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-insoluble 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.

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(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 within the receptor-mediated aggregation of raft-associated proteins such as Src family kinases while excluding others such as tyrosine phosphatase, which initiates phosphorylation signaling cascades. Interestingly, in PUFAs–enriched T cells the Src kinases Lck and Fyn appeared to be excluded from rafts suggesting that PUFAs 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 François 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 minimize the contamination of platelets. PBMC were then adjusted to a concentration of 2 \times 10^6 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 trypsin 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 Ib IIa 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 \times 10^6 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 transmethylation was carried out at 100 °C in the reaction mixture at 90 min in screw-capped tubes. The reaction was terminated by the addition of acetone and water, and the mixture was then dried under nitrogen. The phosphatidylcholine (HIDPCC, 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 μl of ice-cold chloroform/methanol/HCl (1:1:0.002) and mixing. Phase separation was achieved by further addition of 0.1 v/v HCl, 10% TCA, 10% pyridine, and NaCl. The organic phase was collected, dried under nitrogen, and redissolved in chloroform/methanol containing standard phosphatidylethanolamine as a carrier. Phosphatidylethanolamine was separated on TLC using ethyl acetate/isooctane/acidic acid (9:5:2). Plates were stained with Coomassie Brilliant Blue R, and spots corresponding to phosphatidylethanolamine were scraped off and mixed with Picoflor (Packard Instrument Co.), and the radioactivity was determined by liquid scintillation counting. The radioactivity associated with phosphatidylethanolamines was expressed as percentage of the radioactivity of the substrate (HIDPCC).

P3TP2-dependent PLD activity was determined according to Brown et al. (25). Briefly, 10 μl of mixed lipid vesicles (phosphatidylthanolamine/caveolin/caveolin-phosphatidylcholine, molar ratio 16:1.5:1) with [choline-methyl-3H] dipalmitoylphosphatidylcholine (specific activity 2200 GBq/mmol, PerkinElmer Life Sciences) to give 10^6 cpm per assay was added to 40 μl of cell lysate in a total of 100 μl containing 50 mM Hepes, pH 7.5, 50 mM KCl, 2.5 mM MgCl2, 2 mM CaCl2, 1 mM dithiothreitol. Assays were done with or without 50 μM GDPγS 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 amplify both PLD1 and PLD2. PLD1 sense and antisense primers were 5′-GGGATCCCGTGTGAGGGAATCGTCTGAGGACCC G-3′ and 5′-GGGATCCCTGGTACCGGACTTCTGAGGACCC G-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. These sense and antisense primers were 5′-GGGATCCCGTGTGAGGGAATCGTCTGAGGACCC G-3′ and 5′-GGGATCCCGTGTGAGGGAATCGTCTGAGGACCC G-3′, respectively. Amplification conditions for PLD1 were 94 °C for 45 s, 60 °C for 45 s, and 72 °C for 45 s, and 72 cycles. Amplification conditions for PLD2 were 94 °C for 45 s, 57 °C for 45 s, and 72 °C for 45 s.
45 s, for 35 cycles. PCDNA3 plasmids carrying the hPLD1b and hPLD2 cDNAs originating from Dr. M. Frohman 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 × g for 10 min, resuspended in lysis buffer (20 mM Hepes, 25 mM sucrose, 0.1 mM EDTA, 0.05 mM phenylmethylsulfonyl fluoride, 10 units/ml aprotinin, 2 μg/ml peaptain 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 homogenate was centrifuged at 42,000 × g for 20 min. Particulate and cytosolic fractions (3 volumes) were mixed with 4× 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% (PKC) or 15% (ARF) acrylamide gel and electrotransferred 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× urea. Aspecific antibody-binding sites were saturated by incubating membranes in TBS-T (20 mM Tris, pH 7.5, 150 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). Aspecific binding was blocked with 10% nonfat dry milk 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 (ImagingMaster VDS-CL, Amersham Biosciences) and ImageQuant software.

PI2P was quantitated on dot blots performed as described above using a mouse PI2P 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 ferrihexacyanoferrate method according to Stewart (34).

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

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-stimulated cells compared with the corresponding control cells incubated in the presence of delipidated albumin (Fig. 1A). Gas chromatography analysis of the fatty acid composition of cell phospholipids showed that the relative amount of esterified DHA was concomitantly increased (Fig. 1B). Furthermore, PLD activity was significantly correlated to the percentage of DHA present in cell phospholipids (Fig. 1C, 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. 1D).

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. 2A, 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. 2B). 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. 2C, PBMC lysates did not show any oleate-stimulated PLD activity. In contrast, a marked synthesis of
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 GTPyS. 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 GTPyS addition due to the loss of cytosolic G proteins (Fig. 2D). 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 PKC$_\alpha$ 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 PKC$_\alpha$ expression. Results of Western blot experiments show that DHA enrichment of the cells dose-dependently decreased the PKC$_\alpha$ amount in the cytosolic fractions (Fig. 3A, left) while the amount in the particulate ones was maintained and even increased at the highest DHA concentration used (Fig. 3B, left). This pattern suggests that DHA favors PKC$_\alpha$ translocation in the absence of cell activation. In marked contrast, the PKC$_\alpha$ distribution pattern was totally different when PBMC were further stimulated with ConA after DHA enrichment (Fig. 3C, right). The large PKC$_\alpha$ translocation induced by ConA in non-enriched cells was substantially impaired in DHA-enriched cells. At the highest DHA concentration used for enrichment, PKC$_\alpha$ remained mainly cytosolic. Thus, under the conditions of maximal PLD activation (DHA plus ConA), the translocation of PKC$_\alpha$ 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. 1.** Effect of DHA enrichment on PLD activation and on the DHA content of total phospholipids from human PBMC. A, [³H]arachidonate-labeled PBMC were incubated for 2 h with 5 μg 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, [³H]arachidonate-labeled PBMC were incubated for 2 h with 5 μg HSA (control cells) or with 15 μg 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.
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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 × 10⁶ 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 × 10⁶ 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]DPPC in the absence (−) or presence (+) of 12 µM PIP₃, with or without 50 µM GTP-γS. [3H]Phosphatidylethanol was separated by TLC. D, aliquots of the 100,000 x g pellet fraction (from 8 × 10⁶ cells) were added to reaction mixtures containing substrate phospholipid vesicles (PE/PIP2/PC, 16:1.5:1) and incubated at 37°C for 30 min in the absence (−) or presence (+) of 50 µM GTP-γS with or without recombinant ARF. [3H]Choline released from dipalmitoylphosphatidylcholine-[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. 4A, 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 downregulation. Fig. 4B 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 downregulation (Fig. 4C). 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 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. 5A, 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...
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**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/ml) 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/ml) 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.

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. 5B, 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 μM, 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. 6A shows that the relative 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. 6B). 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. 7A, greater than 80% of the total proteins were recovered in the first three high density fractions containing from 36 to 31% sucrose (Fig. 7D). In con-
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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 ± S.D. of three separate experiments. Data were analyzed by ANOVA, and means were compared by a protected t test. *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.

In contrast, 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, PIP2 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 [3H]phosphatidylcholine, in mixed phospholipid vesicles including PIP2, 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 5% of total (Fig. 7D). PLD2 protein was then immunoprecipitated from each gradient fraction and analyzed by Western blotting using a PLD2-specific antiserum. A ~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, PIP2, 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 hydrolysed 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 (~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 PIP2 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 flotation in a discontinuous sucrose density gradient, and the fractions were analyzed for protein concentration, 5'-nucleotidase activity, GM1, PIP2, cholesterol, phospholipid content, and ARF plus GTP-S-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 PIP2 (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.

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 proteins in different leukemic cell lines. Thus, human myeloid leukemic HL60 have been shown to express only PLD1 and mouse lymphocytic leukemia 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 PIP2 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...
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

### Table I

| Fatty acids (FA) composition of total phospholipids of non-DIM and DIM membranes isolated from control and DHA-enriched PBMC |
|------------------|--------------------|--------------------|------------------|--------------------|
|                  | Non-DIM membranes  | DIM membranes      |                  |                   |
|                  | Control PBMC       | DHA-enriched PBMC  | Control PBMC     | DHA-enriched PBMC  |
|                  | mol %              | mol %              | mol %            | mol %              |
| 16:0             | 13.47 ± 0.35       | 14.14 ± 0.76       | 38.10 ± 0.45°    | 29.21 ± 6.81      |
| 18:0             | 19.31 ± 2.70       | 19.93 ± 1.98       | 16.45 ± 1.20     | 17.86 ± 0.67      |
| 18:1n-9          | 11.71 ± 0.47       | 11.91 ± 0.65       | 6.73 ± 0.56°     | 6.89 ± 0.76       |
| 18:2n-6          | 9.31 ± 1.30        | 9.43 ± 0.83        | 3.54 ± 0.42°     | 3.85 ± 0.50       |
| 20:0             | 0.16 ± 0.02        | 0.20 ± 0.01        | 1.59 ± 0.17°     | 1.80 ± 0.36       |
| 20:4n-6          | 25.55 ± 2.65       | 25.96 ± 1.39       | 9.32 ± 0.55°     | 11.86 ± 0.97      |
| 20:5n-3          | 0.58 ± 0.07        | 0.60 ± 0.06        | 0.18 ± 0.02°     | 0.25 ± 0.03       |
| 22:0             | 0.13 ± 0.06        | 0.27 ± 0.01        | 4.07 ± 0.19°     | 5.41 ± 0.95       |
| 22:5n-3          | 2.46 ± 0.24        | 2.53 ± 0.24        | 6.73 ± 0.66°     | 8.70 ± 1.14       |
| 24:0             | 0.05 ± 0.03        | 0.09 ± 0.04        | 1.67 ± 0.04°     | 2.84 ± 0.39°      |
| Σsat FA          | 33.15 ± 2.90       | 34.68 ± 1.40       | 63.42 ± 1.11°    | 58.46 ± 3.82      |
| Σn-6 FA          | 39.04 ± 4.52       | 39.63 ± 2.92       | 20.12 ± 0.94°    | 25.83 ± 1.97°     |
| Σn-3 FA          | 7.06 ± 1.13        | 7.84 ± 1.14        | 3.00 ± 0.18°     | 4.37 ± 0.53°      |

*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.
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 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 PIP3 in the gradient fractions using a specific PIP3 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, PIP3, 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, PIP3, 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 1). This increase in polyunsaturated fatty acids at the expense of saturated palmitate is very likely to affect the physicochemical

![Fig. 9. DHA enrichment of human PBMC displaces Lck and ARF proteins from detergent-insoluble microdomains.](image)
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 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 evident when considering the PLD activity profiles of fractionation gradients. However, if PLD1 sequestration into rafts disturbs their organization. This may in turn relieve the inhibitory constraints and allow the measurement of a PLD activity masked in intact cells. As an alternative explanation, if PLD1 changes of the enzyme switched to a different membrane environment based on the present results, it can be proposed that PLD1 caveolin-scaffolding domain inhibits PLD activity (15). On the relationships between DHA-induced PLD activation and exclusion of the rafts by DHA had no effect on GM1 and these assay conditions are expected to induce a maximal active of ARF to non-raft membranes, it cannot be observed in broken membranes. Regarding the above hypotheses, an apparently discrepant result is that the DHA-induced decrease 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 such interaction 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 masked in intact cells. As an alternative explanation, if PLD1 changes of the enzyme switched to a different membrane environment based on the present results, it can be proposed that PLD1 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 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 evident when considering the PLD activity profiles of fractionation gradients. However, if PLD1 sequestration into rafts disturbs their organization. This may in turn relieve the inhibitory constraints and allow the measurement of a PLD activity masked in intact cells. As an alternative explanation, if PLD1 changes of the enzyme switched to a different membrane environment based on the present results, it can be proposed that PLD1 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 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 evident when considering the PLD activity profiles of fractionation gradients. However, if PLD1 sequestration into rafts disturbs their organization. This may in turn relieve the inhibitory constraints and allow the measurement of a PLD activity masked in intact cells. As an alternative explanation, if PLD1 changes of the enzyme switched to a different membrane environment based on the present results, it can be proposed that PLD1 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 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 evident when considering the PLD activity profiles of fractionation gradients. However, if PLD1 sequestration into rafts disturbs their organization. This may in turn relieve the inhibitory constraints and allow the measurement of a PLD activity masked in intact cells. As an alternative explanation, if PLD1 changes of the enzyme switched to a different membrane environment based on the present results, it can be proposed that PLD1 caveolin-scaffolding domain inhibits PLD activity (15). On the