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Platelet-Activating Factor Stimulates Calcium-Dependent Activation of Protein–Tyrosine Kinase Syk in a Human B Cell Line

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In ASK.0 B lymphoblastoid cells, platelet activating factor (PAF) induced a rapid increase in Syk protein–tyrosine kinase activity which was insensitive to pertussis toxin (PTX) but was abolished by the phopholipase C inhibitor, U73122. In parallel, PAF-induced Ca^{2+} mobilization was also insensitive to PTX and was almost completely inhibited by U73122. Incubation of ASK.0 cells with the compounds that increase intracellular Ca^{2+} (i.e., the ionophore A23187, thapsigargin which releases Ca^{2+} from internal store) mimicked the effect of PAF on Syk kinase activity. Loading cells with the intracellular Ca^{2+} chelator, bis-(O-aminophenoxy)-ethane-N,N,N′,N′-tetraacetoxymethyl ester (BAPTA-AM), completely inhibited the activation of Syk kinase in response to PAF, thapsigargin and ionophore. These results suggest that intracellular free Ca^{2+} seems to be critical for PAF-induced activation of Syk kinase in human B lymphoblastoid cells. © 1997 Academic Press

Platelet-activating factor (1-*O*-alkyl-2-acetyl-sn-glycero-3-phosphocholine; PAF), a potent phospholipid molecule, has been shown to play an important role in inflammatory and immune responses, as well as in heart and vascular functions (1-2). PAF exerts its effects through binding to specific receptors in target cells including B lymphocytes (3-4). Recent cloning of the PAF receptor indicates that it is a member of the G-protein-coupled receptor superfamily (5). Upon binding to its receptor, PAF induces stimulation of phosphatidylinositol (PtdIns) turnover, calcium fluxes, activation of protein kinase C (PKC) and phospholipase A2 (PLA2) and eicosanoid production and protein-tyrosine phosphorylation of several proteins (6).

PAF appears to be a unique ligand for B lymphocytes, inducing a very rapid release of Ca^{2+} from internal stores when compared to cross-linking of sIgM, and it does not result in heterologous desensitization of the anti-IgM induced Ca^{2+} response (7). Treatment of B lymphoblastoid cells with PAF also induces a rapid increase in expression of growth-associated nuclear proto-oncogene, *c-fos* and *c-jun* (8), stimulation of immunoglobulin synthesis (9), and activation of both mitogen activated protein kinase (MAPK) and $p90^{sk}$ (10). As is the case with B cell receptor (BCR) cross-linking, PAF stimulates the activation of the Src-family protein-tyrosine kinases (PTKs), Fyn and Lyn, and induces tyrosine-phosphorylation of PLCγ and phosphatidylinositol 3-kinase (PtdIns 3-kinase) in B lymphocytes (11).

The nonreceptor PTK Syk, a member of the Syk/ZAP-70 family of PTKs, is widely expressed in hematopoietic cells (12). Crosslinking of the BCR promotes the phosphorylation of the receptor complex by Src family PTKs, allowing the cytoplasmic PTK Syk to bind to the receptor and undergoes enzymatic activation (13-14). Genetic studies have demonstrated that Syk is necessary both for the proper development of B cells and for the cellular responses of B cells to BCR stimulation (15-17). However, the activity of Syk is rapidly up-regulated by G-protein coupled receptors in platelets (18-19) and in chicken B cells (20). The pathway originating from G-protein coupled receptors in the activation of Syk is unclear.

In view of circumstantial evidence that PTKs play an important role in the signal transduction by PAF receptor in B lymphocytes, we undertook an approach to investigate the involvement of Syk in this process. Our results demonstrate that Syk is rapidly activated

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Abbreviations: PAF, 1-*O*-alkyl-2-acetyl-sn-glycero-3-phosphocholine; PTX, pertussis toxin; BAPTA-AM, bis-(O-aminophenoxy)-ethane-N,N,N′,N′-tetraacetoxy-methyl ester; PtdIns, phosphatidylinositol; PLC, phospholipase C; BCR, B cell receptor; PTK, protein-tyrosine kinase; PKC, protein kinase C; IP3, inositol triphosphate; MAPK, mitogen activated protein kinase; TPA, 12-O-tetradecanoylphorbol 13-acetate.

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after PAF receptor activation and this activation appears to be largely dependent on intracellular free Ca^{2+} in B lymphoblastoid cells.

MATERIALS AND METHODS

Cell culture. ASK.0 cells, an EBV-transformed human B lymphoblastoid cell line, were kindly provided by Dr. William T. Shearer (Baylor College of Medicine, Houston, TX). The cells were maintained in RPMI 1640 containing 10 mM HEPES, 2 mM L-glutamine, penicilline-G (100 IU/ml), streptomycin (100 μg/ml), and 10% heat-inactivated fetal bovine serum at 37°C in a humidified 5% CO2 environment.

Reagents. Platelet-activating factor (C-16), lyso-PAF and PAF antagonist CV-6209 were from Biomol Rsearch Laboratories, Inc. (Plymouth Meeting, PA). Monoclonal anti-Syk antibody was from Wako Chemicals (Japan). Monoclonal anti-phosphotyrosine (4G10) was from Upstate Biotechnology Inc. (Lake Placid, NY). Pertussis toxin was from Kaken Pharmaceutical Co. Ltd. (Japan). U73122 was from Calbiochem (San Diego, CA). Calcium ionophore A23187 and thapsigargin were from nacalai tesque (Japan). Bis-(O-aminophenoxy)-ethane-N,N,N′,N′-tetraacetoxymethyl ester (BAPTA-AM) and EGTA were from Dojin Chemical (Japan).

Immunoprecipitation and in vitro kinase assay. Cells were washed, resuspended at $1x10^7$ cells/ml in Hanks' balanced salt solution (HBSS) containing 10 mM Hepes (pH 7.3) and 0.2% BSA and equilibrated at 37°C for at least 30 min before the start of treatment. After activation by PAF for the indicated times in the figure legends, cells were lysed in lysis buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Nonidet P-40, 50 mM NaF, 5 mM EDTA, 0.1 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride, and 10 μg/ml of each leupeptin and aprotonin) followed by quick centrifugation. Nuclei and cell debris were removed by centrifugation at 16,000 x g for 15 min at 4°C. An equal amount of protein was subjected to immunoprecipitation with monoclonal anti-Syk antibodies prebound to protein A-sepharose beads for 2 h at 4°C. The washed immunoprecipitates were subjected to *in vitro* kinase assay as described previously (12). Autoradiography was obtained with a PhosphoImager (Fuji, Japan).

Immunoblotting. The immunoprecipitates were analyzed on sodium dodecyl sulfate (SDS)-polyacrylamide gels and transferred electrophoretically to polyvinylidene difluoride membranes (Millipore, Bedford, MA). Blots were probed with the indicated antibodies and immunoreactive proteins were developed by the Enhanced Chemiluminescence detection system (Dupont NEN, Boston, MA).

Measurement of intracellular Ca^{2+} level. ASK.0 cells were loaded with Fura-2/AM as described previously (4). After wash, cells were resuspended at 1×10^6 cells/ml in the HBSS. For experiments in Ca^{2+} free medium, no Ca^{2+} was added and 0.5 mM EGTA was included in the medium. Fluorescence was monitored by using a Hitachi spectrofluorimeter F-4500 with standard monitor settings of 340 nm and 380 nm excitation and 510 nm emission.

RESULTS

PAF receptor stimulation induces activation of Syk kinase. To measure the PAF-induced activation of Syk, anti-Syk immunoprecipitates from resting and PAF-treated ASK.0 cells were subjected to *in vitro* kinase assay. The data showed that PAF induced stimulation of Syk activity was rapid, occurring within 15 s, was maximal at 30 s, and then declined to the basal

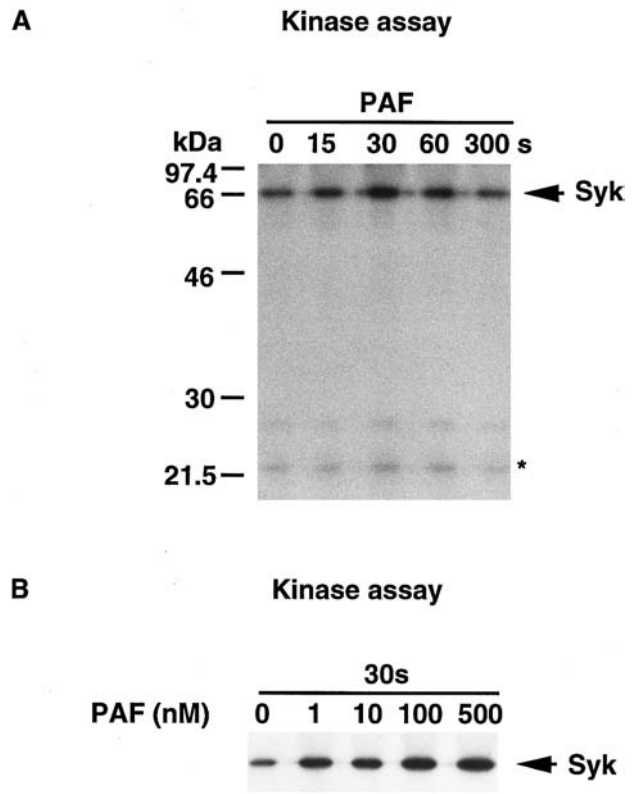

FIG. 1. PAF-induced activation and tyrosine-phosphorylation of Syk in human B cell line ASK.0. (A). Time course. ASK.0 cells were stimulated with PAF (500 nM) for the indicated time. (B). Dose dependence. ASK.0 cells were stimulated with the indicated concentration of PAF for 30 s. The anti-Syk immunoprecipitates were then processed for *in vitro* kinase assay using H2B histone as an exogenous substrate. The samples were sujected to SDS/PAGE and were revealed by autoradiography. The size of molecular weight marker is indicated to the left. An asterisk indicates the position of H2B histone.

levels after 5 min (Fig. 1A). The increase in Syk kinase activity was also confirmed by phosphorylating an exogenous substrate H2B histone. The PAF-induced increase in Syk kinase activity appears to reflect increase in the specific activity of the enzyme, since constant level of Syk protein was immunoprecipitated during PAF stimulation (not shown). The concentration dependence of PAF-stimulated increase in Syk kinase activtiy was initially detectable at 1 nM and maximal at 500 nM (Fig. 1B). The increased activity of Syk was 3-5-fold upon PAF receptors stimulation. The activity was blocked by pretreatment for 10 min with the PAF receptor antagonist CV6209 and lyso-PAF, a structurally similar metabolite of PAF, did not increase Syk kinase activity (not shown).

PAF induced tyrosine-phosphorylation of Syk kinase. To investigate whether PAF increases tyrosine-phosphorylation of Syk, Syk was immunoprecipitated from lysates of PAF-treated and untreated cells with monoclonal antibodies specific to Syk. Immunoblot analysis

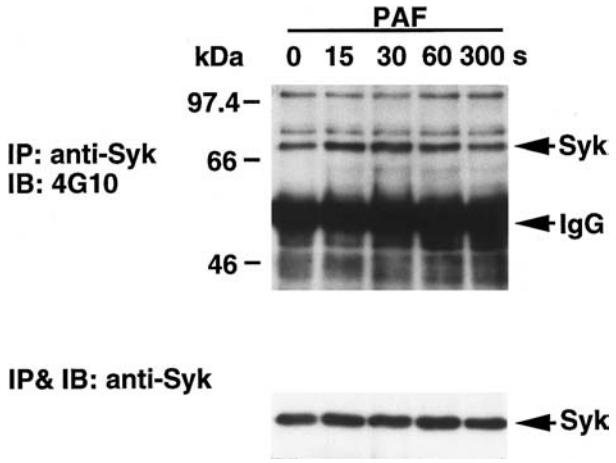

FIG. 2. Time course for PAF-stimulated tyrosine-phosphorylation of Syk in human B cell line ASK.0. ASK.0 cells were stimulated with PAF (100 nM) for the indicated times and harvested with lysis buffer. The anti-Syk immunoprecipitates were analysed by immunoblotting with either anti-phosphotyrosine antibodies (4G10) or anti-Syk antibodies as described under MATERIALS AND METHODS. The size of molecular weight marker is indicated to the left.

of anti-Syk immunoprecipitates with anti-phosphotyrosine antibody showed an increase in tyrosine-phosphorylation of Syk compared to control as shown in Fig. 2 (upper pannel). Anti-Syk immunoblot analysis revealed that the amounts of Syk immunoprecipitated from treated and untreated cells were comparable (Fig. 2, lower pannel).

Effect of pertussis toxin and PLC inhibitor on PAF-induced Syk activity and Ca^{2+} Mobilization. To determine which G-protein mediated signaling is involved in the Syk kinase activation, the effects of pertussis toxin (PTX) and the PtdIns-PLC inhibitor U73122 on PAF-induced Syk activation were examined in B lymphoblastoid cells. Treatment with PTX (1 μg/ml) for 4 h did not affect PAF-induced Syk activation (Fig. 3A). In contrast, U73122 completely suppressed PAF-induced Syk activation without affecting basal Syk kinase activity (Fig. 3B). In parallel with these observation, PAF-induced Ca^{2+} mobilization was also insensitive to PTX and almost completely abolished by U73122 (Fig. 3C). These results suggest that PAF-induced Syk activation is secondary to PLC activation.

Role of Ca^{2+} to the PAF-induced activation of Syk. To evaluate the role of Ca^{2+} in PAF-stimulated Syk kinase activation, ASK.0 cells were pretreated either with EGTA, an extracellular Ca^{2+} chelator, or BAPTA-AM, an intracellular Ca^{2+} chelator, prior to PAF treatment. Extracellular Ca^{2+} chelation by EGTA failed to inhibit PAF-induced Syk kinase activation (Fig. 4A) even though this treatment abolished the PAF-induced sustained phase of Ca^{2+} elevation. In BAPTA-AM-loaded (20 μM) cells, the PAF-stimulated increase in cytosolic Ca^{2+}, measured by fura-2 fluorescence, was reduced by >90% (not shown). Under this condition, BAPTA-AM treatment completely abolished the PAF-stimulated increase in Syk kinase activity (Fig. 4A).

To further evaluate the involvement of Ca^{2+} in Syk activation, ASK.0 cells were treated with other agents that increase intracellular Ca^{2+} level. Calcium ionophore A23187 and the tumor promoter thapsigargin, which promotes Ca^{2+} release from endoplasmic reticulum, increased Syk kinase activity as measured by autophosphorylation assay. Thapsigargin increased Syk kinase activity less potently than PAF or the A23187. Preincubation with EGTA prevented activation of Syk kinase by the A23187; the cell-permeant chelator BAPTA-AM inhibited both the thapsigargin and A23187-stimulated Syk kinase activity (Fig. 4B). Taken together these results suggest that the release of Ca^{2+} from IP3-sensitive stores, rather than Ca^{2+} influx, may play a major role in PAF-induced Syk activation in ASK.0 cells. To test the potential involvement of PKC in PAF-induced Syk activation, ASK.0 cells were treated with 12-O-tetradecanoylphorbol 13-acetate (TPA), an exogenous activator of PKC. Acute TPA treatment (100 ng, 12 h) had no effect on Syk kinase activity (not shown).

DISCUSSION

In the present study we have demonstrated that PAF specifically induces Syk kinase activity in ASK.0 B lymphoblastoid cells as we observed in porcine platelets (19). There was detectable PTK activity of Syk in unstimulated cells, possibly reflecting a state of generalized activation of Epstein-Barr virus-transformed human B lymphocytes we used. The activity of Syk kinase correlated well with phosphotyrosine content.

The type of G-protein involved in the PAF responses may differ from cell to cell and also depend on effector systems because some processes coupled with G-protein are sensitive to PTX while others are resistant (21). In fact, in neutrophils PAF induced tyrosine-phosphorylation in a PTX-sensitive and insensitive manner (22). Recent evidence also suggests that the chemoattractant receptor of neutrophils, N-formyl peptides regulate activation of Lyn kinase and Shc adaptor molecule through PTX-sensitive Gi-protein pathway (23). In contrast, our results demonstrated that PAF-induced Syk activation in ASK.0 B-lymphoblastoid cells was insensitive to PTX and was supressed by the PtdIns-PLC inhibitor, U73122. Taken together, these observations indicate that PtdIns-PLC activation through a PTX-insensitive G-protein may play a crucial role in PAF-induced Syk activation in this B-lymphoblastoid cells. In addition to activating G-protein-dependent PLC isoform (probably PLCβ), PAF receptor also recruits PLCγ1 isoform in this B cell line (11). At present, we cannot determine which PLC contributes this Syk activation processes. The complete abolition of the PAF-evoked rise in $[Ca^{2+}]i$

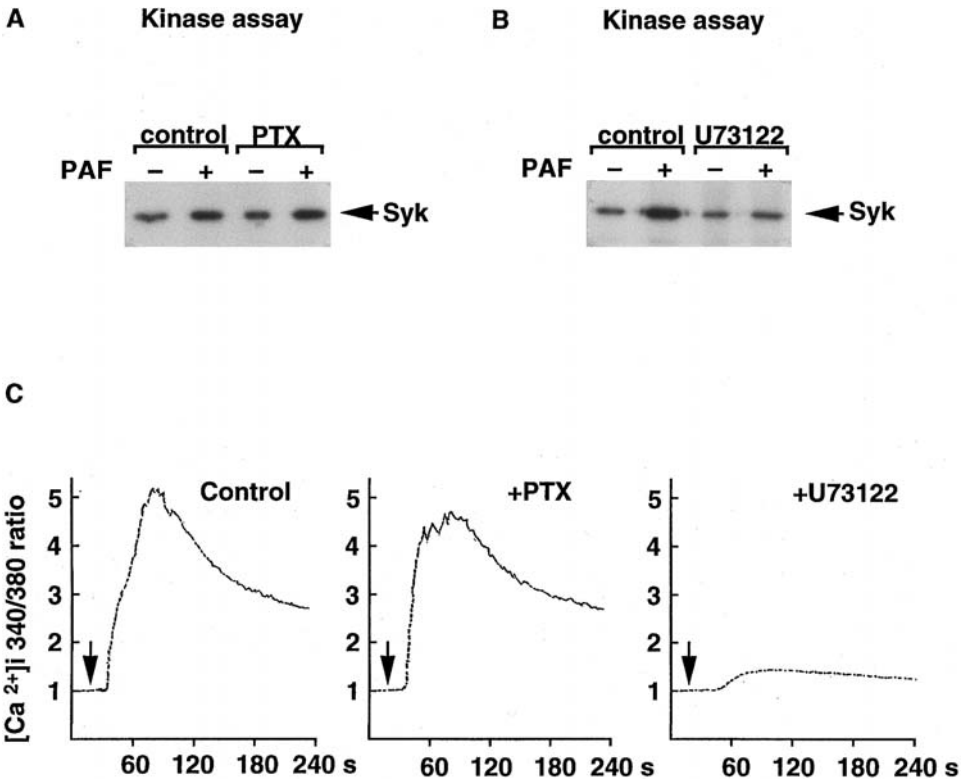

FIG. 3. Effects of pertussis toxin and U73122 on PAF-induced Syk activation and Ca^{2+} mobilization. (A). ASK.0 cells were pretreated with or without 1 μg/ml PTX for 4 h and stimulated with PAF (500 nM) for 30 s. (B). ASK.0 cells were pretreated with or without 5 μM U73122 for 30 min and stimulated with PAF (500 nM) for 30 s. The anti-Syk immunoprecipitates were then processed for *in vitro* kinase assay. The samples were then subjected to SDS/PAGE and were revealed by autoradiography. (C). Fura-2 fluorescence was monitored in ASK.0 cells pretreated either with PTX or U73122 as described above. Cells were stimulated with 100 nM PAF at the times indicated by arrows. Ca^{2+} concentration is reported as the 340/380-nm fluorescence ratio.

by U73122 is consistent with a recent report that the activity of both PLCβ and PLCγ isoforms is inhibited by U73122 (24). Further investgation is necessary for understanding this events.

An addition of Ca^{2+} ionophore A23187 causes an increase in Syk activity in platelets through the elevation of intracellular Ca^{2+} (25). Recent study supports that thrombin may also induce phosphorylation of Syk through the elevation of an intracellular Ca^{2+}, as its response is blocked in the presence of combination of Ro 31-8220, a specific PKC inhibitor and BAPTA-AM (26). PAF induces Ca^{2+}-dependent tyrosine-phosphorylation in human neutrophils and rat Kupffer' cells (24, 27). Our present results showed that Ca^{2+} -dependent Syk activation by PAF was mainly mediated by the release of Ca^{2+} from IP3 -sensitive store, rather than by Ca^{2+} influx through transmembrane, by demonstrating that PAF-induced Syk activation was abolished by both U73122 and BAPTA-AM, whereas it was insesitive to the extracellular Ca^{2+} chelation by EGTA. The effect of Ca^{2+} on Syk is indrect, because the addition of Ca^{2+} to cell lysates does not activate Syk, and thus the mechanism by which Ca^{2+} or Ca^{2+}-dependent signals regulate this kinase remains to be established. In addition to increasing the intracellular calcium, PAF also stimulates PKC activity (27), however our results do not suggest a role for PKC in the regulation of Syk in B-lymphoblastoid cells.

The BCR mediated Ca^{2+} response was almost completely abolished in a Syk deficient chicken B cell line, indicating a crucial role of Syk in the Ca^{2+} response upon BCR activation (15). In contrast to BCR-signaling in which Syk activation is independent of Ca^{2+}, PAF-induced activation of Syk is intracellular Ca^{2+}-dependent. What role Syk plays in Ca^{2+}-dependent pathway remains to be elucidated.

The focal adhesion kinase-related PTK, PYK2, which is highly expressed in brain, was shown to be activated following stimulation of the Gq-coupled bradykinin receptor in PC12 cells and activation of PYK2 is dependent on the cytosolic Ca^{2+} and PKC activity (28). This Ca^{2+}-dependent activation of PYK2 results in significant MAPK activation. It has been shown that Syk is essential for MAPK activation induced by muscarinic acetylcholin receptors transfected in chicken B cells (20). It will be of important to determine whether PAF-induced Ca^{2+}-dependent activation of Syk can regulate the analogous signaling pathway in B lymphocytes.

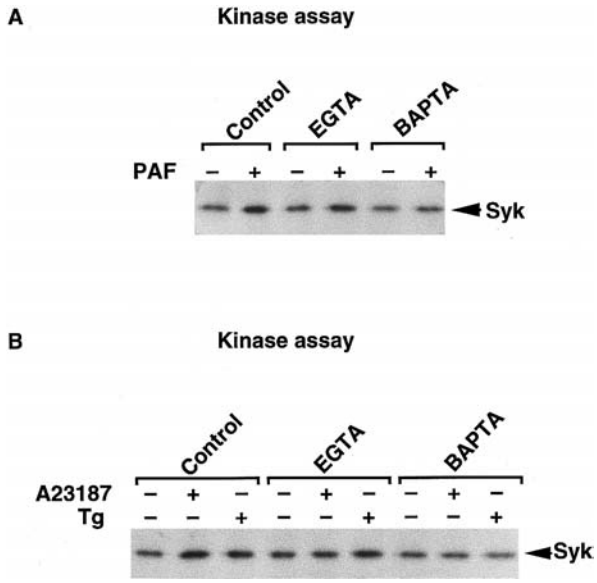

FIG. 4. Role of Ca^{2+} to the PAF-induced Syk activation. (A). ASK.0 cells were pretreated with the extracellular Ca^{2+} chelator EGTA (2 mM) for 5 min, the intracellular Ca^{2+} chelator BAPTA-AM (20 μM) for 30 min and then stimulated with PAF (500 nM) for 30 s. (B). ASK.0 cells were incubated with either ionophore A23187 (1 μM for 30 s), or thapsigargin (Tg, 1 μM for 1 min). In some cases the cells were pretreated with EGTA (2 mM for 5 min) or BAPTA-AM (20 μM for 30 min) as indicated. The anti-Syk immunoprecipitates were then processed for *in vitro* kinase assay as described under MATERIALS AND METHODS. The samples were then subjected to SDS/PAGE and were revealed by an autoradiography.

In conclusion, we have demonstrated that in addition to the activation of Src-family kinases, phospholipid PAF is able to activate Syk kinase in a B lymphoblastoid cell line. Syk activation occurs early in response to PAF receptor ligation and this activation can be regulated by intracellular Ca^{2+} and PKC-independent mechanism. Further studies are necessary to identify the role of Syk kinase and downstream signaling in B lymphocyte activation responses caused by PAF receptor.

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