Cross-linking of the B cell receptor induces activation of phospholipase D through Syk, Btk and phospholipase C-γ2

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Abstract Phospholipase D (PLD) has been proposed to play a key role in the signal transduction of cellular responses to various extracellular signals. Herein we provide biochemical and genetic evidence that cross-linking of the B cell receptor (BCR) induces rapid activation of PLD through a Syk-, Btk- and phospholipase C (PLC)-γ2-dependent pathway in DT40 cells. Activation of PLD upon BCR engagement is completely blocked in Syk- or Btk-deficient cells, but unaffected in Lyn-deficient cells. Furthermore, in PLC-γ2-deficient cells, BCR engagement failed to activate PLD. These results demonstrate that Syk, Btk and PLC-γ2 are essential for BCR-induced PLD activation.

Key words: B cell receptor; Phospholipase D; Syk; Btk; Phospholipase C-γ; Src family

1. Introduction

Stimulation of surface IgM on B cells (B cell receptor; BCR) by antigen or anti-IgM antibody initiates a cascade of biochemical events including protein tyrosine kinase (PTK) activation, phosphatidylinositol 4,5-bisphosphate (PIP_2) hydrolysis, and a Ras-mediated pathway. Three distinct families of non-receptor cytoplasmic PTKs, the Src family PTK Lyn, Syk, and Btk, have been shown to be activated and have an important role in BCR signaling [1,2]. It has also been demonstrated that these enzymes mediate the phosphorylation of at least partially distinct sets of substrates [3,4]. Tyrosine phosphorylation of Shc, which is an adaptor protein in the Ras pathway, is regulated by Lyn and Syk [5], while phospholipase C (PLC)-γ2 activation is mediated by Syk and Btk [4,6]. Tyrosine phosphorylation of PLC-γ2 is responsible for an increase in its catalytic activity [7]. Activated PLC-γ2 catalyzes the hydrolysis of phosphoinositides, leading to the generation of inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol (DG) which, respectively, induce calcium mobilization and protein kinase C (PKC) activation. However, the downstream events of phosphoinositide hydrolysis by PLC-γ2 are largely unknown.

Phospholipase D (PLD) activation in response to a variety of extracellular signals has been observed in many cells and tissues. PLD plays an important role in signal transduction through phosphatidylcholine (PC) hydrolysis to choline and phosphatidic acid (PA) [8]. The latter is a second messenger implicated in the regulation of many signaling proteins [9]. Lysophosphatidic acid (LPA) generated through the action of a specific phospholipase A_2 (PLA_2) on PA is also becoming recognized as a major extracellular signal [10]. PA can also be dephosphorylated by PA phosphohydrolases to DG, which is an activator of certain isoforms of PKC [11]. Although the important role of PLD is well documented, the control of PLD activity is very complex and remains unclear. PKC [12–14], small GTP-binding protein ADP-ribosylation factor (ARF) [15] and RhoA [16] are known as PLD activators in vitro and in vivo. Moreover, many investigations using various inhibitors have suggested the involvement of PTK(s) [12], phosphatidylinositol 3-kinase (PI3-K) [17], trimeric GTP-binding proteins [12,15,18] and PLC [19,20] in PLD activation. However, the exact signal pathway involved in the induction of PLD activity remains to be defined.

Recent gene-targeting experiments have established Lyn-, Syk-, Btk- or PLC-γ2-deficient cell lines derived from avian DT40 B cells [4,6,21], exploiting their unusually high homologous recombination proficiency. Since we found that PLD was activated upon BCR engagement in DT40 cells, using these mutants, a molecular genetic analysis was performed to identify the pathway involved in BCR signal transduction ultimately leading to PLD activation. Herein, we provide genetic and biochemical evidence that Syk, Btk and PLC-γ2 are essential for PLD activation in BCR signaling. This is the first report of the involvement of PLD in BCR signaling.

2. Materials and methods

2.1. Materials

The generation of DT40 Lyn^-, DT40 Syk^-, DT40 Btk^-, DT40 PLC-$\gamma2^-$, wild-type *syk* cDNA-transfected DT40 Syk^- cells (DT40 Syk^-/Syk), wild-type *btk* cDNA-transfected DT40 Btk^- cells (DT40 Btk^-/Btk) and wild-type PLC-γ2 cDNA-transfected DT40 PLC-$\gamma2^-$ cells (DT40 PLC-$\gamma2^-$/PLC-γ2) was carried out as described previously [4,6,21]. Anti-chicken IgM mAb M4 was produced as described previously [22]. Methyl 2,5-dihydroxycinnamate (erbstatin analog) and bisindolylmaleimide (BIM) were obtained from Toronto Research, Inc. and Calbiochem-Novabiochem Corp., respectively. Other chemicals were purchased from commercial sources.

2.2. Cell culture and harvesting

DT40 cells were maintained at a cell density of $0.5–1.5\times10^6$ cells/ml as a suspension in RPMI 1640 medium supplemented with 10% (v/v) fetal calf serum (FCS), 100 U/ml penicillin, and 100 μg/ml streptomycin in a humidified 95% air, 5% CO_2 atmosphere. For experiments, cells were collected by centrifugation, washed once in PBS buffer, and then resuspended in FCS-free RPMI 1640 medium, pH 7.4.

2.3. PLD assay

Cells were preincubated with [^{14}C]lysoPC (0.25 μCi/1×10^7 cells) for

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Abbreviations: BCR, B cell receptor; PTK, protein tyrosine kinase; PIP_2, phosphatidylinositol 4,5-bisphosphate; PLC, phospholipase C; IP_3, inositol 1,4,5-trisphosphate; DG, diacylglycerol; PKC, protein kinase C; PLD, phospholipase D; PC, phosphatidylcholine; PA, phosphatidic acid; ARF, ADP-ribosylation factor; PEt, phosphatidylethanol; FCS, fetal calf serum; BIM, bisindolylmaleimide; TCR, T cell receptor

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1 h at 37°C. The labeled cell suspension (1×10^6 cells/100 μl) was transferred to the reaction mixture (100 μl) containing ethanol (1%) and 6 μg/ml anti-IgM (M4) in FCS-free RPMI 1640 medium, pH 7.4. The mixture was incubated for additional 30 min at 37°C. The reaction was stopped by adding 1 ml of ice-cold chloroform/methanol/HCl 1:1:0.006 (v/v). Phase separation and lipid extraction were carried out as described previously [23]. Samples were spotted on a Silica Gel 60 TLC plate (Merck) and were developed with the upper phase of a mixture of ethyl acetate/2,2,4-trimethylpentane/acetic acid/water (13:2:3:10) as a solvent. The radioactive spot of phosphatidylethanol (PEt) was quantified using a Fujix Bio Imaging Analyzer, BAS 2000. PLD activity was expressed as percentage of the radioactivity in [^{14}C]PEt with the total radioactivity found in all spots in that lane as 100%.

2.4. Chicken PLD1 cDNA

Total RNA was isolated from DT40 cells by the acid guanidine thiocyanate method as described previously [24] and reverse transcribed using oligo(dT) primers. Primers used for amplification of chicken PLD1 derived from DT40 cell were synthesized as follows: primer (sense) was 5′-TGGGCTCACCATGAGAA-3′; primer (antisense) was 5′-GTCATGCCAGGGCATCCGGGG-3′. Amplification was carried out at 94°C for 1 min, 50°C for 1 min, and 72°C for 1 min at 30 cycles. Amplified DNA fragments were labeled with [^{32}P]dCTP and used in Northern hybridization as probes.

2.5. Northern blot analysis

RNAs were prepared from various types of DT40 cells using the acid guanidine thiocyanate method as described previously [24]. Total RNA (20 μg) was separated in 1.2% formaldehyde gel, transferred to Hybond-N (Amersham Pharmacia Biotech) membrane and probed with ^{32}P-labeled chicken PLD1 cDNA.

3. Results and discussion

PLD hydrolyzes phospholipids generating PA and free choline. However, in the presence of a primary alcohol such as ethanol, the enzyme catalyzes a transphosphatidylation reaction that transfers the phosphatidyl moiety to the alcohol, yielding a phosphatidylalcohol, which is metabolically stable. This transphosphatidylation reaction is a unique feature of PLD and constitutes the basis for a sensitive and specific assay to detect PLD activity. Thus, PLD activation was investigated in [^{14}C]lysoPC-labeled avian DT40 B cells following BCR stimulation with anti-IgM (M4) in the presence of ethanol. As shown in Fig. 1A, TLC analyses of lipid extracts derived from BCR-induced DT40 cells exposed to ethanol revealed a time-dependent increase in the production of PEt. This result demonstrates the presence of PLD activity in DT40 cells, which can be stimulated upon BCR engagement. As shown in Fig. 1B, the formation of PEt can be detected within 3 min and the total amount of PEt reaches a plateau at 10 min following BCR stimulation. Since BCR engagement induces the rapid activation of three distinct families of nonreceptor cytoplasmic PTKs, the Src family PTK Lyn, Syk, and Btk, within 3 min [4,6], subsequently PLD activation may occur following the activation of these PTKs. As shown in Fig. 1C, the dose dependence of M4-mediated PEt formation demonstrated that PLD was maximally activated following stimulation with M4 at concentration of 4–10 μg/ml, a range suboptimal for the activation of PTKs [4,6]. Thus, we hypothesized that BCR-induced PLD activation may be dependent on PTK activation. Indeed, in a preliminary experiment, a PTK inhibitor, erbstatin analog, drastically reduced BCR-induced PLD activation in DT40 B cells (data not shown).

To further investigate which PTK(s) is (are) involved in

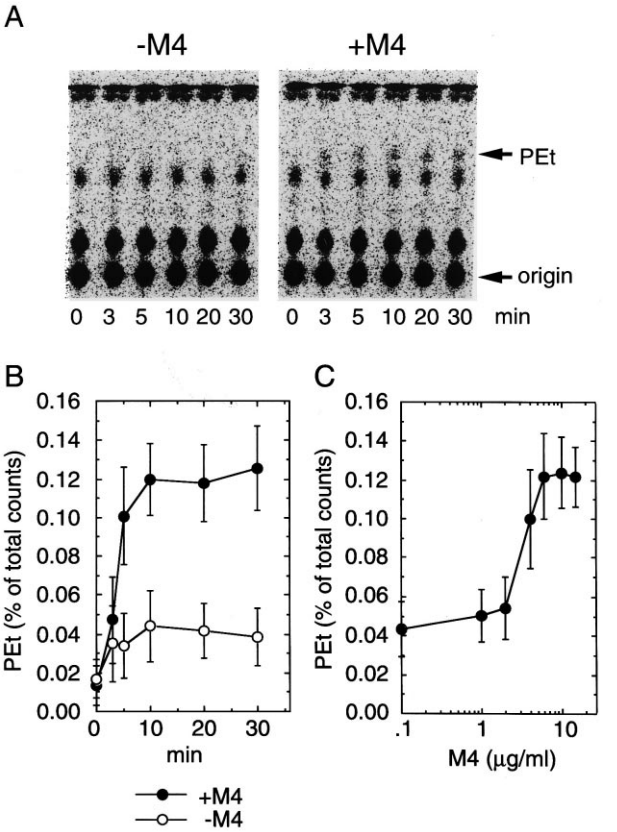

Fig. 1. PLD activation in DT40 cells following BCR stimulation. A: TLC analysis pattern of lipid extracts derived from anti-IgM (M4)-stimulated DT40 cells. B: Radioactivity in the PEt fraction in A was determined. Metabolically labeled wild-type DT40 cells with [^{14}C]lysoPC were incubated for the indicated times (min) at 37°C with (●) or without (○) 6 μg/ml M4. C: Incubation for 30 min at 37°C with indicated dose of M4. PLD activity was measured as described in Section 2. 0 min: M4 or vehicle was added after extraction by chloroform/methanol/HCl 1:1:0.006 (v/v). Results are the means ± S.D. of five independent experiments.

PLD activation, we have analyzed the BCR-induced PLD activation in each of the DT40 B cells deficient in Lyn, Syk and Btk [4,6]. As shown in Fig. 2A, PLD activation upon BCR engagement was completely blocked in DT40 Syk$^-$, DT40 Btk$^-$ cells and restored in DT40 Syk$^-$/Syk and DT40 Btk$^-$/Btk cells. Unexpectedly, in DT40 Lyn$^-$ cells, PLD activation, following BCR stimulation, was found to be unaffected. Through these PTKs, both the Ras pathway and the PLC-γ2 pathway are known to be major downstream events in BCR signaling. Since it has been suggested that the BCR-induced Ras pathway is blocked in Lyn-deficient cells [5,25], it appears that PLD activation is independent of the Src family PTK Ras pathway. This striking point contrasts with the previous observation that in fibroblast cells v-Src activates PLD through Ras [26]. On the other hand, PLC-γ2 activity is regulated by Syk and Btk through their concerted actions, and the activation of PLC-γ2 upon BCR engagement is shown to occur normally in Lyn-deficient cells, but completely abolished in Syk- or Btk-deficient cells [4,6]. Together, these observations prompted us to analyze PLD activation in PLC-γ2-deficient cells. As shown in Fig. 2B, DT40 PLC-γ2$^-$ cells, BCR engagement failed to activate PLD and was restored in DT40 PLC-γ2$^-$/PLC-γ2 cells. These results demonstrated

that Syk, Btk, but not Lyn, and subsequent activation of PLC-γ2 are required in the signaling pathway from the BCR to the induction of PLD activation. Since Northern blot analysis indicated no relation between PLD activities and PLD1 mRNA levels, the blocking of PLD activity is not due to a difference of PLD1 expression (Fig. 2C).

Activated PLC-γ2 catalyzes the hydrolysis of PIP_2, leading to calcium mobilization and PKC activation. To investigate whether PKC activation is involved in PLD activation in BCR signaling, the effect of the specific PKC inhibitor BIM on BCR-induced PLD activation was examined. As shown in Fig. 3, at 10 μM, BIM inhibited phorbol ester-induced PLD activation in DT40 cells (data not shown), and drastically inhibited M4-induced PLD activation. Indeed, this result is consistent with previous observation that at 1–10 μM, BIM efficiently blocks EGF-induced PLD activation in intact Swiss 3T3 cells [27]. Although it is known that the conventional α- and β-isozymes of PKC activate PLD without kinase activities in vitro [14], our result indicates that PKC activities are required for PLD activation in intact DT40 cells, suggesting that PKC may modulate PLD activity without direct interaction. Recently, it has been reported that PKCμ was activated upon BCR cross-linking [28], and that PKCβ-deficient mice showed impaired humoral immune responses in their B cells [29]. These findings imply that certain PKC isozymes are functionally important in BCR signaling.

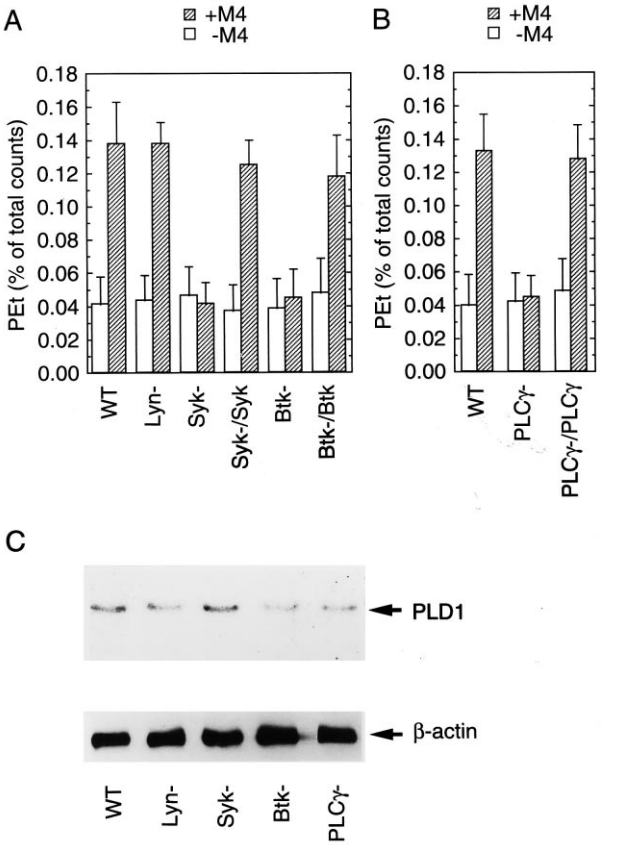

Fig. 2. BCR-induced PLD activation and Northern blot analysis for PLD1 in various DT40 mutants. A: PLD activation in wild-type (WT), Lyn^-, Syk^-, Syk^-/Syk, Btk^-, Btk^-/Btk DT40 cells. These cells were labeled with [^{14}C]lysoPC and incubated for 30 min at 37°C with (hatched bar) or without (open bar) 6 μg/ml anti-IgM (M4). B: PLD activation in wild-type (WT), PLC-$\gamma2^-$ (PLCγ−), PLC-$\gamma2^-$/PLC-γ2 (PLCγ−/PLCγ) DT40 cells. PLD activity was measured as described in Section 2. Results are the means ± S.D. of five separate experiments. C: mRNA expression was analyzed by Northern blot analysis using chicken cDNA probe for PLD1 (top) or β-actin (bottom).

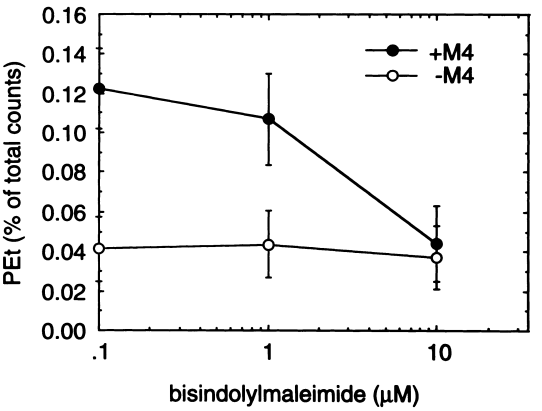

Fig. 3. Inhibition of BCR-induced PLD activation by the PKC-specific inhibitor bisindolylmaleimide. Labeled DT40 cells with [^{14}C]lysoPC were preincubated for 20 min at 37°C with bisindolylmaleimide as indicated, followed by incubation for 30 min at 37°C with (●) or without (○) 6 μg/ml anti-IgM (M4). PLD activity was measured as described in Section 2. Results are the means ± S.D. of five separate experiments.

In other cell systems, the PTK-dependent pathway involved in PLD activation is still controversial. For instance, PLD activation induced by the stimulation of the PDGF receptor has been suggested to be downstream of PDGF-mediated tyrosine phosphorylation of PLC-γ, PIP_2 hydrolysis and PKC activation [20]. In the case of the T cell receptor (TCR), another antigen receptor similar to the BCR, TCR-induced PLD activation has been shown to be abrogated by PTK and PKC inhibitors [30]. These observations support the PTK-PLC-γ-dependent pathway for PLD activation. On the other hand, Reid et al. found substantial activation of PLD activity in response to concentrations of anti-CD3 which led to cell activation, but undetectable levels of IP_3 generation, suggesting that at least one form of CD3-induced PLD activation is PIP_2 hydrolysis-independent in Jurkat T cells [31]. Moreover, Koike et al. found that treatment of rat peritoneal mast cells with stem cell factor activates PLD in the absence of IP_3 formation [32]. Melendez et al. also observed that Fcγ receptor I-induced PLD activation is not coupled to PLC in the monocyte [33]. Therefore, it appears that PLD activation may result from many different mechanisms, and our results genetically demonstrate the Syk-, Btk- and PLC-γ2-dependent activation of PLD in BCR signaling.

Regarding the function of PLD in BCR signaling, PLD has been proposed to play a key role in the transduction of proliferative responses of a wide range of mitogens and growth factors. PA, a product of PLD, has been shown to promote the induction of the proto-oncogenes c-*fos* and c-*myc* [34]. Interestingly, the induction of c-*myc* following BCR engagement was observed in wild and Lyn-deficient DT40 cells, but not in Syk- or PLC-γ2-deficient DT40 cells [35]. Furthermore, very recently, Gilbert et al. reported that mitogenic stimulation of BCR is coupled to a novel non-PC-hydrolyzing PLD (phosphatidylinositol-specific PLD) activity and that this PLD activation may play a role in mature B cell survival and proliferation [36]. Indeed, butanol, a PLD inhibitor, induced

apoptosis in DT40 cells (data not shown). Thus, these data suggest that PLD activity plays an important role in the BCR-induced survival, protection from apoptosis and proliferation. However, it is possible that BCR-induced PLD activation transduces early signals associated with BCR-mediated growth arrest or apoptosis in immature B cells since the vast majority of DT40 cells are programmed for cell death by apoptosis. The exact role of PLD remains unclear.

We are currently attempting to establish a PLD-deficient cell line by gene targeting in DT40 B cells. The genetic analysis of this mutant will provide a major tool in the physiological investigation of the functions of PLD.

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