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Role of Phospholipase Cβ3 Phosphorylation in the Desensitization of Cellular Responses to Platelet-activating Factor*

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Platelet-activating factor (PAF) stimulates a diverse array of cellular responses through receptors coupled to G proteins that activate phospholipase C (PLC). Truncation of the cytoplasmic tail of the receptor to remove phosphorylation sites (mutant PAF receptor, mPAFR) results in enhancement of PAF-stimulated responses. Here we demonstrate that PAF or phorbol 12-myristate 13-acetate (PMA) pretreatment inhibited wild type PAFR-induced PLC-mediated responses by ~90%, whereas these responses to the phosphorylation-deficient mPAFR were inhibited by ~50%, despite normal G protein coupling, suggesting a distal inhibitory locus. PAF and PMA, as well as a membrane permeable cyclic AMP analog, stimulated phosphorylation of PLCβ3. A protein kinase C (PKC) inhibitor blocked phosphorylation of PLCβ3 stimulated by PAF and PMA but not by cAMP. Activation of protein kinase A (PKA) by cAMP did not result in inhibition of Ca^{2+} mobilization stimulated by PAF. In contrast, cAMP did inhibit the response to formylpeptide chemoattractant receptor. These data suggest that homologous desensitization of PAF-mediated responses is regulated via phosphorylation at two levels in the signaling pathway, one at the receptor and the other at PLCβ3 mediated by PKC but not by PKA. Phosphorylation of PLCβ3 by PKA could explain the inhibition of formylpeptide chemoattractant receptor signaling by cAMP. As PAF and formylpeptide chemoattractant receptors activate PLC via different G proteins, phosphorylation of PLCβ3 by PKC and PKA could provide distinct regulatory control for classes of G protein-coupled receptors.

Platelet-activating factor (1-*O*-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine, PAF)[1] plays important roles in inflammation and has physiological effects on cardiovascular, reproductive, and central nervous systems (1). PAF mediates its effects via the activation of a seven transmembrane domain G protein-coupled receptor (2, 3). One of the consequences of PAF receptor activation is the phospholipase C (PLC)-mediated hydrolysis of phosphatidylinositol 4,5-bisphosphate leading to the activation of protein kinase C (PKC) and mobilization of intracellular Ca^{2+} (4). Molecular cloning has revealed three classes of PLC: PLCβ, PLCγ, and PLCδ, and each of these occur in several isoforms (5, 6). All four PLC β isoforms are activated to varying extent by α subunit of a class of G proteins known as G_q (7–10). PLCβ2 and PLCβ3 are also activated by the βγ subunit of the G_i family of G proteins (11–13). Tyrosine phosphorylation of PLCγ is required for its activation, but the mechanism by which PLCδ is activated is unknown (5). PAFR couples to both pertussis toxin (Ptx)-sensitive and Ptx-insensitive G proteins in a variety of cells (4, 14, 15). In B cells and platelets, PAF activates PLCγ (16, 17), but the identity of PLCs activated by PAF in phagocytic leukocytes is unknown.

In phagocytes, PAF-stimulated G protein activation and Ca^{2+} mobilization are desensitized by prior treatment of cells with PAF and other chemoattractants such as the formylated peptides, C5a and interleukin-8 (18). This laboratory has developed stably transfected RBL-2H3 cells to study molecular mechanisms of leukocyte chemoattractant receptor regulation (15, 19–21). Using wild type and phosphorylation-deficient PAFR expressed in this cell line, we now describe the novel finding that homologous desensitization of PAF-mediated phosphoinositide hydrolysis and Ca^{2+} mobilization is mediated via two processes; one at the level of receptor phosphorylation and the other at the level of PLC activation apparently by PKC-dependent phosphorylation of PLCβ3.

EXPERIMENTAL PROCEDURES

Materials—Bisindolylmaleimide or GF 109203X (BIM), 8-(4-chlorophenylthio)-adenosine 3′-cyclic monophosphate (Cpt-cAMP) and the tyrosine kinase inhibitor PP1 were purchased from Calbiochem. Affinity purified polyclonal antibodies against PLCβ1, PLCβ2, and PLCβ3 were obtained from Santa Cruz Biotechnology. ECL Western blot analysis kit was purchased from Amersham Corp. Mouse monoclonal IgE and antigen (dinitrophenylated BSA) were generously provided by Drs. Juan Rivera and Henry Metzger (National Institutes of Health). All other materials were obtained from sources previously described (15).

Cell Culture, Phosphoinositide Hydrolysis, and Ca^{2+} Mobilization—RBL-2H3 cells stably expressing ~3×10^4 receptors for PAFR and mPAFR were used (21). The cells were maintained in Dulbecco's modified Eagle's medium supplemented with 15% fetal bovine serum, glutamine (2 mM), penicillin (100 units/ml), and streptomycin (100 mg/ml) (19). For phosphoinositide hydrolysis, cells (5×10^4 cells/well) were subcultured overnight in 96-well tissue culture plate with 1 μCi/ml of [^{3}H]inositol in an inositol-free medium supplemented with 10% dialyzed fetal bovine serum. Cells were washed with HEPES-buffered saline containing 10 mM LiCl and 0.1% BSA and stimulated, and the generation of total [^{3}H]inositol phosphates was determined. For Ca^{2+} mobilization, cells (3×10^6) were loaded with 1 μM indo-1/AM in the presence of 1 μM pluoronic acid for 30 min at room temperature. Cells were washed and resuspended in 1.5 ml HEPES-buffered saline, and intracellular Ca^{2+} mobilization was determined as described previously (19).

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[1] The abbreviations used are: PAF, platelet-activating factor; PAFR, PAF receptor; mPAFR, mutant PAFR; PLC, phospholipase C; PMA, phorbol 12-myristate 13-acetate; RBL, rat basophilic leukemia; Cpt-cAMP, 8-(4-chlorophenylthio)-adenosine 3′-cyclic monophosphate; PKA, protein kinase A, PKC; protein kinase C; BIM, bisindolylmaleimide; Ptx, pertussis toxin; BSA, bovine serum albumin; PAGE, polyacrylamide gel electrophoresis; GTPγS, guanosine 5′-3-*O*-(thio)triphosphate.

TABLE I
Desensitization of PAF-mediated Ca^{2+} mobilization

RBL-2H3 cells (3×10^6) expressing mPAFR or PAFR were loaded with indo-1 and stimulated with PAF (10 nM), and peak Ca^{2+} mobilization was determined as described (19). For homologous desensitization, PAF-stimulated cells were washed, and 5 min later they were restimulated with PAF (10 nM) and peak Ca^{2+} mobilization was determined. Cells were also preincubated with PMA (100 nM), and 5 min later they were stimulated with PAF (10 nM) and peak Ca^{2+} mobilization was determined. The basal unstimulated intracellular Ca^{2+} concentration was 150 ± 10 and was subtracted from the values shown, which are the means ± S.E. of three different experiments.

Cells/treatment	Ca^{2+} mobilization	Desensitization
	nM	*%*
mPAFR		
PAF → PAF	517 ± 15 → 240 ± 19	53
PMA → PAF	0 ± 0 → 264 ± 12	51
PAFR		
PAF → PAF	617 ± 40 → 48 ± 4	92
PMA → PAF	0 ± 0 → 43 ± 5	93

Western Blotting and Immunoprecipitation of PLCβ3—For Western blotting, lysate (150 μg protein) from RBL-2H3 cells, and for comparison, lysates from bovine brain, COS cells, and HL-60 cells were resolved on a 6% SDS-PAGE gel. The proteins were transferred to nitrocellulose membrane, blocked with 3% milk, and incubated with 1.5 μg/ml of different anti-PLCβ antibodies. The immunoreactive proteins were visualized using ECL Western blotting detection system. Phosphorylation of PLCβ3 was performed essentially as described for the epitope-tagged chemoattractant receptors (19). Briefly, RBL-2H3 cells (3×10^6) were subcultured overnight in 60-mm tissue culture dishes. The following day, cells were washed twice with 5 ml of phosphate-free Dulbecco's modified Eagle's medium and incubated in the same medium plus [^{32}P]orthophosphate (150 μCi/dish) for 90 min. The labeled cells were stimulated, and PLCβ3 was immunoprecipitated from cell lysate with anti-PLCβ3 antibody. The proteins were resolved on a 6% SDS-PAGE gel and visualized by autoradiography.

RESULTS AND DISCUSSION

The mechanism of PAF receptor desensitization was studied using the rat basophilic leukemia (RBL-2H3) cells expressing wild type PAFR and a mPAFR in which the cytoplasmic tail was truncated to delete potential phosphorylation sites (21). In contrast to PAFR, mPAFR was resistant to both ligand and PKC-induced receptor phosphorylation and desensitization of G protein coupling, as measured by [^{35}S]GTPγS binding to membranes (21, 22). However, when indo-1-loaded cells expressing mPAFR were stimulated with PAF (10 nM), washed, and restimulated with PAF (10 nM), the peak Ca^{2+} response was still homologously desensitized by ~50% (Table I). PMA also inhibited mPAFR-mediated Ca^{2+} mobilization by ~50%, suggesting a locus of inhibition distal to G protein activation. In cells expressing wild type PAFR, both PAF and PMA inhibited PAF-induced Ca^{2+} mobilization by >90%. PMA also caused substantial inhibition of mPAF-induced generation of inositol phosphates and completely blocked the response to PAF (Fig. 1). To further test the role of PKC on PAFR desensitization, cells expressing mPAFR and PAFR were treated with PMA (100 nM) overnight to deplete PKC or incubated with the PKC-inhibitor bisindolylmaleimide for 10 min to block the activity of the enzyme. PAF-stimulated generation of inositol phosphates was then determined. Under both of these conditions, PAF-induced responses were enhanced 2–3-fold in cells expressing mPAFR (Fig. 1). Overnight PMA treatment did not enhance the response to PAF in cells expressing PAFR, but bisindolylmaleimide did potentiate the response to PAF. Because PMA-induced PKC depletion involves activation of the enzyme, it was possible that the differential effect of PMA on mPAFR and PAFR cells resulted from PKC-induced phosphorylation and down-regulation of PAFR but not mPAFR. Indeed, treatment of cells with PMA (100 nM, overnight) resulted in ~50% decrease of the wild type PAFR expression but had no effect on mPAFR as measured by the PAFR antagonist [^{3}H]WEB 2086 binding to intact cells (data not shown). These data suggest that homologous desensitization of PAF-mediated cellular responses is mediated by two processes. One involves receptor phosphorylation, presumably by PKC and a G protein-coupled receptor kinase, which uncouple the receptor from G proteins (21). In addition, a new mechanism was identified as a consequence of PKC-induced modification of a component distal to G protein activation but proximal to the generation of inositol phosphates.

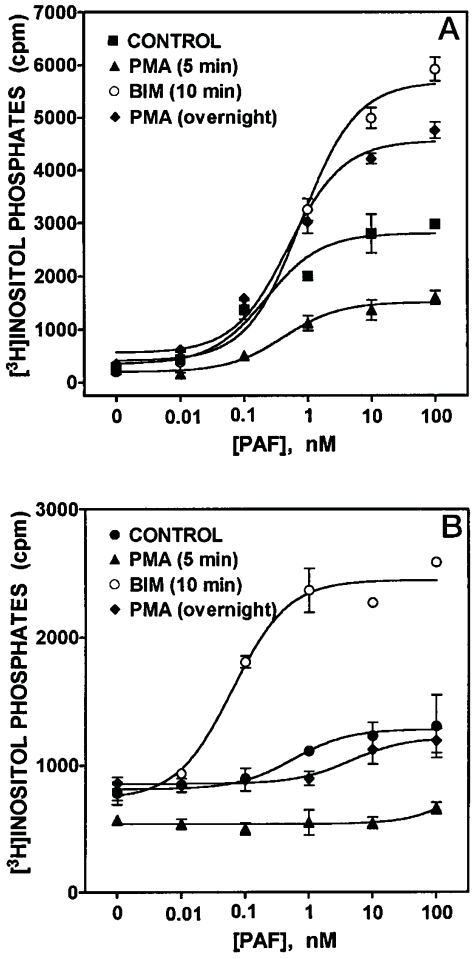

FIG. 1. **Regulation of PAF-stimulated generation of [^{3}H]inositol phosphates by PKC.** RBL-2H3 cells expressing mPAFR (*A*) and PAFR (*B*) were cultured overnight with [^{3}H]inositol in the absence (*control*, ■, ●) and the presence of PMA (100 nM, ◆). The next day, cells were washed in a HEPES-buffered saline containing 10 mM LiCl, preincubated with the same buffer (■, ●, ◆), PMA (100 nM, ▲), or BIM (10 μM, ○) and then stimulated with different concentrations of PAF, and the generation of total [^{3}H]inositol phosphates were determined. Data are the means ± S.E. of one of three similar experiments performed in triplicate.

Although most G protein-coupled receptors activate PLCβ, angiotensin receptors in smooth muscle cells and PAF receptors in B cells and platelets cause tyrosine phosphorylation and activation of PLCγ (16, 17, 23). The molecular mechanism by which G protein-coupled receptors activate PLCγ has yet to be determined. PMA, which causes serine phosphorylation of PLCγ (24), leads to reduction of both tyrosine phosphorylation of PLCγ and generation of inositol phosphates stimulated by cross-linking of cell surface IgE receptors with antigen (25, 26). This suggests that if PAF activates PLCγ, a similar modification of PLCγ by PKC could be involved in its homologous desensitization. However, the tyrosine kinase inhibitor PP1 (27), which blocked antigen (IgE)-stimulated Ca^{2+} mobilization

TABLE II
Effects of tyrosine phosphorylation inhibition or cAMP elevation on PAF, fMLP, and IgE-mediated Ca^{2+} mobilization

RBL-2H3 cells coexpressing fMLP and PAF receptors (21) were incubated with IgE (0.2 μg/ml) overnight to saturate cell surface IgE receptors. The following day cells were loaded with indo-1 for 30 min in the absence (control) and the presence of the tyrosine kinase inhibitor, PP1 (10 μM), and Ca^{2+} mobilization in response to PAF (0.2 nM), fMLP (1.0 nM), and antigen, dinitrophenylated (DNP) BSA (1 ng/ml) was determined. Indo-1-loaded cells were also preincubated with the membrane permeable cAMP analog, cpt-cAMP (1 mM, 5 min), and its effect on peak Ca^{2+} mobilization was tested. Values are the means ± S.E. of four experiments. The numbers in parenthesis represent the percentage of inhibition of response as compared with control.

Stimulation	Control	PP1 (10 μM)	cpt-cAMP (1 mM)
PAF	495 ± 23	531 ± 51 (−7)	548 ± 25 (−10)
fMLP	249 ± 3.5	250 ± 3 (0)	44 ± 12 (82)
DNP BSA/IgE	376 ± 22	90 ± 11 (76)	103 ± 3 (72)

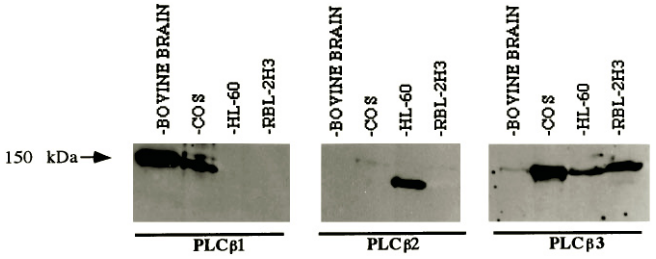

FIG. 2. **Identification of PLCβ isozymes expressed in RBL-2H3 cells.** Lysate (150 μg protein) from RBL-2H3 cells, bovine brain, COS, and HL-60 cells were resolved on a 6% SDS-gel and analyzed by Western blotting using antibodies directed against PLCβ1, PLCβ2, and PLCβ3. This experiment was repeated three times with similar results.

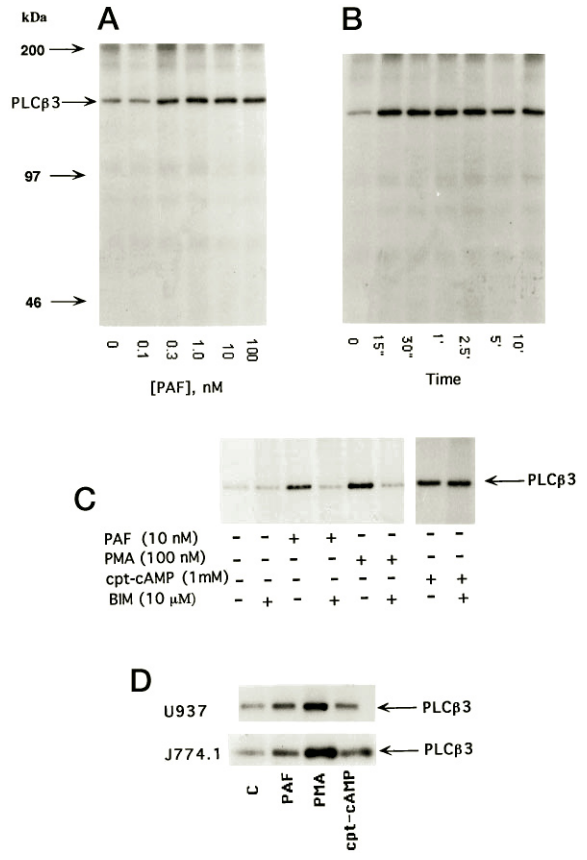

FIG. 3. **Phosphorylation of PLCβ3.** ^{32}P-labeled RBL-2H3 cells expressing PAFR were stimulated with different concentrations of PAF for 5 min (*A*) or with a fixed concentration of PAF (10 nM) for different times (*B*). *C*, effects of bisindolylmaleimide on PLCβ3 phosphorylation. ^{32}P-Labeled cells expressing PAFR were preincubated with BIM (10 μM; 10 min) and stimulated with PAF (10 nM), PMA (100 nM), or cpt-cAMP (1 mM) for 5 min. *D*, U937 cells and J774.1 cells were labeled with ^{32}P as described for RBL-2H3 cells, stimulated with PAF (10 nM), PMA (100 nM), and cpt-cAMP (1 mM) for 5 min. Cells were lysed, immunoprecipitated with anti-PLCβ3 antibody, and analyzed by SDS-PAGE and autoradiography. Results shown are from one of four similar experiments.

by >70%, presumably by inhibiting tyrosine phosphorylation of PLCγ, had no effect on PAF-mediated responses (Table II). Elevation of intracellular cAMP has also been shown to cause serine phosphorylation of PLCγ and inhibition of antigen-stimulated responses (24). As shown in Table II, the membrane permeable cAMP analog 8-(4-chlorophenylthio)-adenosine 3′-cylic monophosphate (cpt-cAMP) substantially inhibited IgE-mediated Ca^{2+} mobilization but had no effect on the PAF-mediated response. These data suggest that unlike B cells and platelets (16, 17) PAF does not activate PLCγ in RBL-2H3 cells and that PKA does not down-regulate PAF-mediated responses.

We therefore sought to determine whether a PLCβ isoform is activated by PAF in RBL-2H3 cells. To identify the PLCβ isoforms expressed in RBL-2H3 cells, their lysates and lysates from bovine brain, COS and HL-60 cells were separated by SDS-PAGE, and the presence of PLCβ1, PLCβ2, and PLCβ3 was determined by Western blotting using PLCβ isoform-specific antibodies. All cells tested, including RBL-2H3 cells, expressed PLCβ3 in different amounts (Fig. 2). Although PLCβ1 and PLCβ2 were expressed at high levels in brain and HL-60 cells, respectively, neither could be detected in RBL-2H3 cells (Fig. 2).

Studies with purified G proteins and PLC revealed that PLCβ1 and PLCβ3 are equally responsive to activation by $G\alpha_q$ (28). It was therefore possible that in RBL-2H3 cells, which do not express PLCβ1, PAF activates PLCβ3 and that PKC-induced modification of this enzyme accounts for the distal component of PAFR homologous desensitization. To test this hypothesis, cells were labeled with [^{32}P]orthophosphate and stimulated with PAF, and cell lysate was immunoprecipitated with PLCβ3-specific antibody. As shown in Fig. 3 (*A* and *B*), PAF caused phosphorylation of PLCβ3 in a dose- and time-dependent manner. PLCβ3 phosphorylation was detectable at physiologic PAF concentration of 0.3 nM and was maximum by 10 nM. PAF-induced PLCβ3 phosphorylation was rapid, reached a maximum by 15 s, and remained elevated for 10 min. To determine whether there was a correlation between PKC-mediated PLCβ3 phosphorylation and inhibition of PAF-stimulated responses, cells were treated with or without the PKC inhibitor bisindolylmaleimide, stimulated with PAF (10 nM) and PMA (100 nM), and PLCβ3 phosphorylation was determined. As shown in Fig. 3*C*, PAF- and PMA-induced PLCβ3 phosphorylation was almost completely inhibited by bisindolylmaleimide. Cpt-cAMP also phosphorylated PLCβ3 presumably through activation of PKA, and this phosphorylation was not inhibited by bisindolylmaleimide. PLCβ3 phosphorylation was also tested in two other cell lines that endogenously express PAF receptors. These were the human monocyte-like U937 cells and the murine macrophage-like J774.1 cells. In both cases, PAF, PMA, and cpt-cAMP stimulated phosphorylation of PLCβ3 (Fig. 3*D*).

The effect of cAMP elevation on PLC activation was determined using the RBL-2H3 cells coexpressing PAF and formylpeptide receptors. In such cells, cpt-cAMP did not inhibit Ca^{2+} mobilization to PAF, although it did inhibit the response of the formylpeptide receptor (Table II). Furthermore, in cells expressing mPAFR, cpt-cAMP (1 mM, 5 min) had no effect on PAF-stimulated generation of [^3H]inositol phosphates, whereas PMA (100 nM, 5 min) blocked this response by ~50%.

Preincubation of cells with cpt-cAMP had no effect on PMA-mediated inhibition of PAF-stimulated response (data not shown). These data support the hypothesis that PLCβ3 is likely activated by PAFR and that PLCβ3 phosphorylation by PKC, but not by PKA, is responsible for one component of PAFR homologous desensitization.

PLCβ1 is phosphorylated by PKC (29), and PLCβ2 is phosphorylated by PKA (30). The present study revealed that both PKC and PKA phosphorylate of PLCβ3 *in vivo* (Fig. 3*C*). More importantly, phosphorylation of PLCβ3 by the different protein kinases appears to be associated with distinct functional consequences. Although phosphorylation of PLCβ3 by PKC resulted in the inhibition of responses stimulated by PAF, its phosphorylation by PKA was not associated with such inhibition. On the other hand, PKA activation by cpt-cAMP inhibited formylpeptide receptor-mediated responses. In RBL-2H3 cells PAF receptors activate PLC predominantly via a Ptx-insensitive G protein, whereas formylpeptide-mediated response is completely blocked by Ptx (15, 19). Therefore, differential inhibition of responses to PAF and formylpeptide by PKC and PKA is likely due to the different G protein subunits these receptors utilize to activate PLC. It was demonstrated *in vitro* that Gαq and G$\beta\gamma$ released from Ptx-sensitive G protein interact with distinct regions of PLCβ3 to activate the enzyme (28). This led Smrcka and Sternweis to propose that different receptors that utilize G_q or G_i could activate PLCβ3 by producing Gαq or G$\beta\gamma$ (28). The data presented herein support this hypothesis and provide a molecular basis for the differential regulation of Gαq- and Gβγ-mediated activation of PLCβ3. It is likely that PKC and PKA phosphorylate PLCβ3 on distinct sites that block its activation by Gαq and Gβγ, respectively.

Similar to RBL-2H3 cells, many human cell lines and tissues express PLCβ3 but not PLCβ1 or PLCβ2 (9, 11, 28, 31). Given the duality of PLCβ3 activation by both $G\alpha_q$ and Gβγ and its ubiquitous tissue distribution, phosphorylation of PLCβ3 by PKC and PKA may be general mechanisms by which functions of different G protein/PLC-coupled receptors are regulated.

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