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# Phospholipid turnover during phagocytosis in human polymorphonuclear leucocytes

Merche GARCÍA GIL,* Fernando ALONSO,† Vicente ALVAREZ CHIVA,*
Mariano SÁNCHEZ CRESPO† and José M. MATO*

**Departamento de Metabolismo, Nutrición y Hormonas, and †Departamento de Nefrología, Fundación Jiménez Díaz, Avda. Reyes Católicos 2, Madrid 3, Spain*

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We have previously observed that the phagocytosis of zymosan particles coated with complement by human polymorphonuclear leucocytes is accompanied by a time- and dose-dependent inhibition of phosphatidylcholine synthesis by transmethylation [García Gil, Alonso, Sánchez Crespo & Mato (1981) *Biochem. Biophys. Res. Commun.* **101**, 740–748]. The present studies show that phosphatidylcholine synthesis by a cholinephosphotransferase reaction is enhanced, up to 3-fold, during phagocytosis by polymorphonuclear cells. This effect was tested by both measuring the incorporation of radioactivity into phosphatidylcholine in cells labelled with [*Me*-$^{14}$C]choline, and by assaying the activity of CDP-choline:diacylglycerol cholinephosphotransferase. The time course of CDP-choline:diacylglycerol cholinephosphotransferase activation by zymosan mirrors the inhibition of phospholipid methyltransferase activity previously reported. The extent of incorporation of radioactivity into phosphatidylcholine induced by various doses of zymosan correlates with the physiological response of the cells to this stimulus. This effect was specific for phosphatidylcholine, and phosphatidylethanolamine turnover was not affected by zymosan. The purpose of this enhanced phosphatidylcholine synthesis is not to provide phospholipid molecules rich in arachidonic acid. The present studies show that about 80% of the arachidonic acid generated in response to zymosan derives from phosphatidylinositol. A transient accumulation of arachidonoyldiacylglycerol has also been observed, which indicates that a phospholipase C is responsible, at least in part, for the generation of arachidonic acid. Finally, isobutylmethylxanthine and quinacrine, inhibitors of phosphatidylinositol turnover, inhibit both arachidonic acid generation and phagocytosis, indicating a function for this pathway during this process.

PtdCho can be synthesized either by a cholinephosphotransferase reaction or by transmethylation. The first pathway involves the transfer of a phosphocholine group from CDP-choline to a 1,2-diacylglycerol molecule (Kennedy & Weiss, 1956). The second pathway involves the addition of three methyl groups into the amino group of a PtdEtn molecule, with *S*-adenosylmethionine as the methyl donor (Bremer & Greenberg, 1961). The purpose of these two pathways for PtdCho synthesis remains unclear. In rat hepatocytes cyclic AMP stimulates PtdCho synthesis by transmethylation (Castaño *et al.*, 1980, 1981) and inhibits PtdCho synthesis by a cholinephosphotransferase reaction (Pelech *et al.*, 1981). Previously we have shown that the addition of opsonized zymosan to human polymorphonuclear leucocytes inhibits the synthesis of PtdCho by transmethylation (García Gil *et al.*, 1981). In the present paper we have studied the effect of zymosan addition to human polymorphonuclear cells on PtdCho synthesis by a cholinephosphotransferase reaction and observed (1) an increased incorporation of choline into PtdCho in cells prelabelled with [*Me*-$^{14}$C]choline and (2) an activation of the enzyme CDP-choline:diacylglycerol cholinephosphotransferase (EC 2.7.8.2). In order to know whether the previously reported inhibition of PtdCho synthesis by transmethylation was due to a shortage

Abbreviations used: PtdIns, phosphatidylinositol; PtdA, phosphatidic acid; PtdCho, phosphatidylcholine; PtdEtn, phosphatidylethanolamine; lyso-PtdCho, lysophosphatidylcholine; PtdSer, phosphatidylserine; PAF, platelet activating factor; IMX, 1-isobutyl-3-methylxanthine.



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of the substrate PtdEtn, the effect of zymosan on PtdEtn synthesis in cells labelled with [1-$^3$H]ethanolamine or [3-$^3$H]serine was also studied. Finally, we have studied the effect of zymosan addition on arachidonic acid release by polymorphonuclear cells. Arachidonic acid has been shown to be released by polymorphonuclear cells in response to zymosan (Waite *et al.*, 1949; Walsh *et al.*, 1981). However, whereas Waite *et al.* (1949) reported that arachidonic acid was mainly provided by PtdCho hydrolysis, Walsh *et al.* (1981) recently concluded that PtdIns was the main source of arachidonic acid. Our results indicate that PtdIns, and not PtdCho, is the main source of arachidonic acid during phagocytosis in polymorphonuclear leucocytes.

## Materials and methods

### *Materials*

[*Me*-$^{14}$C]Choline choride (sp. radioactivity 59.8 Ci/mmol), L-[3-$^3$H]serine (sp. radioactivity 28 Ci/mmol), [1-$^3$H]ethanolamine (sp. radioactivity 23 Ci/mmol), CDP-[*Me*-$^{14}$C]choline (sp. radioactivity 58 Ci/mmol), [5,6,8,9,11,12,14,15-$^3$H]-arachidonic acid (sp. radioactivity 120 Ci/mmol) and [1-$^{14}$C]oleic acid (61 Ci/mmol) were from Amersham International. Lipid standards, CDP-choline and IMX were from Sigma. Silica gel-60 plates were from Merck.

### *Methods*

Human polymorphonuclear leucocytes were isolated from venous blood as described by Sánchez Crespo *et al.* (1980) using polypropylene tubes and 5 mM-EDTA as anticoagulant. The final cell population represented more than 97% polymorphonuclear leucocytes, of which more than 98% were viable by Trypan Blue exclusion. Platelet contamination was always less than 0.1%. The cells were finally resuspended in 6 mM-Hepes [4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid] buffer, pH 7.4, containing 136.8 mM-NaCl, 2.61 mM-KCl, 1.3 mM-$CaCl_2$, 1 mM-$MgCl_2$, 0.1% glucose and 0.25% bovine serum albumin.

*Incorporation of [Me-$^{14}$C]choline, [1-$^3$H]ethanolamine and [3-$^3$H]serine into phospholipids.* Polymorphonuclear cells at a density of $1 \times 10^7$ cells per ml were labelled with [*Me*-$^{14}$C]choline (2 $\mu$Ci/ml) for 30 min at 37°C and at the end of this period washed three times. After washing, the cell suspension was resuspended in Hepes buffer at a density of $10^7$ cells per ml and incubated for 15 min at 37°C. At the end of this period the cell suspension was stimulated with opsonized zymosan (1 mg/ml) and samples containing $4 \times 10^6$ cells were taken at various times, pipetted into precooled tubes and centrifuged 5 min at 400 *g* at 4°C. Phospholipids were extracted from the pellet with 2.9 ml of methanol/chloroform/water (10:10:9, by vol.), the aqueous phase treated three times with 2 ml of methanol/chloroform (1:1, v/v) and the pooled organic fraction dried under a stream of $N_2$ at 22°C. PtdCho and lyso-PtdCho were quantified after separation by t.l.c. on silica gel-60 plates (García Gil *et al.*, 1980). [1-$^3$H]Ethanolamine and [3-$^3$H]serine were added at concentrations of respectively 2 $\mu$Ci/ml and 20 $\mu$Ci/ml. After 30 min incubation the cell suspension was treated as described above. PtdEtn, PtdCho and PtdSer were quantified after separation by t.l.c. on silica gel-60 plates as described by García Gil *et al.* (1980).

*Assay of CDP-choline:diacylglycerol cholinephosphotransferase.* Polymorphonuclear cells were incubated for 15 min at 37°C at a density of $10^7$ cells per ml and at the end of this period stimulated with zymosan (2 mg/ml). At various times after stimulation, samples containing $27 \times 10^6$ cells were centrifuged for 5 min at 400 *g* at 4°C in precooled tubes and resuspended in 200 $\mu$l of Tris/HCl (1 M), pH 8.5. After sonication for 15 s in a Branson B-12 sonifier equipped with microtip set at position 3, the homogenate was incubated at 37°C in a medium containing 500 mM-Tris/HCl, pH 8.5, 0.5 mM-EGTA, 10 mM-$MgCl_2$, 100 nM-CDP-choline and 0.5 $\mu$Ci of CDP-[*Me*-$^{14}$C]choline in a final volume of 500 $\mu$l (Renooij & Snyder, 1981). At various times 100 $\mu$l samples were pipetted into 2 ml of chloroform/methanol/2 M-HCl (6:3:1, by vol.), the organic phase washed three times with 0.5 M-KCl in methanol, dried and counted for radioactivity as described previously (García Gil *et al.*, 1980). Results are expressed as fmol of [*Me*-$^{14}$C]choline groups transferred/min per $10^7$ cells.

*Generation of arachidonic acid and arachidonoyldiacylglycerol in response to zymosan.* Polymorphonuclear cells were washed once in Hepes buffer containing 0.25% delipidated albumin and resuspended in the same buffer, containing 0.5 $\mu$Ci of [$^3$H]arachidonic acid/ml, at a density of $3 \times 10^7$ cells per ml. After 30 min incubation at 37°C, the cell suspension was washed three times with Hepes buffer containing delipidated albumin and resuspended in the same buffer at a density of $10^7$ cells per ml. After incubation for 15 min at 37°C, zymosan (2 mg/ml) was added and 1 ml samples were pipetted into tubes containing 3.75 ml of chloroform/methanol (1:2, v/v). Then 1.25 ml of chloroform and 1.25 ml of 2 M-KCl, containing 5 mM-EDTA were added to form two phases (Billah *et al.*, 1980). The organic phase was then removed, dried under $N_2$ and the labelled lipids were quantified after separation by t.l.c. Neutral lipids were separated in ligroin/diethyl ether/acetic acid (50:50:1, by vol.) and phospholipids in chloroform/methanol/acetic acid/0.1 M-sodium borate (Schrey & Rubin, 1979; Billah *et al.*, 1980). [1-$^{14}$C]Oleic acid was added at a concentration of

0.5 $\mu$Ci/ml. After 30 min incubation at 37°C, the cell suspension was treated as indicated for arachidonic acid.

*Assay of PAF and phagocytosis.* PAF and phagocytosis of zymosan particles were assayed as described by Sánchez Crespo *et al.* (1980) and Alonso *et al.* (1982). PAF levels are expressed as the percentage of radioactivity released by platelets prelabelled with 5-hydroxy[$^3$H]tryptamine in response to 100 $\mu$l samples.

## Results

### *Zymosan stimulates the synthesis of PtdCho by a cholinephosphotransferase reaction*

In cells labelled with [*Me*-$^{14}$C]choline, PtdCho incorporated about 6.5-fold more radioactivity than lyso-PtdCho (results not shown). Zymosan increases the incorporation of [*Me*-$^{14}$C]choline groups into PtdCho in a time- and dose-dependent manner (Figs. 1 and 2), whereas the labelling of lyso-PtdCho is not significantly changed. The increase in PtdCho labelling is linear with time for about 15 min after zymosan addition. Maximal stimulation of PtdCho labelling, about 3-fold, is observed with 5 mg of zymosan/ml. The extent of stimulation of PtdCho labelling induced by various concentrations of zymosan correlates well with the release of PAF ($r = 0.892$; Fig. 2). A similar correlation was observed with $\beta$-glucuronidase release and zymosan uptake (results not shown). The enzymic synthesis of PtdCho, as measured by the formation of [*choline*-$^{14}$C]PtdCho after the addition of CDP-[*Me*-$^{14}$C]-choline, is increased in homogenates obtained from cells stimulated with zymosan when compared with control cells (Fig. 3). PtdCho is the only phos-

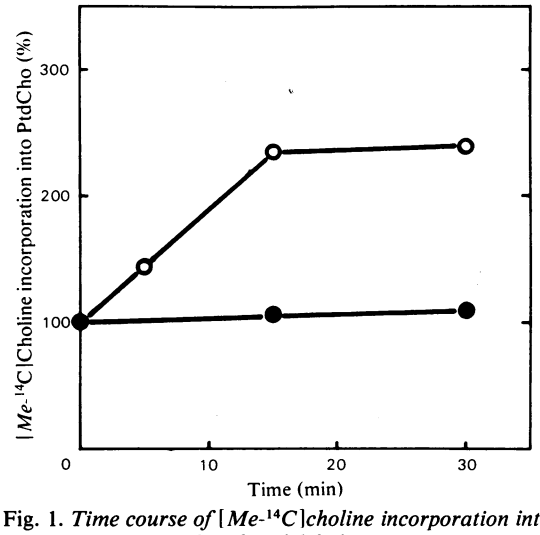


Fig. 1. *Time course of* [*Me*-$^{14}$*C*]*choline incorporation into phosphatidylcholine*

At zero time, cells were stimulated with 1 mg of zymosan/ml (○) or buffer (●), samples taken at the indicated times and the incorporation of [*Me*-$^{14}$C]choline into PtdCho measured as described in the Materials and methods section. The graph shown is the average of three independent experiments; 100% corresponds to 1665 d.p.m./$10^7$ cells.

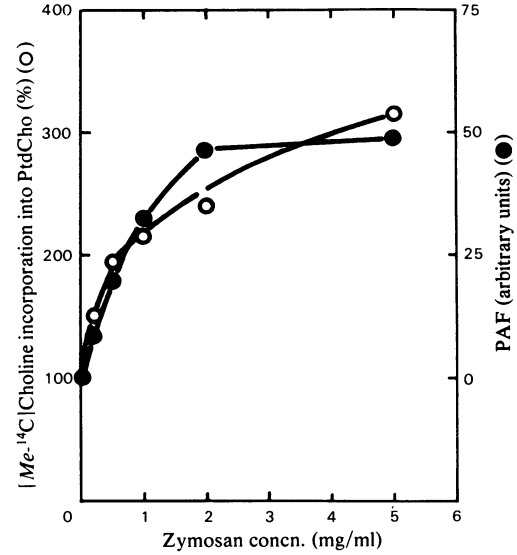


Fig. 2. *Correlation between the extent of* [*Me*-$^{14}$*C*]-*choline incorporation into PtdCho induced by various doses of zymosan and the liberation of PAF*

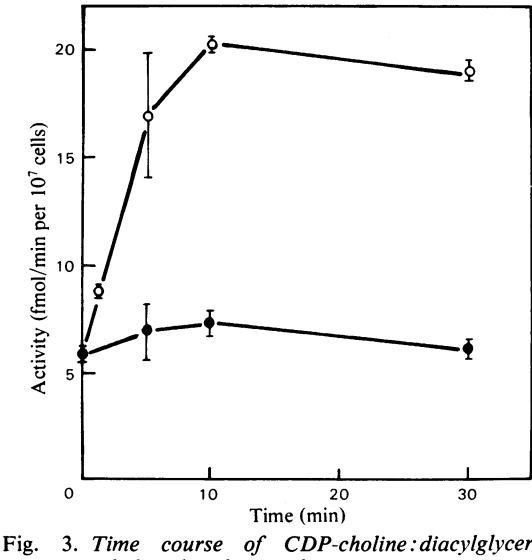


Fig. 3. *Time course of CDP-choline:diacylglycerol cholinephosphotransferase activation*

At zero time, cells were stimulated with 2 mg of zymosan/ml (○) or buffer (●), samples taken and the enzymic activity assayed as described in the Materials and methods section. Data represent means ± S.D. for three independent experiments.

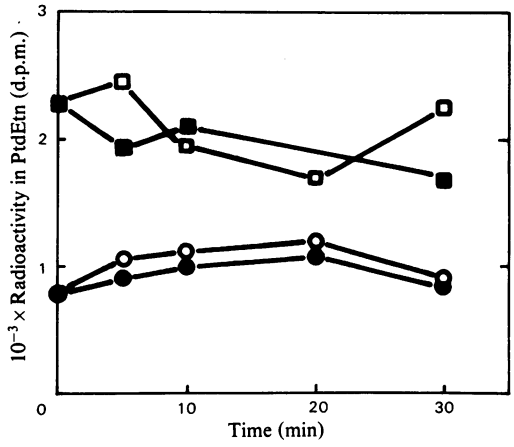


Fig. 4. *Time course of the incorporation of radioactivity into PtdEtn in cells labelled with [1-$^3$H]ethanolamine or [3-$^3$H]serine after zymosan addition*

At zero time, cells were stimulated with zymosan (2 mg/ml; ○, □), or buffer (●, ■), samples taken at the indicated time and the incorporation of radioactivity into PtdEtn measured as described in the Materials and methods section. ○ and ●, Cells labelled with [1-$^3$H]ethanolamine; □ and ■, cells labelled with [3-$^3$H]serine. The graph shown is the average of three independent experiments.

pholipid labelled under these experimental conditions (results not shown). PtdEtn levels measured by the incorporation of labelled ethanolamine or by the decarboxylation of labelled PtdSer remain unchanged after zymosan addition (Fig. 4).

*Specific production of arachidonic acid by zymosan*

When polymorphonuclear cells labelled with [$^3$H]arachidonic acid are challenged with zymosan (1 mg/ml) the production of arachidonic acid increases in a time-dependent manner (Fig. 5). However, in cells labelled with [1-$^{14}$C]oleic acid zymosan does not produce oleic acid (Fig. 5). The production of labelled diacylglycerol is initially increased but decreases at later times after the addition of zymosan (Fig. 6). The addition of zymosan (1 mg/ml; 10 min incubation) depletes PtdIns (about 25%) and PtdCho (about 14%) of labelled arachidonic acid (Fig. 7). As the amount of arachidonic acid incorporated into PtdIns is about 2.5-fold greater than that incorporated into PtdCho (Fig. 7), these results indicate that 80% of the arachidonic acid generated in response to zymosan originates from PtdIns.

*Quinacrine and IMX inhibit the formation of arachidonic acid in response to zymosan*

Quinacrine (0.1 mM) inhibits by about 50% the zymosan (15 min incubation)-induced arachidonic acid formation and phagocytosis (Table 1). IMX, a

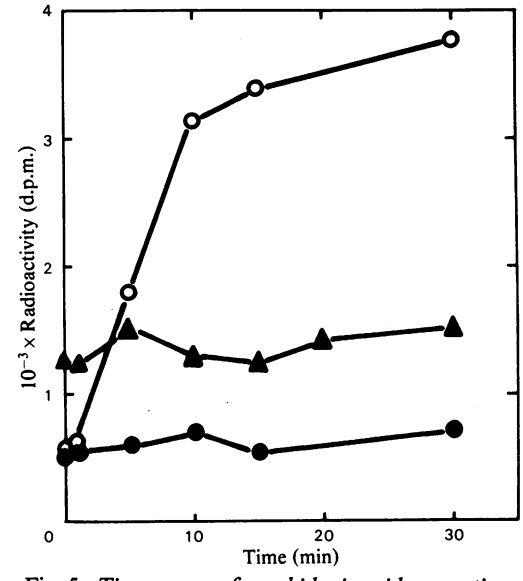


Fig. 5. *Time course of arachidonic acid generation*

At zero time, cells were stimulated with 1 mg of zymosan/ml (○) or buffer (●), samples taken at the indicated times and the amount of [$^3$H]arachidonic acid determined as described in the Materials and methods section. △ shows the levels of [1-$^{14}$C]oleic acid in cells labelled with this compound and then stimulated with 1 mg of zymosan/ml. The graph shown is the average of three independent experiments.

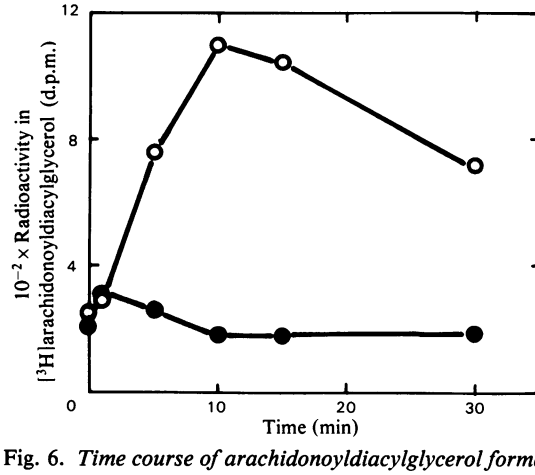


Fig. 6. *Time course of arachidonoyldiacylglycerol formation*

At zero time, cells were stimulated with 1 mg of zymosan/ml (○) or buffer (●), samples taken at the indicated times and the amount of [$^3$H]arachidonoyldiacylglycerol determined as described in the Materials and methods section. The graph is the average of three independent experiments.

phosphodiesterase inhibitor, also inhibits the phagocytic response in human polymorphonuclear leucocytes (Alonso *et al.*, 1982). In the presence of

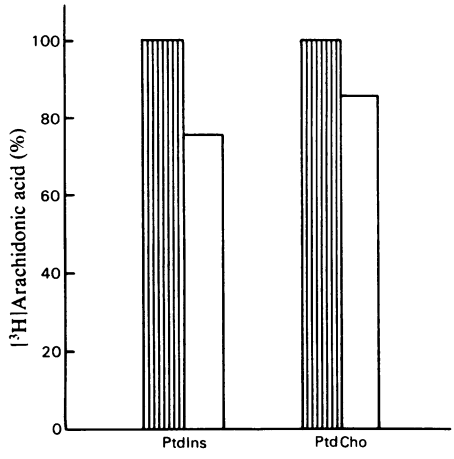


Fig. 7. *Levels of arachidonoylglycerophosphoinositol and arachidonoyl glycerophosphocholine in polymorphonuclear cells prelabelled with [3H]arachidonic acid and stimulated with zymosan (1 mg/ml, 10 min incubation) or buffer alone*

□, Zymosan; ▥, buffer; 100% corresponds to 16500d.p.m. for PtdIns and 6600d.p.m. for PtdCho. The graph shown is the average of three independent experiments.

Table 1. *Effect of quinacrine and IMX on arachidonic acid release and phagocytosis in response to zymosan in human polymorphonuclear cells*

Samples of polymorphonuclear cells were incubated and the release of arachidonic acid or the uptake of zymosan particles were measured as described in the Materials and methods section 15min after the addition of 2mg of zymosan/ml. Quinacrine (0.1mM) or IMX (50μM) were added 5min before zymosan stimulation. Results are averages of three independent experiments.

| Additions | Arachidonic acid (%) | Phagocytosis (%) |
|---|---|---|
| None | 23 | — |
| Zymosan | 100 | 100 |
| Zymosan + quinacrine | 49 | 68 |
| Zymosan + IMX | 66 | 61 |

50μM-IMX, arachidonic acid generation in response to zymosan (15min incubation) decreases by about 40% (Table 1).

## Discussion

The present studies show that PtdCho synthesis by a cholinephosphotransferase reaction is enhanced during phagocytosis in polymorphonuclear cells. The evidence to support this hypothesis includes the following. (1) Zymosan stimulation of polymorphonuclear cells labelled with [*Me*-$^{14}$C]-choline induces an accumulation of radioactivity into PtdCho. (2) This effect depends on the concentration of zymosan and correlates well with the physiological response (PAF and β-glucuronidase release, zymosan uptake). (3) Zymosan causes a stimulation of the enzyme CDP-choline:diacylglycerol cholinephosphotransferase. Whereas the enzyme activity remains stimulated for about 30min, the accumulation of PtdCho is linear for only 15min. A possible explanation could be a consumption of the labelled CDP-choline available intracellularly. In rat hepatocytes the concentration of CDP-choline is the rate-limiting factor in PtdCho synthesis (Pritchard & Vance, 1981). Previously we have shown that the addition of zymosan to human polymorphonuclear cells inhibits the synthesis of PtdCho by transmethylation (García Gil *et al.*, 1981). The time course of phospholipid methyltransferase inhibition after zymosan addition mirrors the activation of CDP-choline:diacylglycerol transferase shown in the present paper. In rat hepatocytes a similar situation exists and the activation of PtdCho synthesis by transmethylation after cyclic AMP addition (Castaño *et al.*, 1980) is accompanied by an inhibition of the synthesis of PtdCho by a cholinephosphotransferase reaction (Pelech *et al.*, 1981). PtdEtn levels, measured by the incorporation of labelled ethanolamine or by the decarboxylation of labelled PtdSer, remained unchanged after zymosan addition. These results indicate that the trigger for PtdCho synthesis by a cholinephosphotransferase reaction is not a shortage in the concentration of the substrate of the transmethylation reaction. The purpose of this differential regulation of PtdCho synthesis remains unclear. It has been postulated (Hirata & Axelrod, 1980) that the transmethylation pathway would provide PtdCho molecules rich in arachidonic acid. However, in human polymorphonuclear cells, arachidonic acid is released (Waite *et al.*, 1949; Walsh *et al.*, 1981), though the synthesis of PtdCho by transmethylation is diminished (García Gil *et al.*, 1981). A similar situation has been recently reported in thrombin-stimulated platelets (Bell *et al.*, 1978; Shattil *et al.*, 1981). Our present data, in agreement with those of Walsh *et al.* (1981), indicate that about 80% of the arachidonic acid generated in response to zymosan originates from PtdIns turnover. This evidence includes the following. (1) Zymosan stimulation depletes PtdIns of arachidonic acid. (2) Zymosan stimulation causes a transient accumulation of arachidonoyldiacylglycerol, the product of phospholipase C action. (3) Arachidonic acid release is inhibited by prior treatment of polymorphonuclear cells with IMX, an agent that blocks PtdIns turnover by raising cyclic AMP levels (Lapetina & Cuatrecasas, 1979; Lapetina *et al.*, 1981). Many tissues exhibit a similar enhanced PtdIns turnover when stimulated (Michell, 1975). Previous experiments with labelled inositol and phosphate

Kornovsky & Wallach, 1961; Sastry & Hokin, 1966; Tou & Stjernholm, 1974) were suggestive of a stimulated PtdIns turnover during phagocytosis in polymorphonuclear leucocytes. Walsh *et al.* (1981) were not able to detect an increase in arachidonoyldiacylglycerol during zymosan stimulation and therefore concluded that a phospholipase $A_2$ acting on PtdIns, rather than a phospholipase C, was responsible for arachidonic acid generation. However, these same authors could not detect an increased formation of lyso-PtdIns, the product of phospholipase $A_2$, after stimulation. The production of arachidonoyldiacylglycerol is transient and about 4-fold lower than that of arachidonic acid, which makes the measurement of this metabolite of PtdIns difficult. We therefore suggest that both phospholipase $A_2$ and phospholipase C are involved in the generation of arachidonic acid during phagocytosis in human polymorphonuclear cells. Finally, the simultaneous inhibition by quinacrine and IMX, inhibitors of the PtdIns turnover (Lapetina & Cuatrecasas, 1979; Matsuzama & Hostetler, 1980; Brockman *et al.*, 1980), of arachidonic acid generation and phagocytosis, indicate a function for this pathway during this process. Although arachidonic acid is the precursor of numerous metabolites, arachidonoyldiacylglycerol is a potential candidate to modulate protein kinase activity, as it has been shown in other systems. (Kishimoto *et al.*, 1980). With respect to the increased synthesis of PtdCho, its function may be the building of membranes during phagocytosis. If so, PtdEtn, the second most abundant phospholipid within membranes, would not be used for PtdCho synthesis by transmethylation but to build membranes.

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