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A novel Lyn-Protein Kinase Cδ/ε-Protein Kinase D axis is activated in B cells by signalosome-independent alternate pathway BCR signaling

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Summary

B cell receptor (BCR) signaling initiates multiple activities critical for B cell function. Recently we identified an alternate BCR signaling pathway, produced by IL-4, that is signalosome-independent, unlike the classical signalosome-dependent pathway, and that leads to activation of the MAP kinase, ERK. In the present study we questioned whether alternate pathway signaling extends to other key downstream events, especially PKD activation. We found that the IL-4-induced alternate pathway for BCR signaling does result in PKD and PKD substrate phosphorylation, and that alternate pathway phosphorylation of HDAC5/7 and other key substrates require PKD. Furthermore, we found that tyrosine phosphorylation of PKCδ/ε occurs as a result of alternate but not classical pathway signaling and is required for phosphorylation of PKD and PKD substrates. This result identifies PKCδ/ε tyrosine phosphorylation as a unique outcome of the alternate pathway. The alternate pathway is mediated by Lyn which is not required for classical pathway signaling and we found that Lyn associates directly with PKCδ/ε and is required for phosphorylation of PKCδ/ε and of PKD. These results indicate that IL-4 influences B cell activation by inducing a novel signaling pathway from BCR to Lyn to PKCδ/ε to PKD.

Keywords

BCR; cytokine; signal transduction

Introduction

B cell antigen receptor (BCR) signaling is critical for B cell development, survival, activation, and differentiation. Upon antigen engagement, a BCR-triggered signaling cascade is initiated that begins with activation of src family tyrosine kinases (SFK), includes stimulation of signalosome elements that include PI3K, PLCγ2 and PKC, and proceeds to

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distal events that include MAP kinase (MAPK) activation and transcription factor expression [1, 2].

B cell-derived antibodies are critical for pathogen clearance. For T cell-dependent immune responses, the germinal center is the main site in which B cell expansion, affinity maturation, and selection by FDC harbored antigens takes place and antibody-secreting plasma cells and memory B cells are generated [3, 4]. Germinal center formation relies on optimal B cell activation, which in turn depends on the integration of signaling from encountered pathogens and T cell help. T cells provide two principal forms of help: co-stimulatory molecules such as CD40L [5] and cytokines such as Interleukin-4 [6–9]. Deficiency of these molecules results in defective germinal center structure and immunoglobulin expression. IL-4 in particular is essential for B cell differentiation and activation in vitro or in vivo [9–13].

Recently, we demonstrated that IL-4 produces a signalosome-independent alternate BCR signaling pathway that leads to ERK activation [14]. The alternate signaling pathway operates in parallel with the classical, signalosome-dependent pathway, so that BCR triggering in IL-4-treated B cells produces much more pERK than BCR triggering in naïve B cells [14–16]. In contrast to classical BCR signaling for which Lyn activity is dispensable [17], the IL-4-induced alternate BCR signaling pathway is specifically mediated by, and depends on, Lyn [15, 16].

The protein kinase D (PKD) family comprises three members including PKD1, PKD2, and PKD3, which have been implicated in activities as diverse as cell activation, proliferation, motility and adhesion. PKD2 is the major isoform expressed in B lymphocytes [18] and is phosphorylated by novel PKC kinases PKCδ and PKCε that are in turn activated in response to BCR signaling. PKD plays an essential role in the regulation of class II histone deacetylases in B (and T) cells [19, 20]. Histone deacetylases are important molecules that regulate chromatin accessibility to transcription factors and control gene expression [21–23], and thus PKD is thought to play a key role in the transcriptional reprogramming that follows antigen receptor engagement and signalosome-dependent intracellular signaling.

In the present study we questioned whether alternate pathway signaling propagates to PKD activation, which directly affects chromatin dynamics, or whether downstream alternate pathway events terminate in, and are limited to, ERK. We found that PKD phosphorylation is produced by Lyn-dependent alternate pathway signaling through a novel pathway from BCR to Lyn to PKCδ/ε to PKD.

Results

PKD activation is amplified in IL-4-treated B cells

PKD is a target of classical BCR signaling and its activation is mediated by signalosome elements [24, 25]. In view of our previous delineation of a distinct signalosome-independent, alternate BCR signaling pathway leading to ERK activation in IL-4-treated B cells [14, 15], we questioned whether BCR-triggered PKD activation is also influenced by IL-4. To address this issue, we stimulated naive B cells and IL-4-treated B cells with anti-Ig in the presence

or absence of the PI-3K inhibitor, Ly294002, and then examined PKD phosphorylation on Ser744/748 by western blotting with a polyclonal antibody. Results are shown in Figure 1A. Consistent with previous reports, BCR engagement in naïve B cells stimulated PKD phosphorylation that was blocked by inhibition of PI-3K [25]. However, we found that anti-Ig stimulated substantially more phosphorylated PKD in IL-4-treated B cells as compared to naïve B cells, and that about half of the BCR-triggered pPKD in IL-4-treated B cells was Ly294002-resistant and thus PI-3K-independent. Notably IL-4 treatment had no effect on the level of PKD protein expression in B cells (Figure S1). These results recapitulate our earlier results on BCR-triggered pERK, and suggest that the increased level of pPKD produced by BCR engagement in IL-4-treated B cells results from integration of signalosome-dependent classical and signalosome-independent alternate pathways leading to PKD.

Activated PKD recognizes and phosphorylates substrates with the consensus sequence: LXRXXS [26]. PKD substrate phosphorylation was evaluated by utilizing a specific anti-phospho-PKD substrate antibody that recognizes phosphorylated PKD substrates at their consensus motif. Results are in Figure 1B. BCR engagement in naïve B cells resulted in phosphorylation of several PKD substrates that was sensitive to inhibition by Ly294002. However, after IL-4-pretreatment, BCR engagement produced more phosphorylation of PKD substrates. This enhanced phosphorylation of PKD substrates was Ly resistant. More importantly, one PKD substrate was phosphorylated only in IL-4-pretreated B cells as indicated by the star. The consensus sequence LXRXXS can also be phosphorylated by other kinases such as MAPKAPK2 [27]. To assure that IL-4-enhanced phosphorylation of PKD substrates is mediated by PKD, B cells were isolated from PKD2 mutant and littermate control mice. Naïve or IL-4-treated B cells were stimulated with anti-Ig in the presence or absence of the PI-3K inhibitor, Ly294002, and then phosphorylation of PKD substrates was examined by western blotting. As shown in Figure 1C, PKD2 mutation resulted in decreased phosphorylation of PKD substrates in anti-Ig-stimulated naïve B cells, suggesting that PKD2 mediates phosphorylation of PKD substrates along with other kinases during conventional BCR signaling. Importantly, IL-4-enhancement of BCR-mediated phosphorylation of PKD substrates (including the novel PKD substrate) was eliminated by PKD2 mutation. These results indicate that IL-4 treatment elevates anti-Ig-initiated PKD activation and its downstream substrate phosphorylation.

Previous work [19] has shown that HDAC5 and HDAC7 are activated by BCR stimulation in a PKD-dependent manner. We asked whether IL-4-induced alternate pathway BCR signaling affects PKD-mediated HDAC5 and HDAC7 phosphorylation in B cells. To examine this issue, naive B cells and IL-4-treated B cells were stimulated with anti-Ig in the presence or absence of the PI-3K inhibitor, Ly294002, and then examined for phosphorylation of HDAC5 and HDAC7 by western blotting with an anti-phospho-HDAC5/7 antibody. BCR engagement in naïve B cells resulted in HDAC5/7 phosphorylation that was sensitive to inhibition by Ly294002. However, after IL-4 treatment, BCR engagement produced more phosphorylation of HDAC5/7 that was Ly-resistant. As with other substrates, PKD2 mutation resulted in decreased phosphorylation of HDAC5/7 in naïve B cells stimulated with anti-Ig. Most importantly, IL-4-enhancement of BCR-mediated HDAC5/7 phosphorylation was eliminated by PKD2 mutation (Figure 1C). These results suggest that the IL-4-induced alternate BCR signaling pathway leads to not only enhanced, Ly-resistant, activation of PKD

and phosphorylation of PKD substrates including HDAC5/7, but also a novel PKD substrate phosphorylation.

Novel PKC family members are required for enhanced PKD activation in IL-4-treated B cells

To examine the role of PKC family members in producing PI-3K-independent activation of PKD, we stimulated purified B cells with anti-Ig in the presence or absence of either of two broad-spectrum PKC inhibitors, Go6983 and GF109203X, both of which inhibit conventional and novel PKC kinases. Results are shown in Figure 2. As expected [28], two inhibitors completely abrogated BCR-stimulated PKD activation in naïve B cells. In IL-4-treated B cells, we found that all BCR-stimulated PKD activation was blocked by inhibition of PKC, including that which was resistant to inhibition by Ly294002. Thus, PKC family members are required for BCR-triggered PKD activation in naïve or IL-4-treated B cells.

In naïve B cells, BCR signaling depends on PKCβ, a conventional PKC and signalosome component. To determine which sub-group of PKC is involved in producing PI-3K-independent PKD phosphorylation in anti-Ig-stimulated IL-4-treated B cells, we combined Ly294002 with the narrow-spectrum PKC inhibitor, Go6976, which predominantly interferes with the conventional kinases, PKCα and PKCβ. Go6976, as well as general PKC inhibitors (Go6983 and GF109203X), was used at a minimal dose that completely blocked BCR-induced IKBα degradation (Figure S2). We found that Ly294002-resistant PKD activation stimulated by anti-Ig in IL-4 treated B cells is also resistant to Go6976. These results indicate that novel PKCs (Go6983/GF109203X-sensitive, Go6976-insensitive) are required for alternate, signalosome-independent PKD activation in IL-4-treated B cells.

PKCδ and PKCε are tyrosine phosphorylated upon BCR stimulation in IL-4 treated B cells

Activation and phosphorylation of PKD is mediated by novel PKC family members [29], particularly the prototypical novel PKC, PKCδ, which is activated by src kinase-mediated tyrosine phosphorylation in multiple cell lines [30–33]. Because the alternate pathway for BCR signaling produced by IL-4 requires the src kinase, Lyn, we questioned whether alternate pathway signaling for PKD might be mediated by tyrosine phosphorylation of PKCδ. To address this issue, we stimulated naive B cells and IL-4-treated B cells with anti-Ig in the presence or absence of the PI-3K inhibitor, Ly294002, and then examined immunoprecipitated PKCδ for tyrosine phosphorylation by western blotting. Results are shown in Figure 3. Naïve B cells expressed little or no tyrosine phosphorylated PKCδ before or after BCR engagement, whereas, in contrast, we found that anti-Ig stimulated substantial amounts of pTyrPKCδ in IL-4-treated B cells and this induced phosphorylation was not affected by inhibition of PI-3K. To provide additional evidence for PKCδ tyrosine phosphorylation, we probed cell lysates with an antibody specific for PKCδ phosphorylated at tyrosine311 by western blotting. Here again naïve B cells expressed little or no tyrosine pTyr311PKCδ after BCR engagement, whereas, in contrast, we found that anti-Ig stimulated substantial amounts of pTyr311PKCδ in IL-4-treated B cells and this phosphorylation of tyrosine311 was not affected by inhibition of PI-3K.

In addition to PKCδ, B cells express PKCε, which, like PKCδ, might be capable of phosphorylating and activating PKD. To determine whether PKCε is inducibly tyrosine phosphorylated like PKCδ, we stimulated naive B cells and IL-4-treated B cells with anti-Ig in the presence or absence of the PI-3K inhibitor, Ly294002, and then examined immunoprecipitated PKCε for tyrosine phosphorylation by western blotting. Naïve B cells expressed little or no tyrosine phosphorylated PKCε before or after BCR engagement, whereas, in contrast, we found that anti-Ig stimulated substantial amounts of pTyrPKCε in IL-4-treated B cells and this induced phosphorylation was not affected by inhibition of PI-3K. Thus, both PKCδ and PKCε are targets for phosphotyrosine kinase activity. These results indicate that IL-4-treatment dramatically alters BCR signaling such that BCR triggering in IL-4-treated B cells produces signalosome-independent tyrosine phosphorylation of PKCδ/ε, whereas BCR triggering in naïve B cells fails to do so.

Novel PKCδ/ε is tyrosine phosphorylated in a signalosome-independent manner and is required for PKD activation in IL-4-treated B cells. We then asked whether novel PKCδ/ε directly interacts with PKD. To address this issue, naïve or IL-4-treated B cells were stimulated with anti-Ig in the presence or absence of the PI-3K inhibitor, Ly294002, and then immunoprecipitated PKD was examined for the presence of PKCδ and PKCε by western blotting. Although novel PKC members are required for Ly294002-resistant PKD activation, they do not directly interact with PKD in IL-4-treated B cells with or without BCR stimulation (Figure S3).

Novel PKC tyrosine phosphorylation in IL-4-treated B cells depends on Lyn

Because alternate pathway BCR signaling induced by IL-4 depends on Lyn [14, 15], we questioned whether PI-3K-independent BCR-triggered tyrosine phosphorylation of PKCδ/ε might also depend on Lyn. To address this issue, we stimulated naive and IL-4-treated B cells obtained from Lyn null and from WT littermate control mice with anti-Ig in the presence or absence of the PI-3K inhibitor, Ly294002, and then probed cell lysates with anti-pTyr311PKCδ antibody by western blotting. Results are shown in Figure 4A. As expected, in WT control B cells, anti-Ig produced tyrosine phosphorylation of PKCδ only after IL-4 treatment, and this occurred even when PI-3K was inhibited. In direct contrast, we found that Lyn deficiency completely abrogated tyrosine phosphorylation of PKCδ. Thus, in IL-4-treated B cells, BCR-triggered PKCδ tyrosine phosphorylation that is PI-3K-independent requires Lyn. To determine the connection between Lyn and phosphorylated nPKC (direct vs indirect), we stimulated naive and IL-4-treated B cells with anti-Ig for 2 or 5 minutes, and then examined immunoprecipitated Lyn for the presence of PKCδ and PKCε by western blotting. Results are shown in Figure 4B. We found that after anti-Ig treatment (but not before), both PKCδ and PKCε associated with Lyn. However, this only occurred in IL-4-treated B cells, and not in naïve B cells, the same situation in which PKCδ/ε is tyrosine phosphorylated in a Lyn-dependent manner. These results strongly suggest that alternate pathway BCR signaling for PKCδ/ε tyrosine phosphorylation is mediated by direct interaction between Lyn and nPKC.

PI-3K-independent PKD activation in IL-4-treated B cells depends on Lyn

Because Lyn is directly connected to pTyrPKCδ/ε which is required for activating PKD, we questioned whether PI-3K-independent PKD phosphorylation might be Lyn-dependent as well. To address this issue, we stimulated naive and IL-4-treated B cells obtained from Lyn null and from WT littermate control mice with anti-Ig in the presence or absence of the PI-3K inhibitor, Ly294002, and then probed cell lysates with anti-pPKD antibody by western blotting. Results are shown in Figure 5. As expected, in WT control B cells, anti-Ig produced phosphorylation of PKD that was PI-3K-dependent in naïve B cells and PI-3K-independent in IL-4-treated B cells. In direct contrast, we found that the loss of Lyn completely abrogated PI-3K-independent anti-Ig-induced PKD phosphorylation. Thus, in Lyn null B cells, BCR triggering produces PKD phosphorylation that is always sensitive to Ly294002 and no alternate pathway to PKD exists, even after treatment with IL-4. This recapitulates the results described above, where in the absence of Lyn no alternate pathway to PKDδ/ε tyrosine phosphorylation exists. Thus, in IL-4-treated B cells, alternate pathway signalosome-independent BCR signaling for PKD activation involves a pathway from Lyn to nPKC to PKD.

Discussion

The present results constitute three key findings: 1) IL-4-induced alternate pathway BCR signaling propagates downstream to activate PKD, a kinase well known for its critical functions and which mediates enhanced phosphorylation of HDAC5/7 and other critical substrates; 2) Novel PKC kinases are tyrosine phosphorylated in response to BCR engagement only within the framework of the alternate pathway, and not via the classical pathway, and are responsible for PKD phosphorylation; and, 3) Lyn associates with nPKC and is required for nPKC and PKD phosphorylation, strongly suggesting a direct pathway from Lyn to nPKC to PKD.

BCR signaling is pivotal for B cell development, differentiation, activation, and survival. Much knowledge regarding the nature, constituents and outcomes of BCR signaling cascades has been derived from *in vitro* studies in which potential stimuli are examined one at a time. However, the situation is likely quite different *in vivo*, where in the course of a developing response B cells integrate multiple signals from multiple sources that include specific antigen and T cell help produced by membrane-bound ligands and secreted cytokines. Among the latter, numerous studies have shown that IL-4 is a key regulator of B cell activity and immunoglobulin isotype *in vivo* and *in vitro* [13, 34–37]. Recently we reported that IL-4 reprograms BCR signaling by inducing a signalosome-independent alternate BCR signaling pathway [16]. We showed that the alternate pathway exists in parallel with the classical pathway for BCR signaling in IL-4-treated B cells and that the two pathways coincide in producing ERK phosphorylation. As a result, demonstration of the alternate pathway using pERK as a read-out depends on inhibition or deficiency of classical signaling elements. In the present study we have identified a signaling outcome that is relatively unique to the alternate pathway, namely, tyrosine phosphorylation of PKCδ/ε. This novel PKC tyrosine phosphorylation can thus be used to monitor alternate pathway activity without the need for disturbance of other signaling mediators.

With these new results we can categorize several outcomes of BCR signaling in relation to the alternate pathway: those produced by either the alternate or the classical pathway acting alone (eg, ERK phosphorylation); those produced by the combined action of the alternate and classical pathways acting together (eg, osteopontin transcription); and, those produced only by the alternate pathway and not by the classical pathway (eg, nPKC tyrosine phosphorylation).

Alternate pathway activation of PKCδ/ε is especially significant because it leads to PKD activation. Thus, in IL-4-treated B cells, BCR-stimulated pPKD consists of two fractions, one that is signalosome-dependent and blocked by Ly294002 (and is present in stimulated naïve B cells), and one that is signalosome-independent and Ly294002-resistant. In IL-4-treated B cells these fractions together produce PKD activation that is enhanced as a result of the integration of the alternate and classical BCR signaling pathways. PKD plays an essential role in the regulation of class II histone deacetylases in B (and T) cells [19, 20]. In this study, we found that naïve B cells express Ly-sensitive phosphorylation of PKD and PKD substrates upon BCR stimulation. After IL-4-pretreatment, B cells express not only Ly-resistant phosphorylation of PKD and PKD substrates, including HDAC5/7, upon BCR stimulation but also a novel phosphorylated PKD substrate that is not found in BCR stimulated naïve B cells. In view of the role of PKD and HDACs in regulating chromatin dynamics [21], increased levels of phosphorylated PKD and PKD substrates including HDACs are likely to have a significant effect on gene expression.

Lyn is required for BCR-triggered PKD activation via the IL-4-induced alternate pathway as it is for all alternate pathway signaling--this requirement distinguishes the alternate from the classical pathway, the latter of which operates in the complete absence of Lyn [38]. Intriguingly, whereas IL-4 plays an important role in germinal center formation and germinal center B cell expansion [6, 9, 12, 39], Lyn deficient mice fail to generate germinal centers in T cell-dependent immune response [40, 41]. Thus, IL-4 and Lyn appear to bear some similarities in their effects on B cell differentiation. It may be speculated that IL-4 secretion by germinal center Tfh cells reprograms BCR signaling by inducing alternate pathway BCR signaling in which Lyn plays an indispensable role, resulting in a proper germinal center reaction. In this view, the integration of the alternate and classical BCR signaling pathways favors optimal B cell activation, expansion, and differentiation, relying on chromatin remodeling produced by PKD and HDAC, to produce an effective serological immune response.

Materials and Methods

Animals

Male BALB/cByJ mice, C57BL/6 mice, and PKD2 ($S_{707}A/S_{711}A$) mutant mice at 8–14 weeks of age were obtained from The Jackson Laboratory. Lyn-deficient mice on the C57BL/6 background have been described [15]. All mice were cared for and handled in accordance with National Institutes of Health and institutional guidelines, and studies with these mice were approved by an institutional review committee.

Lymphocyte isolation

B cells were prepared from spleen cell suspensions by negative selection using magnetic-activated cell sorting (Miltenyi Biotec).

B cell stimulation

Purified B cells were cultured at 2 X 10^6 per ml in RPMI 1640 medium containing 10% FCS, 10 mM HEPES, 2 mM L-glutamine, 0.1 mg/ml penicillin and streptomycin, and 50 μM 2-ME as previously described [42]. B cells were stimulated by $F(ab')_2$ goat anti-mouse IgM at 15 μg/ml (anti-Ig) after incubation in medium for 3 h, or after treatment with IL-4 at 10 ng/ml for 24 h, followed by incubation in medium for 3 h. Inhibitors were added 1 h before stimulation with anti-Ig.

Western immunoblot analysis

Proteins were extracted from B cell pellets with radioimmunoprecipitation assay lysis buffer and equal amounts of protein for each condition (15–30 μg) were subjected to SDS-PAGE followed by immunoblotting as described previously [43]. Immunoreactive proteins were detected by ECL (Amersham Biosciences). Immunoblots were stripped and reprobed with control Ab to verify that equal amounts of protein were loaded in each lane.

Immunoprecipitation

Naive or IL-4-treated B cells were stimulated with 15 μg/ml $F(ab')_2$ fragments of anti-IgM antibody for the indicated times, and the reactions were terminated with ice-cold PBS. The cells were centrifuged and resuspended in NP-40 lysis buffer (1% Nonidet P-40, 20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 2 mM Na_3VO_4, 2 mM EDTA) or digitonin lysis buffer (1% digitonin, 20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 2 mM Na_3VO_4, 2 mM EDTA). The lysates were centrifuged and the supernatants were subjected to immunoprecipitation. Samples were immunoprecipitated with antibodies against PKCδ or PKCε in NP-40 buffer, or Lyn in digitonin buffer, at 4°C overnight. Immunoprecipitates were boiled and subjected to 8% SDS-PAGE separation.

Reagents

Affinity-purified $F(ab')_2$ fragments of polyclonal goat anti-mouse IgM (anti-Ig) was obtained from Jackson ImmunoResearch Laboratories. Anti-phospho-ERK1/2 (Thr202/Tyr204), anti-ERK1/2, anti-phospho-PKD (Ser744/748), anti-phospho-(Ser/Thr) PKD substrate, anti-PKD, anti-Phospho-HDAC4 (Ser632)/HDAC5 (Ser498)/HDAC7 (Ser486), anti-phospho-PKCδ (Tyr311), and secondary Abs for immunoblotting were obtained from Cell Signaling Technology. Anti-phosphotyrosine antibody (4G10) was obtained from Millipore. Anti-PKCδ, anti-PKCε, and anti-Lyn antibodies were obtained from Santa Cruz Biotechnology. Anti-actin antibody was obtained from Sigma-Aldrich. Protein A/G ultralink resin was obtained from Thermo Scientific. Ly294002, Go6983, Go6976, and GF109203X were obtained from Calbiochem. Recombinant murine IL-4 was obtained from BD Pharmingen.

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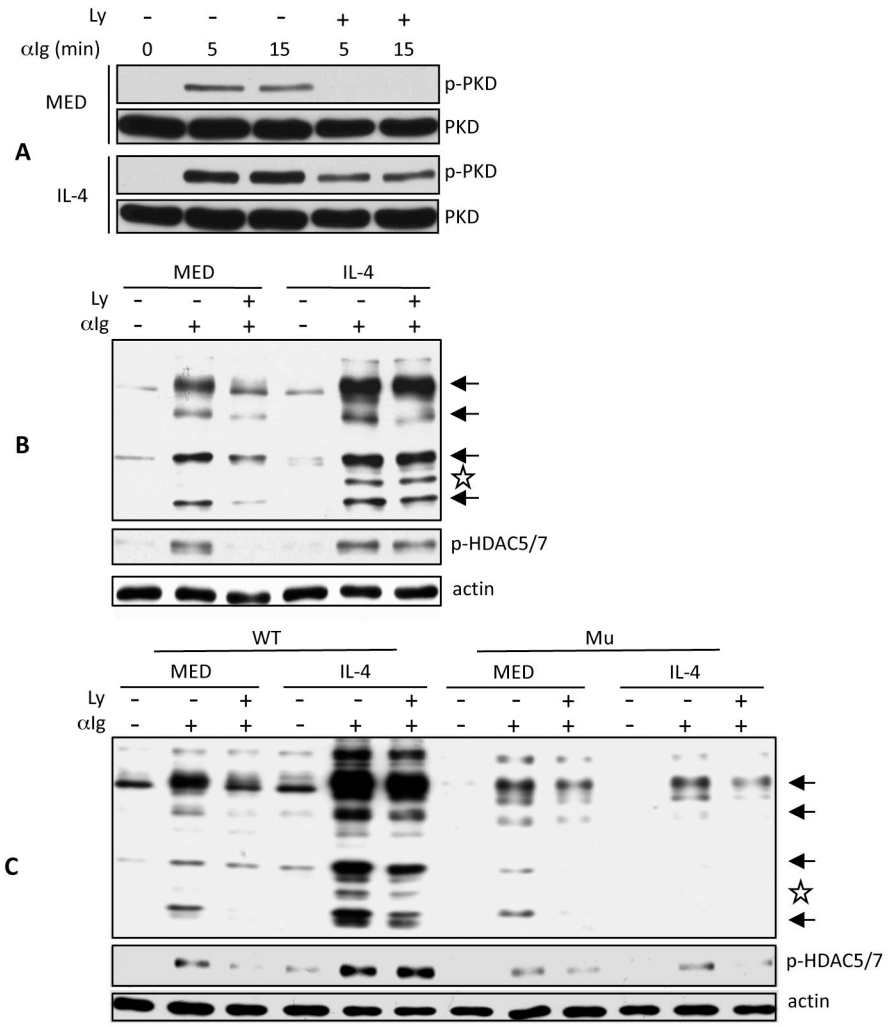

Figure 1.

PKD activation is amplified in IL-4-treated B cells. **A**. B cells were cultured in medium alone (MED, *top panels*) or with IL-4 at 10 ng/ml for 24 h (IL-4, *bottom panels*), and then stimulated with anti-Ig at 15 μg/ml (αIg) for 0, 5, or 15 min. B cells were exposed to the PI3K inhibitor, Ly294002 at 20 μM (Ly) for 60 min before addition of anti-Ig. Whole cell extracts were prepared and western blotted as described in *Materials and Methods* with anti-phospho-PKD Ab. Blots were stripped and reprobed with anti-PKD Ab. One of three comparable experiments is shown. **B**. B cells were cultured in medium alone (MED, *left panels*) or with IL-4 at 10 ng/ml for 24 h (IL-4, *right panels*), and then stimulated with anti-Ig at 15 μg/ml (αIg) for 0 (−) or 5 (+) min. B cells were exposed to the PI3K inhibitor, Ly294002 at 20 μM (Ly) for 60 min before addition of anti-Ig. Whole cell extracts were prepared and western blotted with anti-phospho-PKD substrate antibody or anti-phospho-HDAC4 (Ser632)/HDAC5 (Ser498)/HDAC7 (Ser486) antibody. Blots were stripped and reprobed with anti-actin antibody. One of three comparable experiments is shown. **C**. B cells purified from PKD2 mutant (Mu) and littermate control (WT) mice were cultured in medium alone or with IL-4 and stimulated with anti-Ig for 0 (−) or 5 (+) min in the presence

(+) or absence (−) of Ly294002. Whole cell extracts were western blotted with anti-phospho-PKD substrate antibody or anti-phospho-HDAC4 (Ser632)/HDAC5 (Ser498)/HDAC7 (Ser486) antibody. Blots were stripped and reprobed with anti-actin antibody. One of three comparable experiments is shown.

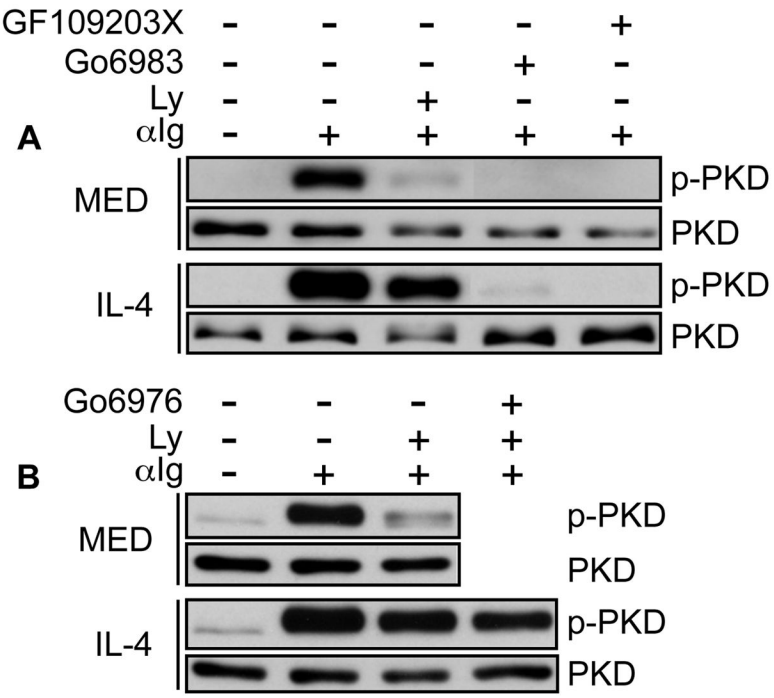

Figure 2.

PKD activation in IL-4-treated B cells is mediated by novel PKC family members. **A.** B cells were cultured in medium alone or with IL-4 and stimulated with anti-Ig for 0 (−) or 5 (+) min. B cells were exposed to the PI3K inhibitor (Ly294002 (Ly) (20 μM)) or broad PKC inhibitors (G06983 (2μM) or GF109203X (5μM)) for 60 min before addition of anti-Ig. Whole cell extracts were prepared and western blotted as described in *Materials and Methods* with anti-phospho-PKD Ab. Blots were stripped and reprobed with anti-PKD Ab. **B.** B cells were cultured in medium alone or with IL-4 and stimulated with anti-Ig for 0 (−) or 5 (+) min. B cells were exposed to the PI3K inhibitor (Ly294002 (Ly) (20 μM)) or a combination of the conventional PKC inhibitor Go6976 (0.5μM) and Ly for 60 min before addition of anti-Ig. Whole cell extracts were prepared and western blotted as above. One of three comparable experiments is shown for both A and B.

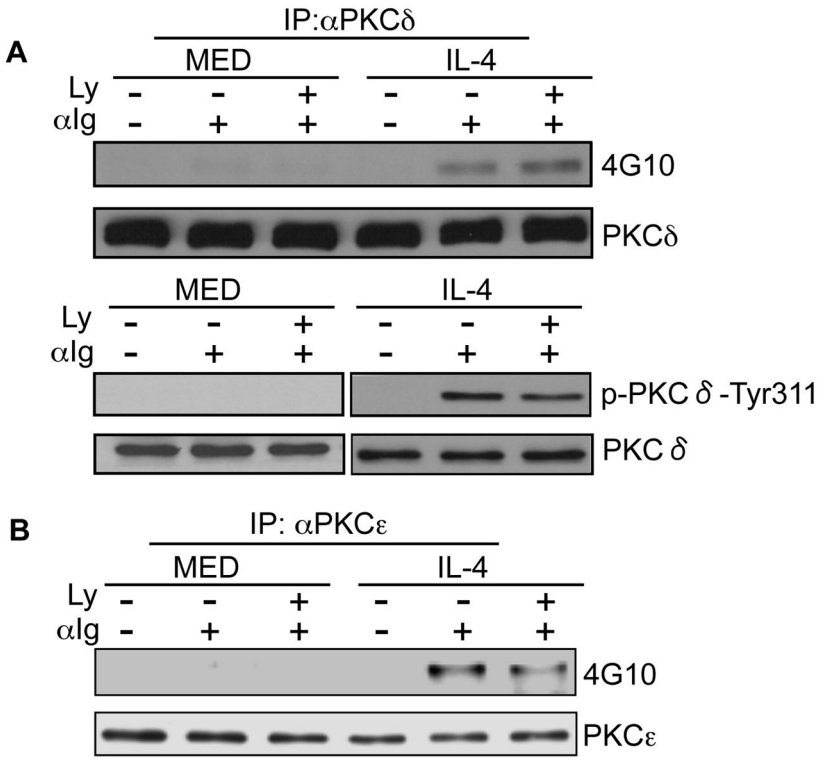

Figure 3.

PKCδ and PKCε are tyrosine phosphorylated upon BCR stimulation in L-4-treated B cells. **A.** B cells were cultured in medium alone or with IL-4 and stimulated with anti-Ig for 0 (−) or 5 (+) min in the presence or absence of Ly294002. Whole cell extracts were immunoprecipitated (IP) with anti-PKCδ Ab. Immunoprecipitates were western blotted with anti-phosphotyrosine Ab 4G10 (*top panels*) or whole cell lysates were western blotted with anti-phospho-PKCδ (Tyr311) Ab (*bottom panels*). Blots were stripped and reprobed with anti-PKCδ Ab. **B.** Whole cell extracts from B cells treated as above were immunoprecipitated (IP) with anti-PKCε Ab. Immunoprecipitates were western blotted with anti-phosphotyrosine Ab 4G10. Blots were stripped and reprobed with anti-PKCε Ab. One of three comparable experiments is shown for both A and B.

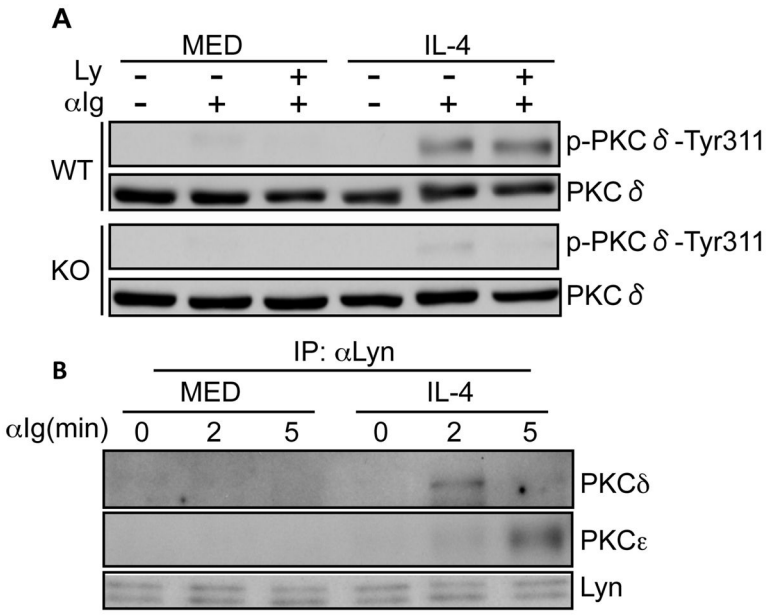

Figure 4.

Novel PKC tyrosine phosphorylation in IL-4-treated B cells depends on Lyn. **A.** B cells purified from Lyn null (KO) and littermate control (WT) mice were cultured in medium alone or with IL-4 and stimulated with anti-Ig for 0 (−) or 5 (+) min in the presence or absence of Ly294002. Whole cell extracts were western blotted with anti-pTyr311PKCδ (*top panels*). Blots were stripped and reprobed with anti-PKCδ Ab (*bottom panels*). **B.** B cells purified from Lyn null and littermate control mice were cultured in medium alone or with IL-4 and then stimulated with anti-Ig for 0, 2, or 5 min. Whole cell extracts were immunoprecipitated (IP) with ant-Lyn Ab. Immunoprecipitates were western blotted with anti-PKCδ(*top panel*) or PKCε (*middle panel*). Blots were stripped and reprobed with anti-Lyn Ab (*bottom panel*). One of three comparable experiments is shown for both A and B.

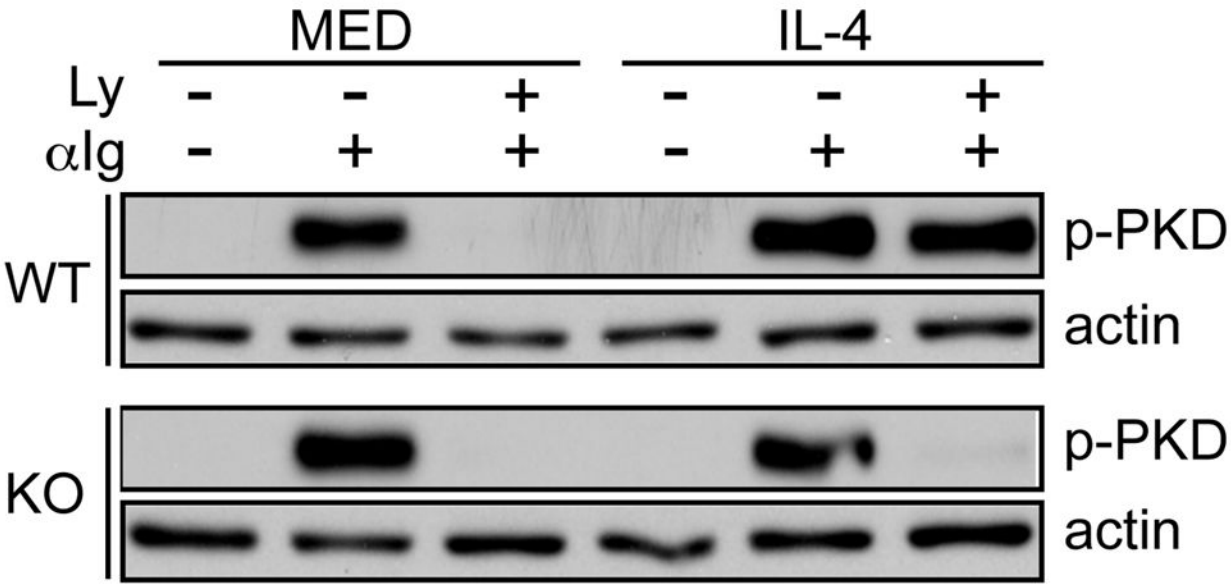

Figure 5.

PI3K-independent PKD activation in IL-4-treated B cells depends on Lyn. B cells purified from Lyn null (KO) and littermate control (WT) mice were cultured in medium alone or with IL-4 and stimulated with anti-Ig for 0 (−) or 5 (+) min in the presence or absence of Ly294002. Whole cell extracts were western blotted with anti-phospho-PKD (Ser744/748) Ab (*top panels*). Blots were stripped and reprobed with anti-PKD Ab (*bottom panels*). One of two comparable experiments is shown.