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Phospholipase D as a potential target for the antiproliferative effects of polyunsaturated fatty acids in rat thymocytes

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Abstract

The effects of various saturated and unsaturated fatty acids (FAs)on the proliferative response and phospholipase D (PLD) activity of rat thymocytes were investigated. When added to culture medium as complexes with albumin, all the FAs tested, except stearic acid, inhibited the ConA-induced thymocyte proliferation, eicosapentaenoic (20:5n-3) and docosahexaenoic (22:6n-3) acids being the most inhibitory. Apart from 22:6n-3 which slightly increased the percentage of late apoptotic and necrotic thymocytes in the presence of mitogen, none of the FAs induced significant apoptosis or necrosis. A short 2-h preincubation of rat thymocytes in the presence of FA–albumin complexes was sufficient to induce a significant enrichment of cell phospholipids with each FA and to stimulate thymocyte PLD activity. However, 20:5n-3 was inactive despite a large enrichment in phospholipids. Furthermore, the PLD activity of activated thymocytes was negatively correlated to the proliferative response, with the exception of 20:5n-3-supplemented cells. These results support further our current hypothesis that PLD activity conveys antiproliferative signals in lymphoid cells, and suggest that 20:5n-3 inhibits thymocyte proliferation by a particular mechanism unrelated to that of the other FAs.

Keywords: Phospholipase D; Rat thymocyte proliferation; Immune response; n-3 fatty acids; n-6 fatty acids

1. Introduction

It is now well recognized that dietary fats affect immune functions both in laboratory animals and humans [1]. High-fat diets generally suppress lymphocyte functions compared with low-fat diets, but the extent of impairment depends on the level and type of fat. Thus, saturated and n-6 poly-unsaturated fatty acids (PUFAs) are usually described as inactive or weakly active on lymphocyte proliferation, cytokine production or natural killer cell activity, whereas n-3 PUFAs have marked inhibitory effects [1,2]. In contrast, when fatty acids (FAs) are exogenously added to lymphocyte culture medium, most of them inhibit the mitogen-stimulated proliferation of lymphocytes isolated from rodent lymphoid tissues or human peripheral blood [2]. Among the various mechanisms that have been proposed to explain their immunosuppressive effects, alterations of mitogenic signaling pathways have received increasing attention during the past few years [1,3,4]. In this regard, phospholipase D (PLD) that catalyzes the hydrolysis of phosphatidylcholine (PC) to generate the second messenger phosphatidic acid (PA) [5,6] might be a relevant target of FAs. Indeed, we have previously shown that docosahexaenoic acid (22:6n-3) [7,8] or the monohydroxylated derivative of arachidonic acid (20:4n-6), 12-hydroxy eicosatetraenoic acid (12-HETE) [9] stimulated the PLD activity of ConA-stimulated human lymphocytes. Several other groups have reported PLD-dependent FA effects in neutrophils [10], smooth muscle cells [11] or macrophages [12]. The functional consequences of PLD activation on mitogenic signaling clearly depends on the cell type considered. Although in most cell types PLD activation seems to favor cell proliferation, opposite effects

Abbreviations: 7-AAD, 7-aminoactinomycin; ConA, concanavalin A; 22:6n-3, docosahexaenoic acid; 20:5n-3, eicosapentaenoic acid; 22:5n-3, docosapentaenoic acid; FA, fatty acid; FCS, fetal calf serum; PA, phosphatidic acid; PC, phosphatidylcholine; PUFA, polyunsaturated fatty acid; HSA, human serum albumin; 18:2n-6, linoleic acid; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; 14:0, myristic acid; 18:1n-9, oleic acid; 16:0, palmitic acid; PLD, phospholipase D; 18:0, stearic acid.

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are observed in lymphoid cells. The mitogenic activation of normal lymphocytes is known to induce a large increase in PA level occurring during the first minutes of stimulation. However, this early peak can be attributed to PA derived from diacylglycerols (DAG) through the sequential activation of phospholipase C-γ and DAG kinase [13,14]. In contrast, when lymphocyte membranes were enriched with docosahexaenoate or 12-HETE prior to mitogenic stimulation, PLD was activated, leading to an inhibition of the proliferation response. The hypothesis that PLD is antiproliferative in lymphoid cells has been strongly reinforced by our recent work showing that overexpression of the PLD1 isoform in Jurkat cells inhibits IL-2 mRNA expression in response to phorbol 12-myristate 13-acetate (PMA) and ionomycin activation [15].

The aim of the present study was to address the question of a possible link between the effects of various saturated and unsaturated FAs on the proliferative response of rat thymocytes and their effects on cellular PLD activity.

2. Material and methods

2.1. Preparation of rat thymocytes

Thymus glands were removed immediately and placed into 0.15 mol/L NaCl. They were cleaned of adherent connective tissue and dilacerated gently in a loosely fitting glass/glass homogeniser. Tissue remnants were removed by filtering the cells through nylon gauze. Thymic lymphocytes were separated by Ficoll-Hypaque gradient centrifugation. The cells were then washed with RPMI 1640 medium. All steps were conducted at room temperature. Under such conditions, cell viability was consistently >95%, as revealed by the Trypan blue exclusion test. Cells were suspended at a concentration of 4×10^7 cells/ml, and cell suspensions were incubated for 60 min at 37°C before starting the experiments.

2.2. Preparation of FA–albumin complexes

For thymocyte proliferation assays, different amounts of ethanolic solutions containing the different FAs were evaporated to dryness under reduced pressure. Decomplemented fetal calf serum (FCS) (Gibco, Cergy Pontoise, France) was added to give final FA-to-albumin ratio of 0.5, 1, 1.5, 2 and 3. The mixtures were incubated overnight under nitrogen at 37°C. For thymocyte PLD assays, different amounts of ethanolic solutions containing the different FAs were evaporated to dryness under reduced pressure. Delipidated human serum albumin (HSA) 5 μmol/L in RPMI 1640 medium was added to give final FA concentrations of 5 and 15 μmol/L corresponding to FA-to-albumin ratio of 1 and 3. The mixtures were incubated under nitrogen for 4 h at 37°C.

2.3. Cell proliferation

Thymic lymphocytes were cultured in 96-well culture plates at a cell density of 2.5×10^6 cells/ml in a final volume of 110 μL of RPMI 1640 supplemented with 2 mmol/L glutamine, 0.1 mg/ml penicillin/streptomycin and 10% (v/v) FCS previously enriched with the different FAs, as described above, or nonenriched FCS. At the initiation of culture, 2.5 μg/ml of concanavalin A (ConA) (Sigma-Aldrich, Saint Quentin Fallavier, France) was added to the cell suspensions. Control cells were incubated in the same conditions without mitogen. Cultures were incubated at 37°C in a humidified air/CO_2 (95/5) atmosphere. After 68-h incubation, cell proliferation was measured by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) colorimetric assay (Roche Diagnostics, Meylan, France) as described previously by Mosmann [16]. Briefly, 10 μL of the MTT labeling reagent (0.5 mg/ml final concentration) was added to each well. The cells were incubated for further 4 h. Solubilization solution 100 μL was then added, and plates were allowed to stand overnight at 37°C in a humidified atmosphere. The optical density (OD) was measured the next day at 550- and 690-nm wavelength using an enzyme-linked immunosorbent assay plate reader (PowerWave X; Biotek Instruments, Winooski, VT). The number of viable cells was directly correlated to the difference of absorbance measured at 550 and 690 nm. Cell proliferation was measured as the difference of absorbance between assays with and without ConA. Results were normalized relative to their respective controls (thymocytes incubated with nonenriched FCS) taken as 100.

2.4. Thymocyte apoptosis analyses

Thymocyte apoptosis/necrosis was examined using annexin-V staining to label externalized phosphatidylserine and 7-aminoactinomycin (7-AAD) to label necrotic cells, as previously described by Durant et al. [17]. Thymic lymphocytes were cultured as described above in the presence of FA-enriched or nonenriched (controls) FCS, with or without ConA for 68 h. Then, cells were washed with RPMI. Aliquots of cell suspensions (5×10^5 cells) were suspended in 100 μL of annexin-V binding buffer. 7-AAD 10 μL and 5 μL of phycoerythrin (PE)-conjugated annexin-V (Becton Dickinson, Le Pont de Claix, France) were added for 15 min at room temperature in the dark. Cells were resuspended in 400 μL of annexin-V binding buffer. Annexin-V-PE (FL2) and 7-AAD (FL3) labeling were analyzed by flow cytometry (FACSCalibur cytofluorometer, Becton Dickinson). Single positive annexin-V+/7-AAD− and annexin-V−/7-AAD+ cells are considered as early apoptotic and dead cells, respectively, whereas double-positive annexin−V+/7-AAD+ cells are thought to be in a late stage of apoptosis. Thymocytes treated with dexamethasone (0.4 μg/ml for 16 h) and hydrogen peroxide (10 mmol/L for 16 h) were used as apoptosis and necrosis controls, respectively [18].

2.5. Thymocyte PLD assay

PLD was determined on the basis of its transphosphatidylation activity. Thymocytes were first labeled with

[^{3}H]arachidonic acid (37 kBq/ml, specific activity 7400 GBq/mmol; Amersham Biosciences, Orsay, France) for 1 h at 37°C. After extensive washing, labeled cells suspended at a density of 4×10^7 cells/ml were incubated with the different FA–HSA complexes for 2 h at 37°C. Control cells were incubated in the same conditions with HSA alone. In some experiments, cells were incubated with linoleic acid (18:2n-6)–HSA or eicosapentaenoic acid (20:5n-3)–HSA complexes (FA/HSA ratio=3) or with HSA alone for 16 h, then labeled with [^{3}H]arachidonic acid and washed to eliminate unincorporated radioactivity prior to PLD activity measurement. For PLD assays, cell suspensions were incubated for 20 min at 37°C in the presence or absence of 1% 1-butanol. ConA 1 μg per 10^6 cells (or RPMI) was added, and cell suspensions were further incubated for 5 min at 37°C. Incubations were terminated by addition of ethanol and acidification of the medium to pH 3–4 with 2 mol/L HCl. Lipids were extracted with chloroform/ethanol (6/3, v/v) according to Boukhchache and Lagarde [19] in the presence of 50 μmol/L butylhydroxylated toluene (BHT). Phosphatidylbutanol (PBut) was separated on bidimensional thin layer chromatography (Silica Gel G60 plates, Merck, Darmstadt, Germany) using chloroform/methanol/28% ammonia (65/35/5.5, v/v) for the first migration, and ethyl acetate/isooctane/acetic acid (9/5/2, v/v) for migration in the second dimension. Spots stained by Coomassie Brilliant Blue R were scraped off, mixed with Picofluor (PerkinElmer, Courtaboeuf,

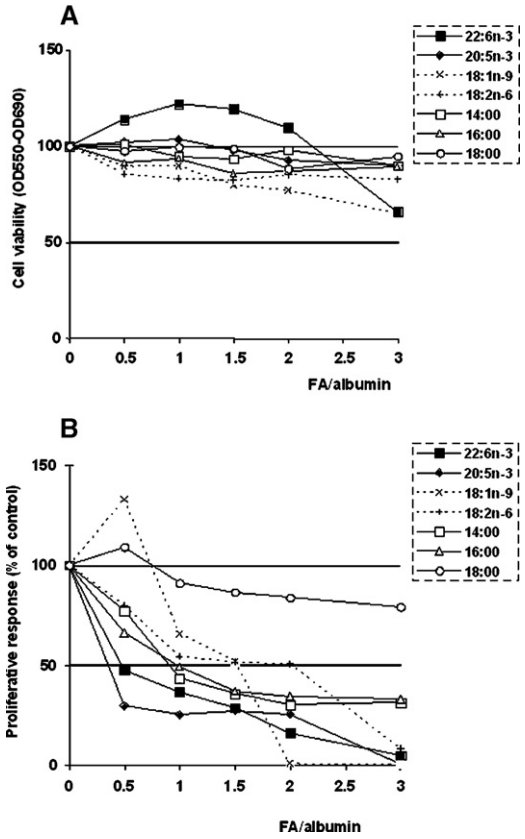

Fig. 1. In vitro effects of FAs on thymocyte viability and proliferation. Thymocytes were cultured in 96-well microtitre plates (2.5×10^5 cells/well) with FAs complexed to the albumin of FCS at different FA-to-albumin ratio ranging from 0.5 to 3 (15 to 90 μmol/L FAs) in the absence (A) or presence of 2.5 μg/ml ConA (B). After 68 h of culture, cells were incubated with 10 μL of MTT solution for 4 h and solubilized according to the manufacturer's recommendations. Absorbances were monitored at 550 and 690 nm. (A) Cell viability measured in the absence of ConA as the difference of absorbances ($A_{550}-A_{690\ nm}$). and normalized relative to controls without FA taken as 100. (B) Cell proliferation measured as the difference of absorbance between assays with and without ConA and normalized relative to controls without FA taken as 100. Results are means of three separate experiments performed with six replicates. For clarity purpose, S.E.M. less than 10% of the means are not shown on the graphs.

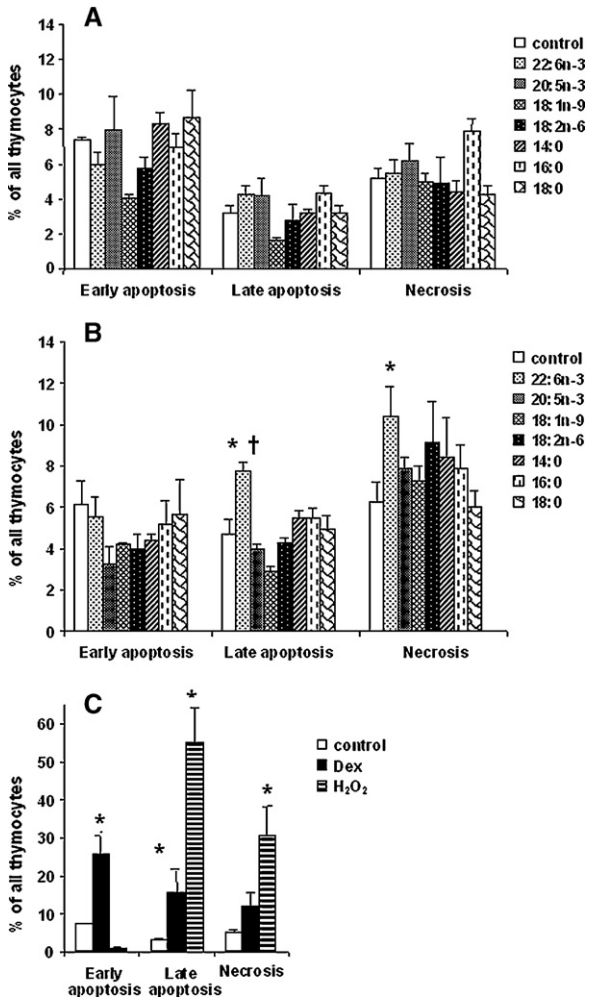

Fig. 2. In vitro effects of FAs on thymocyte apoptosis and necrosis. Thymocytes were cultured in 96-well microtitre plates (2.5×10^5 cells/well) with FAs complexed to the albumin of FCS at a FA-to-albumin ratio of 3 in the absence (A) or presence of 2.5 μg/ml ConA (B). After 68 h of culture, cells were washed with RPMI, resuspended in annexin-V binding buffer and labeled with 7-AAD and annexin-V, as described in Materials and methods. The histograms show the relative proportions of thymocytes in different phases of apoptosis/necrosis in unstimulated (A) and ConA-stimulated thymocytes (B). Results are means of two separate experiments performed in duplicate. *Significantly different from control nonenriched thymocytes; †Significantly different from all other FA-enriched thymocytes ($P<.05$). (C) Thymocytes were incubated for 16 h in the presence of 0.4 μg/ml dexamethasone or 10 mmol/L hydrogen peroxide before washing and labeling with 7-AAD and annexin-V. *Significantly different from control thymocytes ($P<.05$).

France), and the radioactivity of the spots corresponding to PBut was determined by liquid scintillation counting. PLD activity was expressed as the percentage of total phospholipid radioactivity incorporated into PBut.

2.6. Gas chromatography analyses of the FA composition of thymocyte phospholipids

The FA enrichment of thymocyte phospholipids, following the 2 h incubation with the various FA–HSA complexes, was determined by gas chromatography. Thymocyte total lipids were extracted with chloroform/methanol (2/3, v/v) in the presence of 50 μmol/L of BHT according to the method of Bligh and Dyer [20] and separated on silica gel G 60 plates (Merck, Darmstadt, Germany) with the solvent system hexane/diethyl ether/acetic acid (70:30:1, 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 under an N_2 atmosphere at 100°C for 90 min in the screw-capped tubes. The reaction was terminated by the addition of 1.5 volume ice-cold 5% (w/v) K_2CO_3, and the FA 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°C to 160°C at 2°C/min and from 160°C 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 psi (5520 Pa). Peaks were identified using standard FA methyl esters.

2.7. Statistical methods

Values are presented as means± S.E.M. of *n* independent experiments. Data were compared by analysis of variance (ANOVA) (Statview II for Macintosh), followed by protected *t* test. *P* values of .05 or less were considered statistically significant. For PLD measurements, data were evaluated by a two-way ANOVA. Calculated *F* values are given in the legends of Fig. 2. They were considered as significant when the calculated *P* value was less than .05.

3. Results

3.1. In vitro effects of FAs on thymocyte proliferation

We first examined the effects of the different FAs, added to culture medium as complexes with the albumin of FCS, on thymocyte proliferation. In preliminary experiments, we observed that a concentration of 2.5 μg ConA/ml gave maximal proliferation (not shown). This ConA concentration was used in subsequent experiments. For each FA concentration, parallel assays were performed without mitogen, which allowed to estimate the influence of FAs on cell viability at the end of the culture period. As shown in Fig. 1A, none of the FAs significantly affected cell viability in the absence of mitogen up to a FA-to-albumin ratio of 2. At a higher ratio (FA/albumin=3), only oleic acid (18:1n-9) significantly decreased cell viability by 35%, the lowering effect of 22:6n-3 being not significant. The overall lack of cytotoxicity of FAs was confirmed by annexin-V and 7-AAD labeling experiments. After 68 h of culture, none of the FAs significantly modified the percentages of early apoptotic, late apoptotic and necrotic cells, as compared with unstimulated control cells incubated in the presence of nonenriched serum (Fig. 2A). All FAs, except stearic acid (18:0), significantly inhibited ConA-induced thymocyte proliferation ($P \leq .05$) (Fig. 1B). Largest inhibitions were observed for FAs from the n-3 family whatever the FA-to-albumin ratio considered. From data shown on Fig. 1B, the FA-to-albumin ratios giving 50% inhibition of thymocyte proliferation can be extrapolated as follows: 20:5n-3, 0.35; 22:6n-3, 0.5; myristic acid (14:0), 0.9; palmitic acid (16:0), 1; 18:1n-9, 1.5 and 18:2n-6,

Table 1A
FA composition (mol %) of thymocyte phospholipids after enrichment with n-3 FAs

FAs*	Control	22:6n-3		20:5n-3	
		FA/HSA=1	FA/HSA=3	FA/HSA=1	FA/HSA=3
14:0	0.58±0.12	0.72±0.47	0.93±0.04	0.85±0.47	0.88±0.11
16:0	30.12±0.55	29.47±0.42	31.31±0.85	29.89±1.40	29.18±1.68
18:0	19.86±0.30	19.41±0.28	18.64±0.15	19.46±0.40	19.06±0.22
18:1n-9	10.69±0.24	10.89±0.28	10.41±0.22	10.29±0.35	10.15±0.32
18:2n-6	7.35±0.28	7.70±0.97	7.74±0.98	6.76±0.35	6.66±0.26
20:4n-6	23.29±0.55	23.16±0.97	22.01±1.36	23.26±1.15	23.60±1.10
20:5n-3	$0.07±0.03^{a}$	$0.12±0.06^{a}$	$0.16±0.01^{a}$	$1.33±0.15^{b}$	$2.67±0.27^{c}$
22:5n-3	$0.10±0.03^{a}$	$0.10±0.05^{a}$	$0.05±0.05^{a}$	$0.24±0.12^{b}$	$0.40±0.06^{b}$
22:6n-3	$0.49±0.07^{a}$	$0.80±0.23^{ab}$	$1.34±0.38^{c}$	$0.42±0.06^{a}$	$0.44±0.06^{a}$
SFA	50.55±0.56	49.60±0.57	50.88±0.98	50.20±1.80	49.12±1.95
∑n-6 PUFA	30.65±0.41	30.85±0.37	29.80±1.30	30.02±1.46	30.26±1.30
∑n-3 PUFA	$0.64±0.10^{a}$	$1.02±0.31^{abc}$	$1.55±0.32^{b}$	$2.00±0.32^{c}$	$3.50±0.37^{d}$
∑monounsat	18.15±0.30	18.87±0.40	17.81±0.34	17.78±0.20	17.12±0.39
n-6/n-3	$48.86±5.24^{a}$	$36.50±10.83^{ab}$	$21.31±5.22^{bc}$	$15.43±2.17^{bc}$	$8.10±0.13^{c}$

Values are means± S.E.M. ($n=3$). Different superscript letters denote significant differences ($P<.05$). If no superscript appears in a row, the values are not statistically different. ND, not detected; SFA, saturated fatty acids; ∑monounsat=∑18:1n-9+18:1n-7 (not shown).

* Only selected fatty acids are presented.

Table 1B
FA composition (mol %) of thymocyte phospholipids after enrichment with 18:1n-9 or 18:2n-6

FAs*	Control	18:1n-9		18:2n-6	
		FA/HSA=1	FA/HSA=3	FA/HSA=1	FA/HSA=3
14:0	0.58±0.12	0.10±0.10	0.73±0.16	0.88±0.10	0.78±0.14
16:0	30.12±0.55	29.52±0.36	29.48±1.75	31.16±1.69	29.74±2.09
18:0	19.86±0.32	19.03±0.65	18.77±0.35	18.78±0.64	18.84±0.38
18:1n-9	10.70±0.24	11.02±0.37	11.49±0.68	10.89±1.00	11.10±1.01
18:2n-6	7.35±0.28[a]	7.81±0.12[abc]	7.03±0.42[ac]	8.83±0.89[b]	9.33±0.62 [b]
20:4n-6	23.29±0.55	24.08±1.15	24.40±0.72	21.00±0.71	21.74±0.22
20:5n-3	0.07±0.03	0.23±0.13	0.17±0.03	0.13±0.06	0.14±0.07
22:5n-3	0.10±0.03	0.29±0.06	0.18±0.02	0.13±0.06	0.13±0.07
22:6n-3	0.48±0.07	0.51±0.01	0.42±0.05	0.46±0.05	0.52±0.04
SFA	50.55±0.56	48.65±0.19	48.99±1.64	50.84±1.90	49.35±2.08
∑n-6 PUFA	30.65±0.41	31.90±1.03	31.44±1.10	29.82±0.72	31.04±0.67
∑n-3 PUFA	0.64±0.10	1.03±0.19	0.76±0.08	0.71±0.17	0.79±0.15
∑monounsat	18.15±0.30	18.43±0.65	18.81±0.65	18.64±1.12	18.79±1.30
n-6/n-3	48.87±5.23	32.45±7.12	42.38±3.43	50.07±15.96	42,27±8.35

Values are means± S.E.M. ($n=3$). Different superscript letters denote significant differences ($P<.05$). If no superscript appears in a row, the values are not statistically different. ND, not detected; SFA, saturated fatty acids; ∑monounsat=∑18:1n-9+18:1n-7 (not shown).

* Only selected fatty acids are presented.

2. It is noteworthy that at the highest FA-to-albumin ratio tested (FA/albumin=3), all the unsaturated FAs were more inhibitory than saturated ones, whereas at ratios lower than 2, 14:0 and 16:0 were more active than 18:1n-9 and 18:2n-6. Interestingly, in the presence of ConA and for an FA-to-albumin ratio of 3, only 22:6n-3 significantly increased the percentage of late apoptotic (from 4.7% to 7.7%) and necrotic (from 6.2% to 10.4%) cells, as compared to control cells incubated in the absence of FA (Fig. 2B). It is noteworthy that 20:5n-3, which was the most potent inhibitor of ConA-induced thymocyte proliferation, did not induce any apoptosis or necrosis.

3.2. In vitro effects of FAs on thymocyte phospholipase D activity

Radiolabeled thymocytes were first incubated with the different FAs complexed to albumin (ratio, 1 and 3) for 2 h, then stimulated or not with ConA for 5 min and PLD activity was determined in unstimulated and ConA-stimulated thymocytes. As shown in Tables 1A, 1B, 1C, all the FAs except 18:1n-9 and 18:0 were efficiently incorporated in phospholipids at the end of the preincubation period. For the FA-to-albumin ratio of 1, the relative amount of the considered FA was increased by 1.2-fold for 18:2n-6, 19-fold for 20:5n-3, 1.6-fold for 22:6n-3, and by 1.7-fold for 14:0. At the highest ratio of 3, the relative increases were 1.3-fold for 18:2n-6, 38-fold for 20:5n-3, 2.7-fold for 22:6n-3, and 4.6-fold for 14:0. It is noteworthy that 20:5n-3 was also efficiently elongated to docosapentaenoic acid (22:5n-3) by thymocytes during the 2-h incubation, which induced a drastic lowering of the ratio of n-3 to n-6 in phospholipids.

Evaluation of PLD data (Fig. 3) by a two-way analysis of variance indicates that 22:6n-3, 18:2n-6 and 14:0 significantly increased PLD activity ($P=.0001$, .0002 and .027,

Table 1C
FA composition (mol %) of thymocyte phospholipids after enrichment with saturated FAs

FAs*	Control	14:0		18:0	
		FA/HSA=1	FA/HSA=3	FA/HSA=1	FA/HSA=3
14:0	0.58±0.12[a]	1.01±0.23[b]	2.65±0.27[c]	0.68±0.10[ab]	0.47±0.14[ab]
16:0	30.12±0.55[a]	25.43±1.54[b]	26.10±1.38[b]	27.46±1.68[ab]	25.08±2.09[ab]
18:0	19.86±0.33	19.06±1.07	18.60±0.74	19.56±0.65	20.55±0.39
18:1n-9	10.70±0.24	11.40±1.13	10.76±0.89	11.23±1.00	10.99±1.02
18:2n-6	7.35±0.28	7.84±0.41	7.73±0.43	7.61±0.89	7.67±0.62
20:4n-6	23.29±0.55	27.35±1.05	26.29±1.27	26.43±0.73	28.11±0,24
20:5n-3	0.07±0.03	ND	0.08±0.08	ND	ND
22:5n-3	0.10±0.03	ND	ND	ND	ND
22:6n-3	0.49±0.07	0.39±0.20	0.35±0.18	0.34±0.05	0.39±0.04
SFA	50.55±0.56[a]	45.51±0.70[b]	47.34±0.81[b]	47.69±1.89[ab]	46.11±2.07[ab]
∑n-6 PUFA	30.65±0.41	35.19±0.80	34.02±0.93	34.04±0.74	35.79±0.70
∑n-3 PUFA	0.64±0.10	0.58±0.04	0.65±0.16	0.34±0.11	0.39±0.08
∑monounsat	18.15±0.30	19.30±0.60	18.20±0.60	17.92±1.12	17.71±1.31
n-6/n-3	48.87±5.23	61.20±6.72	57.19±16.40	99.18±12.88	90,84±5.25

Values are means± S.E.M. ($n=3$). Different superscript letters denote significant differences ($P<.05$). If no superscript appears in a row, the values are not statistically different.

* Only selected fatty acids are presented.

respectively), and that ConA was effective to increase PLD activity in the presence of each FA ($P \leq .001$). The effect of 18:1n-9 did not reach statistical significance, whereas both 20:5n-3 and 18:0 were inactive. It is noteworthy that 20:5n-3 did not modify the PLD activity of rat thymocytes despite a very large enrichment in cell phospholipids. For 18:2n-6 which effectively stimulated PLD activity and 20:5n-3 which proved to be inactive, we have verified that longer incubation in the presence of the FA–albumin complexes did not modify the pattern of PLD activation. Thus, ConA-stimulated thymocytes enriched in 18:2n-6 for 2 h or for 16 h exhibited comparable increases in PLD activity (5.6-fold vs. 4.6-fold, respectively), as compared to unstimulated control thymocytes. Besides, 20:5n-3 remained inactive on PLD activity after 16 h of enrichment (1.7-fold vs. 1.6-fold increase with respect to unstimulated control values, after 2 h and 16 h of enrichment, respectively). Interestingly, PLD activity of ConA-stimulated thymocytes, with the exception of 20:5n-3-supplemented cells, was negatively correlated to the proliferative response (Fig. 4). These results support further our current hypothesis that PLD activity conveys antiproliferative signals in lymphoid cells [8,15] and suggest that 20:5n-3 inhibits thymocyte proliferation by a particular mechanism unrelated to that of the other FAs.

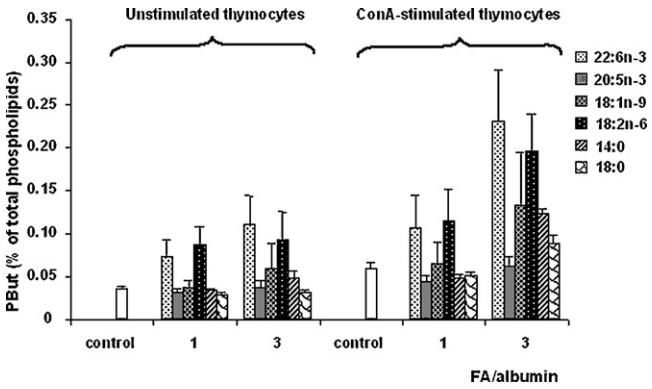

Fig. 3. In vitro effects of FAs on thymocyte phospholipase D activity. [^{3}H]-arachidonate-labeled thymocytes suspended at a cell density of 40×10^6 cells/ml were incubated with FA–albumin complexes (FA-to-albumin ratio of 1 and 3 corresponding to 5 and 15 μmol/L FA) or with albumin alone (control) for 2 h at 37°C. 1-butanol (1% final concentration) was added, and cell suspensions were further incubated for 20 min. Cells were then stimulated or not with 1 μg of ConA per 10^6 cells for 5 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 in Materials and methods. The radioactivity associated to PBut was measured by liquid scintillation counting. Results are expressed as percentages of total phospholipid radioactivity incorporated into PBut and are means±S.E.M. of three separate experiments. Data were evaluated by a two-way ANOVA. For 22:6n-3 data, F for 22:6n-3 effect was 12.90 (22:6n-3 vs. control: $P=.0001$) and F for the effect of ConA was 18.31 (basal vs. ConA-stimulated: $P=.0002$). For 20:5n-3 data, F for 20:5n-3 effect was 0.44 (20:5n-3 vs. control: non significant) and F for the effect of ConA was 8.94 (basal vs. ConA-stimulated: $P=.007$). For 18:1n-9 data, F for 18:1n-9 effect was 2.38 (18:1n-9 vs. control: non significant) and F for the effect of ConA was 18.94 (basal vs. ConA-stimulated: $P=.002$). For 18:2n-6 data, F for 18:2n-6 effect was 15.53 (18:2n-6 vs. control: $P=.0002$) and F for the effect of ConA was 24.13 (basal vs. ConA-stimulated: $P<.0001$). For 14:0 data, F for 14:0 effect was 4.33 (14:0 vs. control: $P=.027$) and F for the effect of ConA was 23.02 (basal vs. ConA-stimulated: $P=.0001$). For 18:0 data, F for 18:0 effect was 0.30 (18:0 vs. control: P=non significant) and F for the effect of ConA was 9.11 (basal vs. ConA-stimulated: $P<.007$).

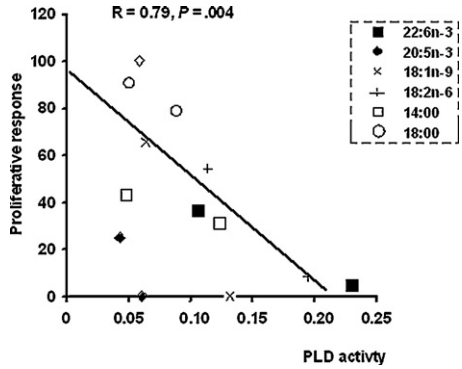

Fig. 4. Inverse linear relationship between the PLD activity of ConA-stimulated thymocyte and ConA-induced proliferation (data from Figs. 1 and 2; $P=.004$, $r=.79$, $n=10$).

4. Discussion

Although unsaturated free FAs have been shown to induce death of a variety of cell types including lymphocytes [21], rat thymocytes proved to be relatively resistant to this treatment. Indeed, when FAs were delivered to the thymocyte culture medium complexed to albumin, none of them decreased cell viability by more than 23% after 68 h of culture up to a FA-to-albumin ratio of 2. It is noteworthy that this ratio corresponds to an effective free FA concentration in the nanomolar range [22]. Interestingly, when thymocytes were cultured in the presence of 22:6n-3 delivered at a FA-to-albumin ratio lower than 2, the number of live cells was even higher than that observed in control cultures. These data suggest that 22:6n-3 is able either to protect thymocytes against natural cell death or to stimulate spontaneous cell growth. At the highest FA-to-albumin ratio of 3, only 18:1n-9 significantly reduced the number of live cells by 35%, as estimated by the MTT colorimetric assay. However, even 18:1n-9 did not increase the percentages of apoptotic or necrotic thymocytes above those of control thymocytes incubated without FAs. This discrepancy might be explained either by an interference of high 18:1n-9 concentration with the MTT test or to effects of the FAs on cell metabolism.

Among the FAs tested, both 20:5n-3 and 22:6n-3 were the most potent to inhibit the ConA-induced thymocyte proliferation, whereas 18:0 was the least efficient whatever the FA-to-albumin ratio. These results are in line with those previously reported in lymphocytes isolated from various rodent lymphoid tissues [23,24] and from human peripheral blood ([25–27]; for review, see Ref. [2]). Although saturated FAs are usually reported to have little effect on in vitro lymphocyte proliferation [1,2], surprisingly, both 14:0 and 16:0 were more inhibitory than 18:1n-9 or 18:2n-6

at FA-to-albumin ratio ≤2 (corresponding to 60 μmol/L FA). At the highest ratio of 3, all unsaturated FAs were more inhibitory than the saturated ones, as usually expected. Interestingly, when added to ConA-stimulated thymocytes, only 22:6n-3 slightly increased the percentage of late apoptotic and necrotic cells, suggesting that apoptosis/necrosis contributed for a small part to the observed inhibition of lymphoproliferation. In contrast, for all the other FAs, the decreased lymphocyte responses that we observed could actually be attributed to an inhibition of cell proliferation and not to FA toxicity. Controversial results ranging from inhibition to stimulation have been reported concerning the effects of FAs on cell apoptosis [28]. These inconsistencies can be attributed to differences in methods used to evaluate apoptosis, concentrations of FAs and serum and the cell models used. Indeed, it has been shown that normal human lymphocytes are, by far, more resistant to the apoptotic effects of FAs than transformed cell lines such as Jurkat T or Raji B cells [29]. Although highly sensitive to the apoptotic effects of dexamethasone and to the necrotic effects of hydrogen peroxide, as shown in Fig. 2C, rat thymocytes proved to be only marginally sensitive to the apoptotic effects of FAs.

A short 2-h incubation of rat thymocytes with individual FAs complexed to defatted HSA (ratios 1 and 3) was sufficient to induce incorporation of the respective FA in cell phospholipids, with the exception of 18:0 and 18:1n-9, which were barely detectable in the supplemented cells after the preincubation period. Zurier et al. [30] have also reported significant incorporation of FAs in unstimulated human lymphocytes during a short 15-min incubation period. The lack of significant incorporation of 18:0 and 18:1n-9 in thymocyte phospholipids contrasts with the huge increase in the proportion of 20:5n-3 in the corresponding supplemented cells. These results are at variance with those of Calder et al. [31], showing that in cervical lymph node lymphocytes, all FAs were incorporated at a similar low rate in the absence of mitogen. They could be explained when considering the mechanisms of phospholipid remodelling in these cells. Indeed, rodent thymocytes are known to possess an efficient acyl CoA:lysophosphatidate acyl transferase. This enzyme can operate in reverse to cleave fatty acyl moieties from the glycerol backbone to generate lysophospholipids, which can then be reacylated [32]. The acylation of phospholipids by this pathway is known to be faster with CoA esters of 20-carbon polyunsaturated FAs than with unsaturated 18-carbon acyl CoA, whereas saturated acyl CoA are relatively poor substrates of the enzyme [33]. Among the FAs tested, 22:6n-3 and 18:2n-6 were the most efficient to potentiate the PLD stimulating effect of ConA. Since early reports showing that unsaturated FAs, but not the saturated ones, stimulated the activity of heart sarcolemmal PLD [34] or recombinant PLD2 in cell-free assays [35], only few reports have addressed the question of FA effects on the PLD activity of intact cells. For example, the treatment of smooth muscle cells isolated from diabetic pigs by oleate or linoleate have been shown to potentiate insulin-like growth factor 1 PLD-stimulating effects [11]. We have previously shown that docosahexaenoate enrichment of human lymphocytes stimulates the activity of the PLD1 isoform through exclusion of the protein from lipid rafts [8]. More recently, Wang and Oram [12] have shown that linoleate was able to stimulate PLD activity of BHK (baby hamster kidney) cells within 15 min of treatment, whereas stearate was inactive. Although docosahexaenoate-induced PLD activation in human lymphocytes was clearly related to esterification in raft phospholipids, no clear relationship between the degree of FA incorporation in phospholipids and PLD activation could be found in rat thymocytes. Indeed, 20:5n-3, which exhibited the highest incorporation in thymocyte phospholipids, had no effect on the PLD activity of either unstimulated or ConA-stimulated rat thymocytes. These results are in good agreement with those of our previous study showing that 22:6n-3, but not 20:5n-3, stimulated the PLD activity of human mononuclear cells [7]. 20:5n-3 was also found inactive toward the PLD activity of human neutrophils, whereas arachidonate induced a 2-fold increase at micromolar concentrations [10].

An interesting result of the present study was the negative linear relationship between the PLD activity of ConA-stimulated thymocytes and the proliferation response. This indicates that PLD activation occurring in the early steps of the mitogenic process inhibited the late proliferation response. By analysing PA molecular species, we have previously shown that PA produced within the first 30 min of thymocyte stimulation by ConA was originating from phosphoinositides through a phospholipase C (PLC)-DAG kinase pathway [36]. It may be hypothesized that the molecular species of PA generated through PC hydrolysis have reduced signaling properties, as compared with the more unsaturated ones issued from phosphatidylinositol bisphosphate (PIP2) hydrolysis [37]. In line with the above results, we have already demonstrated that PLD1 overexpression markedly lowers Jurkat cell response to PMA and ionomycin activation [15]. On the whole, the present results support further the current hypothesis that PLD activation conveys antiproliferative signals in lymphoid cells. In this regard, 18:2n-6 and 22:6n-3 are especially active both as PLD activators and inhibitors of lymphocyte proliferation.

Acknowledgments

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