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# IN HUMAN MONOCYTES INTERLEUKIN-1 STIMULATES A PHOSPHOLIPASE C ACTIVE ON PHOSPHATIDYLCHOLINE AND INACTIVE ON PHOSPHATIDYLINOSITOL

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**Abstract**—Interleukin-1 (IL-1) can initiate the synthesis of prostaglandins which in turn act as endogenous modulators of IL-1 production. The human monocyte/macrophage synthesizes various eicosanoids through the activation of the cellular phospholipase system. Cell stimulation results in the activation of phospholipase $A_2$ ($PLA_2$) whose major substrate is phosphatidylcholine (PC) and the release of the eicosanoid precursor arachidonic acid (AA) from PC. Another pathway is the stimulation of a phospholipase C (PLC) mainly active on phosphoinositides and the resulting formation of inositol phosphates (IPs) and diacylglycerol (DAG). Phospholipids other than phosphoinositides can also be hydrolysed by PLC to give rise to DAG. Studies have shown that IL-1 does not activate the IP pathway, but it primarily stimulates a PLC linked to phosphatidylethanolamine in cultured rat mesangial cells, and a PLC linked to PC in Jurkart cells. We have stimulated human monocytes with IL-1 and calcium ionophore A23187 and we have observed their effect on the phospholipase system. The results indicate that IL-1 does not activate the formation of IPs in cells labeled with [$^3$H]*myo*-inositol. In contrast, in cells labeled with [$^3$H]AA, IL-1 causes the formation of DAG associated with the hydrolysis of PC. Moreover, after stimulation with IL-1 there is no accumulation of free AA which would indicate that there has been no activation of $PLA_2$, which occurs instead with A23187 stimulation. These data suggest that, in monocytes, IL-1 does not directly stimulate a $PLA_2$ or a PLC active on phosphatidylinositol; instead it primarily stimulates a PLC active on PC.

Interleukin-1 (IL-1‡), a small polypeptide whose primary sources are monocytes and macrophages, is synthesized by a wide variety of cells. These include keratinocytes, mesangial cells, endothelial and smooth muscle cells, and some T lymphocyte cell lines. It is produced after infection, injury or antigenic challenge, and its biological effects are therefore manifested in almost every tissue and organ [1, 2].

One of the most important biological properties of IL-1 is its ability to initiate prostaglandin (PG) synthesis. When cultured endothelial cells are exposed to IL-1 they synthesize $PGE_2$, $PGI_2$ and platelet-activating factor [3, 4]. IL-1 induces the production of $PGE_2$ in synovial cells and chondrocytes *in vitro* [5]. Treatment of rat glomerular mesangial cells with recombinant human IL-1 induces $PGE_2$ synthesis and activation of a phospholipase $A_2$ ($PLA_2$) [6–8]. PGs seem to act as endogenous mediators of IL-1 production and there are data which support the concept that IL-1 can regulate its own production through a self-induced inhibitor, $PGE_2$ [9, 10].

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‡ Abbreviations: IL-1, interleukin-1; PG, prostaglandin; $PLA_2$, phospholipase $A_2$; AA arachidonic acid; PC, phosphatidylcholine; PL, phospholipid; PLC, phospholipase C; DAG, diacylglycerol; PE, phosphatidylethanolamine; IP, inositol phosphate; FFA, free fatty acids; PBS, phosphate-buffered saline; BSA, bovine serum albumin.

The monocyte/macrophage remains an important source of IL-1. In addition, the human monocyte/macrophage is capable of synthesizing a variety of cyclooxygenase/lipoxygenase-derived eicosanoids [11–14]. Since $PGE_2$ was found to be the major PG synthesized by human peripheral blood monocytes in culture [11], it would seem that the regulation of various aspects of immunologic responses by the monocyte may depend in part on the release of substances such as PG [15–18].

Eicosanoids have been implicated as components of IL-1-stimulated transmembrane signal pathways. The eicosanoid synthesis is made possible through the activation of the cellular phospholipase system [19]. An important source of arachidonic acid (AA) is phosphatidylcholine (PC) after hydrolysis by $PLA_2$; the AA produced is then metabolized to a variety of eicosanoids. Phospholipids (PLs) can also be hydrolysed by phospholipase C (PLC) releasing diacylglycerol (DAG) and by phospholipase D to give rise directly to phosphatidic acid [20]. Work done with rat mesangial cells seems to suggest that IL-1 activates a PLC predominantly linked to phosphatidylethanolamine (PE) [21]. It has also been shown that IL-1 stimulates rapid DAG and phosphorylcholine production from PC in the absence of phosphatidylinositol turnover in Jurkart cells [22].

The purpose of the present work was to study the activation of cellular phospholipases in human blood monocytes in response to stimulation with IL-1. This

object was pursued by measuring the products of PL hydrolysis resulting from the enzymatic activity. The first part deals with the production of inositol phosphates (IPs); the second part deals with the measurement of the loss of radioactivity by PL and the radioactivity associated with the products of PL hydrolysis: free fatty acids (FFA) and DAG. The results indicate that much of the radioactive AA taken up by the cells was incorporated in PC and PE and that PC was implicated in the loss of radioactivity after IL-1 stimulation of the cells. There was an increase in the production of radioactive DAG following stimulation with IL-1, in the absence of changes in the amount of radioactivity in FFA. In contrast the calcium ionophore A23187 stimulated the accumulation of radioactive FFA, without concomitant production of DAG. In addition, IL-1 completely failed to generate radioactive IPs by [$^3$H]-*myo*-inositol-labeled cells.

## MATERIALS AND METHODS

### *Materials*

Dextran T 500 and Ficoll-Paque were from Pharmacia (Uppsala, Sweden). RPMI 1640 medium and phosphate-buffered saline (PBS) were from Gibco BRL, produced by Technologies Ltd (Paisley, U.K.). *myo*-[2-$^3$H]Inositol with PT-271 (18.9 Ci/mmol) and [$^3$H]AA (209.6 Ci/mmol) were purchased from Amersham International (Amersham, U.K.). Bovine serum albumin (BSA), lithium chloride and calcium ionophore A23187 were from the Sigma Chemical Co. (St Louis, MO, U.S.A.). IL-1$\beta$ was a recombinant preparation kindly supplied by Sclavo S.p.A. (Siena, Italy). Ultima Gold, a universal liquid scintillation cocktail, was purchased from Packard, a Canberra Company (Groningen, The Netherlands).

### *Preparation of cells*

Venous blood (300 mL) from three healthy human volunteers was collected for three different experiments using sodium citrate as anticoagulant (3.8%, 1:9 v/v). Blood from a single subject was used for each experiment. The blood was centrifuged at 150 *g* for 18 min; the platelet-rich plasma thus obtained was discarded. The remainder was sedimented on dextran T500 (1:1) and the leukocyte-rich fraction was separated by centrifugation on Ficoll-Paque gradients [23] at 600 *g* for 20 min. The lymphocyte–monocyte layer which appears as a ring between the phases was removed, washed and suspended in RPMI 1640 medium containing 10% heat-inactivated human serum. Monocytes were then separated from the lymphocytes by allowing them to adhere to the surface of plastic Petri plates. The plates were incubated for 90 min at 37° in 5% $CO_2$ atmosphere. The monocytes were recovered by scraping the plates, washed several times with PBS (Dulbecco's formula without $Ca^{2+}$ and $Mg^{2+}$) containing 0.5% BSA and centrifuged at 4°. At the end the monocytes were resuspended in PBS containing 0.25% BSA and characterized by evaluating the non-specific esterase activity. The purity of the preparations was in the range of 95–97%. Cell counts were obtained by phase-contrast microscopy; the number of monocytes obtained from 300 mL of blood was around $60 \times 10^6$. Cell viability was determined by Trypan blue exclusion.

### *Labeling with* [$^3$H]myo-*inositol and stimulation of IP production*

Monocytes ($10 \times 10^6$/mL) were incubated for 4 hr at 37° with 10 $\mu$Ci/mL [$^3$H]*myo*-inositol. After incubation the cells were centrifuged, the pellet was washed twice with PBS and then resuspended in PBS. One millilitre samples ($10 \times 10^6$ cells) were incubated with stirring at 37° in PBS containing 1 mM $CaCl_2$ and 1 mM $MgCl_2$ for 1 min. This was followed by the addition of 10 mM LiCl and the incubation was continued for one more minute. Lithium was added to stop the activity of phosphatases which convert $IP_3$ generated by hydrolysis of phosphatidylinositol 4,5-bisphosphate ($PIP_2$) in $IP_2$ and IP. The cells were incubated with IL-1 (100 pM) for 2 min or with calcium ionophore A23187 (5 $\mu$M) for 2 and 15 min. Since higher concentrations (500 pM) of IL-1 did not stimulate further production of IPs, the concentration of IL-1 to be used in these experiments was fixed at 100 pM. These conditions for IL-1 were also in accordance with those used previously in the literature [21, 22].

At the end of the incubation period the samples were quantitatively emptied in plastic test-tubes containing 3 mL of ice-cold chloroform/methanol/HCl 100/200/2. The samples were homogenized using a Politron Ultra-turrax T25 (Jankel & Kunkel IKA-Labortechnik Stanfen, Germany); 3 mL of chloroform and 0.6 mL $H_2O$ were added in order to bring the concentrations of chloroform/methanol to the ratio of 2/1. The two phases were allowed to separate at −20° for 2 hr.

The levels of radioactive IPs in the aqueous phase were measured after chromatographic separation and counting of the radioactivity [24]. After evaporation of the aqueous phase, the residue was taken up in 3 mL of distilled $H_2O$ and was applied on anion-exchange columns Dowex AG-1 × 8, 100–200 mesh. The following fractions were collected: [$^3$H]inositol and [$^3$H]glycerophosphorylinositol (16 mL of 60 mM ammonium formate, 5 mM disodium tetraborate); IPs (16 mL of 200 mM ammonium formate, 100 mM formic acid); $IP_2$ (20 mL of 400 mM ammonium formate, 100 mM formic acid); $IP_3$ (12 mL of 1 M ammonium formate, 100 mM formic acid). Two millilitre aliquots from each fraction were used for measuring the radioactivity in a liquid scintillation counter after addition of 10 mL of scintillation fluid for aqueous phase.

The organic phase was also evaporated under $N_2$ and the residue was taken up in a given volume of chloroform/methanol 2/1. Aliquots from each sample were used to measure the radioactivity by liquid scintillation counting after addition of 10 mL scintillation fluid (Ultima Gold, Canberra Packard).

### *Measurement of PL hydrolysis in* [$^3$H]*AA-labeled cells*

The activity of $PLA_2$ which hydrolyses the AA in position 2 of the glycerol in the PL of the cellular membranes, was measured by following the reduction in the incorporation of the radioactivity in total PLs

after stimulation of monocytes prelabeled with [$^3$H]-AA. The radioactivity associated with the individual PL before and after stimulation was also measured. In addition, cells prelabeled with AA were also used for the detection of DAG production by the activation of PLC.

Monocytes were resuspended in PBS at a concentration of $20 \times 10^6$ cells/mL and were incubated with 5 $\mu$Ci/ml [$^3$H]AA for 37° for 2 hr. At the end of the incubation period the cells were centrifuged and the pellet washed once with PBS containing 0.5% BSA. There followed two more washings with PBS without BSA. The cells were resuspended in PBS at a concentration of $20 \times 10^6$ cells/mL. Samples (0.5 mL) were incubated with stirring at 37°. Cells were incubated with IL-1$\beta$ or with calcium ionophore A23187. The cells were extracted with chloroform/methanol 2:1 in the presence of 1 $\mu$g/mL butylated hydroxy toluene as antioxidant; after washing of the extract [25] and separation of the phases, the radioactive metabolites were analysed by chromatographic techniques.

After separation of the phases the organic or lipid phase was evaporated under $N_2$, the residue was taken up in a given volume of chloroform/methanol 2/1 and aliquots from each sample were counted in a liquid scintillation counter. This measurement was utilized to determine the extent of incorporation of radioactive AA in cell lipids. Aliquots from the aqueous phase were also counted and the radioactivity measured in the total fraction (less than 5% of the total) was added to that measured in the organic phase. The total radioactivity value was used to determine the percentage of radioactivity incorporated in the cells.

The radioactivity in the following lipid fractions was determined after separation of lipids by TLC and HPLC.

*Total phospholipids.* Solvent: ethylacetate/isoctane/acetic acid/$H_2O$ (90/50/20/100) upper phase. In this system, DAG and the metabolites of the cyclo and lipoxygenase pathways move away from the origin where only the phospholipids remain. In addition aliquots of the lipid extract were spotted on TLC plates and spots were counted without being subjected to chromatography.

*DAG.* Two-dimensional chromatography on TLC silica plates. The solvent in the first dimension consisted of hexane/diethyl ether 90/10; in the second dimension the solvent was chloroform/methanol/ammonia 95/5/0.8. The system is effective in separating DAG and metabolites of the lipoxygenase pathway (hydroxyeicosatetraenoic acid and leukotrienes $B_4$). In the first dimension DAG does not move from the origin; it does move instead in the second dimension and can thus be identified.

*FFA.* Monodimensional chromatography on TLC silica plates. The solvent consisted of hexane/diethyl ether/acetic acid 80/20/1.5.

*Measurement of radioactivity.* The silica spots identified on the TLC, after the described separation, were scraped off the plates, placed on vials and counted after addition of 1 mL 50% methanol and 10 mL of scintillation fluid. The presence of radioactive products was also detected by scanning the chromatographic plate with an appropriate scanner (Dunnschicht-Scanner II LB 2722-2 Berthold, Dortmund, Germany).

*PLs.* PLs were separated by HPLC essentially according to the method described by Patton *et al.* [26] using a two-pump system with the following solvents. Solvent A: acetonitrile/ hexane/ methanol/ *ortho*-phosphoric acid 920/30/30/9. Solvent B: acetonitrile/hexane/methanol 920/30/30. A flow rate of 1.5 mL/min was used for both pumps. Step 1, solvent A 30%, solvent B 70% for the first 5 min; step 2, solvent A 100% for the following 60 min. (Column: LiChrospher Si 100, 10 $\mu$m, Hibar, E. Merck, Darmstadt, Germany; HPLC by Jasco Model 880-PU, Japan Spectroscopic Co., Tokyo 192, Japan). Detection of the radioactivity associated with PLs was achieved by using an on-line radioactivity detector (Flo-one beta, A-200, Radiomatic Instruments and Chemical Co., A Canberra Company, Tampa, FL, U.S.A.) equipped with a 500 $\mu$L flow cell, using Flo-Scint-A as scintillant. The Flo-one beta microprocessor/computer controlled detectors are fully automated instruments, designed to detect and quantitate radioactivity in a flowing system. A complete data reduction software package makes it possible to receive final data in required units of measurement. The net counts may be further processed into cpm, or dpm with special in-built flow-counting calculations.

The data presented in each figure on IP production and PL hydrolysis represent the results obtained in three different experiments; for each experiment duplicate analyses of three different samples were carried out. Significance was determined using the Student's *t*-test.

## RESULTS

Figure 1A illustrates the levels of radioactivity in IPs generated by monocytes incubated with [$^3$H]-*myo*-inositol under basal conditions and after stimulation for 2 min with IL-1 and with calcium ionophore A23187. Incubation with IL-1 resulted in virtually no change in the radioactive levels of IP, $IP_2$ and $IP_3$ over basal values. It must be pointed out that in non-stimulated cells, after 2 min of incubation, values for IP were rather high with respect to those for $IP_3$ and $IP_2$. In contrast, after stimulation with A23187 there was a large increase in the radioactivity of the $IP_2$ and $IP_3$ fractions but a relatively smaller increase in the radioactivity of the IP fraction.

The absence of IP stimulation found after 2 min incubation of monocytes with IL-1 was confirmed also after a longer period of incubation (15 min) (Fig. 1B). Accumulation of labeled $IP_2$ and IP was observed instead after 15 min stimulation of [$^3$H]-*myo*-inositol labeled cells with A23187 (Fig. 1B). The absence of IP stimulation after incubation with IL-1 prompted us to investigate the pathways of PL hydrolysis in cells prelabeled with AA and stimulated with the two agonists.

In monocytes incubated with [$^3$H]AA, it was found that the greater part of the radioactivity (75%) was incorporated into PLs as assessed by TLC separation of non-polar lipids from polar lipids.

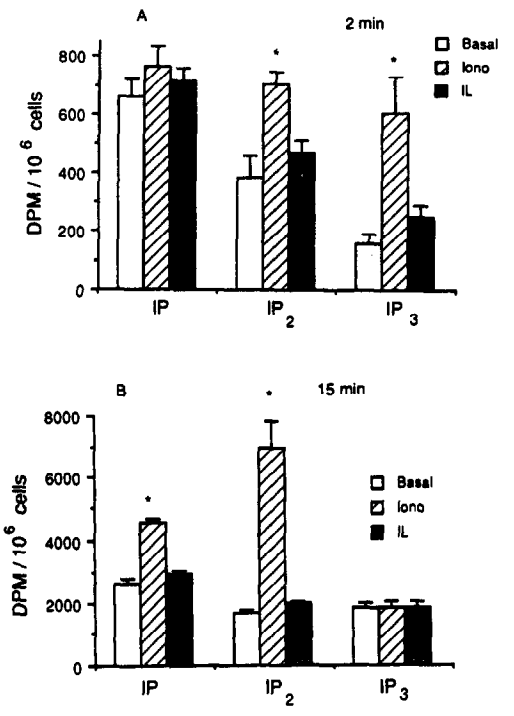


Fig. 1. (A) Levels of radioactive IPs in monocytes prelabeled with [$^3$H]*myo*-inositol after 2 min stimulation with 5 $\mu$M calcium ionophore A23187 and 100 pM IL-1. (B) Levels of radioactive IPs in monocytes prelabeled with [$^3$H]*myo*-inositol after 15 min stimulation with 5 $\mu$M calcium ionophore A23187 and 100 pM IL-1. Error bars represent SEs of the mean values; significance was determined using Student's *t*-test.

After stimulation with IL-1 for a period of 2 min there was a decrease (9.8%) in the amount of radioactivity in the total PLs. In the case of the calcium ionophore A23187 there was almost twice (18.3%) the decrease in the amount of radioactivity during the same incubation period. At this point the loss of radioactivity by the individual classes of PL was measured by HPLC separation and detection of radioactivity in the separated compounds. A typical tracing of the radioactivity distribution of cell PLs at the end of the 2 hr incubation period is shown in Fig. 2. PC and PE appeared to be the major contributors to the loss of radioactivity by total PLs.

In Fig. 3, the results obtained for the labeling of PC and PE before and after stimulation are illustrated. IL-1 caused significant (12%) reduction in the levels of radioactive PC (left panel); in contrast, the levels of radioactivity in PE did not decrease significantly (right panel). Stimulation with A23187 caused a more intense loss of radioactivity by the PC fraction (20%) and an even greater loss of radioactivity by the PE fraction (29%).

Figure 4 illustrates the changes in radioactivity found in DAG and FFA in non-stimulated cells and after stimulation with IL-1 in comparison with A23187. The determination of the radioactivity incorporated in DAG reveals that there was a great increase in the production of DAG in cells stimulated with IL-1 for 2 min whereas there was no parallel increase in the amount of radioactive DAG in cells stimulated with A23187. At 2 min, IL-1 had no effect on the production of FFA while the calcium ionophore A23187 greatly stimulated their formation.

## DISCUSSION

The presence in monocytes of a PLC specifically active on phosphoinositides was demonstrated by the accumulation of IPs after incubation with the calcium ionophore A23187. As can be seen in Fig. 1A and B, there was a 3-fold increase in the levels of $IP_3$ at 2 min over basal values; at 15 min, the levels of $IP_2$ were markedly elevated while the IP levels were also somewhat increased. The absolute levels of total IPs at 15 min stimulation were about 5–6-fold higher than at 2 min with a concomitant

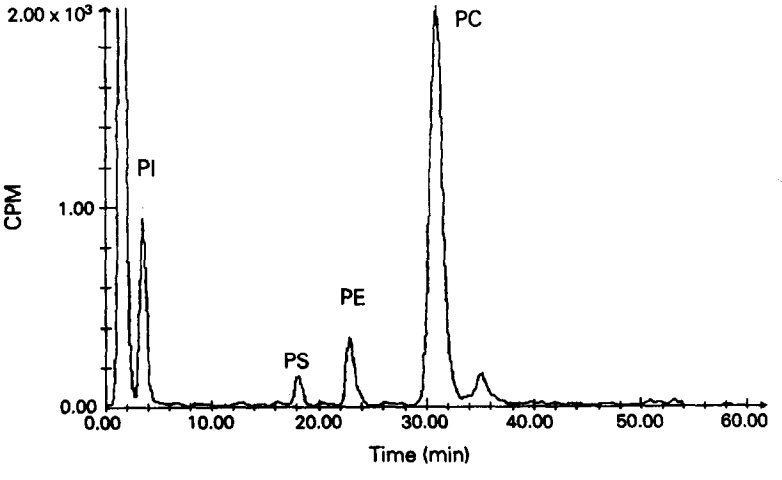


Fig. 2. HPLC profile of [$^3$H]AA-labeled PL from human monocytes.

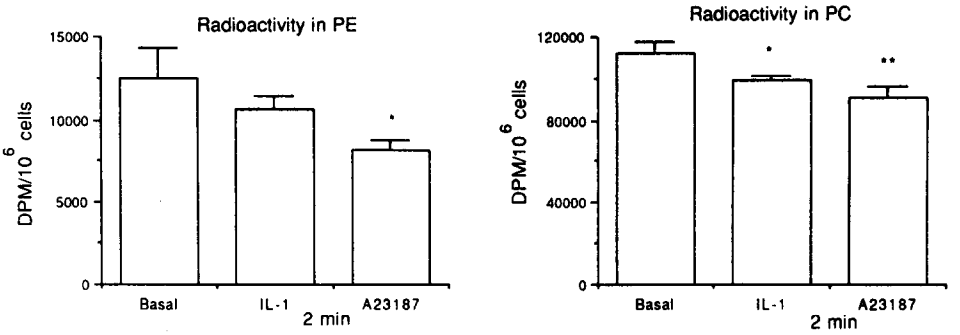


Fig. 3. Loss of radioactivity by PC and PE in monocytes prelabeled with [$^3$H]AA after 2 min stimulation with 100 pM IL-1 and 5 $\mu$M calcium ionophore A23187. Error bars represent SEs of the mean values; significance was determined using Student's $t$-test.

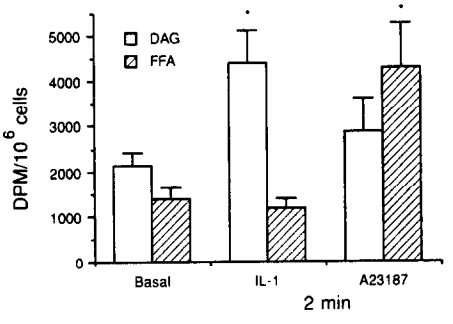


Fig. 4. Release of radioactive DAG and FFA by monocytes prelabeled with [$^3$H]AA after 2 min stimulation with 100 pM IL-1 and 5 $\mu$M calcium ionophore A23187. Error bars represent SEs of the mean values; significance was determined using Student's $t$-test.

redistribution of radioactivity from $IP_3$ to $IP_2$ and IP. The activation of the phosphoinositide-specific PLC by the calcium ionophore is in agreement with previous results [24]. In this type of assay IL-1 did not show any activity, as already described for other types of cells [27].

Although we have no information on the possible stimulation of phosphoinositide hydrolysis by IL-1 at the earlier time points, the lack of accumulation of any of the individual IPs at 2 min, in contrast with the marked elevation of $IP_3$ and $IP_2$ at this same time point induced by the calcium ionophore, minimizes the possibility that the IP pathway was significantly implicated.

When monocytes were incubated with [$^3$H]AA, cell lipids very actively incorporated up to 40% of the total radioactivity incubated with the samples. About 65% of the radioactivity taken up by the cells was recovered in the PLs; the PC and PE fractions contained about 40 to 50% of the PL label. The incubation with the calcium ionophore A23187 besides stimulating an overall PL hydrolysis resulted in the formation of free AA without significant labeling of DAG. The lack of detectable accumulation of labeled DAG, after stimulation by the potent agent A23187 and in spite of the simultaneous activation of the IP pathway, could be a consequence of further processing by lipases, which would be greatly stimulated by the massive increase in cytosolic calcium induced by the calcium ionophore [28].

The loss of radioactivity by PL after IL-1 stimulation was rather selective for PC, in contrast with the effect of A23187 stimulation, and was associated with significant DAG accumulation. These combined effects would suggest that a PLC active on PC was stimulated by IL-1. A more direct determination of a PLC acting on PC would be obtained by measuring the release of phosphorylcholine from cells labeled with [$^3$H-*methyl*]-choline chloride. We have tried to measure this process in monocytes labeled with [$^3$H-*methyl*]-choline chloride but we have found that a very high proportion, more than 80% of the radioactivity recovered after a 4 hr incubation, was associated with phosphorylcholine even in non-stimulated cells. This suggests that choline is actively phosphorylated and released from the cells even in the absence of any PLC stimulation. Release of labeled phosphorylcholine from human T cell Jurkart lines after IL-1 stimulation has been described [22] but labeling of cell PC with exogenous [$^3$H-*methyl*]-choline chloride was obtained after 48 hr of incubation. It is obvious that long periods of incubation cannot be used with monocytes obtained from human subjects. For this reason this approach could not be applied to study PC hydrolysis through a PLC pathway. An alternative approach would be to characterize the fatty acid profile of DAG released after IL-1 stimulation in order to ascertain the possible PL precursor.

The data discussed here allow one to postulate an effect of IL-1 on the activation of selective phospholipases, different from $PLA_2$ and from PLC active on phosphoinositides. The loss of radioactivity by PC points to the activation of a $PLA_2$ by IL-1 but the lack of release of FFA seems to indicate that one is not dealing with a type $A_2$ phospholipase. The large increase in DAG after stimulation of [$^3$H]-AA-labeled cells with IL-1 suggests that the production of such a compound was mediated by the hydrolysis of PLs, mainly PC, through a PLC.

These observations agree with a similar observation with other cell types [21, 22] that IL-1 stimulated PLCs active on PC [22] and on PE [21].

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