Prolonged activation of phospholipase D in Chinese hamster ovary cells expressing platelet-activating-factor receptor lacking cytoplasmic C-terminal tail

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The mechanism and role of phospholipase D (PLD) activation by platelet-activating factor (PAF) were examined with Chinese hamster ovary cells stably expressing wild-type PAF receptor (WT-H cells) and truncated PAF receptor lacking the C-terminal cytoplasmic tail (D-H cells). Treatment of D-H cells with PAF resulted in the rapid formation of $\text{Ins}(1,4,5)\text{P}_3$, which was followed by a sustained phase for more than 10 min. In these cells, PAF-induced PLD activation lasted for more than 20 min. In contrast, PLD activation in WT-H cells was transient. PAF stimulation caused the biphasic formation of 1,2-diacylglycerol (DG) in both types of cell. The first phase was rapid and transient, coinciding with the $\text{Ins}(1,4,5)\text{P}_3$ peak. The second sustained phase of DG formation was attenuated by butanol, which produces phosphatidylbutanol at the expense of phosphatidic acid (PA) by transphosphatidyltransfer activity of PLD, and by propranolol, a selective inhibitor for PA phosphohydrolase catalysing the conversion of PA into DG. The DG level returned nearly to basal at 20 min after PAF stimulation in

WT-H cells, whereas in D-H cells the elevated DG level was sustained for more than 20 min. The profile of translocation of protein kinase $\text{C}\alpha$ (PKC α) to membrane was similar to that of DG formation. In WT-H cells, PKC α was transiently associated with membranes and then returned to the cytosol. However, in D-H cells PKC α was rapidly translocated to and remained in membranes for more than 20 min. Butanol suppressed this sustained translocation of PKC α . Furthermore the mRNA levels of *c-fos* and *c-jun* by PAF in WT-H cells were much lower than those in D-H cells. Propranolol and butanol at concentrations that inhibited the formation of DG suppressed the PAF-induced mRNA expression of *c-fos* and *c-jun*. Taken together, the prolonged PLD activation in D-H cells confirmed a primary role for phospholipase C/PKC in PLD activation by PAF. Furthermore the results obtained here suggest that sustained PLD activation in turn leads to chronic activation and membrane translocation of PKC α , which might play an important role in the expression of *c-fos* and *c-jun*.

INTRODUCTION

Cell-surface receptor-mediated phospholipase D (PLD) activation has been shown to be involved in diverse cellular functions [1–5] and mitogenesis [5,6]. Hydrolysis of phosphatidylcholine (PC) by PLD produces phosphatidic acid (PA) and choline. PA can be further hydrolysed via PA phosphohydrolase (PAP) to produce 1,2-diacylglycerol (DG). DG functions as an activator for protein kinase C (PKC). Although DG can be produced from PC by PC-specific phospholipase C (PC-PLC), it is convincingly suggested that it can also be produced by the sequential actions of PLD and PAP. The production of DG by the latter pathway might be involved in the sustained activation of PKC and would take a part in prolonged cellular responses [1,4,7] and gene expression [8–10].

Platelet-activating factor (PAF) has an important role in mediating physiological and pathophysiological responses [11–13]. cDNA cloning revealed that PAF receptor is a member of GTP-binding protein-coupled receptors [14–16]. PAF has been shown to cause PLD activation in neutrophils [17], promonocytic U937 cells [18] and renal mesangial cell [19], and also the expression of transcription factors in a human B-cell line [20] and Chinese hamster ovary (CHO) cells [21]. In CHO cells

transfected with cloned PAF receptor (WT-H cells), PAF activates a variety of signal transduction systems [22] including PLD activation [23,24]. However, the regulatory mechanism for PAF-induced PLD activation remains to be clarified.

Several regulatory factors have been proposed to regulate PLD activity, including low-molecular-mass GTP-binding proteins, Ca^{2+} ions, PKC and protein tyrosine kinase [1–4]. It has been shown that the platelet-derived growth factor-mediated PLD activation occurs downstream of PLC γ and PKC [25–27]. PLD activation through a PLC/PKC-dependent pathway has also been demonstrated in other cell systems [28–30]. We have previously suggested the possibility that, in WT-H cells, PAF-induced PLD activation might be mediated by the PLC–PKC pathway [23]. To gain more direct insight into the proposed mechanism, we have used CHO cells expressing the PAF receptor lacking the C-terminal cytoplasmic tail (D-H cells), because these cells exert sustained PLC activation by PAF [31].

The results obtained in the present study showed prolonged PLD activation in D-H cells, indicating a primary role for the PLC–PKC pathway in PAF-induced PLD activation. The prolonged PLD activation in turn resulted in the sustained translocation of PKC α from cytosol to membranes. Furthermore PAF-induced *c-fos* and *c-jun* mRNA expression was suppressed

Abbreviations used: CHO, Chinese hamster ovary; DG, 1,2-diacylglycerol; D-H cells, CHO cells expressing C-terminal deletion mutant PAF receptor; PA, phosphatidic acid; PAF, platelet-activating factor (1-O-alkyl-2-acetyl-sn-glycero-3-phosphocholine); PAP, phosphatidic acid phosphohydrolase; PtdBut, phosphatidylbutanol; PC, phosphatidylcholine; PKC, protein kinase C; PLC, phospholipase C; PLD, phospholipase D; WT-H, CHO cells expressing guinea-pig PAF receptor.

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by propranolol and butanol. Thus it is tempting to speculate that PLD might play a role in expression of immediate-early genes, *c-fos* and *c-jun*, in CHO cells stimulated with PAF.

EXPERIMENTAL

Materials

PAF (C-16) was purchased from Cascade Biochem. (Reading, Berks., U.K.). Propranolol was from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Authentic phosphatidylbutanol (PtdBut) was purchased from Avanti Polar-Lipids (Alabaster, AL, U.S.A.). Fetal calf serum was from Irvine Scientific (Santa Ana, CA, U.S.A.). Ham's F-12 nutrient mixture and genitcin were from Gibco (Grand Island, NY, U.S.A.). Anti-PKC α , β , γ , δ , ϵ and ζ antibodies (rabbit polyclonal) were from Santa Cruz Biotech (Santa Cruz, CA, U.S.A.). Goat anti-(rabbit IgG) horseradish peroxidase-coupled secondary antibody was obtained from Tago (Burlingame, CA, U.S.A.). TLC plates (LK6D) were purchased from Whatman (Clifton, NJ, U.S.A.). Immobilon-P membrane filters were from Millipore. [9,10- ^3H]Palmitic acid (52.4 Ci/mmol) and enhanced chemiluminescence system used for Western blotting were from Amersham International (Little Chalfont, Bucks., U.K.). The RNA extraction reagent isogen was obtained from Nippon gene (Tokyo, Japan). Gene screen plus hybridization membranes were from DuPont-NEN (Boston, MA, U.S.A.). Other reagents were of A. R. grade.

Cell culture

WT-H cells [22–24] and D-H cells [31] were cultured in growth medium composed of Ham's F-12 nutrient mixture (containing 0.2 g/l genitcin) supplemented with 10% (v/v) fetal calf serum in a humidified air/CO₂ (19:1) atmosphere.

Lipid analysis

WT-H cells and D-H cells seeded in 60-mm-diameter dishes (5×10^5 cells in 4 ml per dish) were labelled with [^3H]palmitic acid (1 $\mu\text{Ci/ml}$) for 24 h. After washing twice with a modified Krebs–Ringer buffer containing 125 mM NaCl, 5 mM KCl, 1.2 mM KH₂PO₄, 1.2 mM MgSO₄, 2 mM CaCl₂, 6 mM glucose, 0.1% BSA and 25 mM Hepes, pH 7.4, cells were preincubated in Krebs–Ringer buffer at 37 °C for 30 min and then stimulated with PAF. Lipids were extracted essentially by the method of Bligh and Dyer [32]. Authentic PtdBut, DG and PA were added to each sample as internal standards. Lipid samples were dried under nitrogen gas, redissolved in chloroform/methanol (6:1, v/v) and applied to TLC plates (LK6D). For the analysis of [^3H]PtdBut and [^3H]PA the plates were developed with an upper phase of ethyl acetate/iso-octane/acetic acid/water (130:20:30:100, by vol.) [33]. For the analysis of [^3H]DG the plates were developed with chloroform/acetone (96:4, v/v) [34]. Each spot on the plates revealed in iodine vapour was scraped off the plates and the radioactivity was measured in a liquid-scintillation counter (Beckman LS-6500). The formation of [^3H]PtdBut, [^3H]DG and [^3H]PA was expressed as a percentage of the total radioactivity recovered from the TLC plates.

Immunoblot analysis

Cells grown in a 60-mm-diameter dish were stimulated as described above. The reaction was terminated by aspiration of the incubation buffer followed by two immediate washes with ice-cold PBS. Cells were suspended in ice-cold lysis buffer [20 mM Tris/HCl (pH 7.4)/10 mM EGTA/2 mM EDTA/5 mM dithiothreitol/1 mM PMSF/20 $\mu\text{g/ml}$ leupeptin] and sonicated with

Astrason ultrasonic processor for 30 s. After removal of nuclei and unbroken cells, the homogenate was centrifuged at 100 000 *g* for 60 min to obtain the cytosol (supernatant) and the membrane (pellet) fractions. The pellet was extracted with lysis buffer containing 1% (w/v) Triton X-100 and sonicated. After incubation in an ice bath for 30 min, the samples were centrifuged at 14 000 *g* for 30 min to obtain the membrane extract. Proteins were separated by SDS/PAGE [7.5% (w/v) gel] and then transferred on Immobilon-P membrane. After the membranes had been blocked with 3% (v/v) milk, immunoblot analysis was performed with anti-PKC antibodies [24]. Blots were probed with the enhanced chemiluminescence reagents. The bands corresponding to individual PKC isoenzymes were quantified by a densitometer (Atto, Densitograph series 1).

Northern blot analysis

Cells grown in a 100-mm-diameter dish were stimulated as described above. Total cellular RNA was extracted with isogen in accordance with the manufacturer's protocol, and 20 μg aliquots were subjected to electrophoresis on 1.2% (v/v) formaldehyde denaturing agarose gels and transferred to Gene Screen Plus membranes by capillary blotting. To fix RNA the membranes were baked for 2 h at 80 °C in a vacuum oven and then hybridized with ^{32}P -labelled *c-fos* or *c-jun* cDNA probe at 42 °C for 16 h. After being washed twice in $2 \times \text{SSC}$ [0.3 M NaCl/0.03 M trisodium citrate (pH 7.0)] containing 0.1% SDS, the membranes were exposed to Kodak X-Omat film with intensifying screens at –80 °C. The density of each band was measured by a densitometer (Atto, Densitograph series 1).

RESULTS

PAF-induced PLD activation in WT-H and D-H cells

We have examined the PAF-induced activation of PLD by measuring the production of PtdBut, a unique product of its transphosphatidyl activity [23,24], in which butanol acts as phosphatidyl group acceptor. [^3H]PtdBut formation is a specific, well established and widely used marker for PLD activity. PtdBut

Figure 1 Time course and concentration dependence of PAF-induced PLD activity in WT-H and D-H cells

WT-H cells (●) and D-H cells (○) labelled with [^3H]palmitic acid were stimulated with 100 nM PAF for the indicated periods (A) or for 5 min with the indicated concentrations of PAF (B) in the presence of 0.3% (v/v) butanol. [^3H]PtdBut ([^3H]PtdBut) formation was measured as described in the Experimental section. The amount of [^3H]PtdBut is expressed as a percentage of the total radioactivity recovered from the TLC plates. Results shown are means $\pm$ S.E.M. for two separate experiments, each performed in duplicate.

Figure 2 Time-dependent changes in PLD activity in response to PAF in WT-H and D-H cells

WT-H (●) and D-H cells (○) labelled with [3 H]palmitic acid were stimulated with 100 nM PAF for the indicated periods. Butanol (0.3%, v/v) was added for the last 1 min of treatment with PAF. [3 H]PtdBut ([3 H]PBut) formation was measured as described in the Experimental section. The amount of [3 H]PtdBut is expressed as a percentage of the total radioactivity recovered from the TLC plates. Results shown are means $\pm$ S.E.M. for two separate experiments, each performed in duplicate.

is metabolically stable and accumulates in cells. In the presence of 0.3% (v/v) butanol, PAF induced [3 H]PtdBut formation in a time-dependent and concentration-dependent manner in both WT-H and D-H cells prelabelled with [3 H]palmitic acid (1 μ Ci/ml) for 24 h (Figure 1). PAF (100 nM)-induced [3 H]PtdBut formation in WT-H cells was detectable within 1 min and reached a plateau at 10 min. In contrast, the level of [3 H]PtdBut in D-H cells increased continuously over 20 min of PAF stimulation. PAF-induced [3 H]PtdBut formation in WT-H cells reached its maximal level at 100 nM, whereas it showed a continuous increase in D-H cells.

In the next experiment, the temporal changes in PLD activity were examined in WT-H cells and D-H cells (Figure 2). Cells were stimulated with 100 nM PAF for various periods of time and then exposed to 0.3% (v/v) butanol for the last 1 min. [3 H]PtdBut formation reached a peak at 1 min and returned to the basal level at around 5 min after stimulation in WT-H cells. In contrast, [3 H]PtdBut formation in D-H cells reached a peak at 2 min, followed by a plateau phase, suggesting persistent PLD activation. Taken together, PAF-mediated PLD activity was higher and more prolonged in D-H cells than in WT-H cells.

PAF-mediated PA and DG formation in WT-H cells and D-H cells

PA, an immediate product of PLD, was accumulated in these two types of cell in a time-dependent manner (Figure 3A). PAF-stimulated PA formation in D-H cells was higher than that in WT-H cells.

The addition of PAF to WT-H and D-H cells resulted in a biphasic increase in the level of DG (Figure 3B). The first DG peak in both WT-H and D-H cells was attained within 30 s of stimulation with PAF. This initial rapid DG peak was well correlated with the initial peak of Ins(1,4,5) P_3 formation, indicating PLC activation (results not shown). In both cell types Ins(1,4,5) P_3 reached a maximum within 20 s (results not shown), as reported previously [31]. In WT-H cells the rapid and transient increase was followed by a prompt decrease and returned almost to the basal level after 10 min. However, Ins(1,4,5) P_3 formation in D-

Figure 3 Time course of PAF-induced [3 H]PA and [3 H]DG formation in WT-H and D-H cells

WT-H cells (●) and D-H cells (○) labelled with [3 H]palmitic acid were stimulated with 100 nM PAF for the indicated periods. The formation of [3 H]PA (A) or [3 H]DG (B) was measured as described in the Experimental section. The amounts of [3 H]PA and [3 H]DG are expressed as percentages of the total radioactivity recovered from the TLC plates. Results shown are means $\pm$ S.E.M. for two separate experiments, each performed in duplicate.

Figure 4 Effect of butanol on [3 H]PtdBut, [3 H]PA and [3 H]DG formation in WT-H and D-H cells

WT-H cells (●, ○) and D-H cells (■, □) labelled with [3 H]palmitic acid were stimulated for 5 min with 100 nM PAF (●, ■) or buffer alone (○, □) in the presence of the indicated concentrations of butanol. [3 H]PtdBut ([3 H]PBut) (A), [3 H]PA (B) and [3 H]DG (C) were measured as described in the Experimental section. The amounts of [3 H]PtdBut, [3 H]DG and [3 H]PA are expressed as percentages of the total radioactivity recovered from the TLC plates. Results shown are means $\pm$ S.E.M. for two separate experiments, each performed in duplicate.

H cells was observed to continue for more than 10 min [31]. The peak of the second phase of DG in WT-H cells was obtained at 5 min and declined thereafter. D-H cells exhibited the second phase of DG accumulation, which was sustained for more than 20 min (Figure 3B).

The second peak of DG can be produced either by the hydrolysis of PC via PC-PLC or by PAP's action on PA generated via PLD. To define the pathway(s) for the supply of DG and PA, the temporal formation of these two products was measured after treatment of the cells with butanol or propranolol. PAF-induced [3 H]PtdBut formation was dependent on butanol concentration and reached a maximal level at 0.3% (v/v) butanol in both WT-H and D-H cells (Figure 4A). In contrast, PAF-induced [3 H]PA formation was decreased in a concentration-

Figure 5 Effect of propranolol on formation of [3 H]DG and [3 H]PA in WT-H and D-H cells

WT-H cells (A, C) and D-H cells (B, D) labelled with [3 H]palmitic acid were preincubated in the absence (□) or presence (■) of 250 μ M propranolol for 30 min and then stimulated with 100 nM PAF for the indicated periods. [3 H]DG (A, B) and [3 H]PA (C, D) were measured as described in the Experimental section. The amounts of [3 H]DG and [3 H]PA are expressed as percentages of the total radioactivity recovered from the TLC plates. Results shown are means $\pm$ S.E.M. for two separate experiments, each performed in duplicate.

dependent manner in both cells (Figure 4B). These results indicate that, with increasing butanol concentration, PAF-induced PtdBut formation occurs at the expense of PA. Under the same condition, DG formation was also decreased in a concentration-dependent manner (Figure 4C). Thus it was conceivable that DG was derived at least in part by the PLD–PAP pathway. To this end, the effect of propranolol was examined because it inhibits PAP without affecting PLD activity [4,35]. Propranolol inhibited the PAF-induced formation of DG with a reciprocal increase in PA formation in a concentration-dependent manner. As shown in Figure 5, propranolol (250 μ M) decreased the second peak of PAF-induced DG formation in both WT-H and D-H cells, but it had no effect on the first peak of DG formation via PLC. In accordance with the preventive effect on DG production, propranolol significantly augmented the sustained level of PA formation in both WT-H (Figure 5C) and D-H cells (Figure 5D). These findings suggest that the PAF-induced second phase of DG formation is mainly via the activation of PLD.

Translocation of PKC isoenzymes in WT-H and D-H cells

PKC α , ϵ and ζ isoenzymes were detected in both WT-H and D-H cells by immunoblot analysis, whereas PKC β , γ and δ isoenzymes were undetectable (results not shown). In the resting state, most of the isoenzymes PKC α , ϵ and ζ were present in the cytosol fractions in both types of cells. We have previously shown that on stimulation of WT-H cells with PAF, there

Figure 6 PAF-mediated translocation of PKC isoenzymes in WT-H and D-H cells

WT-H and D-H cells were stimulated with 100 nM PAF for the indicated periods. Proteins in membrane and cytosol fractions were subjected to electrophoresis and transferred to Immobilon-P membrane filters. Filters were probed with anti-PKC α (A) or -PKC ϵ (B) antibodies. (C, D) Changes in membrane-associated PKC isoenzymes in WT-H (●) and D-H (○) cells, expressed as percentages of untreated control cells. Quantification of PKC α (C) and PKC ϵ (D) was performed with a densitometer. Details are given in the Experimental section. Results presented are representative of two experiments, which gave essentially identical results.

occurred a rapid translocation of PKC α and PKC ϵ from cytosol to membranes within 30 s [24]. Of much interest was that the level of membrane-associated PKC α displayed an increase in WT-H cells that was transient, but a sustained elevation in D-H cells (Figure 6). In contrast, the translocation of PKC ϵ occurred rapidly and then associated with membrane for at least 20 min in both WT-H and D-H cells. The subcellular distribution of PKC ζ remained unaltered in response to 100 nM PAF in both cell types (results not shown). The sustained translocation of PKC α to membranes was suppressed by butanol, which prevented the second sustained phase of DG production (Figure 7).

Effects of propranolol and butanol on PAF-induced transcription factors *c-fos* and *c-jun* expression

Despite numerous studies there is little or no direct evidence with which to assess the physiological role of PLD. Recently the possible involvement of PLD in expression of immediate-early genes (*c-fos* and *c-jun*) was shown in gonadotropin-releasing hormone-stimulated pituitary cells [9], T lymphoid Jurkat cells activated by anti-CD3 antibody [8], and hepatocyte growth factor-stimulated rat hepatocytes [10]. To gain some insight into the role of PLD activation in the PAF-mediated signalling, we have examined expression of the transcription factor genes *c-fos* and *c-jun*. The expression of *c-fos* and *c-jun* in D-H cells was much higher (approx. 10-fold) than that in WT-H cells (Figure 8). Propranolol (250 μ M) and butanol (0.3%, v/v), at concentrations that effectively inhibited the formation of DG, suppressed PAF-induced mRNA levels of *c-fos* and *c-jun*. It should be noted that the inhibitory effect was much greater in D-H cells than in WT-H cells.

Figure 7 Effect of butanol on PAF-induced translocation of PKC α in WT-H and D-H cells

WT-H and D-H cells were incubated with or without 0.3% (v/v) butanol for 5 min and then stimulated with 100 nM PAF for the indicated periods. Proteins in membrane and cytosol fractions were subjected to electrophoresis and transferred to Immobilon-P membrane filters. Filters were probed with anti-PKC α antibodies (A). Quantification of PKC α was performed with a densitometer (B). Details are given in the Experimental section. Results presented are representative of two experiments, which gave essentially identical results.

Figure 8 Inhibition of PAF-induced *c-fos* and *c-jun* mRNA expression by propranolol and butanol

WT-H cells (A) and D-H cells (B) were preincubated with 250 μ M propranolol for 30 min (lanes c) or 0.3% (v/v) butanol for 5 min (lanes d) and then stimulated with 100 nM PAF for 45 min (lanes b, c and d). The levels of *c-fos* and *c-jun* were quantified by Northern blotting. The incorporation of ethidium bromide in the RNA samples permitted the detection of the 28 S rRNA band by UV transillumination, indicating the equal loading with RNA in all lanes of the gel. Lower panels: quantification of *c-fos* and *c-jun* was performed with a densitometer. Details are given in the Experimental section. Results shown are representative of three different experiments.

DISCUSSION

PAF stimulates multiple signalling pathways via its own GTP-binding protein-coupled receptor superfamily with seven hydrophobic transmembrane segments. It has been shown that PAF

stimulates PLD in various cell types [17–19,36], including WT-H cells [23,24]. Although there now are numerous studies dealing with the regulatory mechanism(s) and function(s) of PLD activity, no precise mechanism and physiological role of PLD activation have been defined. We have previously shown that PLD activation in WT-H cells was dependent on Ca²⁺ ions, PKC α and an unidentified protein tyrosine kinase [23,24]. However, the exact mechanism underlying the PAF-mediated PLD activation has not yet been disclosed. In the present study we have extended the previous work [23,24] to achieve a better understanding of the activation mechanism of PLD with special reference to PKC, and also examined the effect of PLD on the mRNA expression of *c-fos* and *c-jun*. For this purpose we have used a CHO cell line (D-H cells) that expresses a mutated PAF receptor lacking the C-terminal cytoplasmic tail [31], because this cell has no capability for desensitization, thereby resulting in the sustained elevation of Ins(1,4,5)P₃ and intracellular Ca²⁺ concentrations by prolonged activation of PLC.

PAF produced a biphasic DG formation in both WT-H and D-H cells (Figure 2B). The first peak was consistent with the peak of Ins(1,4,5)P₃ formation [22], indicating that the first peak of DG accumulation is derived from inositol phospholipids via PLC activation. The second peak of DG accumulation persisted for several minutes in WT-H cells and for more than 20 min in D-H cells (Figure 3B). It has been described that the second phase of DG is derived from the hydrolysis of PC rather than PtdIns [1,6,7]. Hydrolysis of PC by PLD leads to the accumulation of PA in stimulated cells. The sequential actions of PLD and PAP have been shown to have a significant role in the prolonged increase in DG level in several cell lines [1,4,6,7,9]. When PLD was activated in the presence of butanol, [³H]PtdBut was produced by its transphosphatidyl activity at the expense of PA. Accordingly, the second sustained formation of DG was prevented by butanol (Figure 4). Propranolol has been known to inhibit PAP without affecting PLD activity and it has often been used to investigate the role of PA-derived DG in the signalling pathway [4,35,37–40]. This agent inhibited the second phase of DG (Figure 5A) and increased the formation of PA (Figure 5C) in both WT-H and D-H cells. In addition, the increase in PA preceded the second phase of that of DG (Figure 3). These findings support the notion that the second sustained phase of DG accumulation is produced mainly by the sequential actions of PLD and PAP.

The transient increase of [³H]PtdBut formation in WT-H cells indicates that PLD activation is rapidly desensitized after the application of PAF (Figure 2) [23]. Desensitization of receptor-mediated PLD activation has been studied in several cell types [41–46]. Recent reports have shown that PAF receptors are desensitized in RBL-2H3 cells [47] and guinea-pig ileal smooth-muscle cells [48]. In contrast, [³H]PtdBut formation in PAF-stimulated D-H cells was much more prolonged than that in WT-H cells (Figure 1). In the previous study it was shown that in D-H cells, which express the mutated PAF receptor lacking the C-terminal cytoplasmic tail, stimulation with PAF induced the sustained elevation of Ins(1,4,5)P₃ and intracellular Ca²⁺ concentrations [31]. In contrast, we have previously shown that PAF-induced PLD activation is dependent on PKC α [24]. In the present study the rapid transient translocation of PKC α to membranes occurred concomitantly with the temporal changes of [³H]PtdBut formation (Figures 2 and 6) in WT-H cells. In marked contrast, in PAF-stimulated D-H cells PKC α had a sustained association with membrane (Figure 6), which was well correlated with the second sustained phase of DG. This prolonged association of PKC α to membranes was suppressed by butanol, which inhibited the formation of DG by the PLD–PAP pathway.

These results support the notion that the elevated levels of PC-derived DG lead to constitutive membrane translocation and activation of PKC α . Chronic PKC α activation in D-H cells in turn causes the sustained activation of PLD.

PLD has been suggested to be involved in the regulation of cell growth, because it is activated by mitogenic signals [4,6,9]. The relationship between PLD and the expression of immediate-early genes (*c-fos* and *c-jun*) has recently been observed in pituitary cells [9], T lymphoid Jurkat cells [8] and rat hepatocytes [10]. It is possible that a functional role exists for PLD activation in gene expression such as *c-fos* and *c-jun*. Schulam et al. [20] reported that PAF stimulated *c-fos* mRNA expression in a B-cell line. In the present study it was shown that PAF promoted *c-fos* and *c-jun* expression in both WT-H and D-H cells, and also that the expressed levels of *c-fos* and *c-jun* in D-H cells are much higher than those in WT-H cells (Figure 8). Moreover, in the presence of either propranolol or butanol, PAF-induced expressions of *c-fos* and *c-jun* mRNA species were almost completely abolished in D-H cells (Figure 8). Under these conditions, butanol prevented the formation of both DG and PA (Figure 4), whereas propranolol preferentially suppressed the formation of the sustained DG with an increase in PA (Figure 5). Therefore it can be concluded that the sustained phase of DG produced via the PLD-PAP pathway promotes the expression of these two genes. A similar relationship between PLD-mediated DG formation and the expression of these immediate-early genes was obtained in hepatocyte growth factor-stimulated hepatocytes [10].

Taking the results together, we find that in CHO (WT-H) cells the first transient DG peak from PtdInsP₂ was produced within 1 min by PLC activation and the produced DG together with Ca²⁺ released by Ins(1,4,5)P₃-stimulated PKC α , leading to the activation of PLD. The second DG phase, due to the sequential actions of PLD and PAP, then resulted in the further activation of PKC α . In marked contrast with WT-H cells expressing wild-type PAF receptor, which was susceptible to desensitization, in D-H cells expressing the mutated receptor, which was defective in desensitization of the PLC-PKC α pathway, the constitutive membrane translocation and activation of PKC α due to the elevated DG level led to the prolonged activation of PLD, which in turn gave rise to the sustained high level of DG. This DG recruited the further activation of PKC-dependent PLD. Furthermore it was shown that the expression of *c-fos* and *c-jun* is much higher in D-H cells, suggesting that chronic activation of PKC α might somehow be involved in expression of these immediate-early genes. Further work is required to elucidate the role of PLD in the signalling pathway to the transcriptional gene expression.

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