Contribution of phosphoinositides and phosphatidylcholines to the production of phosphatidic acid upon concanavalin A stimulation of rat thymocytes

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Abstract Stimulation of rat thymocytes by concanavalin A (Con A) results in a very early increase of the cellular level of phosphatidic acid (PA), while that of diacylglycerol (DAG) was not affected. As the biological activity of PA is very likely to be determined by its molecular species composition, the present study aims to investigate the pathways leading to the production of PA in Con A-stimulated rat thymocytes. Prelabeling the cells with [^{3}H]arachidonic acid, [^{3}H]myristic acid, [^{3}H]choline, or [^{14}C]lysophosphatidylcholine allowed us to determine that PA is formed by both phosphoinositide (PIs) and phosphatidylcholine (PC) hydrolysis. We then investigated whether PA derived from PC was formed by phospholipase C (PLC) or phospholipase D (PLD) hydrolysis. In the presence of 1-butanol, the production of phosphatidylbutanol was only observed in tetradecanoyl phorbol acetate (TPA)-stimulated cells. The use of a specific PC phospholipase C inhibitor resulted in a decrease of Con A-stimulated PA production in cells labeled with [^{3}H]myristate. When cells were labeled with [^{3}H]choline, only TPA stimulation induced a release of labeled choline. All together, these experiments suggest that PA is originated from two phospholipid sources, predominantly PI via PLC hydrolysis and to a lesser extent PC, by PLC hydrolysis also. Molecular species analyses by reverse phase HPLC are in agreement with this hypothesis, as diacyl-GP molecular species composition is similar to that of diacyl-GPC and DAG in resting cells, but resembles that of diacyl-GPI in Con A-treated cells. Thus, in stimulated cells, the amount of 18:0/20:4 species doubled while those of saturated and monounsaturated species decreased.—**El Bawab, S., O. Macovschi, C. Thevenon, A. Goncalves, G. Nemoz, M. Lagarde, and A-F. Prigent.** Contribution of phosphoinositides and phosphatidylcholines to the production of phosphatidic acid upon concanavalin A stimulation of rat thymocytes. *J. Lipid Res.* 1996. **37:** 2098–2108.

Supplementary key words phosphatidic acid • diacylglycerol • thymocytes • mitogenic stimulation • phospholipid molecular species • HPLC • phospholipase C • phospholipase D

T cell activation is initiated upon ligation of the TcR/CD3 receptor complex by antigens, anti-CD3 antibodies, or lectins. It is well established now that stimulation of the cellular lipid metabolism plays a central role in a large number of receptor-mediated responses. One of these biochemical events is the rapid hydrolysis of phosphatidylinositol bisphosphate (PIP_2) by a specific phospholipase C (PLC), leading to the generation of two second messenger molecules, diacylglycerol (DAG) and inositol 1,4,5-trisphosphate (1–3). DAG is an activator of protein kinase C (PKC), a large family of isoenzymes (4). There are at least three known classes of PLC (β, γ, δ), which are expressed in a cell type-specific manner (5, 6). In lymphocytes, the PLCγ1 isoform that predominates is phosphorylated by receptor-linked tyrosine kinase of the src family during activation (7–10). Another possible pathway of DAG generation is the hydrolysis of phosphatidylcholine (PC) by a specific PLC (11). However, the molecular species composition of the DAG generated from PC is different from that of DAG originated from PIP_2 and conflicting data exist concerning the signalling activity of these DAG species (12–14).

DAG can be phosphorylated by DAG kinase and generate phosphatidic acid (15) for which increasing evidence shows a role as second messenger in various activation processes. PA can also be formed by phospholipase D (PLD) hydrolysis of PC (1, 16) or acylation of a lysophosphatidic acid pool by lysoPA-acyltrans-

Abbreviations: AA, arachidonic acid; Con A, concanavalin A; DAG, diacylglycerol; diacyl-GP, diacyl-glycerophosphate; diacyl-GPC, diacyl-glycerophosphocholine; diacyl-GPI, diacyl-glycerophosphoinositol; DRG, diradylglycerol; 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; NL, neutral lipids; TPA, tetradecanoyl phorbol acetate.

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ferase (17). Recent data have shown that in mesangial cells and Madin-Darby canine cells, PA is formed by PLD hydrolysis of phosphatidylethanolamine (18) and phosphatidylinositol (19). It is not known whether the different agonists mobilize the same or different phospholipid pools in the same cell. Thus, the question of the presence of distinct agonist-sensitive phospholipid pools, even phospholipid subclasses, remains to be answered. Several roles have been proposed for PA, including the stimulation of enzyme activities (20–22), the induction of expression of protooncogene mRNAs, and the stimulation of DNA synthesis (23–26). The fatty acid composition of this molecule is likely to determine its efficiency as second messenger as reported in several studies (27, 28). In a previous work, we have shown that the PA cellular mass increased during concanavalin A (Con A) stimulation, together with an increase in its arachidonic acid content and a decrease in its palmitic acid content (29).

Cyclic AMP is another signalling molecule implicated in lymphocyte proliferation. Several studies have confirmed that a early rise in cAMP level blocked the mitogenic signal transduction at several points (30–32). Different reports have suggested that the cAMP-dependent protein kinase inhibits the MAP kinase activity by phosphorylating proteins implicated in its activation cascade (33, 34). Similarly, in 3T3 cells (35) and in Jurkat T-cells (36, 37), the phosphorylation of PLCγ1 by cAMP-dependent protein kinase or by PKC alters the interaction of this phospholipase with protein tyrosine kinase, hence leading to inhibition of its activity. Cyclic AMP level is tightly controlled by the cyclic nucleotide phosphodiesterase enzyme family, and in rat thymocytes, Con A stimulation induced an early activation of the cAMP-specific PDE isoforms (38). With regard to recent results from our laboratory, showing that exogenous PA (from egg yolk) or PA endogenously formed in Con A-stimulated thymocytes markedly stimulated the cAMP specific PDE 4 (20; S. El Bawab, O. Macovschi, C. Sette, M. Conti, M. Lagarde, G. Némoz, and A. F. Prigent, unpublished results), the determination of the synthesis pathway of PA in activated cells may be of physiological relevance. In the present study, we have investigated the contribution of PLD and PLC pathways to the formation of PA in rat thymocytes upon mitogenic stimulation. The comparison of the molecular species composition of PA formed in activated thymocytes with that of potential phospholipid precursors can help to establish the pathway of PA formation. Thus, we have analyzed by reverse phase HPLC the molecular species composition of PC, PI, PA, and DAG in resting and Con A-stimulated cells. Another approach used was to evaluate PA levels, after a selective labeling of the potential phospholipid precursors, or after a selective inhibition of enzymes involved in the pathways of PA formation. The present results indicate that stimulation of rat thymocytes with the phorbol ester TPA produces increased PA synthesis by PLD hydrolysis of PC. On the other hand, when cells are stimulated by the mitogenic lectin Con A, PA is formed by PLC hydrolysis of two different phospholipids, PIP_2 and PC.

MATERIALS AND METHODS

Chemicals

Lymphocyte separation medium Histopaque 1077, RPMI 1640, glutamine, bovine serum albumin, concanavalin A, D609, TPA, and lipid standards were from Sigma (La Verpillère, France). [^{3}H]arachidonic acid (213 Ci/mmol), [^{3}H]myristic acid (54 Ci/mmol), [methyl-^{3}H]choline (84 Ci/mmol), [palmitoyl-1-^{14}C]lysophosphatidylcholine (57 mCi/mmol) were from Amersham (Les Ulis, France). Silicagel G 60 and LK5 plates were from Merck (Darmstadt, Germany) and Whatman (Clifton, OH), respectively. Acetonitrile was of HPLC grade; other solvents were of analytical grade and were purchased from SDS (Peypin, France).

Isolation of rat thymocytes

Male Sprague-Dawley rats (250–300 g) were killed by decapitation. Thymus glands were removed, cleaned of connective tissues, placed into 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 out twice with 0.15 M NaCl and resuspended into 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 prior to starting the experiments.

Cell labeling and mitogenic stimulation

Isolated thymocytes were incubated in RPMI 1640 medium at 37°C, in the presence of 1.25 μCi/ml [^{3}H]arachidonic acid for 1 h, 1 μCi/ml [^{3}H]myristic acid for 2 h, or 0.2 μCi/ml [^{14}C]lysoPC for 5 min. When cells were labeled with [^{3}H]choline (4 μCi/ml), the incubation was carried out in choline-free Hanks' medium and

the incubation time was prolonged to 4 h. After washing the cells with the incubation medium, thymocytes were incubated at 37°C for different periods of time in the absence (controls) or presence of Con A (1 μg/10^6 cells) or TPA (100 nM). In experiments measuring phosphatidylalcohol formation, or where the PC-PLC inhibitor was used, labeled thymocytes were first preincubated with the alcohol (0.5%) for 5 min or the inhibitor (50 μg/ml) for 1 h, and then activated by mitogens. The stimulation was terminated by acidifying the cell suspension with 2 N HCl (pH 3–4) followed by lipid extraction with chloroform methanol according to Bligh and Dyer (39).

Lipid analyses

Lipid extracts were analyzed by TLC on silica gel G60 plates. DAG was isolated on plates pretreated with 0.25 M boric acid in 50% ethanol solution as described by Welsh and Schmeichel (40) with hexane–diethylether–acetic acid 50:50:1. PA was separated with chloroform–methanol–acetic acid–water 80:15:8:0.5; under these conditions PA migrated with an R_f of 0.82 ± 0.03. Production of [^{3}H]arachidonic acid-labeled PA and DAG was quantified with a TLC radioactivity scan analyzer (Berthold ZM). In other experiments PA was separated by two-dimensional migration, the first solvent being chloroform–methanol–ammonia 65:35:5.5, and the second one being chloroform–acetone–methanol–acetic acid–water 30:40:10:10:5. The second run was done with ethyl acetate–isooctane–acetic acid 90:50:20 for the separation of phosphatidylalcohol. Phosphatidylcholine and phosphatidylinositol were separated with chloroform–methanol–methylamine 60:30:5. Spots corresponding to phospholipids and neutral lipids were visualized by Coomassie blue staining as described by Nakamura and Handa (41), scraped off from the plate, and assayed for radioactive content by liquid scintillation counting.

Analysis of the water-soluble metabolites, choline and phosphocholine

The Bligh and Dyer aqueous phase was evaporated to dryness, resuspended in 100 μl methanol–water 1:1 and further applied on LK5 Whatman plates to separate choline and phosphorylcholine. The mobile phase was methanol–water–ammonia 70:50:5 (two runs). The area of the plate containing choline, phosphocholine, and other radioactive metabolites was scraped off and quantitated by liquid scintillation counting.

Analysis of lipid molecular species during Con A stimulation

Preparation of samples for HPLC. Phospholipids separated on TLC as described above were extracted, dried under nitrogen, and vesicles were obtained by sonication with 1 ml of incubation buffer (Tris-borate, pH 7.5). After addition of 1 ml diethylether, phospholipids were hydrolyzed overnight by PLC from *Bacillus cereus* (50 U/200 μg phospholipids) at room temperature. The generated diradylglycerols (DRG) were then treated with 9-anthroyl chloride as described by Takamura and Kito (42). The anthroyl-derivatives were then separated into alkenylacyl, alkylacyl, and diacyl subclasses by TLC using toluene–hexane–diethylether 50:45:5 as the mobile phase. The corresponding bands were scraped off, extracted 3 times with hexane–diethylether 1:1 and the solvent was evaporated under N_2 stream.

HPLC separation of diacyl molecular species. Separation of molecular species within each subclass was achieved with a Kontron 320 HPLC system fitted with a C18 precolumn and a 250 × 4.6 mm, 5 μm Hypersil analytical column (H5CC18-25F). Molecular species were quantified by fluorometry with excitation at 360 nm and emission at 460 nm using a Kontron SFM 25 detector and a PC Integration Pack. The eluent was acetonitrile–propan-1-ol 90:10, and the flow rate was 1.5 ml/min. Authentic standard molecular species were prepared as described above from commercially available phospholipids. Individual molecular species present in samples collected from HPLC were also identified as FAME by GLC after transmethylation with 10% BF_3 in MeOH.

Statistical analysis

Data were analyzed by ANOVA using the STATVIEW II program for Macintosh. They were considered as significant when the P value was 0.05 or lower.

RESULTS

Cell labeling experiments

In Table 1 are presented results obtained from experiments of thymocyte labeling with [^{3}H]arachidonic acid, [^{3}H]myristic acid, [^{3}H]choline, or [^{14}C]lysoPC. Arachidonic acid labeled all phospholipids, whereas myristic acid and lysoPC labeled mainly PC, allowing thus the identification and assay of PC-derived PA. Kinetics of thymocyte labeling with [^{3}H]choline in choline-free

TABLE 1. Distribution of labeled arachidonic acid, myristic acid, lysoPC, and choline in thymocyte lipid fractions

	[3H] Arachidonic Acid	[3H] Myristic Acid	[14C] Lyso PC	[3H] Choline
PC	58.70 ± 1.70	86.30 ± 1.00	81.90 ± 2.40	54.59 ± 1.41
PI/PS	12.90 ± 0.40	0.72 ± 0.05	1.60 ± 0.14	
PA	0.30 ± 0.03	0.05 ± 0.002	0.39 ± 0.08	
PE	23.50 ± 1.00	5.40 ± 0.30	5.73 ± 0.33	
NL	3.50 ± 0.30	7.40 ± 0.90	5.40 ± 0.40	
Choline				2.38 ± 0.14
Phosphocholine				30.68 ± 1.63
Choline metabolites				13.11 ± 0.80

Thymocytes were labeled with [3H]arachidonic acid, [3H]myristic acid, [14C]lyso PC, or [3H]choline for periods of time indicated in Methods, and lipids were analyzed by TLC. The various phospholipids were identified by comparison with appropriate standards after Coomassie blue staining, then scraped off, and the radioactivity was measured by liquid scintillation counting. Results are expressed as % of the total incorporated radioactivity, and are means ± SEM of four experiments.

Hanks' medium reached a plateau after 2 h incubation (not shown). The [^{3}H]choline incorporation yield was higher than 60% and remained unchanged for at least 4 h afterwards. TLC analysis of the organic phase after Bligh and Dyer extraction, showed that more than 98% of the radioactivity was incorporated into PC. In the aqueous phase, the radioactivity was mainly associated to phosphocholine and to unidentified labeled metabolites, indicating a rapid choline turn-over. In the subsequent experiments, cells were labeled for 4 h with [^{3}H]choline.

[^{3}H]arachidonic acid-labeled PA and DAG production

[^{3}H]arachidonic acid-labeled cells were activated by Con A for different periods of time. The time course of the Con A effect upon the labeled DAG and PA production is shown in **Fig. 1.** Stimulation of cells caused a rapid increase in PA production detectable within seconds and peaking at 5 min; as we have previously reported (29), the increase in [^{3}H]arachidonic acid-labeled PA was greater than the increase of PA mass. DAG production was only marginally affected during the same period. On the other hand, no phosphatidylethanol formation could be observed in the presence of ethanol (not shown). As phosphoinositides are known to contain high levels of arachidonic acid, these data suggested a predominant participation of the PIs via PLC pathway to the early PA synthesis upon Con A stimulation with slight if any participation of PC-PLC or PC-PLD activities. In order to test this hypothesis, both enzyme activities were assayed in unstimulated and Con A-stimulated thymocytes.

Activation of PLD in TPA- but not in Con A-stimulated cells

PLD activity was assayed in control and stimulated thymocytes prelabeled with [^{3}H]myristic acid or [^{14}C]lysoPC, which preferentially labeled PC, as shown in Table 1. A slight but significant increase in [^{3}H]myristic acid-labeled PA was observed in Con A-stimulated cells (**Fig. 2**); the same increase in PA was observed when cells were labeled with [^{14}C]lysoPC (results not shown). In parallel, TPA stimulation induced a 2-fold increase in ^{3}H-labeled PA. When experiments were carried out in

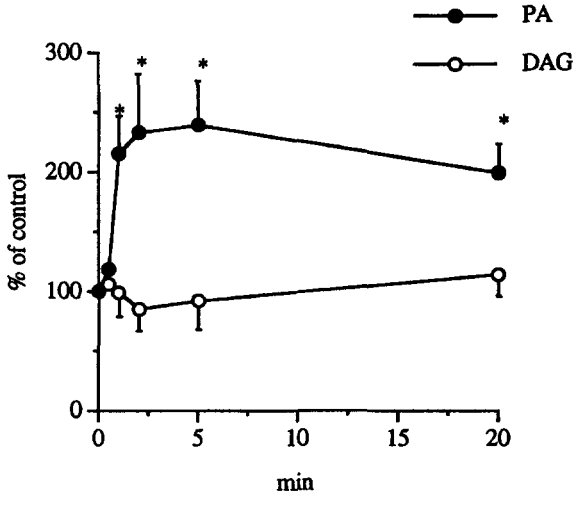

Fig. 1. Time-course of PA and DAG production in [^{3}H]arachidonic acid-labeled thymocytes upon Con A activation. Thymocytes were labeled for 1 h with [^{3}H]AA, then stimulated for the indicated periods of time by Con A (1 μg/10^6 cells). Results are expressed as percentages of PA and DAG measured in unstimulated cells (control), and are means ± SEM of five experiments. *Significantly different from control, $P < 0.05$.

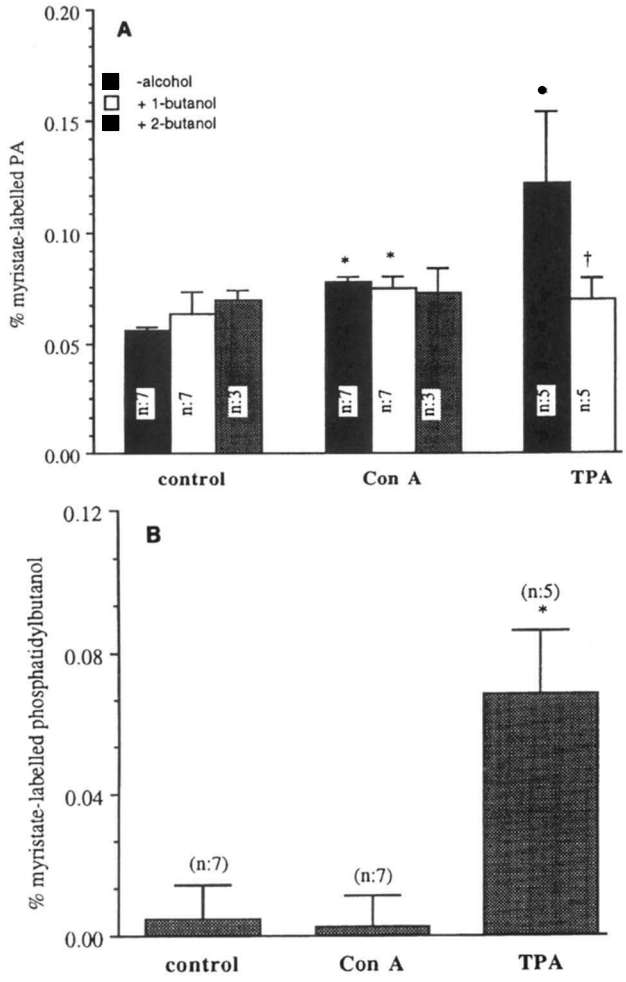

Fig. 2. Stimulation of PLD activity in TPA but not in Con A-stimulated cells. Cells were labeled for 2 h with [^{3}H]myristic acid, then stimulated for 5 min with Con A (1 μg/10^6 cells) or 20 min with TPA (100 nM) in the absence or presence of 0.5% 1-butanol or 2-butanol. Lipids were extracted and analyzed by TLC as described in Methods. Production of ^{3}H-labeled PA (A), and ^{3}H-labeled phosphatidylbutanol (B) is expressed as % of the total incorporated radioactivity. Results are means ± SEM. *Significantly different from control, $P < 0.05$; †significantly different from level measured in the absence of 1-butanol, $P < 0.05$.

the presence of 0.5% 1-butanol, the Con A-induced PA formation was only marginally decreased (Fig. 2A), and no significant phosphatidylbutanol production was observed (Fig. 2B). PA levels were also measured in the presence of 2-butanol, which is not an acceptor in the transphosphatidylation reaction mediated by PLD. Similar results were obtained, indicating that the very slight decrease of PA levels measured in 1-butanol-treated cells was not caused by the transphosphatidylation activity of PLD (Fig. 2A). On the contrary, TPA stimulated PLD activity as shown by the 10-fold increase in phosphatidylbutanol and the decrease of PA production (Fig. 2A, B). In [^{3}H]choline-labeled cells, PLD activity was followed and quantified by the production of [^{3}H]choline. As shown in **Fig. 3,** when cells were stimulated with TPA, a significant increase in [^{3}H]choline was observed, while no significant production of [^{3}H]choline was observed in the Con A-stimulated cells as compared to control cells (Fig. 3).

Inhibition of PA formation in Con A-stimulated cells by a specific PC-PLC inhibitor

Another possible PC-derived pathway for PA production upon Con A stimulation is PC-PLC hydrolysis followed by the phosphorylation of DAG to PA by DAG kinase. We were not able to measure significant increase of [^{3}H]phosphocholine or a decrease of [^{3}H]PC in cells stimulated for 5 min by Con A (results not shown). As these observations could result from a rapid turnover of choline and phosphocholine, we performed another set of experiments with a PC-PLC inhibitor. Pretreatment of [^{3}H]myristate-labeled thymocytes with the PC PLC inhibitor D609 (50 μg/ml) for 1 h before stimulation by Con A resulted in a decreased PA production. Indeed, the 50% increased labeled PA obtained after 5 min of Con A stimulation was significantly abolished when cells were first treated with D609 (**Fig. 4A**). As the specificity of this inhibitor might be questionable, we performed the same experiments in cells labeled with arachidonic acid. As shown in Fig. 4B, the Con A-induced PA level was only slightly lowered as compared to the marked decrease observed in cells labeled with myristic acid. These observations indicate that D609 did not affect the synthesis of PA derived from PI, but rather, inhibited

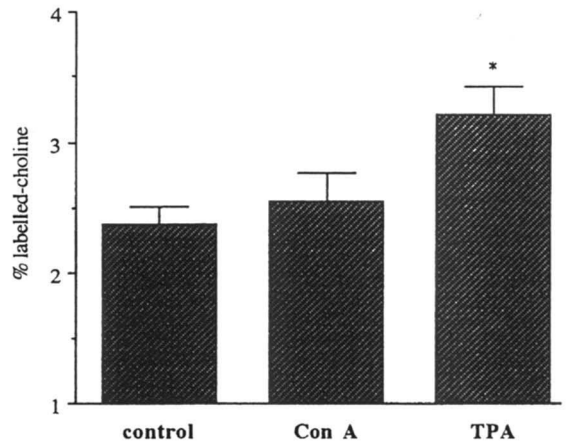

Fig. 3. Measurement of [^{3}H]choline levels in Con A- or TPA-stimulated cells. Cells were labeled for 4 h with [^{3}H]choline, then stimulated for 5 min by Con A (1 μg/10^6 cells) or 20 min with TPA (100 nM). Activation was terminated by lipid extraction as described in Methods. The organic phase (containing PC) and aqueous phase (containing choline and metabolites) were analyzed by TLC. Results represent [^{3}H]choline levels and are expressed as % of the total incorporated radioactivity (aqueous + organic phases). Results are means ± SEM of 5 experiments. *Significantly different from control, $P < 0.05$.

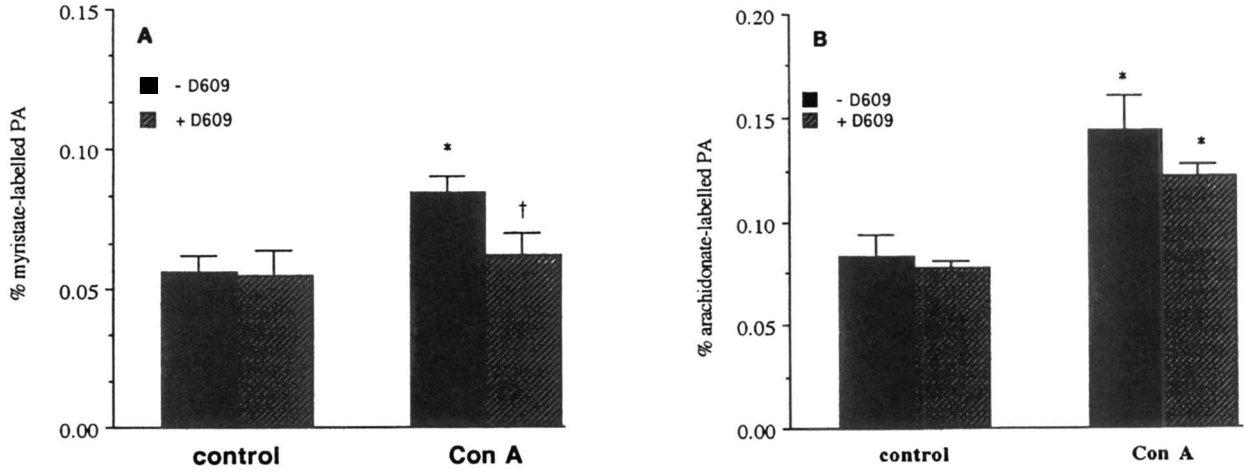

Fig. 4. Effects of the PC-PLC inhibitor D609 on the Con A-induced formation of PA. Cells were labeled with [^{3}H]myristic acid (A) or with [^{3}H]arachidonic acid (B), then preincubated with D609 (50 μg/ml) for 1 h prior to stimulation by Con A for 5 min. Results are expressed as % of the total incorporated radioactivity and are means ± SEM of 5 experiments in (A) and 3 experiments in (B). *Significantly different from control, $P < 0.05$; †significantly different from values in the absence of D609, $P < 0.05$.

the production of PA derived from PC through inhibition of PC specific PLC enzyme.

Molecular species analysis of DAG and diacyl-GPC, -GPI, -GP in control and Con A-stimulated cells

Anthroyl derivatives of DAG and phospholipids obtained as described in Methods were analyzed by reverse phase HPLC. In addition to the high resolution of the HPLC separation, the use of the anthroyl derivatives allowed a highly sensitive fluorometric detection (0.1 ng of each molecular species could be detected). Molecular species of DAG, PA, as well as the phospholipids eventually implicated in their production (through phospholipase action) upon Con A stimulation, were analyzed. An example of HPLC separation profile of diacyl-GP from control cells is given in **Fig. 5.** More than 95% of PA molecular species were of the diacyl subclass and 18 species have been identified using appropriate standards (**Table 2**). As expected, the molecular species composition of diacyl-GP issued from PA and DAG were similar in control cells, these two molecules being interconvertible through the action of DAG kinase and PA phosphohydrolase (Table 2). The diacyl-GPC pattern was similar to that of DAG and diacyl-GP from resting cells (Table 2). Nevertheless, some minor differences are noted. Thus, the 18:0/20:4 (peak 9) and the 16:0/18:0 (peak 17) species were relatively less important in diacyl-GPC than in DAG or diacyl-GP, whereas 16:0/18:1 + 18:0/18:2 (peak 12) and 16:0/16:0 (peak 13) species were present in higher proportions. The diacyl-GPI profile contained mainly arachidonate species, the 18:0/20:4 being the most important. Upon Con A stimulation, the same species as in controls have been identified in all lipid classes studied. Their relative proportions in diacyl-GPC, diacyl-GPI, and DAG were not different between controls and Con A-stimulated cells (results not shown). A quite different profile was observed for the molecular species of diacyl-GP. Thus, although the composition of the molecular species of diacyl-GP in resting cells was similar to that of diacyl-GPC, the molecular species composition of the newly formed PA resembles that of diacyl-GPI but not diacyl-GPC. Thus, as shown in Table 2, 18:0/20:4-GP was significantly increased while species containing palmitate (16:0/16:0-GP, 16:0/18:0-GP, 16:0/18:2-GP) as well as stearate (18:0/18:0-GP and 16:0/18:1-GP plus 18:0/18:2-GP) decreased when compared to unstimulated cells. Pessin, Baldassare, and Raben (43) have compared profiles of diglycerides to phospholipids by calculating the correlation coefficient defined as follows:

$$\frac{\Sigma(A \cdot B)}{\sqrt{(\Sigma A^2) \cdot (\Sigma B^2)}} \qquad (44)$$

where in our experiments, for molecular species i to n, Ai is the percentage of a particular species (i) in a specific phospholipid (PC, PI) profile, and Bi is the percentage of this species (i) in PA profile. **Table 3** presents results obtained by comparing molecular species of PA from stimulated and unstimulated cells to profiles of PI and PC. Thus, the PA profile correlates with that of PC in resting cells (correlation coefficient = 0.907). In Con A-stimulated cells, although the PA profile matches well that of PI (corr. coeff = 0.883), it also correlates with that of PC (corr. coeff = 0.758). In both cases, the correlation coefficient markedly differs from 1, indicating a contribution of the other phospholipid. On the other hand,

the increase percentages of labeled PA were 140% and 50% when cells were labeled with arachidonic acid and myristic acid, respectively, and stimulated by Con A. If the myristate-labeled PA represented the PA derived from PC, and the arachidonic acid-labeled PA that derived from both phospholipids (PC and PI), then the contribution of PC to the formation of PA upon stimulation would be ≈35% (50/140 × 100). Thus, a theoretical calculation can be made by assuming an increase of 0.88 nmol/3 × 10^6 cells PA in Con A-stimulated cells as we have reported in (29), and a contribution to the newly formed PA of 35% and 65% from PC and PI, respectively. As shown in **Fig. 6,** the profile of the theoretical PA matches very well that of PA in Con A-stimulated thymocytes, and a correlation coefficient of 0.966 between both profiles can be calculated. Some discrepancy can be observed with the 18:0/20:4 and 18:1/20:4 species, but the former could be explained by a contribution from PIP_2 containing high levels of 18:0/20:4. In conclusion, these analyses support the participation of PIs (≈65%) and PC (≈35%) to the production of PA upon Con A stimulation of rat thymocytes.

DISCUSSION

Stimulation of rat thymocytes by Con A resulted in an early increase of the cellular PA content that peaked at 5 min. We have previously reported that this increase is also accompanied by a change in PA fatty acid composition consisting of an enrichment in unsaturated fatty acids, particularly arachidonic acid, at the expense of saturated fatty acids (29). On the other hand, the production of PA decreased when cells were activated by Con A in the presence of R59022, a DAG kinase inhibitor. These results, as well as the high content of the newly formed PA in arachidonic acid, strongly suggested that PA was most probably issued from the

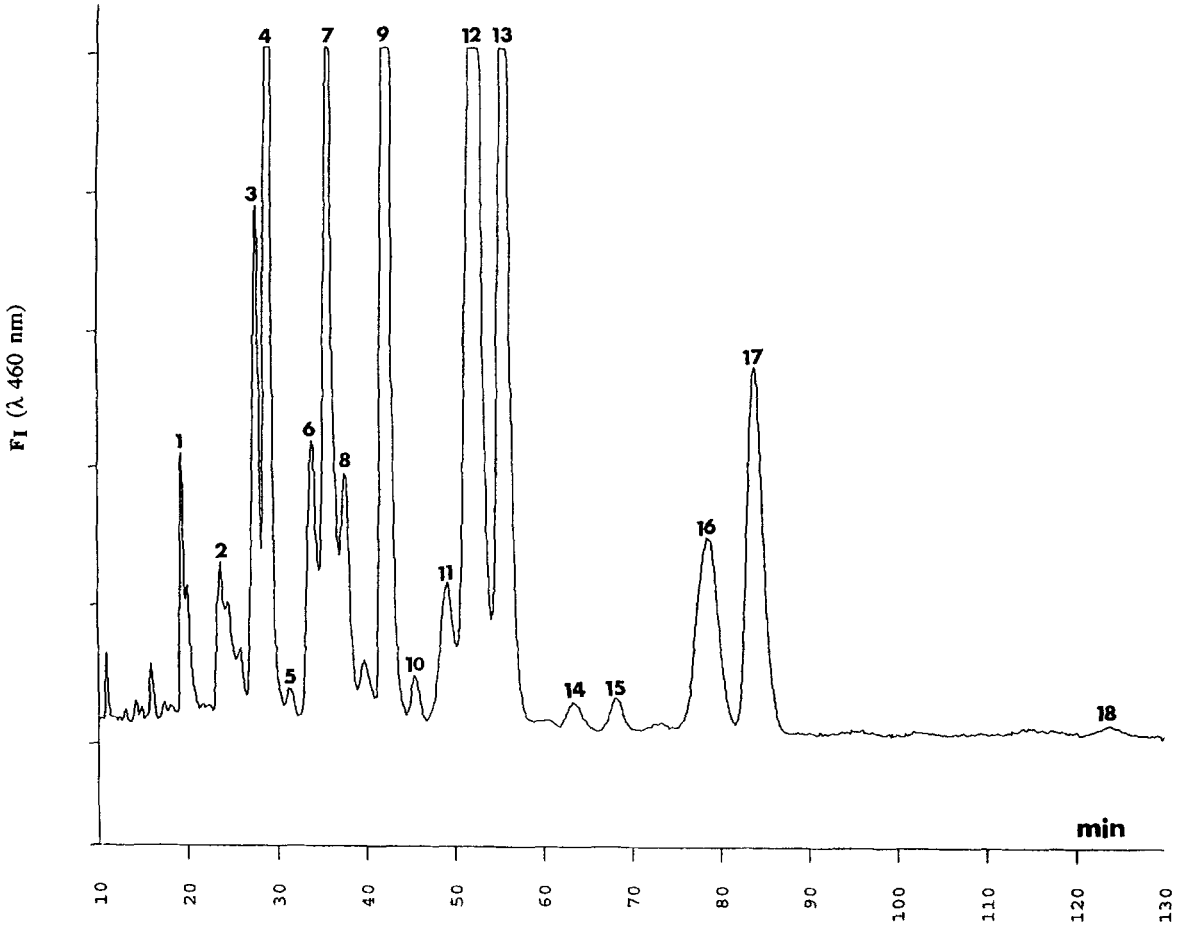

Fig. 5. HPLC separation profile of anthroyl-diacylglycerol molecular species from phosphatidic acid isolated from rat thymocytes. Thymocyte lipids were extracted with chloroform–methanol. PA was separated from phospholipids on TLC, hydrolyzed by PLC, and the DRGs species were anthroylated and separated by reverse phase HPLC as described in Methods. The identity of the peaks is given in Table 2.

PI-PLC-DAG kinase pathway. Nevertheless, this increase of arachidonic acid-containing PA (+0.13 nmol/3 × 10^6 cells) could not explain the total increase of PA mass (+0.88 nmol/3 × 10^6 cells; ref 29), thus PA formation through other pathways could not be excluded. Previous studies in our laboratory have shown that in Con A-stimulated thymocytes, the lysoPA acyltransferase activity was very low and produced weak amounts of PA, which could not explain the important increase observed (A. Goncalves and A. F. Prigent unpublished results). Thus, in the present study we have investigated two other possible pathways of PA formation by PLC or PLD hydrolysis of membrane PC and/or PE. HPLC analysis of PA molecular species revealed that PA, both in resting and stimulated cells, did not contain species of the alkenylacyl subclass, thus indicating that plasmalogen PE did not participate to the production of PA. This analysis also indicated that in resting cells the diacyl-GP molecular species composition resembles that of DAG and diacyl-GPC. When cells were stimulated by Con A, the relative amounts of the diacyl-GP molecular species were markedly changed. Examination of each molecular species revealed a selective increase in the 18:0/20:4 species which is especially abundant in PI. These results favor phosphoinositides as a source of PA formation. The enrichment of PA with the 18:0/20:4 species in Con A-stimulated thymocytes could also be explained by the presence of a membrane-bound DAG kinase, specific

TABLE 2. Comparison of the molecular species of diacyl-GPC, -GPI, -GP, and DAG

Peak No.	Molecular Species	PC (n=3) Control Cells	DAG (n=3) Control Cells	PA (n=7) Control Cells	PA (n=7) Stimulated Cells	PI (n=3) Control Cells
	mol %					
1	18:2/20:4					
	16:1/20:4	1.73 ± 0.67	1.48 ± 0.25	1.15 ± 0.34	0.85 ± 0.14	0.29 ± 0.22
	16:0/20:5					
2	18:2/18:2					
	16:1/22:4	2.48 ± 0.61	3.13 ± 0.41	2.04 ± 0.49	1.22 ± 0.25	0.18 ± 0.09
	16:0/22:6					
3	18:1/20:4	4.00 ± 0.06	4.27 ± 0.64	3.46 ± 0.82	3.87 ± 0.65	15.43 ± 0.01
4	16:0/20:4	6.98 ± 0.51	5.94 ± 0.50	6.38 ± 0.99	7.23 ± 0.76	7.99 ± 1.30
5	18:1/22:5	0.42 ± 0.15	0.74 ± 0.13	1.09 ± 0.42	0.95 ± 0.29	0.97 ± 0.76
6	18:1/18:2	5.63 ± 0.37	5.76 ± 0.60	3.08 ± 0.62	2.44 ± 0.28	2.23 ± 0.28
7	16:0/18:2	11.17 ± 0.19	11.11 ± 0.70	8.04 ± 1.00	6.24 ± 0.59	3.20 ± 0.49
8	16:0/22:4					
	16:0/20:3	4.65 ± 0.15	4.17 ± 0.25	2.28 ± 0.23	1.77 ± 0.17	2.88 ± 0.67
9	18:0/20:4	5.06 ± 0.34	10.15 ± 0.80	11.15 ± 1.38	25.28 ± 1.76[a]	32.64 ± 5.06
10	18:0/22:5	1.25 ± 0.27	0.78 ± 0.03	0.63 ± 0.10	0.62 ± 0.04	0.63 ± 0.12
11	18:1/18:1	3.43 ± 0.16	4.06 ± 0.23	2.68 ± 0.35	2.26 ± 0.18	2.42 ± 0.10
12	16:0/18:1					
	18:0/18:2	23.24 ± 1.35	19.44 ± 1.80	18.64 ± 1.16	15.84 ± 0.87	8.60 ± 0.20
13	16:0/16:0	15.92 ± 1.06	11.36 ± 0.60	12.30 ± 1.69	10.37 ± 1.04	7.29 ± 1.40
14	18:0/20:3	0.21 ± 0.20	0.42 ± 0.09	0.67 ± 0.10	0.79 ± 0.09	3.04 ± 0.80
15	18:1/20:1					
	18:0/20:2	1.36 ± 0.28	0.84 ± 0.08	0.88 ± 0.12	0.55 ± 0.07	0.31 ± 0.12
16	18:0/18:1	5.09 ± 0.67	5.84 ± 0.37	9.35 ± 2.29	7.49 ± 1.61	1.87 ± 0.11
17	16:0/18:0	2.86 ± 0.42	6.52 ± 1.41	11.97 ± 2.55	8.66 ± 1.35	2.33 ± 1.41
18	18:0/18:0	-	0.16 ± 0.10	1.48 ± 1.0	0.79 ± 0.34	-
	unidentified	1.42 ± 0.16	1.32 ± 0.17	1.35 ± 0.47	1.63 ± 0.46	4.42 ± 1.31

The molecular species were identified from their retention time of HPLC and by GLC analysis of the fatty acids after transmethylation of the collected peaks. Data are means ± SEM; n, number of experiments. The peak numbers correspond to those in Fig. 5.

[a]Significantly different from control, $P < 0.01$.

TABLE 3. Comparison of molecular species profiles from PA in unstimulated and Con A-stimulated thymocytes to profiles from PC and PI

	Correlation Coefficient	
	PC	PI
PA from control cells	0.907	0.654
PA from ConA-stimulated cells	0.758	0.883

Molecular species profiles of PA from resting or Con A-stimulated thymocytes were compared to profiles of PC and PI. This comparison was performed by calculating the correlation coefficient between PA profiles and the profile of the potential precursor phospholipids as described in Results.

for arachidonate species (45). If this was the case, then a variation of DAG molecular species should be observed, particularly a decrease in arachidonate species. Our results are not in agreement with this hypothesis as DAG molecular species composition is not affected by Con A treatment (5 min), indicating rather that all DAG species are phosphorylated by the DAG kinase to generate PA. The participation of different DAG kinase isoenzymes from different cell compartments can be assumed. Indeed, in rat T-lymphocytes, a 83 KD DAG kinase is highly expressed but the location of this isoform in these cells is unknown (46). The membrane-bound DAG kinase selective for arachidonate species could be responsible for the formation of PA species which could be responsible for the regeneration of the phosphoinositide pool, while other isoenzymes generated PA species implicated in signal transduction. On the other hand, when the PC pool was selectively labeled with [^{3}H]myristic acid, or [^{14}C]lysoPC, Con A stimulation induced a significant increase (50%) in PA that was lower than the increase observed for the arachidonic acid-labeled thymocytes (140%). This increase most probably represents the PA issued from the hydrolysis of PC by PLC but not by PLD. In fact, this latter pathway was induced in thymocytes stimulated by TPA, as evaluated by the measurement of labeled phosphatidylalcohol formation and the release of [^{3}H]choline, both undetectable in response to Con A stimulation. The involvement of a PC-PLC pathway is supported further by the results obtained with D609, a potent PC-PLC inhibitor. This molecule decreased the production of labeled PA when cells were labeled with [^{3}H]myristic acid and then stimulated by Con A, but had only a marginal effect in cells labeled with arachidonic acid. The comparison of the molecular species profiles indicated that the PA profile from Con A-stimulated cells matches very well a theoretical PA profile issued from the participation of PI (65%) and PC (35%). Taken together, all these results indicate that upon Con A stimulation of rat thymocytes the increase of the cellular PA level can be assigned to the contribution of two different pathways originating from two phospholipid precursors, PC and PIs. In view of these data, the Con A-stimulated cells should produce PA molecular species different from those produced in TPA-stimulated cells.

Thus, as shown in this study, the PA newly formed in Con A-stimulated cells is highly enriched with the PI-specific species (18:0/20:4). This is relevant to our observation that 18:0/20:4-GP is the best activator of PDE-4A5

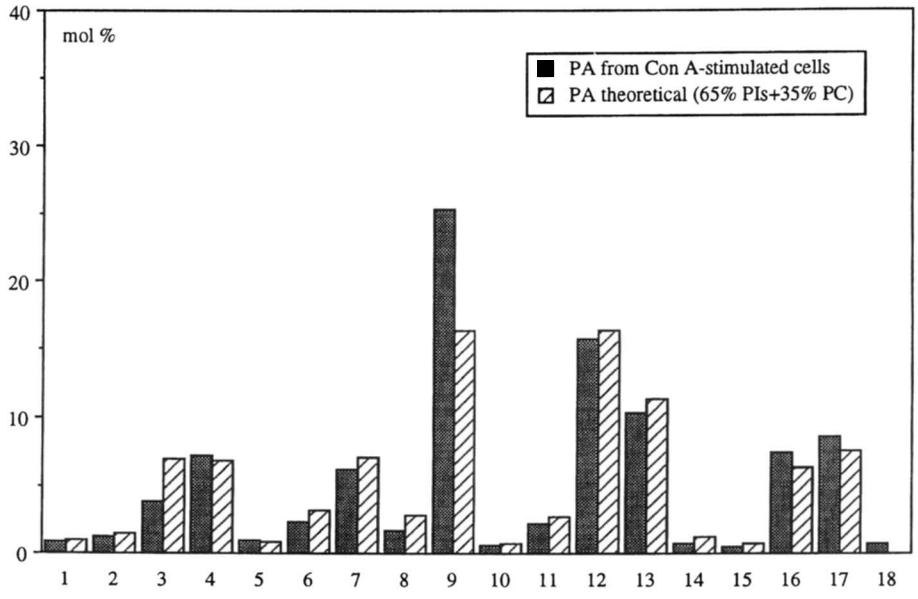

Fig. 6. Comparison of the molecular species PA profile from Con A-stimulated cells to a theoretical PA profile. The theoretical PA profile was calculated by the addition to 0.97 nmol PA present in control cells, to 0.88 nmol of newly synthesized PA (ref. 29) derived from PC (35%) and PI (65%). The correlation coefficient between both profiles equals 0.966.

isoform in vitro (S. El Bawab, O. Macovschi, C. Sette, M. Conti, M. Lagarde, G. Némoz, and A. F. Prigent, unpublished results). Overall, these results suggest that PA formation in stimulated T-cells may activate the cAMP specific PDE to lower and/or modulate the intracellular cAMP levels, and counteract the down-regulating effect of cAMP on the mitogenic activation in these cells.

Literature data increasingly implicate PA as a positive effector in mitogenic signalling. For example, by activating MAP kinase (47) and GAP (27), PLC γ1 (21), PI-4P kinase (22), PA stimulates the signalling cascades that are induced by each of these enzymes in different cells. Our observations underline the importance of determining at which moment and under which conditions the PC-PLD pathway is induced in thymocytes.

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