-HSA. Total RNA was prepared using TRIzol reagent (GIBCO BRL), and first-strand cDNA was synthesized using 5 μg total RNA with M-MLV Reverse Transcriptase (Toyobo). Specific PCR primers and amplification conditions for cytokine gene expression are described in Supplemental Materials and methods.

Lipid raft preparation. Preparation of lipid rafts was performed as described previously with some modifications (64). In brief, 2×10^7 BMMCs sensitized with anti-DNP IgE were incubated for 2 min at 37°C with or without DNP. Cells were washed and pellets were lysed in 1 ml MBS buffer (0.5% Triton X-100, 25 mM MES, pH 6.5, 150 mM NaCl, 1 mM Na_3VO_4, and protease inhibitor cocktail) and incubated for 30 min on ice. Subsequent steps were performed at 4°C. Lysates were mixed with 0.5 ml 85% sucrose in MBS, transferred to 5PA centrifuge tubes (Hitachi), and overlaid with 2.4 ml 35% sucrose followed by 1.5 ml 5% sucrose. After centrifugation for 18 h at 200,000 *g* in a Hitachi P55ST2 rotor at 4°C, 10 0.5-ml fractions were collected starting at the top of the gradient. For analysis of protein composition, aliquots were mixed directly with 4× SDS-PAGE sample buffer and analyzed by Western blotting. Distribution of FcεRI and LAT in lipid rafts was determined by densitometric analysis of immunoblots using Dolphin-1D software (Kurabo).

Online supplemental material. Primers used for cytokine gene expression and procedures for the generation of PIPKIα-deficient mice and the analysis of FcεRI surface expression, actin cytoskeleton assessments, and immunoblotting are available in Supplemental Materials and methods. Fig. S1 shows the effects of latrunculin and jasplakinolide on ionomycin-induced degranulation in WT and *PIPKIα*$^{-/-}$ BMMCs. Fig. S2 shows methyl-β-cyclodextrin inhibition of FcεRI-mediated activation of p38 and ERK. Online supplemental material is available at http://www.jem.org/cgi/content/full/jem.20041891/DC1.

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