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Differential regulation of phospholipase Cγ subtypes through FcεRI, high affinity IgE receptor

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Abstract

The high affinity IgE receptor (FcεRI) usually exists as a tetramer composed of αβγ2 subunits. The COOH-tail of β and γ subunits contains consensus sequence termed 'immunoreceptor tyrosine-based activation motif' (ITAM). Tyrosine phosphorylated ITAM interacts with signaling proteins that contain the Src homology domain, forming a main amplifying and signaling route for FcεRI. Unlike the COOH-tail, the functional role of NH_2-tail of β subunit in the signaling of FcεRI is not clear because it lacks the ITAM sequences. To study the roles of NH_2-tail of β subunit, the cDNA library of RBL-2H3 cells was screened by yeast two-hybrid assay, and the NH_2-tail of the β subunit was found to interact with phospholipase Cγ2 (PLCγ2) but not with PLCγ1. Since both PLCγ1 and PLCγ2 are expressed in RBL-2H3 cells and they possess identical cellular functions, the functional meaning of the protein–protein interaction between PLCγ2 and NH_2-tail of β subunit was studied by comparing the regulatory pathways that control the FcεRI-mediated tyrosine phosphorylation of the two enzymes. Our study shows that PI3-kinase and PMA-sensitive PKCs were required exclusively for the FcεRI-mediated tyrosine phosphorylation of PLCγ1. Also the FcεRI-mediated tyrosine phosphorylation of PLCγ1 was more sensitive to the inhibitors of Src and Syk kinases. These results therefore suggest that PLCγ1 is involved in dynamic regulation of protein kinase C activity and inositol triphosphate levels in response to cellular needs. In contrast, PLCγ2, through continuous interaction with the NH_2-tail of β subunit, co-localizes with FcεRI in the same signaling domain, and maintains the basal cellular PLC activity.

Keywords: FcεRI; Phospholipase Cγ; Yeast two-hybrid; Protein kinase C; Phosphatidylinositol 3-kinase; Src; Syk

The β and γ subunits of FcεRI have three cytoplasmic domains that are long enough to mediate intracellular signaling. These are the NH_2-tail of β subunit and the COOH-tails of β and γ subunits. Functional roles of the COOH-tails of β and γ subunits are relatively well understood. Two disulfide-linked γ subunits transduce signals generated by antigen binding, and β subunit activation serves to amplify γ subunit signaling [1]. Antigenic cross-linking of FcεRI initiates a series of tyrosine phosphorylation events [2] involving immunoreceptor tyrosine-based activation motifs (ITAMS) of the β and γ subunits [3], which then provide SH2 docking sites at tyrosine-phosphorylated consensus sequences for protein tyrosine kinases (PTKs) such as Lyn [4], Syk [5], and Fyn [6–8]. Since the NH_2-tail of β subunit that lacks ITAM is long enough to mediate intracellular signaling, it is conceivable that elucidation of putative functional roles of the NH_2-tail of β subunit might reveal novel signaling components and provide a more complete version of the FcεRI-mediated signaling pathway.

Both subtypes of phospholipase Cγ (PLCγ1 and PLCγ2), which possess identical cellular functions, are expressed in mast cells including RBL-2H3 cells. However, there has been no clue to explain the physiological

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meaning of the redundant expression of two PLCγ subtypes in the same cell. In this study, it was found that the NH_2-tail of β subunit interacts with PLCγ2. Through comparative characterization of the regulatory mechanisms controlling FcεRI-mediated tyrosine phosphorylation of PLCγ1 and PLCγ2 subtypes, here we provide an answer for the physiological meaning of the interaction between FcεRI and PLCγ2, and for the relevance of the existence of two closely related PLCγ subtypes in mast cells.

Materials and methods

Materials. *Saccharomyces cerevisiae* strains (L40 and AMR70), yeast vectors, and the constructs (pLexA, pLexA-lamin, and pVP16) were provided by Dr. Hollenberg (Oregon Health Sciences University, USA) [9]. Anti-phosphotyrosine (PY-20), anti-PLCγ2, and anti-Syk antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-rabbit or anti-mouse antibody conjugated to alkaline phosphatase and antibodies to PLCγ1 were purchased from Sigma Chemical Co. (St. Louis, MI, USA). Anti-mouse IgE antibodies were from Pharminogen (San Diego, CA, USA) or Calbiochem (San Diego, CA, USA). 2-(4-Morpholinyl)-8-phenyl-4H-1-benzopiran-4-one (wortmannin), phorbol-12-myristate-13-acetate (PMA), 3,4,3′, 5′-tetrahydroxy-*trans*-stilbene (piceatannol), and 4-amino-5-(4-chlorophenyl)-7-(*t*-butyl)pyrazolo[3,4-d]pyrimidine (PP2) were purchased from Sigma Chemical Co. 12-(2-cyanoethyl)-6,7,12,13-tetrahydro-13-methyl-5-oxo-5H-indolo [2-3a] pyrrolo [3,4-c]-carbazol (Gö6976) and 3-[1-(3-dimethylamino-propyl)-5-methoxy-1H-indol-3-yl] 4-(1H-indol-3-yl)pyrrolidine-2,5-dione (Gö6983) were purchased from Calbiochem.

Cell culture. Rat basophilic leukocyte (RBL-2H3) cells were maintained as monolayer cultures in EMEM (Invitrogen, Carlsbad, CA, USA) containing 10% fetal bovine serum (Hyclone, Logan, UT, USA) and 10 μg/ml gentamicin (Sigma Chemical Co.). DNP-specific mouse monoclonal IgE was obtained from the hybridoma cell line IGEL b4 (ATCC, Manassas, USA) cultured in DMEM (Invitrogen) with 10% fetal bovine serum and 10 μg/ml gentamicin.

Library construction and screening. The cDNA library was constructed as fusion proteins between a nuclear VP16 acidic activation domain and cDNA fragments from RBL-2H3 cells. cDNA fragments greater than 1 kb were ligated into *Not*I site of pVP16 vector. Detailed procedures regarding the library construction can be found elsewhere [10].

The cytoplasmic part of β-subunit-NH_2-tail subcloned into pLexA was used as a bait to screen the cDNA library and the library screening was conducted as previously described [10]. Briefly, the *S. cerevisiae* L40 strain containing the bait was transformed with the 100 μg library DNA and about 2×10^7 transformants were selected on artificial medium lacking tryptophan, leucine, and histidine. False positive clones were eliminated by β-galactosidase assay and by testing the interaction with lamin, a protein used to eliminate nonspecific interaction due to its sticky nature. The library plasmids were recovered from specifically interacting colonies and sequenced with automatic DNA sequencer using the dye terminating method (Perkin–Elmer 310).

Generation of plasmid constructs. Three bait constructs (COOH-tails of β and γ subunits, NH_2-tail of β subunit) were amplified using primers shown in Table 1, and subcloned into the *Eco*RI site of pLexA. The NH_2-tail of β subunit was further subdivided into two regions (amino acids 1–30 and 31–59), which were subcloned into pLexA.

Co-immunoprecipitation of PLCγ with FcεRI in RBL-2H3 cells. Immunoprecipitation was conducted according to Paolini et al. [11]. Cells were sensitized with DNP-specific mouse IgE (0.5 μg/ml) overnight and treated with 100 ng/ml DNP-BSA in Pipes buffer (119 mM NaCl, 5 mM KCl, 0.4 mM $MgCl_2$, 40 mM NaOH, and 25 mM Pipes, pH 7.2) for 1 min at 37 °C. Cells were solubilized in borate buffer (200 mM boric acid, 160 mM NaCl, 0.3% Triton X-100, and 1% BSA, pH 8.0) and protease inhibitors (4 μg/ml leupeptin and aprotinin, 1 mM PMSF) for 15 min with continuous shaking. Cell extract was centrifuged at 45,000*g* for 30 min, and the postnuclear supernatants were immunoprecipitated with anti-IgE antibodies bound to protein G–agarose beads. The immunoprecipitates were electrophoretically analyzed on the 8% SDS–PAGE gel, transferred to nitrocellulose or PVDF membrane, and the immunoblot was probed with antibodies to PLCγ1 or PLCγ2.

Phosphorylation studies using antibodies specific for phosphorylated amino acids. RBL-2H3 cells were cultured in serum-free EMEM for 6 h and lysed in RIPA buffer containing protease and phosphatase inhibitors (1 mM EDTA, 1 mM sodium orthovanadate, and 5 mM sodium fluoride) for 15 min, scraped, and centrifuged at 48,000*g* for 30 min. Postnuclear supernatants were immunoprecipitated with antibodies against phosphotyrosine (PY-20) coupled to the protein G–agarose beads for 4 h, electrophoretically analyzed on 8% SDS–PAGE gel, and transferred to nitrocellulose or PVDF membrane. The immunoblot was probed with antibodies to PLCγ1 or PLCγ2.

Results and discussion

Library screening

Of the 2×10^7 yeast transformants screened for the bait NH_2-tail of β subunit of FcεRI, 48 were His^+, and 16 out

Table 1
Primers used for the construction of bait plasmids in yeast two-hybrid analysis

Primers[a] (5′–3′)	Bait
(1) ATATGAATTCCAGGTCCGAAAGGC (2) ACGTGAATTCTTGGGGTGGTTTCTCATG	PLexA-γC: the cytoplasmic loop part of γ subunit-COOH tail
(3) GTATGAATTCATGGACACAGAAAATAAG (4) GATTGAATTCCTCCAACTCTTTCTTC	pLexA-βN: the cytoplasmic part of β subunit-NH_2 tail
(5) ACGTGAATTCGAGCGTAGTAAGG (6) TCACGAATTCTTATGAAACCACTGG	pLexA-βC: the cytoplasmic part of β subunit-COOH terminal
(3) GTATGAATTCATGGACACAGAAAATAAG (7) ATATGAATTCGAGTTCAATGTCAGGTGCGC	pLexA-βN (1–30)
(4) GATTGAATTCCTCCAACTCTTTCTTC (8) GATCGAATTCTTGGAAGCGTCCCCTCCTGC	pLexA-βN (31–59)

[a] Underlined represent the engineered *Eco*RI restriction sites.

of these were β-galactosidase positive ($LacZ^+$). For these 16 colonies, the bait was removed and lamin (a sticky protein) was introduced to sort out specifically interacting proteins from false positives. Of 16 Trp^+ Leu^+ diploids containing lex-lamin, 15 was β-galactosidase-positive, suggesting the non-specific transactivation of LacZ reporter construct in the absence of the bait but in the presence of lex-lamin fusion protein. Remaining one clone was sequenced and revealed to be PLCγ2 [12]. PLCγ2 seems to be essential for mast cell degranulation [13].

PLCγ2 specifically interacts with the NH_2-tail of FcεRI-β subunit

The functional role of NH_2-tail of FcεRI-β subunit has been mysterious for a long period of time. This region is composed of about 60 amino acids, which are long enough to be involved in signaling of FcεRI but are not expected to mediate dynamic regulations due to the absence of ITAM region.

The specificity of the interaction of PLCγ2 with the NH_2-tail of β subunit was tested by yeast two-hybrid assays. For this, three yeast constructs (in pLexA vector) were prepared for the three cytoplasmic tails of FcεRI (COOH-tails of β and γ subunits, NH_2-tail of β subunit) as described in Materials and methods. When these constructs were introduced into the *S. cerevisiae* (L40) containing the PLCγ2, only the L40 cells transformed with NH_2-tail of β subunit survived in the culture medium lacking His (Fig. 1A). This suggests that PLCγ2 specifically interacts with the NH_2-tail of β subunit.

In addition to L40 cells, the interaction between FcεRI and PLCγ2 was also studied in RBL-2H3 cells. For this, RBL-2H3 cells were sensitized with IgE overnight, treated with vehicle or 1 μg/ml DNP-BSA, gently solubilized, and the whole "anti-IgE–IgE–FcεRI complex" was immunoprecipitated by conjugating anti-IgE with protein G beads. When the immunoprecipitates were analyzed by SDS–PAGE, FcεRI was found to co-immunoprecipitate with PLCγ2 but not with PLCγ1 (Fig. 1B).

To locate more specific region involved in the binding with PLCγ2, the NH_2-tail of β subunit was further divided into two regions (amino acids 1–30 and 31–59), and their interactions with PLCγ2 were tested as described before. As shown in Fig. 1C, PLCγ2 interacts with the β subunit closer to the transmembrane region (amino acids 31–59).

In agreement with the results from our study, Fukamachi et al. [14] reported that, regardless of the cellular activation states, neither β nor γ subunit of FcεRI was co-precipitated when the cell extracts of RBL-2H3 cells were immunoprecipitated with antibodies to PLCγ1, suggesting that β and γ subunits do not interact with PLCγ1. On the other hand, a more recent study reported that PLCγ1 preferentially interacts with the synthesized diphosphorylated ITAM of the β subunit peptide fragment [8]. Though there is no clear explanation to these contradicting observations, it could be speculated that the overall conformations of FcεRI β or γ subunits could be different depending on whether they exist as a whole folded protein or as peptide fragments. In accordance with this, recent electron microscopic studies clearly showed that a portion of PLCγ2 inherently interacted with the plasma membrane, and after antigenic stimulation, PLCγ2, but not PLCγ1, co-localized with FcεRI-β subunit in osmiophillic patches [15,16].

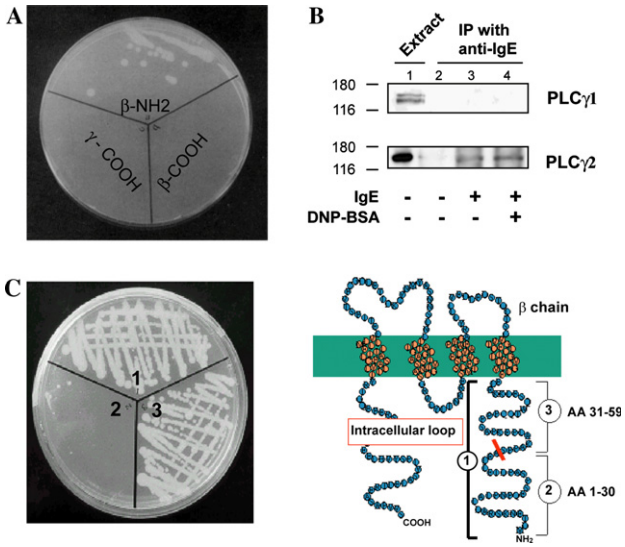

Fig. 1. Interaction between PLCγ2 and the NH_2-tail of FcεRI-β subunit. (A) The COOH-tails of β and γ subunits (β-COOH, γ-COOH), and NH_2-tail of β subunit (β-NH_2) subcloned in pLexA were introduced into *S. cerevisiae* (L40) containing the cDNA of PLCγ2 in pVP16 (pVP16-PLCγ2), and incubated in the culture medium lacking histidine at 30 °C for 3–5 days. (B) Co-immunoprecipitation of PLCγ1 and PLCγ2 with FcεRI in RBL-2H3 cells. Immunoprecipitation was conducted as described in Materials and methods, and it was analyzed on 8% SDS–PAGE gel. The immunoblot was probed with PLCγ1 antibody, washed with TBS-T for 2 days, and then re-probed with PLCγ2 antibody. PLCγ1 was exposed 1 h to overnight and PLCγ2 was exposed between 5 and 10 min. Lane (1): positive control—cell extract of RBL-2H3 cells; lane (2): negative control—incomplete immunoprecipitation, anti-IgE antibody was omitted in the immunoprecipitation; lane (3): resting cells—immunoprecipitation from the RBL-2H3 cells sensitized with IgE overnight; and lane (4): activated cells—immunoprecipitation from the RBL-2H3 cells treated with IgE overnight and stimulated with DNP-BSA for 1 min. (C) Localization of specific interaction site of β subunit with PLCγ2 in yeast. The specific portions of the intracellular FcεRI-β subunit were subcloned into pLexA. These constructs were transformed into yeast containing cDNA of PLCγ2 in pVP16 (pVP16-PLCγ2) and incubated on His^- plates at 30 °C for 3 days. (1) pLexA-cytoplasmic β-NH_2 (1–59) of FcεRI + pVP16-PLCγ2; (2) pLexA-cytoplasmic β-NH_2 (1–30) of FcεRI + pVP16-PLCγ2; and (3) pLexA-cytoplasmic β-NH_2 (31–59) of FcεRI + pVP16-PLCγ2.

RBL-2H3 cells express two subtypes of PLCγ that are closely related to each other in their structure and cellular functions [17], production of IP3 and DAG from PIP2. Interestingly, our yeast two-hybrid studies with NH_2-tail of β subunit as the bait identified only PLCγ2 as the protein that binds to this region. It will be

therefore interesting to examine why RBL-2H3 cells express both enzymes and what is the meaning of interaction between FcεRI and PLCγ2 in terms of their cellular functions. To address this, we comparatively characterized the regulatory mechanisms controlling FcεRI-mediated tyrosine phosphorylation of PLCγ subtypes.

PI3-kinase is partially involved in the tyrosine phosphorylation of PLCγ1 but not that of PLCγ2

Effects of cross-linking of FcεRI on the phosphorylation status of PLCγ subtypes were studied using antibodies specific for phosphorylated-tyrosine. As shown in Fig. 2A (lane 2 vs 3), PLCγ1 and PLCγ2 were phosphorylated at tyrosine residues in 1 min of antigenic stimulation.

As a first step for the elucidation of the signaling pathways connecting FcεRI and PLCγ subtypes, the involvement of phosphatidylinositol 3-kinase (PI3-kinase) was tested. As shown in Fig. 2B, wortmannin, an inhibitor of PI3-kinase, partially inhibited FcεRI-mediated tyrosine phosphorylation of PLCγ1 (50.5% decrease at 30 nM wortmannin) but rather increased that of PLCγ2, suggesting that the signaling through PLCγ2 might be enhanced due to the attenuation of route to PLCγ1. These results confirm the results from previous studies [18–20], and illustrate the specificity of antibodies for PLCγ1 and PLCγ2.

PLCγ1 is more sensitive to the inhibitors of Src and Syk than PLCγ2

Lyn, a Src family non-receptor type protein-tyrosine kinase, is known to be involved in the initial stage of FcεRI signaling that results in the activation of Syk. The relationship between Src and PLCγ subtypes has been reported previously; PLCγ1 and PLCγ2 can be phosphorylated by Src-family protein tyrosine kinases in vitro [21]. Requirement of Syk for the tyrosine phosphorylation of PLCγ1 and PLCγ2 was also reported in Syk-knockout cells [22].

For the determination of the role of Src in FcεRI-mediated activation of PLCγ subtypes, effects of PP2 (a Src kinase inhibitor) on the tyrosine phosphorylation of PLCγ1 and PLCγ2 were tested. As a positive control we also used the effects of Syk kinase inhibitor (piceatannol) on the phosphorylation of the two PLCγ subtypes. Inhibition of Src kinase resulted in the inhibition of FcεRI-mediated tyrosine phosphorylation of Syk (Fig. 3A, lane 4 vs 5), confirming that Src is the upstream signaling component Syk. Effects of Src and Syk kinase inhibitors on the FcεRI-mediated tyrosine phosphorylations of PLCγ1 and PLCγ2 were quantitatively different. PP2 almost completely blocked the tyrosine phosphorylation of PLCγ1 at 10 μM, in contrast, the tyrosine phosphorylation of PLCγ2 was noticeably inhibited only at 30 μM (Fig. 3A). Similar results were observed with piceatannol, a Syk inhibitor (100% inhibition of PLCγ1 at 10 μM piceatannol but only 30% inhibition of PLCγ2 at the same concentration) (Fig. 3B, lane 3 vs 4). These results suggest that PLCγ1 may be more intimately connected with Src and Syk kinases than PLCγ2.

PMA-sensitive protein kinase C subtypes are required for the FcεRI-mediated tyrosine phosphorylation of PLCγ1 but not of PLCγ2

It is known that at least six different PKC subtypes are present in RBL-2H3 cells: conventional

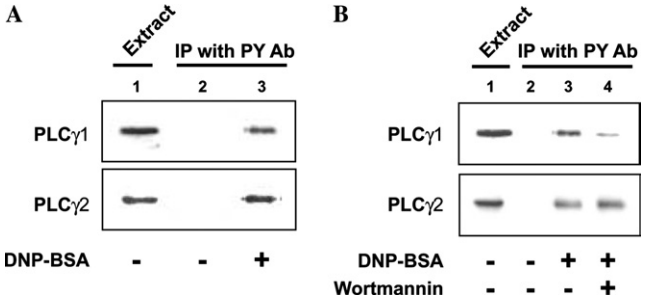

Fig. 2. Effects of PI3-kinase inhibition on the FcεRI-mediated tyrosine phosphorylation of PLCγ subtypes. (A) RBL-2H3 cells were treated with IgE in serum-free EMEM for 6 h stimulated with DNP-BSA (1 μg/ml, 2 min) and treated with RIPA buffer on ice for 15 min. The cell extract was centrifuged at 45,000*g* at 4 °C for 30 min and the supernatant was immunoprecipitated with phosphotyrosine bound to protein G beads. The immunoprecipitates were immunoblotted with antibodies to PLCγ1 or PLCγ2. (B) Effects of the PI3-kinase inhibitor on the FcεRI-mediated tyrosine phosphorylation of PLCγ1 and PLCγ2. Cells were treated with 30 nM wortmannin for 20 min and stimulated with DNP-BSA (1 μg/ml, 2 min). Immunoprecipitation and immunoblotting procedures were conducted in the same way as in (A).

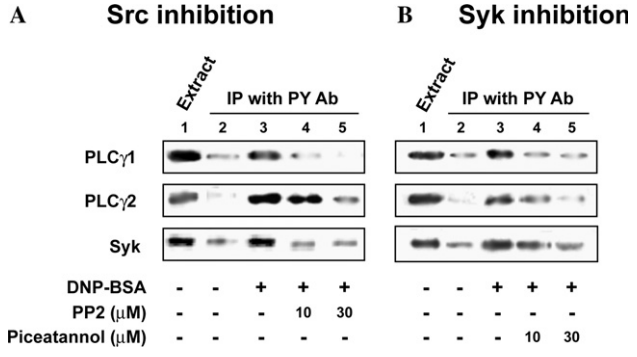

Fig. 3. Effects of protein kinase inhibitors on the FcεRI-mediated tyrosine phosphorylation of PLCγ subtypes. (A) Effects of Src kinase inhibition on the antigen-induced tyrosine phosphorylation of PLCγ subtypes. RBL-2H3 cells were treated with IgE in serum-free EMEM for 6 h. Cells were treated with 10 or 30 μM PP2 for 30 min and stimulated with DNP-BSA (1 μg/ml, 2 min). Immunoprecipitation and immunoblotting were conducted as described in Fig. 1A, with the exception that the blot was also probed with antibodies for Syk. (B) Effects of Syk inhibitor on the antigen-induced tyrosine phosphorylation of PLCγ subtypes. RBL-2H3 cells were treated with 10 and 30 μM piceatannol for 30 min. Immunoprecipitation and immunoblotting were conducted as described in Fig. 3A.

calcium-dependent PKC-α and PKC-β; novel calcium-independent PKC-δ, -ε, and -θ; and atypical PKC-ξ [23,24]. PKC-β, -δ, and -θ mediate positive signals for secretion, while α and ε subtypes mediate inhibitory signals for PLCγ activity [13,24]. However, the relationship between PKCs and PLCγ subtypes is not yet clearly established. Here we determined their contributions to the tyrosine phosphorylation of PLCγ1 and PLCγ2.

Intimate interaction of PLCγ subtypes with PKCs showed complicated patterns in our studies, probably because RBL-2H3 cells express two subtypes of PLCγ and six different subtypes of PKCs. Gö6976, which effectively inhibits PKC-α and PKC-β in RBL-2H3 cells, showed inhibitory effects on tyrosine phosphorylation of both PLCγ1 and PLCγ2 (Fig. 4A), suggesting that the stimulatory activity of PKC-β may be stronger than the inhibitory activity of PKC-α on PLCγ subtypes. On the other hand, the atypical PKC inhibitor Gö6983, which inhibits PKC-α, -β, -δ, and -ξ, strongly potentiated the tyrosine phosphorylation of both PLCγ1 and PLCγ2 (Fig. 4B) (84.1 ± 5.6% increase at 0.5 μM, 167.0 ± 15.7% increase at 1.0 μM for PLCγ1; 124.5 ± 2.1% increase at 0.5 μM, and 300.0 ± 29.7% increase at 1.0 μM for PLCγ2). Gö6983 did not affect the FcεRI-mediated tyrosine phosphorylation of Syk (data not shown), suggesting that the effects of PKCs on PLCγ activities might not involve the upstream signaling components. Considering that inhibition of PKC-α and -β results in the inhibition of both PLCγ subtypes (from Gö6976 results), and PKC-δ has a positive effect on PLCγ subtypes, the results from Gö6983 treatments suggest that PKC-ξ might have strong negative feedback activities on PLCγ1 and PLCγ2.

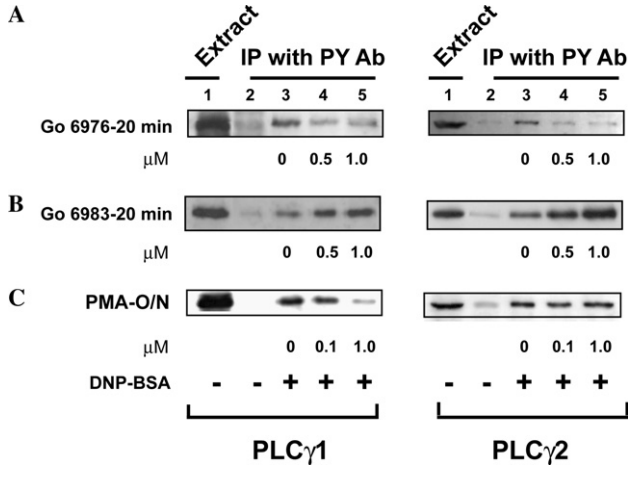

Fig. 4. Effects of regulation of protein kinase C on FcεRI-mediated tyrosine phosphorylation of PLCγ subtypes. RBL-2H3 cells were treated with IgE in serum-free EMEM for 6 h. Cells were treated with (A) 0.5 and 1 μM Gö6976 for 20 min, (B) 0.5 and 1 μM Gö6983 for 20 min, and (C) 0.1 and 1.0 μM PMA overnight (O/N). Immunoprecipitation and immunoblotting were conducted as described in Fig. 2A.

The depletion of PMA-sensitive PKCs by overnight treatment with PMA dose-dependently inhibited the tyrosine phosphorylation of PLCγ1 but not that of PLCγ2 (Fig. 4C). The FcεRI-mediated tyrosine phosphorylation of Syk was not affected by treatment with PKC inhibitors or by PMA (data not shown). When we analyze the results from Gö6976 treatment (conventional PKCs, PKC-α and -β isoforms are affected; both PLCγ isoforms are inhibited) and PMA overnight treatment (PKC-α, -β, -δ, -ε, and -θ are affected; only PLCγ1 is inhibited), and because PKC-δ but not PKC-ε isoform has positive effects on PLC activity, it is likely that PKC-δ isotype, a novel PKC isoform, is specifically required for the activation of PLCγ1. These results might explain the differential physiological roles of PLCγ1 and PLCγ2 on the activation of PKC subtypes and production of inositol triphosphates. Since activation of PLCγ1 relies on the PKC subtypes whose activities depend on cellular diacylglycerol, it is expected that PLCγ1 will be exponentially activated once it is triggered through FcεRI or other cellular pathways.

Wortmannin treatment, which selectively inhibited PLCγ1, significantly but only partially inhibited the degranulation events of RBL-2H3 cells (measured by β-hexosaminidase release) at nanomolar ranges: 34.1 ± 1.7% at 1 nM, 37.1 ± 0.94% at 3 nM, and 43.2 ± 2.1% at 10 nM (data not shown). In contrast, PP2 and piceatannol, which inhibited both PLCγ1 and PLCγ2, showed more profound inhibitory activities on mast cell degranulation (67% and 78% at 50 μM, respectively). PP2 and piceatannol but not wortmannin could completely inhibited mast cell degranulation when their concentrations were further increased, suggesting that both PLCγ1 and PLCγ2 are responsible for mast cell degranulation.

The relevance of redundant cellular expression of two subtypes of PLCγ that are closely related to each other both in structure and cellular functions became clearer through our studies. PLCγ1 is readily regulated by various signal inputs (Src, Syk, PI3-kinase, and PMA-sensitive PKCs); however, PLCγ2 by Src and Syk in a less-sensitive manner. In addition, tyrosine phosphorylation of PLCγ2 was not affected by the depletion of cellular PKCs (Fig. 4C) or by genestin treatment (50 and 100 μM, data not shown), showing again that PLCγ2 is rarely modulated by other tyrosine kinase-related signaling components. These different regulatory properties of PLCγ1 and PLCγ2 are further supported by their intracellular trafficking properties. PLCγ1 is cytosolic at resting state and it translocates to the plasma membrane in response to antigenic stimulation. In contrast, PLCγ2 is localized in a perinulcear region near the Golgi and adjacent to the plasma membrane, and does not redistribute appreciably after FcεRI cross-linking [18].

Based on our observations and the previous literature, we propose the signal transduction pathway for

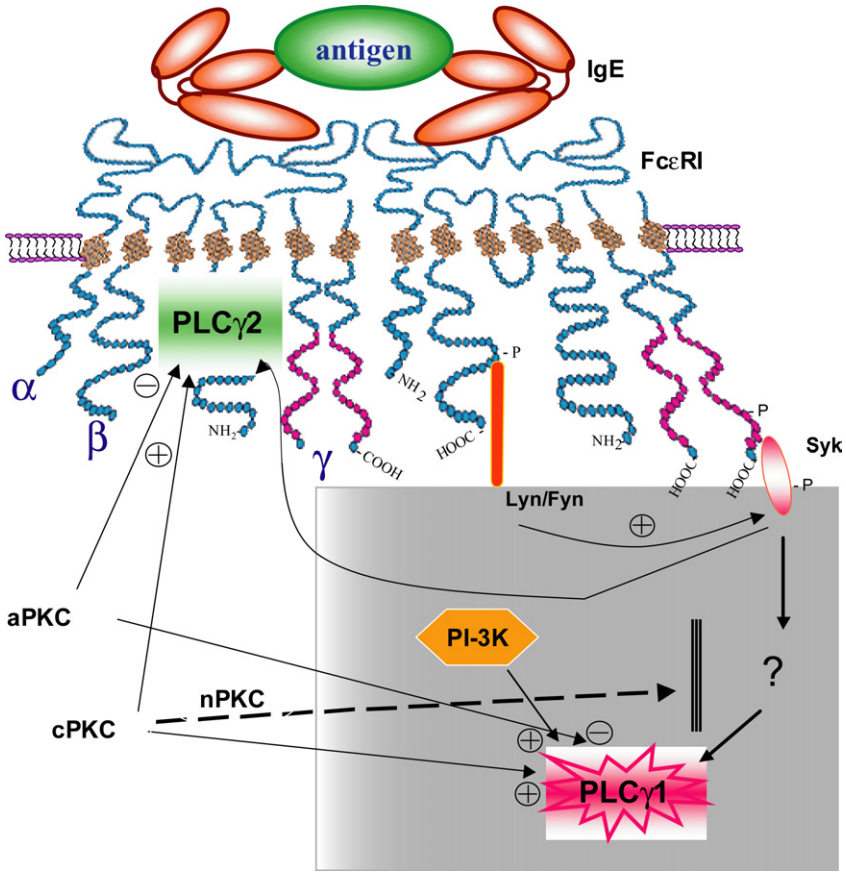

Fig. 5. Proposed signaling transduction pathways of FcεRI for the activation of PLCγ subtypes. FcεRI, high affinity IgE receptor; antigen, DNP-BSA; cPKC, conventional PKC-α, -β, and -γ; nPKC, novel PKC-δ, -ε, and -θ; and aPKC, atypical PKC-ζ. α, β, and γ represent α-, β-, and -γ subunits of FcεRI. NH_2 and COOH represent amino- and carboxy tail. This diagram illustrates the signaling pathways for the FcεRI-mediated activation of PLCγ1 and PLCγ2. PLCγ2 interacts with the NH_2-tail of the β subunit and they co-localize in the osmiophillic patches. Src/Syk are in the upstream of both PLCγ subtypes. PI3-kinase is the upstream signaling component of PLCγ1 but not of PLCγ2. The gray square, which contains PI3-kinase, PLCγ1, and majority of Src/Syk, are excluded from the osmiophillic patches. PKC-α, -β, -δ, and -ε overall have positive influences on PLCγ isoforms but PKC-ζ has strong inhibitory activities. PMA-sensitive PKCs (novel PKCs) are required for the proper activation of PLCγ1.

FcεRI in the activation of PLCγ subtypes as follows (Fig. 5). PLCγ2 is associated with FcεRI in osmiophillic patches while PLCγ1, PI3-kinase, and the majority of Src/Syk are excluded from it [15,16]. Since PLCγ2 is under the intimate influence of FcεRI through direct association with it, we could speculate that PLCγ2 would continuously signal without being noticeably regulated by other signaling components. Majority of Src/Syk are located out of osmiophillic patches, and more intimately influence PLCγ1 than PLCγ2. In addition, PI3-kinase pathway is specifically involved in the regulation of PLCγ1. The activities of PLCγ1 and PLCγ2 are regulated in a positive and negative manner by conventional and atypical PKCs, respectively. FcεRI-mediated tyrosine phosphorylation of PLCγ1 requires intact novel type PMA-sensitive PKCs. Such a characteristic subcellular organization surrounding PLCγ1 and PLCγ2 allows PLCγ1 to be responsible for dynamic regulation of cellular PKC activities and calcium levels. On the other hand, PLCγ2 would be responsible for maintaining the basal levels of PLC activity.

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