The Mechanism of Docosahexaenoic Acid-induced Phospholipase D Activation in Human Lymphocytes Involves Exclusion of the Enzyme from Lipid Rafts*

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Docosahexaenoic acid (DHA), an *n*-3 polyunsaturated fatty acid that inhibits T lymphocyte activation, has been shown to stimulate phospholipase D (PLD) activity in stimulated human peripheral blood mononuclear cells (PBMC). To elucidate the mechanisms underlying the DHA-induced PLD activation, we first characterized the PLD expression pattern of PBMC. We show that these cells express PLD1 and PLD2 at the protein and mRNA level and are devoid of oleate-dependent PLD activity. DHA enrichment of PBMC increased the DHA content of cell phospholipids, which was directly correlated with the extent of PLD activation. The DHA-induced PLD activation was independent of conventional protein kinase C but inhibited by brefeldin A, which suggests ADP-ribosylation factor (ARF)-dependent mechanism. Furthermore, DHA enrichment dose-dependently stimulated ARF translocation to cell membranes. Whereas 50% of the guanosine 5′-3-O-(thio)triphosphate plus ARF-dependent PLD activity and a substantial part of PLD1 protein were located to the detergent-insoluble membranes, so-called rafts, of non-enriched PBMC, DHA treatment strongly displaced them toward detergent-soluble membranes where ARF is present. Collectively, these results suggest that the exclusion of PLD1 from lipid rafts, due to their partial disorganization by DHA, and its relocalization in the vicinity of ARF, is responsible for its activation. This PLD activation might be responsible for the immunosuppressive effect of DHA because it is known to transmit antiproliferative signals in lymphoid cells.

Polyunsaturated fatty acids (PUFAs)[1] are known to modulate immune response, particularly by affecting T cell function

(1, 2). Accordingly, dietary supplementation with *n*-3 fatty acids decreases the production of T cell-derived cytokines and lymphoproliferation (3, 4). Although fish oil-derived fatty acids have found clinical applications for the treatment of various inflammatory diseases (5) and as adjuvant immunosuppressive agents (6), the underlying molecular and cellular mechanisms of PUFA-induced T cell inhibition have not yet been elucidated in detail.

We have reported previously (7) that in docosahexaenoic acid (DHA)-enriched peripheral blood mononuclear cells (PBMC), the mitogenic lectin concanavalin A was able to stimulate phospholipase D (PLD) activity, whereas no stimulation was observed in control cells. PLD acts on phosphatidylcholine (PC) to release phosphatidic acid, a bioactive molecule that has been implicated in the regulation of numerous cellular functions including cell proliferation and apoptosis (8). Two mammalian PLD have been cloned from various human and murine sources. PLD1 has a low basal activity and is activated by ADP-ribosylation factors (ARF), Rho family GTP-binding proteins, and protein kinase C (PKC) in the presence of phosphatidylinositol bisphosphate (PIP_2). PLD2 has a high basal activity in the presence of PIP_2 but is unresponsive to small G proteins and PKC (9, 10). Another PLD form that is dependent on unsaturated fatty acids such as oleate for its activation has also been described, but it has not been cloned yet (10). Interestingly, this oleate-activated PLD seems to be the major form detected in lysates of Jurkat T cells where it could be implicated in the apoptotic process (11). In addition Jurkat T cells also express GTP-dependent PLD activity (12). PLD protein and mRNA expression has been well demonstrated in several established lymphocytic cell lines (13), but only few reports have addressed the PLD of human PBMC. A PLD activity, detected by means of the transphosphatidylation reaction, has been described in these cells (7, 14). However, the identity of the PLD isoforms in presence is not precisely known.

In the past few years, several groups have reported the presence of PLD enzymes in caveolae, which are specialized domains of the plasma membrane related to sphingolipid- and cholesterol-rich microdomains or rafts. Both PLD1 and PLD2 have been found in caveolar membranes depending on the type of cell considered. Thus, PLD1 has been detected in the caveolin-rich membrane fractions of 3Y1 rat fibroblasts (15) and C2C12 mouse skeletal myotubes (16). In human keratinocytes, PLD2 was located in the caveolin-rich low density fractions, whereas PLD1 was clearly excluded from the caveolar fractions

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[1] The abbreviations used are: PUFA, polyunsaturated fatty acid; PLD, phospholipase D; DHA, docosahexaenoic acid; PBMC, peripheral blood mononuclear cells; ARF, ADP-ribosylation factor; GTPγS, guanosine 5′-3-O-(thio)triphosphate; DIM, detergent-insoluble membrane; HSA, human serum albumin; BIM, bisindolylmaleimide; BFA, brefeldin A; DPPC, dipalmitoylphosphatidylcholine; PKC, protein kinase C; PIP_2, phosphatidylinositol bisphosphate; AMPCP, adenosine 5′-(α,β-methylene)diphosphate; PC, phosphatidylcholine; TPA, 12-*O*-

tetradecanoylphorbol-13-acetate; ConA, concanavalin A; RT, reverse transcription; ANOVA, analysis of variance; rARF, recombinant ARF.

(17). In contrast, both PLD1 and PLD2 were found in caveolae of NIH 3T3 cells transfected by activated oncogenic forms of *src*, *ras*, and *raf* (18). Although lymphocytes are devoid of caveolae and caveolin expression (19), their plasma membrane contains typical sphingolipid- and cholesterol-rich microdomains, which have a key role in immune cell signaling (20, 21). It has been shown that upon cross-linking, the T cell antigen receptor associates with lipid rafts and promotes the recruitment and aggregation of raft-associated proteins such as Src family kinases while excluding others such as tyrosine phosphatase, which initiates phosphorylation signaling cascades. Interestingly, in PUFA-enriched T cells the Src kinases Lck and Fyn appeared to be excluded from rafts suggesting that PUFA had partially disrupted these structures (22), which might explain their immunosuppressive effect.

The goal of the present study was to characterize further the PLD activity of human PBMC to investigate the possible association of PLD enzymes with lipid rafts and to determine the mechanisms involved in the increase of PLD activity induced by DHA in mitogen-stimulated cells. In particular, we considered the possibility that the location of PLD isoforms inside or outside lipid rafts might be altered under the influence of DHA with, as a consequence, changes in the impact of T cell stimulation on PLD activity.

EXPERIMENTAL PROCEDURES

Preparation of Human PBMC—Peripheral blood was obtained from healthy subjects who had not taken any medication for 2 weeks prior to blood donation (Etablissement Français du Sang, Lyon, France). Venous blood was drawn into citrate-phosphate-dextran anticoagulant. PBMC were separated by dextran sedimentation and density gradient centrifugation through Histopaque 1077 (Sigma) and then washed three times with RPMI 1640 by low speed centrifugation in order to more thoroughly eliminate the contaminating platelets. PBMC were then adjusted to a concentration of 2×10^7 cells/ml in RPMI 1640 (with Hepes and bicarbonate) medium. All steps were carried out at room temperature. Under such conditions, cell viability established by the trypan blue exclusion test was always greater than 95%. Flow cytometry analyses of cell preparations after staining with specific monoclonal antibodies showed that about 65–70% of the isolated cells were CD3+ T cells (T3 Coulter clone), 4–6% were CD19+ B cells (B4 Coulter clone), 16–24% were CD11b+ monocytes (MO1 Coulter clone), and 4–6% were CD41a+ platelets (GP IIb IIIa, Immunotech, Marseille, France).

DHA Enrichment of the Cells—For the preparation of fatty acid-albumin complexes, docosahexaenoic acid (Sigma) was stored at -20 °C in ethanol solution under nitrogen. Different amounts of the ethanolic solution were evaporated to dryness under reduced pressure. 5 μM human delipidated serum albumin (HSA) in RPMI 1640 medium was added to give final fatty acid concentrations ranging from 5 to 15 μM and an albumin to fatty acid ratio varying from 1 to 3. The mixtures were incubated under nitrogen for 4 h at 37 °C.

DHA enrichment of PBMC was achieved by incubating the cells (2×10^7 cells/ml) for 2 h at 37 °C in RPMI 1640 medium containing increasing concentrations of DHA bound to 5 μM delipidated HSA.

The fatty acid composition of cell phospholipids was analyzed by gas chromatography. Cell lipid extracts or lipids extracted from the gradient fractions were separated on Silica Gel G60 plates (Merck) with the solvent system hexane/diethyl ether/acetic acid (60:40:1, by volume). The silica gel areas corresponding to phospholipids were scraped off and transmethylated. Briefly, 1 volume of 5% H_2SO_4 in methanol was added to the scraped silica gel, and transmethylation was carried out at 100 °C for 90 min in screw-capped tubes. The reaction was terminated by the addition of 1.5 volume of ice-cold 5% (w/v) K_2CO_3, and the fatty acid methyl esters were extracted with isooctane and analyzed using a PerkinElmer Life Sciences chromatograph model 5830, equipped with a capillary column (30 m × 0.32 mm, Supelco) and a flame ionization detection. The column was two-step programmed from 135 to 160 °C at 2 °C/min and from 160 to 205 °C at 1.5 °C/min; the detection temperature was maintained at 250 °C. The vector gas was helium at a pressure of 0.8 pounds/square inch (5520 pascals). Peaks were identified using standard fatty acid methyl esters.

PLD Assay in Intact Cells—PLD was determined on the basis of its transphosphatidylation activity. Freshly isolated cells were incubated for 1 h at 37 °C in the presence of [³H]arachidonic acid (37 kBq/ml, specific activity 7400 GBq/mmol, Amersham Biosciences) in 0.1% ethanol and then washed three times in RPMI 1640 medium. Labeled cells were further enriched with DHA as described above. Control cells were incubated without fatty acid in the presence of 5 μM HSA alone. DHA-treated and control cells were then incubated for 30 min at 37 °C in the presence of 1% butanol or 1% ethanol, and in the absence (unstimulated) or presence of 5 μg/10⁶ cells concanavalin A (+ConA), a specific activator of T cells. In some experiments, control and DHA-enriched cells were stimulated with 0.1 μg/ml OKT3 (Cilag Laboratories, Levallois Perret, France) for 30 min in the presence of 1% butanol. In experiments designed to investigate the influence of inhibitors, the following conditions were used: bisindolylmaleimide (BIM, 1 μM) and calphostin C (126 nM) were added 30 min before ConA activation, and brefeldin A (BFA, 1–25 μg/ml) was added 10 min before ConA activation. Incubations were terminated by addition of ethanol and acidification of the medium to pH 3–4 with 2 N HCl. Lipids were extracted with chloroform/ethanol (6:3, by volume) according to Boukhchache and Lagarde (23) in the presence of 50 μM butylhydroxylated toluene. Phosphatidylalcohols were separated on bidimensional TLC (Silica Gel G60 plates, Merck) using chloroform, methanol, 28% ammonia (65:35:5.5, by volume) for the first migration, and ethyl acetate/isooctane/acetic acid (9:5:2, by volume) for migration in the second dimension. Spots stained by Coomassie Brilliant Blue R were scraped off and mixed with Picofluor (Packard Instrument Co.), and the radioactivity was determined by liquid scintillation counting. The radioactivity associated with phosphatidylalcohols was expressed as percentage of the radioactivity incorporated in total phospholipids.

Cell-free PLD Assay—Oleate-dependent PLD activity was measured as described by Chalifa *et al.* (24). Briefly, the standard reaction mixture (120 μl) contained 50 mM Na-Hepes buffer, pH 7.2, 2.7 mM (10⁷ cpm/assay) [³H]dipalmitoylphosphatidylcholine ([³H]DPPC, specific activity 3700 GBq/mmol, PerkinElmer Life Sciences), 4 mM sodium oleate, 1 mM EGTA, 1 mM MgCl₂, 2% ethanol, and 60–100 μg of proteins from total cell lysates. After 30 min of incubation the reaction was stopped by the addition of 2 ml of ice-cold chloroform/methanol/HCl (1:1:0.002) and mixing. Phase separation was achieved by further addition of 0.1 N HCl, 1 mM EGTA, mixing, and centrifugation (2,000 rpm, 10 min). The organic phase was collected, dried under nitrogen, and redissolved in chloroform/methanol containing standard phosphatidylethanol as a carrier. Phosphatidylethanol was separated on TLC using ethyl acetate/isooctane/acetic acid (9:5:2). Plates were stained by Coomassie Brilliant Blue R, and spots corresponding to phosphatidylethanol were scraped off and mixed with Picofluor, and the radioactivity was determined by liquid scintillation counting. The radioactivity associated with phosphatidylalcohols was expressed as percentage of the radioactivity of the substrate [³H]DPPC.

PIP₂-dependent PLD activity was determined according to Brown *et al.* (25). Briefly, 10 μl of mixed lipid vesicles (phosphatidylethanolamine/phosphatidylinositol bisphosphate/phosphatidylcholine, molar ratio 16:1.5:1) with [*choline-methyl*-³H]dipalmitoylphosphatidylcholine (specific activity 2200 GBq/mmol, PerkinElmer Life Sciences) to give 10⁶ cpm per assay was added to 40 μl of cell lysate in a total of 100 μl containing 50 mM Hepes, pH 7.5, 80 mM KCl, 2.5 mM MgCl₂, 2 mM CaCl₂, 1 mM dithiothreitol. Assays were done with or without 50 μM GTPγS and 1 μM rARF. The final PC concentration was 8 μM. Assays were incubated for 30 min at 37 °C and stopped as described above. After centrifugation, the radioactivity of an aliquot of the aqueous phase was determined by liquid scintillation counting. Recombinant ARF was kindly provided by Dr. Blandine Geny, INSERM U332, Paris, France.

RNA Extraction and Reverse Transcription (RT)-PCR—Total RNA was isolated from human PBMC using Trizol Reagent (Invitrogen) and reverse-transcribed with Moloney murine leukemia virus-reverse transcriptase (Promega, Lyon, France) using random hexamer mixed oligonucleotides. Specific primers for the amplification of PLD1 transcripts were designed on the basis of published human sequences (26) to discriminate between PLD1a and PLD1b. The sense and antisense primers were 5′-GGGATCCGTGTGAAGCGGGTCACTTCAGGACC-G-3′ and 5′-GGGAATTCTCTGGTTTCCCCATGCAGCTCTCCCAC-3′, respectively. Primers to specifically amplify PLD2 transcripts were designed on the basis of published human sequences (27) and chosen in a domain corresponding to the N-terminal part of the protein. The sense and antisense primers were 5′-GGGAATTCGACGGCGACCCCTGAG-AGCCTCTTC-3′ and 5′-GGGAATTCACGGTATTTCTTCTTGGTTGTC-CAGG-3′, respectively. Amplification conditions for PLD1 were 94 °C for 45 s, 60 °C for 45 s, and 72 °C for 45 s, for 35 cycles. Amplification conditions for PLD2 were 94 °C for 45 s, 57 °C for 45 s, and 72 °C for

45 s, for 35 cycles. PCDNA3 plasmids carrying the hPLD1b and hPLD2 cDNAs originating from Dr. M. Frohman's laboratory and kindly provided to us by Dr. Michel Record (Toulouse, France) were used for positive controls.

Preparation of Cytosolic and Particulate Cell Fractions for Western Blotting Experiments—Pelleted cells were washed three times with Phillips' buffer and disrupted by glycerol lysis according to Caldwell *et al.* (28) as described previously (29). After glycerol treatment, the cells were pelleted at $900 \times g$ for 10 min, resuspended in lysis buffer (20 mM Hepes, 25 mM sucrose, 0.1 mM EGTA, 0.05 mM phenylmethylsulfonyl fluoride, 10 units/ml aprotinin, 2 µg/ml pepstatin A, pH 7.4), and stored frozen at -80 °C. After thawing, the cells were homogenized in a glass/Teflon homogenizer (40 strokes at maximal speed), and homogenates were centrifuged at $42,000 \times g$ for 20 min. Particulate and cytosolic fractions (3 volumes) were mixed with $4\times$ concentrated Laemmli buffer (1 volume), and proteins were determined by the method of Schaffner and Weissmann (30) using bovine serum albumin as a standard. Proteins were separated on a 10 (PKCα) or 15% (ARF) acrylamide gel and electrotransfered onto an Immobilon P membrane (Millipore, St. Quentin Yvelines, France). For PLD Western blot analyses, proteins were denatured by 4 M urea in Laemmli buffer and separated on 8% acrylamide gel in 4 M urea. Aspecific antibody-binding sites were saturated by incubating membranes in TBS-T (20 mM Tris, pH 7.6, 137 mM NaCl, 0.1% Tween 20) containing 5% bovine serum albumin, overnight at 4 °C. All following steps were performed in TBS-T at room temperature. After washing, the blots were incubated for 90 min either with an anti-PKCα monoclonal antibody (Santa Cruz Biotechnology, diluted 1:2000) or with an anti-ARF 1:3 polyclonal antibody (sheep IgG, Upstate Biotechnology Inc., diluted 1:1000). The PLD isoforms were detected with PLD1 and PLD2 antisera (dilution 1:2000) prepared by Dr. S. Bourgoin (Laval University, Canada). PLD1 antiserum was raised against the following short human PLD1 peptides: residues 1–16, 144–162, 967–981, and 1027–1040. PLD2 antiserum was raised against the N-terminal sequence of human PLD2 (residues 13–33). After washing, membranes were incubated for 1 h either with a horseradish peroxidase-conjugated goat anti-mouse antibody (1:15,000) or a donkey anti-sheep antibody (Sigma, 1:10,000) or a goat anti-rabbit antibody (Bio-Rad, 1:20,000). Immunoreactive proteins were visualized using the ECL detection system (Amersham Biosciences) and x-ray film autoradiography. Bands were then quantified with a videodensitometric analyzer (Bioprofil, Vilbert-Lourmat, Germany).

Isolation and Characterization of Lipid Rafts from Human PBMC—Lipid rafts were isolated according to the procedure of Montixi *et al.* (31) based on the insolubility of these structures in cold non-ionic detergent, with slight modifications. Briefly, control or DHA-enriched PBMC, either stimulated or not (5×10^8 cells), were homogenized in 1 ml of ice-cold lysis buffer (25 mM Tris, pH 7.5, 150 mM NaCl, 5 mM EDTA) supplemented with a mixture of protease inhibitors (protease inhibitor mixture Sigma) and 1 mM orthovanadate. After centrifugation at $800 \times g$ at 4 °C for 10 min, the post-nuclear supernatant was supplemented with Triton X-100 at a final concentration of 1% for 1 h at 4 °C. The lysate was then adjusted to 1.3 M sucrose by the addition of an equal volume of 2.6 M sucrose and placed at the bottom of an ultracentrifuge tube, and a step sucrose gradient (0.2–0.9 M with 0.1 M steps, 1 ml each) was placed on top. It was centrifuged at $200,000 \times g$ for 16 h in an SW41 rotor (Kontron) at 4 °C. One-ml fractions were recovered from the bottom to the top of the gradient. The sucrose concentration of each fraction was determined with a Brix refractometer (Merck). Aliquots were immediately used for PLD and 5′-nucleotidase assays, and the remaining was stored at -80 °C until lipid analyses.

5′-Nucleotidase activity of the glycosylphosphatidylinositol-anchored CD73, used as a marker of lipid rafts, was assayed as described by Gentry and Olsson (32) with slight modifications. Aliquots of the gradient fractions were incubated with 1 µM [³H]5′AMP (specific activity 722 GBq/mmol, Amersham Biosciences) in 1 ml of reaction mixture containing 60 mM Tris, pH 7.4, 1 µM erythro-9-(2-hydroxy-3-nonyl)adenine to inhibit endogenous adenosine deaminase, 20,000 cpm [¹⁴C]adenosine as an internal standard, with or without 25 µM adenosine 5′-(α,β-methylene)diphosphate (AMPCP) to inhibit 5′-nucleotidase activity, for 30 min at 37 °C. The incubation was terminated by addition of 200 µl of 5% $ZnSO_4$ and 200 µl of 0.3 M $Ba(OH)_2$. After centrifugation, the radioactivity of supernatant containing [³H]adenosine was measured by liquid scintillation counting. 5′-Nucleotidase activity obtained by difference between values with and without AMPCP was expressed as nmol of 5′-AMP hydrolyzed per min per mg of proteins.

GM1 was quantitated on dot blots according to Ilangumaran *et al.* (33). Briefly, 20 µl of each gradient fraction were dotted onto Immobilon

using a Hybri-Dot Manifold apparatus (Bethesda). Membranes were rinsed with distilled water and blocked with 5% bovine serum albumin in TBS-T for 1 h. After three 10-min washes with TBS-T, membranes were incubated with horseradish peroxidase-conjugated cholera toxin in TBS-T containing 1% bovine serum albumin for 90 min and then rinsed 7 times with TBS-T and developed with the ECL reagent. The luminograms were quantitated using cooled digital CCD camera system (ImageMaster VDS-CL, Amersham Biosciences) and ImageQuant software.

PIP_2 was quantitated on dot blots performed as described above using a mouse PIP_2 antiserum (Assay Designs, Euromedex, Souffelweyersheim, France, dilution 1:500) for immunodetection and horseradish peroxidase-conjugated goat anti-mouse antibody diluted 1:10,000 as the secondary antibody.

Total phospholipids were assayed with the ammonium ferrothiocyanate method according to Stewart (34).

Cholesterol was determined enzymatically using a commercial assay kit (Sigma) according to the manufacturer's recommendations.

PLD Activities and Western Blotting—PLD activities were determined on 40-µl aliquots of each gradient fraction as described above. For PLD1 Western blotting, proteins from 400 µl of each fraction were precipitated with ice-cold acetone, and pellets were dissolved in 40 µl of Laemmli buffer containing 4 M urea. PLD2 proteins were immunoprecipitated from 400 µl of gradient fractions using the PLD2 antiserum described above according to Marcil *et al.* (35).

RESULTS

Dose Dependence of the DHA-induced PLD Activation in Stimulated PBMC—To define more precisely the PLD-stimulating effect of DHA that we have observed previously (7) in human PBMC, cells were preincubated for 2 h with increasing concentrations of DHA complexed to human delipidated albumin (DHA to albumin ratio from 0 to 3) before T cell stimulation with ConA in the presence of 1% butanol. A marked and dose-dependent increase of PLD activity was observed in DHA-treated cells compared with control cells incubated in the presence of delipidated albumin (Fig. 1*A*). Gas chromatography analysis of the fatty acid composition of cell phospholipids showed that the relative amount of esterified DHA was concomitantly increased (Fig. 1*B*). Furthermore, PLD activity was significantly correlated to the percentage of DHA present in cell phospholipids (Fig. 1*C*, $r = 0.68$, $p = 0.015$, $n = 12$). These results suggest that the extent of PLD activation was directly related to DHA enrichment of membrane phospholipids. A PLD-stimulating effect of DHA was also observed when PBMC were stimulated with the anti-CD3 monoclonal antibody OKT3, although the extent of PLD activation was lower than that induced by ConA (Fig. 1*D*).

Characterization of PLD in Human PBMC—To investigate the PLD isoform expression pattern of human PBMC, we performed RT-PCR using primers for PLD1, allowing us to discriminate between the two splice variants PLD1a and PLD1b, and primers specific for PLD2. As shown in Fig. 2*A*, human PBMC clearly express the three PLD transcripts. It is noteworthy that the intensity of the PLD1a band appeared to be roughly 2-fold higher than that of PLD1b, suggesting that human PBMC preferentially express the former variant. Western blot analyses of total cell lysates showed that both PLD1 and PLD2 are also expressed at the protein level (Fig. 2*B*). To characterize further the PLD present in human PBMC, we then measured the enzyme activity of cell lysates using specific assay conditions. Although the above results clearly denote the presence of PLD1 and PLD2 in human PBMC, they do not rule out the possible presence of an oleate-dependent enzyme, which has not yet been characterized at the molecular level. Because the PLD activity of human PBMC was up-regulated by DHA enrichment prior to mitogenic activation, it was important to look for an oleate-dependent activity in PBMC lysates. As shown in Fig. 2*C*, PBMC lysates did not show any oleate-stimulated PLD activity. In contrast, a marked synthesis of
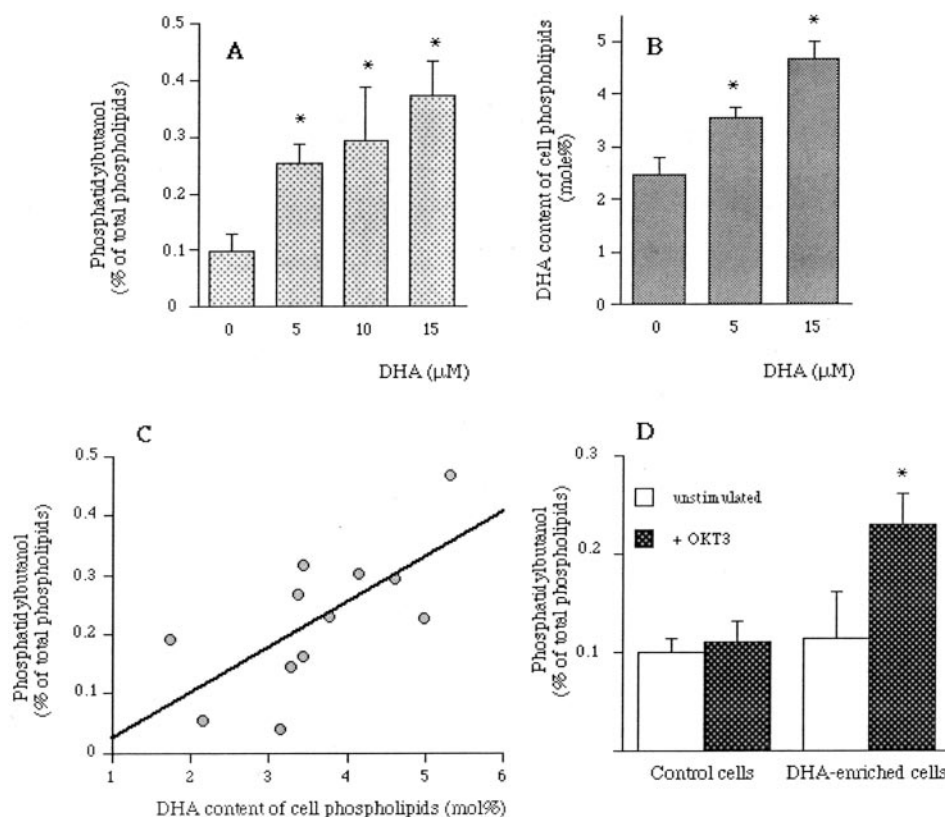
FIG. 1. **Effect of DHA enrichment on PLD activation and on the DHA content of total phospholipids from human PBMC.** *A*, [^{3}H]arachidonate-labeled PBMC were incubated for 2 h with 5 μM HSA or with increasing concentrations of DHA bound to HSA. Cells were then stimulated with ConA (5 μg/10^6 cells) in the presence of 1% butanol for 30 min. At the end of the incubation period, lipids were extracted from cells plus medium, and the lipid extracts were separated by TLC as described under "Experimental Procedures." The radioactivity associated to phosphatidylbutanol was measured by liquid scintillation counting. Results are expressed relative to the radioactivity incorporated in total phospholipids and are means ± S.E. of four separate experiments. Data were analyzed by ANOVA, and means were compared by a protected *t* test. *, significantly different from control cells incubated without DHA. $p < 0.05$. *B*, lipids were extracted and total phospholipids separated on TLC. Phospholipid spots were scraped off, and the fatty acids were transmethylated and analyzed as described under "Experimental Procedures." Results are expressed as mol % and are means ± S.E. of four separate experiments. Data were analyzed by ANOVA and means were compared by a protected *t* test. *, significantly different from control cells incubated without DHA. $p < 0.05$. *C*, the radioactivity associated to phosphatidylbutanol expressed as percent of total phospholipid radioactivity was plotted as a function of the DHA content of cell phospholipids. Data are from *A* and *B*. *D*, [^{3}H]arachidonate-labeled PBMC were incubated for 2 h with 5 μM HSA (control cells) or with 15 μM DHA bound to HSA. Cells were then stimulated or not with the anti-CD3 monoclonal antibody OKT3 (0.1 μg/ml), in the presence of 1% butanol for 30 min. Lipids were extracted and analyzed as described above. Results are means ± S.E. of three separate experiments. *, significantly different from unstimulated control cells. $p < 0.05$.

phosphatidylethanol was observed when PIP$_2$ was included in the substrate phospholipid vesicles confirming the presence of a PIP$_2$-dependent PLD activity in these cells. This PIP$_2$-dependent synthesis was further increased by more than 3.5-fold by GTPγS. This latter result also confirms that a G protein-dependent PLD1 activity is present in PBMC lysates and suggests that the amount of G protein present in lysates is sufficient to allow marked PLD1 activation. The membrane fraction of PBMC exhibited a PIP$_2$-dependent PLD activity that was only modestly increased by GTPγS addition due to the loss of cytosolic G proteins (Fig. 2*D*). Full activation was restored by the addition of recombinant ARF. Taken together these results indicate that PIP$_2$-dependent PLD1a, PLD1b, and PLD2 are present in human PBMC and that these cells are clearly devoid of oleate-dependent PLD activity.

The DHA-induced PLD Activation in ConA-stimulated PBMC Is Independent of PKC—PLD1 activity is known to be regulated through activation by PKC and, in particular, by the PKCα isoform (9). Thus, we sought to determine whether the DHA-induced PLD activation observed in ConA-stimulated PBMC was dependent on PKC. Several studies have shown that polyunsaturated fatty acids can either activate (36) or inhibit (37) protein kinase C activity depending on the cell type considered. It has also been shown that DHA rapidly induces

the translocation of several PKC isoenzymes to the particulate fraction of human monocytes (38). We thus examined whether DHA treatment of PBMC affects PKCα expression. Results of Western blot experiments show that DHA enrichment of the cells dose-dependently decreased the PKCα amount in the cytosolic fractions (Fig. 3*A*, *left*) while the amount in the particulate ones was maintained and even increased at the highest DHA concentration used (Fig. 3*B*, *left*). This pattern suggests that DHA favors PKCα translocation in the absence of cell activation. In marked contrast, the PKCα distribution pattern was totally different when PBMC were further stimulated with ConA after DHA enrichment (Fig. 3, *right*). The large PKCα translocation induced by ConA in non-enriched cells was substantially impaired in DHA-enriched cells. At the highest DHA concentration used for enrichment, PKCα remained mainly cytosolic. Thus, under the conditions of maximal PLD activation (DHA plus ConA), the translocation of PKCα to the particulate fraction was strongly counteracted. This result strongly suggests that the mechanism of DHA-induced PLD activation in stimulated PBMC is PKC independent. To support this hypothesis further, experiments were performed in the presence of PKC inhibitors and in cells treated with the phorbol ester TPA for a prolonged period to down-regulate conventional PKC isoforms. Control or DHA-enriched cells
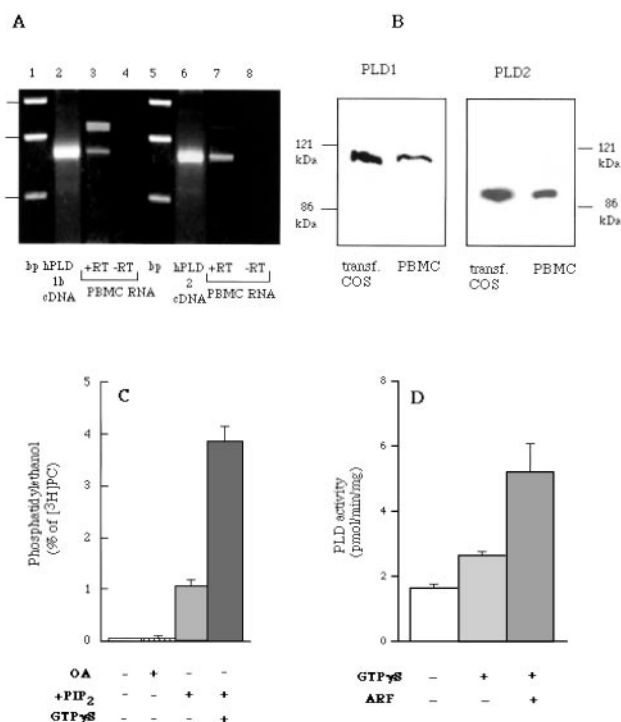
FIG. 2. **Characterization of PLD in human PBMC.** *A*, RT-PCRs were carried out as described under "Experimental Procedures" using specific primers for PLD1, which allowed us to discriminate the splice variants PLD1a and -1b (*lanes 2–4*) and primers for PLD2 (*lanes 6–8*). Positive controls were obtained by using pCDNA3 plasmids carrying hPLD1b (*lane 2*) and hPLD2 (*lane 6*) cDNAs as templates. Negative controls were obtained by using PBMC RNA untreated by reverse transcriptase (*lanes 4* and *8*). *Lane 3*, the larger product of 446 bp and the smaller one of 332 bp derived from PLD1a and PLD1b, respectively; *lane 6*, the band at 329 bp has the expected size for amplification of a PLD2 transcript. *B*, total cell lysates from 6×10^6 PBMC were subjected to SDS-PAGE and analyzed by Western blotting using PLD1 and PLD2 antisera. Lysates from COS-1 cells transfected with pCDNA3-hPLD1b and pCDNA3-hPLD2 were used as positive controls. *C*, aliquots of cell lysates (8×10^6 cells) were added to a reaction mixture containing 2.7 mM [³H]DPPC in the absence (−) or presence (+) of 2 mM sodium oleate or to reaction mixtures containing 8 μM [³H]D-PPC in the absence (−) or presence (+) of 12 μM PIP$_2$, with or without 50 μM GTPγS. [³H]Phosphatidylethanol was separated by TLC. *D*, aliquots of the 100,000 × g pellet fraction (from 8×10^6 cells) were added to reaction mixtures containing substrate phospholipid vesicles (PE/PIP$_2$/PC, 16:1.5:1) and incubated at 37 °C for 30 min in the absence (−) or presence (+) of 50 μM GTPγS with or without recombinant ARF. [³H]Choline released from dipalmitoylphosphatidyl[*methyl*-³H]choline was measured as described under "Experimental Procedures." Data are means ± S.E. of three separate experiments.

were pretreated with the PKC inhibitor BIM that competes with ATP at the catalytic site, prior to ConA activation. As shown in Fig. 4*A*, BIM did not induce any significant PLD inhibition whatever the dose of DHA used for cell enrichment. Similar results were obtained with another PKC inhibitor, calphostin C, that inhibits the binding of phorbol esters to the PKC regulatory domain (not shown). In addition, the DHA-induced PLD activation was not affected by PKC down-regulation. Fig. 4*B* shows that PLD activation was similar in cells pretreated for 14 h with 100 nM TPA prior to ConA activation and in cells incubated for the same period in RPMI alone. In contrast, the strong PLD activation induced by 10 nM TPA in control non-enriched cells was totally lost after PKC down-regulation (Fig. 4*C*). Collectively, these results indicate that PLD activation by DHA in the presence of mitogens neither involves PKC activation nor requires the presence of an intact PKC.

The DHA-induced PLD Activation in ConA-stimulated PBMC Is Dependent on Small G Proteins—To look for the
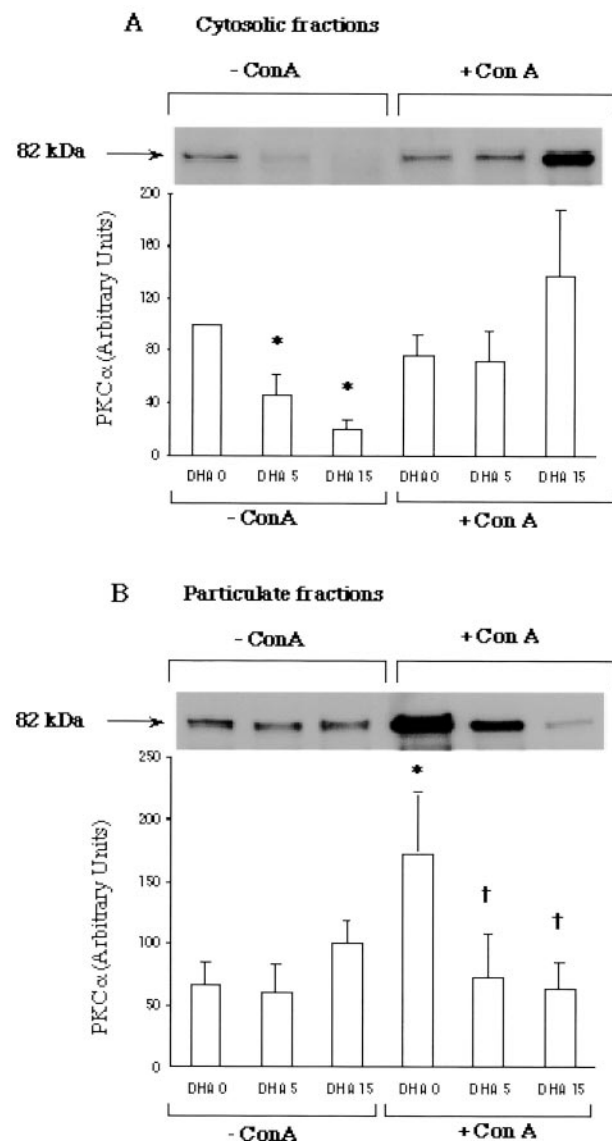

FIG. 3. **Effect of DHA on the translocation of PKCα in human PBMC.** PBMC were incubated for 2 h with 5 μM HSA or with 5 or 15 μM of DHA bound to HSA. Cells were then incubated for 30 min in the absence or presence of ConA (5 μg/10⁶ cells). At the end of incubation, cells were lysed, and the cytosolic and particulate fractions were separated by ultracentrifugation. Cytosolic (*A*) and particulate (*B*) proteins (8 μg per lane) were analyzed by Western blotting, using an anti-PKCα monoclonal antibody for immunodetection. Spots were analyzed by videodensitometry. *Bar diagrams* show results expressed as arbitrary units normalized to values obtained in the cytosol of control unstimulated cells and are means ± S.D. of five separate experiments. *, significantly different from non-enriched unstimulated cells (*DHA 0, −ConA*) $p < 0.05$; †, significantly different from non-enriched stimulated cells (*DHA 0, +ConA*) $p < 0.05$. *Blots* shown in the *upper part* of the two panels are from one typical experiment.

possible involvement of small G proteins in the DHA-induced activation of PLD in stimulated PBMC, DHA-enriched cells were permeabilized with digitonin in the presence or absence of GTPγS before ConA activation. Permeabilization in the absence of GTPγS allows macromolecules such as ARF or Rho to leak out of the cells, whereas such leakage can be prevented when digitonin and GTPγS are added simultaneously. As shown in Fig. 5*A*, cells permeabilized in the absence of GTPγS lost their ability to respond to ConA, whereas PLD activation was maintained when the permeabilization was done in the presence of GTPγS. These observations show that the combined effects of DHA and ConA require soluble factors that are
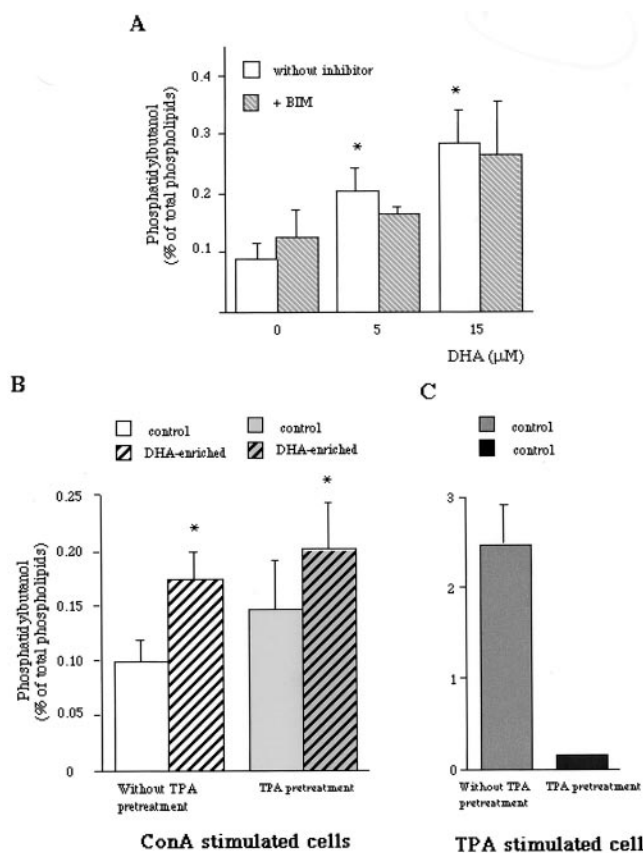
FIG. 4. **The DHA-induced PLD activation in ConA-stimulated PBMC is independent of PKC.** *A*, [³H]arachidonate-labeled PBMC were incubated for 2 h with 5 μM HSA or with 5 or 15 μM of DHA bound to HSA. Cells were then incubated in the absence or presence of 1 μM BIM for 30 min at 37 °C and further stimulated with ConA (5 μg/10⁶ cells), in the presence of 1% butanol for 30 min. Results are expressed relative to the radioactivity incorporated in total phospholipids and are means ± S.E. of four separate experiments. Data were analyzed by ANOVA, and means were compared by a protected *t* test. *, significantly different from control non-enriched cells. $p < 0.05$. *B*, [³H]arachidonate-labeled control or DHA-enriched (5 μM) PBMC were incubated in the absence or presence of 100 nM TPA for 14 h and then stimulated with ConA (5 μg/10⁶ cells) in the presence of 1% butanol for 30 min. Results are expressed relative to the radioactivity incorporated in total phospholipids and are means ± S.E. of five separate experiments. Data were analyzed by ANOVA, and means were compared by a protected *t* test. *, significantly different from control non-enriched cells without TPA pretreatment. $p < 0.05$. *C*, [³H]arachidonate-labeled control PBMC were incubated in the absence or presence of 100 nM TPA for 14 h and then stimulated with 10 nM TPA in the presence of 1% butanol for 30 min. Results are expressed relative to the radioactivity incorporated in total phospholipids and are means ± range of two separate experiments.

FIG. 5. **The DHA-induced PLD activation in ConA-stimulated PBMC is dependent on small G proteins.** *A*, GTPγS is required for ConA activation of PLD in DHA-enriched cells permeabilized with digitonin. [³H]Arachidonate-labeled control or DHA-enriched (5 μM) PBMC were either incubated in RPMI (unpermeabilized) or with 8 μM digitonin in the absence or presence of 100 μM GTPγS and then stimulated with ConA (5 μg/10⁶ cells) in the presence of 1% butanol for 30 min. Results expressed relative to the radioactivity incorporated in total phospholipids are from one experiment representative of two. *B*, effect of brefeldin A on the ConA-induced PLD activation in DHA-enriched PBMC. [³H]Arachidonate-labeled PBMC were incubated for 2 h with 5 μM DHA bound to 5 μM HSA. DHA-enriched cells were then incubated for 10 min before the addition of ConA (5 μg/10⁶ cells) and 1% butanol. Results are expressed relative to the radioactivity incorporated in total phospholipids and are means ± S.E. of five separate experiments. Data were analyzed by ANOVA, and means were compared by a protected *t* test. *, significantly different from control cells incubated without inhibitor, $p < 0.05$; †, significantly different from DHA-enriched cells incubated without inhibitor, $p < 0.05$.

removed during permeabilization. To examine whether ARF proteins could be involved in the DHA-induced PLD activation of stimulated PBMC, DHA-enriched cells were preincubated for 10 min with BFA prior to ConA activation. As shown in Fig. 5*B*, BFA, which acts primarily as an inhibitor of ARF activation, was able to block PLD activation. This inhibitory effect was already observed for a BFA concentration of 5 μg/ml, and the inhibition was total at 10 and 25 μg/ml BFA. These results suggest that ARF is a mediator of PLD activation by ConA in DHA-enriched cells.

DHA Stimulates ARF Translocation—Because ARF activation was correlated with translocation of the protein from cytosol to membranes, cytosolic and particulate fractions were prepared from control and DHA-enriched PBMC either stimulated by ConA or not, and the presence of ARF proteins was investigated by Western blotting. Fig. 6*A* shows that the rela-
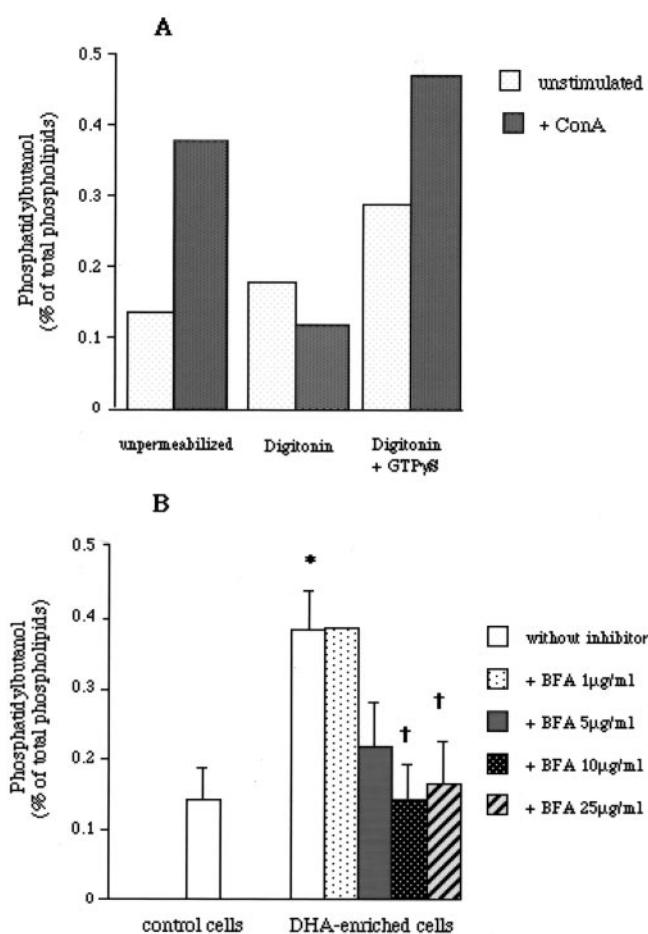
tive amount of ARF in the particulate fraction was about 3-fold higher in DHA-enriched cells stimulated with ConA than in control unstimulated cells. In control PBMC, ConA activation tended to increase the proportion of ARF in the particulate fraction, but this effect did not reach significance. These results suggest that DHA and ConA synergize to translocate ARF to the particulate fractions. In addition, the effect of DHA on ARF translocation was clearly dose-dependent (Fig. 6*B*). Together with BFA inhibition, these results suggest that DHA may activate PLD by favoring ARF translocation to membranes.

PLD1 Activity Is Located to the Detergent-insoluble Membrane (DIM) Domains of PBMC—Detergent-insoluble membrane domains from PBMC were obtained by fractionation of Triton X-100-treated cell lysates on a discontinuous sucrose density gradient. As shown in Fig. 7*A* greater than 80% of the total proteins were recovered in the first three high density fractions containing from 36 to 31% sucrose (Fig. 7*D*). In con-
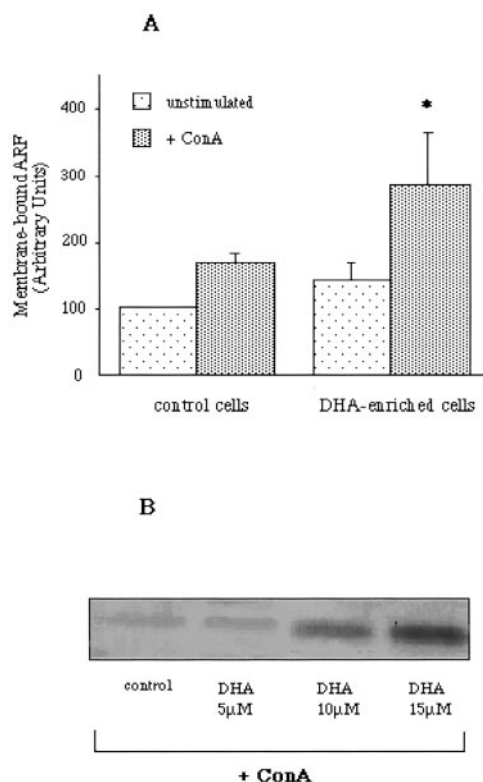
Mechanism of PLD Activation by DHA in Human Lymphocytes

A

B

FIG. 6. **Effect of DHA on ARF translocation.** *A*, PBMC enriched with 15 μM DHA or control PBMC were incubated in the absence or presence of ConA (5 μg/10^6 cells). At the end of the incubation, cells were lysed and the cytosolic and particulate fractions separated by ultracentrifugation. Particulate proteins (10 μg per lane) were analyzed by Western blotting using an anti-ARF polyclonal antibody for immunodetection. Spots were analyzed by videodensitometry. *Bar diagrams* show results expressed as arbitrary units normalized to values obtained for unstimulated control cells and are means $\pm$ S.D. of three separate experiments. Data were analyzed by ANOVA, and means were compared by a protected *t* test. *, significantly different from control unstimulated cells, $p < 0.05$. *B*, PBMC enriched with DHA concentrations increasing from 5 to 15 μM, or control cells were incubated in the presence of ConA (5 μg/10^6 cells) and processed as described above for Western blotting experiments with an anti-ARF antibody. A typical blot representative of three separate experiments is shown.

trast, the main part of the 5'-nucleotidase activity of CD73, a glycosylphosphatidylinositol-anchored enzyme (39), was distributed in the low density fractions containing 21 to 15% sucrose (Fig. 7A). Moreover, the ganglioside GM1 was also distributed in the same low density fractions of the gradient as 5'-nucleotidase activity (Fig. 7A, *inset*). As observed for protein distribution, greater than 70% of the total phospholipids were recovered in the high density sucrose fractions, whereas cholesterol was present both in the high density fractions, which contained Triton X-100-soluble material, and in the low density fractions (Fig. 7B). Thus, the phospholipid to cholesterol molar ratio which was higher than 10 in the high density fractions was decreased to 1–2 in the low density fractions, as it has been observed in detergent-resistant microdomains isolated from Madin-Darby canine kidney cells (40). In contrast, PIP$_2$ was mainly immunodetected in the low density fractions of the gradient (Fig. 7B, *inset*). Taken together these results indicate that the low density fractions fulfill the criteria of detergent-insoluble microdomains. Samples of each gradient fraction were then tested for PLD activity using [*choline-methyl-*³H]phosphatidylcholine, in mixed phospholipid vesicles including PIP$_2$, as a substrate, in the presence or absence of rARF protein plus GTPγS. Basal PLD activity measured in the absence of ARF plus GTPγS was mainly present (76%) in the high

density sucrose fractions, whereas the activity recovered in the low density fractions (6–8) consisted of less than 8% of total (Fig. 7D). PLD2 protein was then immunoprecipitated from each gradient fraction and analyzed by Western blotting using a PLD2-specific antiserum. A $\approx$90-kDa, PLD2-immunoreactive band, in the high density fractions of the gradient, coincided with basal PLD activity (Fig. 7D, *inset*). No PLD2 band could be detected in the low density detergent-insoluble microdomains. In contrast, GTPγS plus ARF-dependent PLD activity, estimated as the difference between assays with and without ARF plus GTPγS, was mainly associated with the GM1, PIP$_2$, and cholesterol-rich low density fractions which also contained most of the 5'-nucleotidase activity. Thus, about 50% of total GTPγS plus ARF-dependent PLD activity from human PBMC were associated with the detergent-insoluble microdomains (Fig. 7C). The highest specific activity was present in fraction 7 (3 nmol of PC hydrolyzed per h/mg of proteins). It was more than 60-fold higher than the specific activity detected in the high density fractions (*fractions 1–4*). Immunoblot analyses of the material precipitated with ice-cold acetone showed the presence of immunoreactive PLD1 ($\approx$115 kDa) in both the high and low density fractions of the gradient (Fig. 7C, *inset*), which suggests a close correlation between PLD1 protein and GTPγS plus ARF-dependent PLD activity.

DHA Enrichment of PBMC Displaces Signaling Proteins Out of the DIMs—When PBMC were first enriched with 15 μM DHA prior to Triton X-100 treatment and fractionation on sucrose gradient, the pattern of GTPγS plus ARF-dependent PLD activity recovered in the gradient fractions was strongly modified. Less than 30% of total activity remained associated to DIMs, and most of the activity appeared in the intermediate fractions from 30 to 25% sucrose (Fig. 8, *A* and *C*). This pattern was paralleled by changes in the distribution of PLD1 protein. The *inset* of Fig. 8A shows that only a faint immunoreactive band remained associated with the low density fraction 6, whereas most part of the immunoreactive PLD1 was recovered in the higher density fractions of the gradient. In contrast, none of the other measured parameters (5'-nucleotidase and basal PLD activities, GM1, cholesterol, total phospholipids) exhibited a modified pattern with respect to control cells (not shown). Although in some experiments PIP$_2$ appeared to be delocalized toward the high density detergent-soluble fractions, such a delocalization could not be reproducibly obtained (not shown). When DHA-enriched PBMC were stimulated with ConA prior to fractionation, GTPγS plus ARF-dependent PLD activity was almost totally excluded from DIM, with only 7% remaining associated to the low density fractions 6–8 (Fig. 8, *B* and *C*). Western blot analyses confirmed that almost the whole PLD1 protein was delocalized to the non-DIM fractions of the gradient (Fig. 8B, *inset*). As observed for unstimulated DHA-enriched cells, neither the distribution of 5'-nucleotidase nor that of basal PLD activities along the gradient fractions was changed as compared with controls (not shown).

It is noteworthy that the extent of DHA enrichment was higher in phospholipids of DIM (+50%) than non-DIM (+20%) membranes (Table I). On the whole, DHA treatment of the cells increased the relative amount of polyunsaturated fatty acids present in phospholipids of the DIM fractions at the expense of the saturated palmitate, which is very likely to selectively increase the fluidity of these domains.

Because Src family tyrosine kinases are known to be associated with the cytoplasmic layer of rafts (41) and because PUFA enrichment of Jurkat cells has been shown to modify the cytoplasmic leaflet of these structures (22), we compared the distribution of the Src family kinase Lck in sucrose gradients prepared from DHA-enriched (Fig. 9B) and control non-en-

FIG. 7. **Characterization of the low density sucrose gradient fractions prepared from control PBMC and association of PLD1 activity to detergent-insoluble microdomains.** Triton X-100 lysates were prepared from control human PBMC and fractionated by floatation in a discontinuous sucrose density gradient, and the fractions were analyzed for protein concentration, 5′-nucleotidase activity, GM1, PIP$_2$, cholesterol, phospholipid content, and ARF plus GTPγS-responsive and unresponsive PIP$_2$-dependent PLD activities as described under "Experimental Procedures." For PLD1 Western blotting, proteins from 400 μl of each fraction were precipitated with ice-cold acetone, and pellets were dissolved in 40 μl of Laemmli buffer containing 4 M urea. PLD2 proteins were immunoprecipitated from 400 μl of gradient fractions prior to Western blotting using a PLD2 antiserum. *A*, distribution of total protein concentration, 5′-nucleotidase activity, and cholera toxin-reactive GM1 (*inset*). *B*, distribution of total phospholipid, cholesterol, and immunoreactive PIP$_2$ (*inset*). *C*, distribution of GTPγS plus ARF-dependent PLD activity and PLD1 protein (*inset*). *D*, sucrose content, basal PLD activity, and PLD2 protein distribution (*inset*). Results are means ± S.E. of six separate experiments. Each *blot* shown in the insets is representative of three.
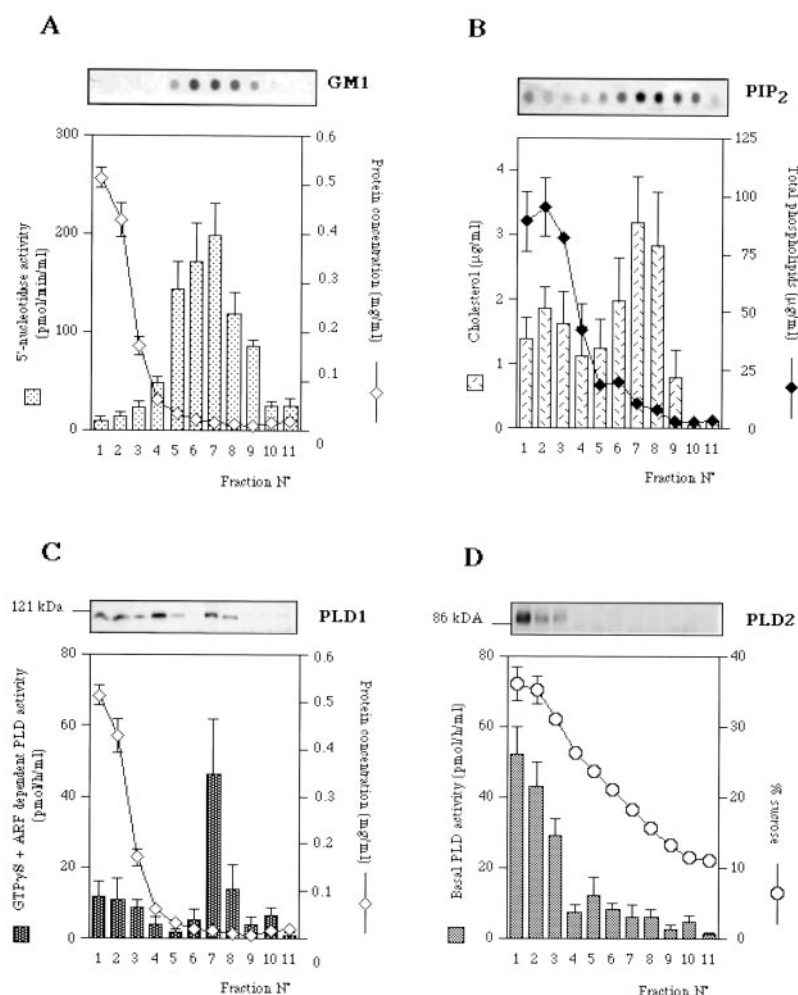
riched PBMC (Fig. 9*A*) stimulated with ConA. Western blot experiments showed that DHA treatment of the cells markedly displaced Lck protein out of the DIMs (fractions 6–8) toward the high density detergent-soluble fractions. In parallel experiments, we also compared the distribution of ARF in the gradient fractions. Although most ARF proteins were found in the high density detergent-soluble fractions, a notable part was also recovered in the DIM fractions of non-enriched PBMC (Fig. 9*C*). Interestingly, in DHA-enriched cells, very little ARF remained associated to rafts, the bulk of the protein being present in the detergent-soluble non-raft fractions (Fig. 9*D*).

DISCUSSION

The present study was undertaken to investigate the potential mechanisms involved in the activation of PLD by the *n*-3 polyunsaturated fatty acid DHA that we have previously observed in mitogen-activated PBMC (7). We confirm here that PLD activation is proportional to DHA enrichment of cell membranes and that it can be observed whatever the mitogenic effector used, concanavalin A or the anti-CD3 monoclonal antibody OKT3 (Fig. 1). The whole results of the present study suggest that DHA activates PLD1 enzyme by an indirect mechanism involving lipid modifications of detergent-insoluble domains within PBMC membranes.

One of the first questions we have addressed concerns the identity of the PLD isoforms present in human PBMC. Information on the expression levels, subcellular localization, and function of PLD in leukocytes is limited. It is usually accepted that human peripheral leukocytes express little or no PLD (13). Variable results have been reported for PLD mRNA and pro-

teins in different leukemic cell lines. Thus, human myeloid leukemic HL60 have been shown to express only PLD1 and mouse lymphocytic leukemic L1210 only PLD2, whereas neither isoform seems to be present in mouse thymoma EL4 (42, 43). Although some authors have only detected PLD2 in Jurkat cells, studies showing that PLD activation by the chemokine RANTES (regulated on activation normal T cell expressed and secreted) requires ARF and RhoA G proteins strongly suggest the presence of PLD1 in these cells (12, 43). In addition, they also contain an oleate-dependent PLD activity, which is increased during actinomycin D-induced apoptosis (11). By using anti-PLD1- and PLD2-specific antibodies, we showed that human PBMC express both isoforms. RT-PCR using specific primers for PLD1 and PLD2 indicated that both 1a and 1b splice variants of PLD1 are expressed together with PLD2 at the mRNA level. The assay of PLD activity in cell lysates confirmed the PIP$_2$ requirement for activity (the hallmark of PLD1 and PLD2) and its partial GTPγS dependence (suggesting the presence of PLD1). Furthermore, it clearly showed the absence of an oleate-dependent activity (Fig. 2).

Because PLD1 activity is known to be regulated by conventional PKC, at least in some types of cells (9, 10), we hypothesized that DHA might favor the recruitment of some PKC isoforms in the vicinity of PLD1. However, our whole results clearly indicate that the DHA-induced PLD activation observed in stimulated PBMC was PKC-independent. Indeed, it was affected neither by the PKC inhibitors calphostin C and BIM nor by PKC down-regulation (Fig. 4). In addition, although DHA decreased the amount of immunoreactive PKCα present

FIG. 8. **DHA enrichment of human PBMC displaces PLD1 activity from detergent-insoluble microdomains.** *A*, human PBMC were enriched with 15 μM DHA before lysis and fractionation of the Triton X-100-treated lysates on a discontinuous sucrose density gradient. The fractions were analyzed for GTPγS plus ARF-dependent PLD activity and PLD1 protein distribution (*inset*) as described under "Experimental Procedures." Results are means $\pm$ S.E. of five separate experiments. The blot shown in the inset is representative of three. *B*, DHA-enriched (15 μM) PBMC were stimulated with ConA (5 μg/10^6 cells) prior to lysis and fractionation. PLD assay and Western blotting were performed as in *A*. Results are means $\pm$ S.E. of five separate experiments. The *blot* shown in the *inset* is representative of three. *C*, the percentage of GTPγS plus ARF-dependent PLD activity remaining associated to detergent-insoluble microdomains in the experiments of Fig. 7*C*, and *A* and *B* of this figure is shown. Data were analyzed by ANOVA, and means were compared by a protected *t* test. *, significantly different from control unstimulated cells, $p < 0.05$.
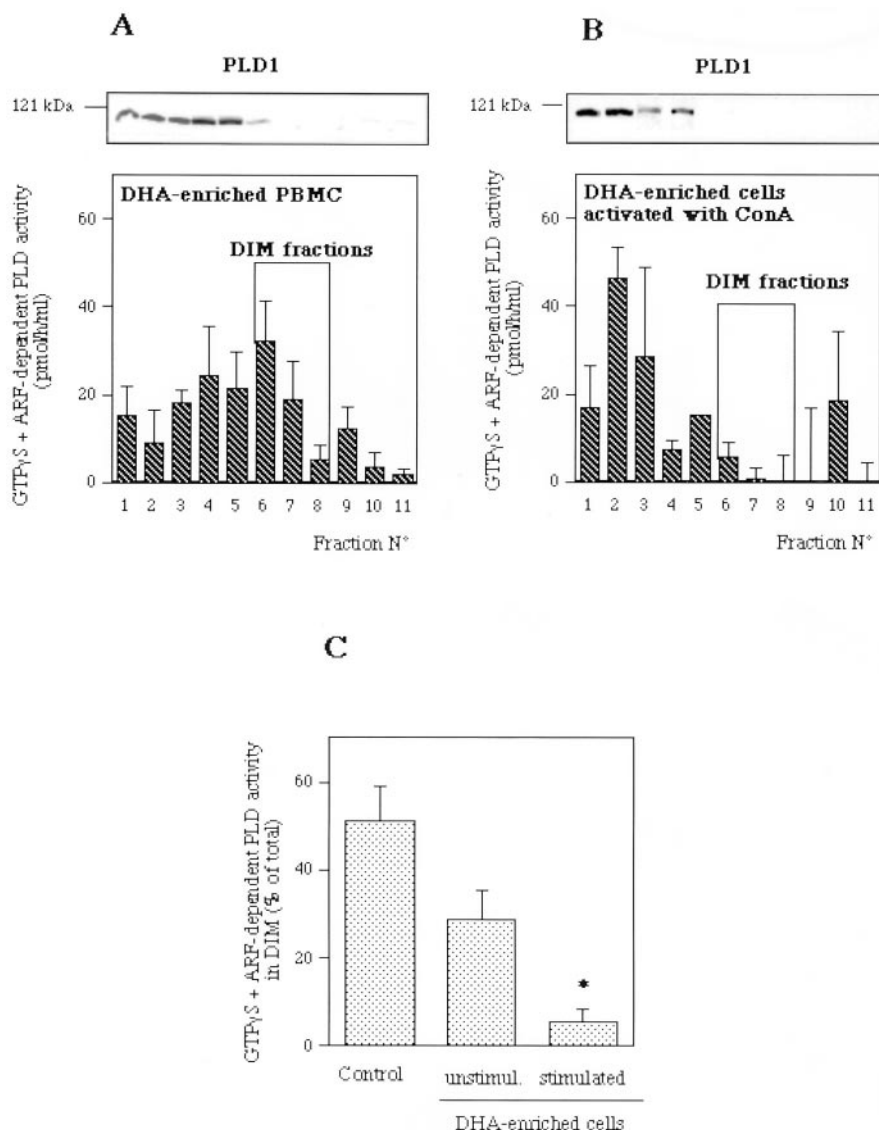
TABLE I

Fatty acid (FA) composition of total phospholipids of non-DIM and DIM membranes isolated from control and DHA-enriched PBMC

Fatty acids	Non-DIM membranes		DIM membranes	
	Control PBMC	DHA-enriched PBMC	Control PBMC	DHA-enriched PBMC
		mol %		
16:0	13.47 $\pm$ 0.35	14.14 $\pm$ 0.76	38.10 $\pm$ 0.45[a]	29.21 $\pm$ 6.81
18:0	19.31 $\pm$ 2.70	19.93 $\pm$ 1.98	16.45 $\pm$ 1.20	17.86 $\pm$ 0.67
18:1n-9	11.71 $\pm$ 0.47	11.91 $\pm$ 0.65	6.73 $\pm$ 0.56[a]	6.89 $\pm$ 0.76
18:2n-6	9.36 $\pm$ 1.30	9.43 $\pm$ 0.83	3.54 $\pm$ 0.42[a]	3.85 $\pm$ 0.50
20:0	0.16 $\pm$ 0.02	0.20 $\pm$ 0.01	1.59 $\pm$ 0.17[a]	1.80 $\pm$ 0.36
20:4n-6	25.55 $\pm$ 2.65	25.96 $\pm$ 1.39	9.32 $\pm$ 0.55[a]	11.86 $\pm$ 0.97
20:5n-3	0.58 $\pm$ 0.07	0.60 $\pm$ 0.06	0.18 $\pm$ 0.02[a]	0.25 $\pm$ 0.03
22:0	0.13 $\pm$ 0.06	0.27 $\pm$ 0.01	4.07 $\pm$ 0.19[a]	5.41 $\pm$ 0.95
22:4n-6	2.46 $\pm$ 0.38	2.53 $\pm$ 0.24	6.73 $\pm$ 0.66[a]	8.70 $\pm$ 1.14
22:5n-3	2.43 $\pm$ 0.38	2.41 $\pm$ 0.33	1.07 $\pm$ 0.04	1.47 $\pm$ 0.23
22:6n-3	4.05 $\pm$ 0.70	4.84 $\pm$ 0.80	1.76 $\pm$ 0.04[a]	2.64 $\pm$ 0.30[b]
24:0	0.05 $\pm$ 0.03	0.09 $\pm$ 0.04	3.04 $\pm$ 0.21[a]	4.18 $\pm$ 1.06
Σsat FA	33.15 $\pm$ 2.90	34.68 $\pm$ 1.40	63.42 $\pm$ 1.11[a]	58.46 $\pm$ 3.82
Σn-6 FA	39.04 $\pm$ 4.52	39.63 $\pm$ 2.92	20.12 $\pm$ 0.94[a]	25.83 $\pm$ 1.97[b]
Σn-3 FA	7.06 $\pm$ 1.13	7.84 $\pm$ 1.14	3.00 $\pm$ 0.18[a]	4.37 $\pm$ 0.53[b]

[a] Significantly different from non-DIM membranes in control PBMC, $p < 0.05$.
[b] Significantly different from control PBMC in DIM membranes, $p < 0.05$.

in the cytosolic fractions of unstimulated cells with a concomitant increase in the proportion of the particulate protein, this effect was entirely reversed after ConA stimulation (Fig. 3). We observed a similar behavior of the PKCβ and atypical PKCζ isoforms (not shown). Thus, in conditions of maximal PLD activation (DHA-enriched and ConA-stimulated cells), most
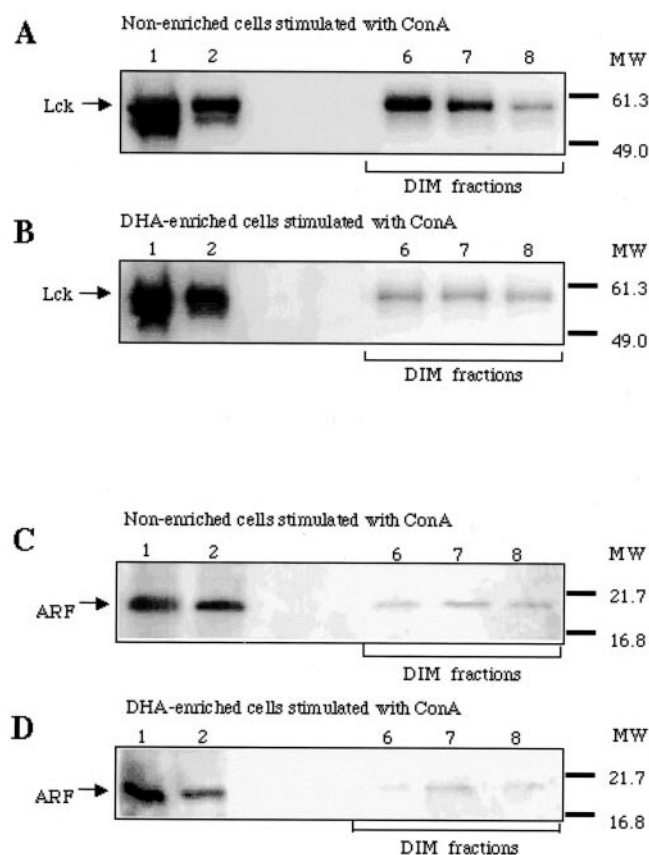
Fig. 9. **DHA enrichment of human PBMC displaces Lck and ARF proteins from detergent-insoluble microdomains.** Control or DHA-enriched (15 μM) PBMC were stimulated with ConA (5 μg/10^6 cells) before lysis and fractionation of the Triton X-100-treated lysates on a discontinuous sucrose density gradient. *A* and *B*, 20 μl of detergent-soluble (non-DIM) and detergent-insoluble (DIM) fractions were analyzed for Lck immunoreactivity. *C* and *D*, 400 μl of gradient fractions were precipitated in ice-cold acetone, and pellets were dissolved in 40 μl of Laemmli buffer and analyzed for ARF immunoreactivity. 5 (*lanes 1* and *2*) and 20 μl (*lanes 6–8*) of total Laemmli extracts were layered.

part of PKC proteins (at least the α, β, and ζ isoforms) were present in the cytosolic compartment, which further supports the lack of PKC involvement in this activation.

The observation that PLD activation was lost when DHA-enriched PBMC were depleted in GTP and small G proteins by permeabilization prior to ConA activation prompted us to investigate the potential involvement of ARF in this mechanism. Our results showing that the fungal metabolite BFA markedly inhibited the DHA-induced PLD activation (Fig. 5) suggest a role for active ARF proteins. Indeed, BFA has been shown to inhibit the exchange activity of several ARF guanine nucleotide-exchange factors, thus preventing the formation of active GTP-bound ARF (44, 45). By using an anti-ARF1–3 antibody in Western blotting experiments, we showed that DHA and ConA synergize to recruit ARF proteins to the particulate fractions of PBMC (Fig. 6). Furthermore, this increase of particulate ARF was dose-dependently related to the DHA enrichment of cell membranes, which further supports the role of ARF in DHA-induced PLD activation. The lack of any oleate-stimulated PLD activity in PBMC lysates together with the present results showing the involvement of ARF proteins strongly suggest that PLD1 was the main target of DHA in mitogen-activated PBMC. However, we cannot totally rule out PLD2 because human PLD2 has also been described as ARF-activable in some cells, although to a lesser extent than PLD1 (46). The present results are at variance with the commonly accepted inhibition of PIP$_2$-

dependent PLD activity by fatty acids (9) and with the results of Kim *et al.* (42) showing that recombinant hPLD2, but not hPLD1, was activated by fatty acids *in vitro*.

Several recent reports (10, 16, 17) have described the presence of PLD activity and/or proteins associated with caveolins in the caveolae of different cell lines. But whether PLD is present in the lipid rafts of cells devoid of caveolins was not known. Although lymphocytes do not express any detectable caveolin, detergent-insoluble rafts closely related to caveolae do exist in these cells where they play a crucial role in the transduction of the mitogenic signals (20, 21). Upon ligation of the T cell antigen receptor, a signaling cascade is initiated via activation of the Src family kinases present in lipid rafts. This, in turn, allows the recruitment of the Syk family kinases and the downstream activation of phospholipase Cγ and the extracellular signal-regulated kinase/mitogen-activated protein kinase cascade, while phosphatases such as CD45 become excluded from the rafts (20, 21). Most studies dealing with the role of lipid rafts in lymphocyte activation have been performed with murine thymocytes and cell lines (31, 33) or with human Jurkat cells (22, 47), and those using human peripheral lymphocytes are very scarce (48). After fractionation of the detergent-treated membranes from PBMC on sucrose density gradient, we used several biochemical markers to characterize glycolipid-enriched complexes floating at a low density during centrifugation. Besides GM1 which is widely used as a raft marker in hematopoietic cells (49), we chose the cell surface antigen CD73 as a glycosylphosphatidylinositol-anchored protein. This cell surface marker is widely distributed within the various B and T cell subpopulations and possesses an intrinsic 5'-nucleotidase activity that can be easily measured (39, 50). In addition, we also determined the localization of PIP$_2$ in the gradient fractions using a specific PIP$_2$ antiserum, because this signaling phospholipid is known to be present in detergent-insoluble, cholesterol-rich membranes (51, 52). The fractions of gradient recovered at a low density, between 15 and 20% sucrose, contained most of GM1, PIP$_2$, and 5'-nucleotidase activity, had a low phospholipid to cholesterol ratio and a very low protein content (Fig. 7) and thus fulfilled the main criteria for lipid rafts (49). An important result of the present study is that these low density fractions originating from cells devoid of caveolae also contain a large proportion of the PLD activity stimulated by rARF in the presence of GTPγS and a substantial part of PLD1 protein. In contrast, the basal PLD activity and PLD2 protein were mainly recovered in the high density fractions containing detergent-solubilized membranes. This allows us to conclude that in human PBMC, PLD1 is largely associated to rafts, as has been observed for caveolae-rich cells such as rat fibroblasts (15) and mouse skeletal myotubes (16), whereas PLD2 is mainly present in non-raft membranes. Because detergent-insoluble membranes isolated from cell lysates most probably originate from the plasma membrane (53), this suggests that a substantial part of PLD1 is located in this compartment. However some detergent-insoluble materials may also derive from intracellular membranes.

DHA enrichment of PBMC before raft isolation did not affect the distribution of GM1, PIP$_2$, or 5'-nucleotidase activity in the gradient fractions, while part of both GTPγS plus ARF-dependent PLD activity and PLD1 protein was delocalized to the high density fractions. The delocalization was almost total when DHA-enriched cells were also stimulated by ConA (Fig. 8). We also observed such a delocalization for Lck and ARF (Fig. 9). It is noteworthy that DHA enrichment of phospholipids was higher in DIM than in non-DIM membranes (Table I). This increase in polyunsaturated fatty acids at the expense of saturated palmitate is very likely to affect the physicochemical

properties and spatial organization of lipid rafts. The fact that alteration of the rafts by DHA had no effect on GM1 and 5′-nucleotidase distribution points out the specificity of DHA effect on PLD1. It is consistent with the results of Stulnig *et al.* (22, 54) showing that *n*-3 PUFAs affect the cytoplasmic face of the lipid leaflet in rafts.

It has been shown that the association of PLD with the caveolin-scaffolding domain inhibits PLD activity (15). On the basis of the present results, it can be proposed that PLD1 association to rafts also impairs its activity and that the DHA-induced PLD activation that we have observed in ConA-stimulated cells results from the PLD1 exclusion from rafts. PLD1 activation might conceivably result either from conformational changes of the enzyme switched to a different membrane environment or from the possibility for PLD1 to interact with ARF, which is present out of the rafts. In support of this hypothesis, DHA enrichment increased the formation of membrane-bound ARF, and this increase only concerns the detergent-soluble non-raft membrane fractions. Regarding the above hypotheses, an apparently discrepant result is that the DHA-induced increase in PLD activity observed in intact cells was no more evident when considering the PLD activity profiles of fractionation gradients. However, if PLD1 sequestration into rafts entails its maintenance under an inactive state due to interactions with the particular lipid environment or to interactions with inhibitory factors present in these structures, it is likely that raft isolation in the presence of detergent and sucrose disturbs their organization. This may in turn relieve the inhibitory constraints and allow the measurement of a PLD activity in DIM fractions of control PBMC, whereas this activity is masked in intact cells. As an alternative explanation, if PLD1 activation in intact cells results from its ability to interact with ARF caused by the DHA-induced displacement of the enzyme out of rafts and by the DHA-induced translocation of cytosolic ARF to non-raft membranes, it cannot be observed in broken cell assays where an excess of exogenous ARF is added. Indeed, these assay conditions are expected to induce a maximal activation of PLD1 trapped into DIMs, which should normally be inactive due to an insufficient amount of activated ARF.

Although further studies aiming at fully delineating the relationships between DHA-induced PLD activation and exclusion of PLD1 from raft microdomains are required, the novel mode of PLD activation described in this study is potentially important to explain the modulation of cell signaling by polyunsaturated fatty acids. Because PLD activation in lymphoid cells is known to transmit antiproliferative signals (55), the partial disorganization of lipid microdomains induced by DHA, leading to the exclusion of Src kinases and PLD1 from rafts, might impair the transduction of the mitogenic signal in DHA-enriched lymphocytes and participate in the immunodepressive effect of this fatty acid.

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