Time-course changes in content and fatty acid composition of phosphatidic acid from rat thymocytes during concanavalin A stimulation

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Several studies have shown the potential role of phosphatidic acid (PA) as a second messenger in different cell types. Thus, PA has been shown to mimic physiological agonists leading to various cellular responses, such as neurotransmitter and hormone release, cell proliferation by modulating DNA or RNA synthesis, the expression of several proto-oncogenes and growth factors, and the stimulation of enzyme activities such as phospholipase C (PLC), protein kinases and cyclic AMP (cAMP) phosphodiesterase. Stimulation of [^{3}H]arachidonate-labelled rat thymocytes with the mitogen lectin concanavalin A (con A) resulted in enhanced production of radiolabelled PA after only 5 min of activation. The radiolabelled PA increase corresponded to a real increase in PA mass as determined by GLC quantification of its fatty acid content. In the presence of ethanol (0.5%), formation of phosphatidylethanol was not observed after 5 min of con A activation. Pretreatment of cells with R 59022 (10 μM), a diacylglycerol (DAG) kinase inhibitor, showed an inhibition in the formation of radiolabelled PA and in PA mass. These results suggest that the PLC–DAG kinase may be the pathway for PA synthesis in the first minutes of mitogenic thymocyte activation. A detailed analysis of the fatty acid composition showed that the relative amount of unsaturated fatty acids was increased in PA from stimulated cells concomitantly with a decrease in saturated ones; in particular, arachidonic acid was increased approximately 2-fold only 2 min after con A addition whereas palmitic acid was decreased for the whole period investigated (20 min). These changes favour the hydolysis of phosphoinositides rather than phosphatidylcholines by PLC. As PA remains a minor phospholipid, these changes are unlikely to affect cell membrane fluidity; but PA being now well recognized as a potential second messenger, its increased content as well as its increased unsaturation in the fatty acyl moiety might modulate several signalling pathways or the activity of enzymes such as cyclic nucleotide phosphodiesterase, controlling in this way the cellular level of cAMP, a negative regulator of blastic transformation.

INTRODUCTION

Rat thymocytes can be activated by lectins such as concanavalin A (con A) which, acting as polyclonal mitogens, give rise to a complex cascade of biochemical events resulting in cell-cycle entry, expression of interleukin-2 (IL-2) receptors, and finally, cell proliferation.

The most critical biochemical events associated with lymphocyte proliferation are thought to occur in the initial minutes of mitogenic lectin action [1]. At the membrane level, hydrolysis of inositol phospholipids [2–4] produces two signalling molecules: inositol 1,4,5-trisphosphate, which releases intracellular calcium, and diacylglycerol (DAG), a protein kinase C activator. There is increasing evidence that phosphatidylcholine (PC) hydrolysis by both phospholipase C (PLC) and phospholipase D (PLD), generating DAG and phosphatidic acid (PA) respectively, can act as another transmembrane signalling pathway [5,6]. PA can be transformed into DAG by phosphatidate phosphohydrolase, and alternatively, DAG can generate PA when phosphorylated by DAG kinase.

Several studies have shown a potential role for PA as a second messenger by itself. In different cell types, PA can mimic physiological agonists leading to various cellular responses such as: neurotransmitter [7] and hormone release [8], cell proliferation by modulating DNA or RNA synthesis, expression of several proto-oncogenes and growth factors [9–12]; it can also act as a second messenger for growth factors and hormones [13,14]. Exogenous PA also mimics several IL-2 actions in T-cell clones [15]. Besides, PA has been shown to stimulate several enzyme activities such as PLC [9,16–19], protein kinases [20,21] and cyclic AMP (cAMP)-phosphodiesterase activity of rat heart [22] or rat liver [23] microsomes.

Recent studies have also shown a physiological interaction between the phosphoinositide and the cAMP-signalling pathways [24]. The influence of cAMP exerted on early intracellular events after lymphocyte stimulation is difficult to interpret, and literature data are rather conflicting, most probably because of the heterogeneity of the cell populations studied as well as their state of activation. Generally, elevation in the cAMP level in lymphoid cells is inhibitory or down-regulatory of immune responses [1,24–28].

Cyclic nucleotide phosphodiesterase (PDE) is the only enzyme system known that is able to hydrolyse cAMP, and in this way, to modulate or terminate the effects of cyclic nucleotides [29]. In fact, increases in PDE activity when lymphocytes are stimulated by mitogenic agents have been observed both several hours [30] and very soon (20–30 min) after exposure of rat thymocytes [31] or human lymphocytes [32] to mitogens. Moreover, the rolipram-sensitive isoform of PDE, which is substantially increased within 30 min following con A stimulation in the cytosol of rat thymic

Abbreviations used: con A, concanavalin A; DAG, diacylglycerol; FAME, fatty acid methyl esters; PA, phosphatidic acid; PC, phosphatidylcholine; PDE, cyclic nucleotide phosphodiesterase; PE, phosphatidylethanolamine; PLC, phospholipase C; PLD, phospholipase D; PI, phosphatidylinositol; PS, phosphatidylserine; cAMP, cyclic AMP; IL-2, interleukin-2.

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lymphocytes [31], is also markedly activated by PA *in vitro* [33]. These data as well as the rapid rise of PA in con A-stimulated lymphocytes, suggest that the link between the phospholipids and the cAMP signalling pathways could be established via PA, which seems able to exert a modulatory effects upon phosphodiesterase activity [33].

Blastic transformation of lymphocytes is also accompanied by changes in the lipid composition of plasma membranes, such as: modification of the molecular ratio of cholesterol to phospholipids [34,35], increased turnover of long-chain fatty acids [36,37] and enrichment in polyunsaturated fatty acids [35,38]. These changes, affecting mainly the major phospholipids [35,38] and being characteristic for each animal species, lead to changes in membrane fluidity and may modulate the activities of membranes or neighbouring proteins implicated in the process of cell proliferation.

The aims of the present work are: (1) to determine the kinetics of enhanced PA synthesis after short periods of mitogenic activation of rat thymocytes; and (2) to study the eventual changes in the fatty acid composition of these PA. The results indicate that a significant increased synthesis of the PA total mass appears after only 2 min of mitogenic activation. Furthermore, a significant accumulation of arachidonic acid and a concomitant decrease of palmitic acid in the PA fatty acid composition were also observed within 2 min of con A action. These variations are discussed in relation to the different possible pathways of PA formation and the mitogenic activation of the cytosolic cAMP-PDE rolipram-sensitive isoenzyme.

MATERIALS AND METHODS

Chemicals

Lymphocyte separation medium Histopaque 1077, RPMI 1640, glutamine, BSA, con A and lipid standards were from Sigma (La Verpillère, France). R 59022 was from Calbiochem (France Biochem). [5,6,8,9,11,12,14,15-^{3}H]Arachidonic acid (specific activity, 213 Ci/mmol) was from Amersham (Les Ulis, France). Silica gel G 60 and LK5 plates were from Merck (Darmstadt, Germany) and Whatman (Clifton, OH, U.S.A.) respectively. All solvents were of analytical grade and were purchased from SDS (Peypin, France).

Isolation of rat thymocytes

Male Sprague–Dawley (250–300 g) were killed by decapitation. Thymus glands were removed, cleaned of connective tissues, placed in 0.15 M NaCl and gently homogenized in a loose-fitting glass homogenizer. After removing tissue remnants by filtering the cell suspension through a nylon gauze, thymic lymphocytes were separated by density gradient centrifugation on Histopaque 1077. The cells were washed twice with 0.15 M NaCl and resuspended in RPMI 1640 medium. All steps were carried out at room temperature. Under such conditions, a homogeneous population of thymocytes was obtained (with 80% having a cell diameter of 4.1–6.5 μm), and the cell viability was consistently greater than 90% as revealed by the Trypan Blue exclusion test. Cells were counted by a Coulter ZM, adjusted to 25×10^6 cells/ml and allowed to rest for 30 min at 37 °C before starting the experiments.

Cell labelling and con A activation

Isolated thymocytes were incubated for 1 h at 37 °C in RPMI 1640 medium in the presence of trace amounts of [^{3}H]arachidonic acid (4–8 nM; 0.05 μCi/10^6 cells). The cells were washed with RPMI 1640 containing 0.2% BSA (w/v) and resuspended in RPMI 1640 containing 2 mM glutamine. The average incorporation of labelled arachidonate in cell lipids was 70%. Cells were then incubated at 37 °C for different periods of time between 30 s and 20 min, in the absence (controls) or presence of 1 μg/10^6 con A stimulated cells. The stimulation was terminated by acidifying the cell suspensions (pH 3–4) with 2 M HCl and the lipid extraction was done according to Bligh and Dyer [39].

In experiments where the DAG kinase inhibitor R 59022 was used, prelabelled thymocytes were first incubated with the inhibitor for 5 min and then activated with con A.

Lipid analysis

The organic phase was further analysed by TLC on silica gel plates. Neutral lipids were separated from phospholipids and fractionated into the different classes on silica gel G 60 plates with the solvent system hexane/diethyl ether/acetic acid (50:50:1, by vol.); in some experiments the phospholipid fraction was extracted from the silica gel with chloroform/methanol/water (5:5:1, by vol.) and further separated on silica gel LK5 plates with the solvent system chloroform/methanol/methylamine 40% (60:30:5, by vol.). In other experiments, the plates were first developed in diethyl ether (100%) to separate the neutral lipids that migrated from the origin to a very narrow band at the solvent front. Then, the plates were air dried for 30 min and developed a second time in the same direction (at 2 cm below the first solvent front) with the solvent system chloroform/methanol/acetic acid/water (60:30:10:1, by vol.). Under these conditions PA migrated with an R_F of 0.82 ± 0.03. Distribution of [^{3}H]arachidonate into lipid classes and phospholipid classes was quantified with a Berthold TLC analyser. Identification of the different lipid classes was made by comparison with appropriate lipid standards.

The fatty acid content and composition of the PA were determined by GLC after TLC separation. The silica gel areas corresponding to PA were scraped off and the fatty acids were transmethylated as described by Morrisson and Smith [40]. Briefly, 500 μl of 10% BF_3 in methanol was added to the scraped silica gel (2 cm^2) suspended in 500 μl of toluene/methanol (2:3, v/v), and the transmethylation was carried out under a nitrogen atmosphere at 100 °C for 90 min in screw-capped tubes. The reaction was terminated by the addition of 1 ml of 5% (w/v) K_2CO_3 and the fatty acid methyl esters (FAME) were extracted with iso-octane and stored at −30 °C under nitrogen. The FAME were analysed using a Perkin-Elmer chromatograph model 5830, equipped with an SP 2380 capillary column (60 m × 0.25 mm; Supelco, Bellefonte, PA, U.S.A.) 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 psi (5.52 kPa). Peaks were identified using standard FAME. For measuring the molar content of fatty acids per 3×10^6 cells, diheptadecanoyl-L-α-phosphatidic acid was added as an internal standard to the cell suspensions before extraction.

Diheptadecanoyl-phosphatidic acid was prepared by the action of PLD type I (Sigma) upon diheptadecanoyl-L-α-phosphatidylcholine, as described by Eibl and Kovatchev [41], purified by TLC with the solvent system chloroform/methanol/acetic acid/water (80:15:8:0.5, by vol.). The PA content was measured by

phosphorus determination as described by Bartlett [42] and the purity was checked by GLC after transmethylation.

Statistical analyses

Data were analysed by analysis of variance using the STATVIEW II program for Macintosh. They were considered as significant when the P value was $\leqslant 0.05$.

RESULTS

Incorporation of [^{3}H]arachidonic acid into rat thymocytes

Thymocytes were incubated in RPMI 1640 in the presence of [^{3}H]arachidonic acid and the incorporation into thymocyte lipids was measured after different periods of time between 15 min and 8 h. The radioactivity associated with total lipids continuously increased to 68–70 % of the initially added radioactivity within the first hour, and no significant changes were observed in the total radioactivity or in its distribution within the main classes of lipids after between 1 h and 4 h (results not shown). Therefore, for subsequent studies, an incubation time of 1 h was chosen for thymocyte labelling. As shown in Table 1, after 1 h of incubation, the arachidonic acid was mainly incorporated into the major phospholipids: PC, phosphatidylethanolamine (PE), phosphatidylinositol (PI) and phosphatidylserine (PS). The PA fraction accounted for only 0.3 % of the total labelling.

Table 1 Distribution of [^{3}H]arachidonic acid in thymocyte lipid fractions

Cells were incubated for 1 h in the presence of [^{3}H]arachidonic acid, washed, and the lipids were extracted using chloroform/methanol as described in the Materials and methods section. TLC was carried out with solvent system I (hexane/diethyl ether/acetic acid, 50:50:1, by vol.) for the fractionation of neutral lipids, and with system II (chloroform/methanol/methylamine 40%, 60:30:5, by vol.) for the fractionation of phospholipids. Radioactivity in the different fractions was determined using a Berthold TLC analyser. Results are expressed as % ± S.E.M. of the total incorporated radioactivity (n = number of experiments). AA, arachidonic acid; PIP, phosphatidylinositol; PIP2, phosphatidylinositol bisphosphate; TG, triacylglycerol; NL, neutral lipid; PL, phospholipid.

Lipid fractions	Radioactivity (%)
Solvent system I ($n = 8$)	
PL	97.00 ± 0.14
DAG	0.38 ± 0.01
AA	0.46 ± 0.01
TG	2.12 ± 0.03
Solvent system II ($n = 6$)	
PIP/PIP$_2$	1.1 ± 0.08
PC	58.7 ± 1.70
PI	6.7 ± 0.30
PS	6.2 ± 0.50
PA	0.3 ± 0.03
PE	23.5 ± 1.00
NL	3.5 ± 0.30

PA and DAG production

Results presented in Table 2 show that con A treatment of [^{3}H]arachidonate-prelabelled rat thymocytes led to a marked increase in labelled PA during the first 20 min of activation. The labelled-PA production was 115 % higher in stimulated cells as soon as 1 min after the addition of con A, peaked at 5 min (+140 %) and then decreased very slowly but still remained significantly superior to that of control cells (+100 %) at 20 min. The DAG production was less noticeably increased by con A, and became significantly higher (+50 %) after 30 min of stimulation, much later than PA (results not shown).

To establish whether the increase in ^{3}H-labelled PA reflects real increases of PA in response to mitogenic activation, we determined PA levels with a PA mass assay. For this, the total fatty acids of PA were quantified by GLC after transmethylation, and the PA mass was calculated assuming two moles of fatty acids per mole of PA. Results presented in Table 3a show that the increase in PA mass tightly paralleled that of [^{3}H]arachidonate-labelled PA. Thus, after 2 min, the PA content of con A-stimulated thymocytes was significantly higher ($P \leqslant 0.05$) as compared with control cells, doubled after 5 min of stimulation ($P \leqslant 0.01$) and was still about 1.5-fold higher than the control value after 20 min ($P \leqslant 0.05$).

Effect of R 59022 on con A-induced PA production

Treatment of thymocytes with the DAG kinase inhibitor R 59022 (10 μM) 5 min before stimulation with con A resulted in a lowering of PA production in the triggered cells. This was observed for both the [^{3}H]arachidonate-labelled PA and the PA mass. Thus, while labelled PA was increased by 130 % after 5 min of stimulation by con A, it was only increased by 32 % when cells were first treated with R 59022. In parallel, the PA mass produced was lowered from 83 to 10 % (Table 4).

Changes in the fatty acid composition of PA from rat thymocytes upon stimulation with con A

The fatty acid composition of PA in control and con A-activated rat thymocytes is shown in Table 3a. The content (pmol/3 × 10^6 cells) of both saturated and unsaturated fatty acids was increased within the first minutes of con A treatment. Saturated fatty acids and the C_{n-3} unsaturated group were significantly increased after between 2 and 5 min of stimulation. The content of C_{n-6} fatty acids (especially $C_{20:4,n-6}$) markedly increased after only 30 s of con A stimulation and was maintained at this high level throughout the period studied (20 min). Finally, the major C_{n-9} fatty acids ($C_{16:1,n-9}$ and $C_{18:1,n-9}$) both showed an important peak at 2 min of activation, then returning to control values. In addition, a reduction in the saturated:unsaturated fatty acids ratio could be observed starting at 30 s of stimulation, suggesting that the increased production of PA was accompanied by an enrichment in unsaturated fatty acids with a concomitant decrease in the proportion of saturated fatty acids. This can readily

Table 2 Kinetics of [^{3}H]arachidonate-labelled PA production in rat thymocytes activated by con A (1 μg/10^6 cells)

Results are expressed as a percentage of PA production measured in unactivated control cells and are the means ± S.E.M. of five experiments; * $P \leqslant 0.05$.

Time (min)	% of labelled PA in con A-stimulated versus control cells
0	100
0.5	118.6 ± 1.6
1	215.5 ± 31.5*
2	232.8 ± 49.5*
5	239.4 ± 37.3*
20	199.3 ± 23.9*

Table 3 Time-course changes in the content and fatty acid composition of PA from rat thymocytes after short times of con A stimulation

Cells were activated for different periods of time by con A (1 μg/10^6 cells) and the lipids were extracted using chloroform/methanol. Phospholipids were separated on TLC, the silica gel areas corresponding to PA were scraped off and transmethylated as described in the Materials and methods section. The FAME were separated by GLC. Results are expressed as pmol/3 $\times$ 10^6 cells in Table 3a and as mol % in Table 3b, and are the means $\pm$ S.E.M. of five experiments. * $P \leqslant 0.05$.; † $P \leqslant 0.01$.

Time (min) ...	0	0.5	2	5	20
Fatty acid					
(a)					
	Content (pmol/3 $\times$ 10^6 cells)				
$C_{16:0}$	482.6 ± 72.9	693.9 ± 307.3	702.5 ± 126.7	760.8 ± 133.5	717.3 ± 185.1
$C_{18:0}$	307.6 ± 42.8	504.0 ± 228.8	621.3 ± 86.1†	564.9 ± 141.1	536.6 ± 193.9
$C_{20:0}$	29.3 ± 4.4	17.6 ± 4.8	31.3 ± 8.2	38.2 ± 10.2	26.5 ± 4.8
$C_{24:0}$	49.2 ± 6.2	53.6 ± 14.0	73.2 ± 11.5	79.5 ± 37.4	39.7 ± 10.4
$C_{16:1,n-9}$	77.5 ± 16.8	75.6 ± 37.9	112.8 ± 32.8	104.5 ± 30.1	96.9 ± 34.4
$C_{18:1,n-9}$	288.9 ± 32.2	360.4 ± 37.3	526.1 ± 56.9†	414.3 ± 118.7	320.2 ± 46.6
$C_{20:1,n-9}$	12.7 ± 3.8	18.9 ± 1.7	30.4 ± 5.1†	34.8 ± 15.4*	10.6 ± 3.5
$C_{16:1,n-7}$	76.0 ± 13.2	114.9 ± 30.6	154.6 ± 39.5*	143.9 ± 44.3	104.8 ± 11.6
$C_{18:1,n-7}$	120.3 ± 18.0	245.3 ± 85.3	202.0 ± 37.3	234.5 ± 98.5	204.6 ± 52.1
$C_{18:2,n-6}$	158.7 ± 19.0	229.8 ± 82.1	288.8 ± 75.6	378.6 ± 186.5	418.1 ± 16.6*
$C_{20:2,n-6}$	24.2 ± 3.3	40.8 ± 7.4	45.6 ± 6.6*	63.4 ± 19.3†	52.8 ± 8.2†
$C_{20:3,n-6}$	39.5 ± 9.2	57.1 ± 16.7	57.2 ± 15.0	60.0 ± 15.1	55.6 ± 6.9
$C_{20:4,n-6}$	79.3 ± 8.2	152.1 ± 9.1*	212.9 ± 27.4†	191.9 ± 67.2†	148.3 ± 12.5*
$C_{22:4,n-6}$	19.4 ± 2.4	37.8 ± 8.3*	38.1 ± 5.4†	35.7 ± 23.7	33.2 ± 7.3
$C_{18:3,n-3}$	12.6 ± 1.6	24.0 ± 3.6	36.6 ± 10.4†	27.9 ± 7.4*	23.9 ± 4.4
$C_{22:6,n-3}$	22.4 ± 3.9	26.2 ± 5.4	41.7 ± 9.0*	53.9 ± 23.1*	24.1 ± 5.6
PA (pmol/3 $\times$ 10^6 cells)	979.7 ± 69.8	1265.7 ± 189.6	1660.8 ± 130.6†	1861.3 ± 501.5†	1375.8 ± 182.2*
Saturated	830.1 ± 91.7	1253.2 ± 527.8	1426.3 ± 206.7*	1463.9 ± 277.2	1266.2 ± 404.6
C_{n-9}	371.9 ± 44.1	411.1 ± 42.1	710.9 ± 80.1†	536.2 ± 108.5	437.1 ± 84.3
C_{n-7}	199.7 ± 25.1	360.3 ± 106.1	356.6 ± 58.7*	378.6 ± 137.7	365.6 ± 91.1
C_{n-6}	350.6 ± 14.3	653.2 ± 228.1	766.8 ± 115.8†	890.2 ± 304.6†	794.7 ± 117.8*
C_{n-3}	31.9 ± 4.3	43.7 ± 5.6	60.4 ± 8.0†	64.4 ± 21.7*	48.1 ± 9.6
(b)					
	Content (mol %)				
$C_{16:0}$	23.6 ± 1.3	18.4 ± 4.9	17.4 ± 1.2†	17.2 ± 1.9*	15.3 ± 1.1†
$C_{18:0}$	17.5 ± 1.0	16.5 ± 4.8	17.3 ± 1.4	16.3 ± 2.5	16.1 ± 2.0
$C_{20:0}$	1.2 ± 0.1	0.9 ± 0.3	1.0 ± 0.2	1.1 ± 0.2	1.1 ± 0.2
$C_{24:0}$	3.5 ± 0.3	3.3 ± 0.3	3.0 ± 0.4	3.3 ± 1.0	2.8 ± 0.4
$C_{16:1,n-9}$	4.5 ± 0.6	2.9 ± 0.5	5.1 ± 1.0	5.6 ± 1.0	4.2 ± 0.4
$C_{18:1,n-9}$	15.5 ± 0.9	15.9 ± 2.3	17.9 ± 1.8	18.9 ± 3.0	15.4 ± 1.7
$C_{20:1,n-9}$	0.8 ± 0.1	0.7 ± 0.1	1.0 ± 0.1	0.4 ± 0.1	0.7 ± 0.3
$C_{16:1,n-7}$	3.7 ± 0.3	3.2 ± 0.8	3.8 ± 0.5	4.0 ± 0.7	3.4 ± 0.3
$C_{18:1,n-7}$	5.7 ± 0.9	3.6 ± 1.6	4.5 ± 0.6	4.9 ± 1.2	4.6 ± 0.7
$C_{18:2,n-6}$	7.4 ± 1.1	9.3 ± 6.3	8.4 ± 1.7	10.2 ± 3.2	10.3 ± 2.8
$C_{20:2,n-6}$	1.4 ± 0.1	1.5 ± 0.3	1.3 ± 0.2	1.7 ± 0.2	1.4 ± 0.2
$C_{20:3,n-6}$	2.5 ± 0.3	2.1 ± 0.4	1.8 ± 0.4	1.6 ± 0.2	1.9 ± 0.4
$C_{20:4,n-6}$	4.2 ± 0.2	4.4 ± 0.6	7.8 ± 1.3†	6.4 ± 1.3†	6.9 ± 0.6†
$C_{22:4,n-6}$	1.3 ± 0.2	1.0 ± 0.3	1.5 ± 0.3	1.9 ± 0.5	1.5 ± 0.4
$C_{18:3,n-3}$	0.7 ± 0.1	0.8 ± 0.1	1.0 ± 0.2	0.9 ± 0.1	1.3 ± 0.3†
$C_{22:6,n-3}$	1.3 ± 0.15	1.2 ± 0.1	1.7 ± 0.5	1.1 ± 0.2	1.3 ± 0.3

Table 4 Effect of R 59022 on PA formation in con A-stimulated thymocytes

Cells prelabelled with [^{3}H]arachidonic acid were incubated for 5 min with R 59022 (10 μM) and then stimulated with con A (1 μg/10^6 cells) for 5 min. Results are the means $\pm$ S.E.M. of six experiments; * $P \leqslant 0.05$.

	Increase of labelled PA in con A-stimulated versus control cells (%)		
	−R59022	+R59022 (10 μM)	Inhibition (%)
Labelled PA	130.6 ± 39.3	32.5 ± 8.1*	75.4 ± 8.9
PA mass	83.5 ± 23.6	9.7 ± 20.5*	79.2 ± 27.5

be observed by expressing results as mol percent of the total fatty acids (Figure 1). Looking in more detail at the time-course changes in relation to the saturated and unsaturated fatty acid series reveals specific patterns (Table 3b). The proportion of C_{n-3} and C_{n-7} fatty acids was not influenced by con A, indicating that their increased contents paralleled the increased content of the PA mass. Within the C_{n-6} fatty acids series, the most important change dealt with a significant increase in arachidonic acid, the proportion of which was enhanced from 4% in the unstimulated thymocytes to about 7% in the con A-treated cells. Conversely, the proportion of 16:0, the major saturated fatty acid, was significantly decreased as soon as 2 min after con A addition (Figure 2).

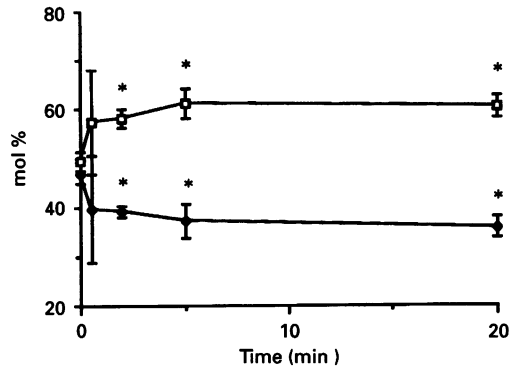

Figure 1 Early changes in the proportions of saturated and unsaturated fatty acids of PA from rat thymocytes during con A stimulation

Time zero corresponds to unstimulated cells. Results are expressed as mol percent and are the means ± S.E.M. of five experiments: ◆, saturated; □, unsaturated. * $P \leqslant 0.05$.

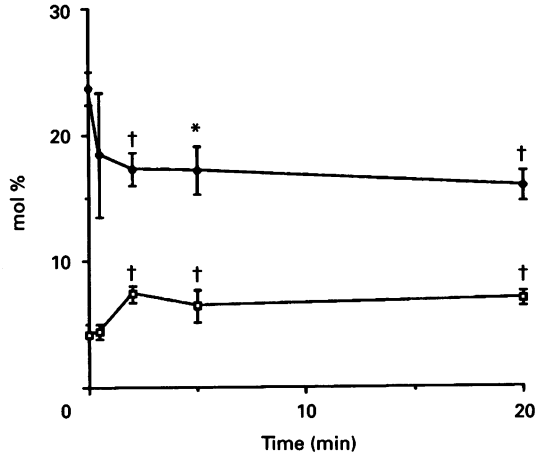

Figure 2 Changes in the proportions of $C_{16:0}$ and $C_{20:4,n-6}$ fatty acids in PA from rat thymocytes during con A activation

Time zero corresponds to unstimulated cells. Results are expressed as mol percent and are the means ± S.E.M. of five experiments: ◆, $C_{16:0}$; □, $C_{20:4,n-6}$. † $P \leqslant 0.01$; * $P \leqslant 0.05$.

DISCUSSION

As shown in several studies [35,38,43], the fatty acid composition of membrane phospholipids changes during blastic transformation of lymphocytes, depending on the species or the type of lymphoid cell. These changes have been shown to occur both in the early steps of the activation process [38,43], leading to an easier signal transduction, or later when they may contribute to the augmentation of the membrane fluidity of T-cells [35].

Earlier observations in our laboratory [33] have shown that PA, a minor phospholipid of rat thymocytes, is increased in the early steps of con A stimulation (30 min). Besides, the cAMP-PDE activity of rat thymocytes can be stimulated *in vitro* by exogenous PA [33], suggesting a possible link between phospholipids and cAMP signalling pathways. It therefore seemed interesting to us to analyse the lipid composition of PA from the con A-stimulated thymocytes in order to establish whether the increased PA formation is accompanied by changes in its fatty acid composition.

The results reported here indicate that con A stimulation of rat thymocytes induces an increased synthesis of PA during the first 20 min of activation with a peak (+140%) between 2 and 5 min. Our results are in good agreement with the early and transient increase of labelled PA observed by Hasegawa-Sasaki and Sasaki [44] in rat lymph node lymphocytes and by Marcoz et al. [33] in rat thymocytes. In addition, the labelled PA increase corresponds to a real increase in PA mass and does not reflect only the increased incorporation of arachidonic acid into PA upon con A stimulation. This increase in PA mass becomes even more striking when the results of Gross et al. [34] are considered. Indeed, by measuring [^{14}C]acetate incorporation into membrane lipids from rat thymocytes, these authors could not detect any significant changes in *de novo* lipid synthesis within the first 3 h of mitogen stimulation of rat thymocytes. In the light of these data, the PA increase observed by others [33,44] and by us in con A-stimulated cells is likely to result from the activation of the phospholipid signalling pathways.

Another interesting observation is the low but significant increase in [^{3}H]arachidonate-labelled DAG after 30 min of stimulation, its mass not being altered, as revealed by measuring DAG with dieicosanoylglycerol as an internal standard (results not shown). An increase in PA in the absence of changes of DAG has also been observed in human platelets stimulated by low concentrations of thrombin [45] or stimulated by ADP [46]. These authors assumed that a weak stimulus of the PI cycle failed to accumulate DAG, and they suggested that phosphatidylinositol bisphosphate breakdown by PLC with DAG formation could be hidden by its rapid conversion into PA, via the DAG kinase action, at least during the first 30 min of activation. Alternatively, PA might be formed by the action of PLD on membrane phospholipids (PC or PE), or by the action of the lysophosphatidate acyl-CoA:acyltransferase upon lyso-PA. The latter pathway has been interestingly associated with IL-1 responses [49] among which the maturation of precursor thymocyte forms is included. Besides, it may be assumed that after 30 min of stimulation, phosphatidate phosphohydrolase is activated, transforming part of the PA into DAG.

In our experiments, the increase in radiolabelled PA was more important (maximum value +140%) than the increase in phosphatidate mass (maximum value +90%). This observation may suggest the following. (1) PA is derived from a single phospholipid pool, containing high proportions of arachidonate and being transformed via a single metabolic pathway. The phosphoinositide PLC-DAG kinase pathway supports this hypothesis rather than the PC counterpart pathway, as phosphoinositides are known to contain high levels of arachidonate and stearate but low levels of palmitate. (2) PA was produced via two different pathways, proceeding at different rates, the arachidonate-rich pool being transformed at a higher rate than the other one. According to the second hypothesis (2), the phosphoinositide PLC-DAG kinase supports the high-rate pathway and the PC-PLD represents the second source of PA.

When cells were pretreated with the DAG kinase inhibitor R 59022, a decrease in the synthesis of both radiolabelled PA and PA mass in con A-activated cells is observed (Table 4). On the other hand, as found by Marcoz et al. [33] and verified also by us (results not shown), formation of phosphatidylethanol could not be detected when cells were stimulated with con A in the presence of 0.5% ethanol. This strongly suggests that PA formation within the first minutes of con A stimulation proceeds via the phosphoinositide PLC-DAG kinase pathway rather than by PLD cleavage of PC or PE.

Analysis of the fatty acid pattern of PA revealed that, concomitantly with its enhanced synthesis, changes in the satu-

ration index of the fatty acyl chains also occur. Thus, an enrichment in unsaturated fatty acids at the expense of saturated ones occurred, which mainly resulted from the accumulation of arachidonic acid and a decrease in palmitic acid. These results are consistent with several literature data. (i) A higher degree of unsaturation of plasma membrane phospholipids in stimulated lymphocytes [37,38]. (ii) A rapid activation of lysophosphatidate acyltransferase, an enzyme responsible for the incorporation of highly unsaturated fatty acids at position 2 of phospholipids [44,47], which is in close structural relationship with the mitogen receptor [48]. This particular pathway seems however unlikely under our conditions, as around 80% of the PA formation was inhibited by the DAG kinase inhibitor. (iii) Preferential incorporation of unsaturated fatty acids into lipids when cells are mitogenically stimulated [36]. The increased arachidonic acid content in PA after con A stimulation also agrees with some literature data showing that mitogen stimulation increased the arachidonic acid content within each of the phospholipid fractions (PC, PE, PI, and PS) during the first 24 h [34].

On the other hand, slight alterations in the structure of PAs are known to cause striking changes in their activities. Thus, Murayama and Ui [16] showed in 3T3 fibroblasts that PAs consisting of unsaturated fatty acids are full agonists, whereas PAs containing saturated fatty acids are antagonists or partial agonists of specific PA receptors located on the outer surface of these cells. In our experiments, both PA mass and PA fatty acid composition were changing in the early steps of the activation process of rat thymocytes, with a transient increase in PA mass and an enrichment in unsaturated fatty acids occurring some 5–20 min after the con A addition.

It is commonly admitted that changes in saturation of the phospholipid fatty acyl chains necessarily modify the physical properties of the membrane, especially the membrane fluidity. These modifications can in turn modulate the activity of membrane constituents implicated in the process of blastic transformation. As PA is not a major component of membrane phospholipids, its contribution to physical changes in the membrane must have little effect. PA is however involved in the activation process, very likely as a modulator of the rolipram-sensitive cAMP-PDE activity [31,33].

In conclusion, significant changes in the PA content and PA fatty acid composition occur very early after the mitogenic activation of rat thymocytes. These are an increase in PA level with an increase in its unsaturation, especially due to the accumulation of arachidonate and the concomitant depletion of palmitic acid. Most probably, this PA is generated via the phosphoinositide PLC-DAG kinase pathway. These changes may be responsible for the activation of a cAMP-specific and rolipram-sensitive isoform (IV) of PDE, an enzyme that controls the lowering of cAMP level in the normal mitogenic response [50,51].

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