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Differential activation by fMet-Leu-Phe and phorbol ester of a plasma membrane phosphatidylcholine-specific phospholipase D in human neutrophil

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Signal transduction involving phosphatidylcholine hydrolysis has been investigated in human neutrophils (PMN) after in situ generation of [^{3}H]alkylacyl-*sn*-glycero-3-phosphocholine ([^{3}H]alkylacyl-GPC) by cell incubation with [^{3}H]alkylacetyl-GPC. When PMN were stimulated with the chemotactic peptide *N*-formyl-Met-Leu-Phe (fMLP) or phorbol myristate acetate (PMA) in the presence of cytochalasin B, both 1-*O*-alkyl-2-acyl-*sn*-glycero-3-phosphate (PA) and 1-*O*-alkyl-2-acyl-*sn*-glycerol (AAG) were generated. On addition of the agonists in the presence of ethanol, phosphatidylethanol (PE) was formed with a concomitant decrease in PA and AAG. These results indicate the presence of a phospholipase D (PLD) acting on phosphatidylcholine in human PMN. The kinetics of hydrolysis were quite different according to the stimulus. Whereas fMLP induced a maximum rise in PA and AAG at 30–45 s, these products began to appear only after 1 min upon cell incubation with PMA. Similar amounts of products were formed at 1 min with fMLP and only at 5 min with PMA. Although similar time courses of PA generation were obtained in the absence of cytochalasin B, AAG were no longer involved and therefore cannot account for intracellular second messenger under physiological conditions. Subcellular distribution studies demonstrated the exclusive location of PA and PE in the plasma membrane. The possible involvement of PA in respiratory burst activation is discussed.

Polymorphonuclear leukocyte; Phospholipase D; Alkylacylglycerophosphocholine; Formylmethionylleucylphenylalanine, *N*-; Phorbol myristate acetate

1. INTRODUCTION

Phosphoinositide hydrolysis by phospholipase C is a general process involved in signal transduction [1], leading to the generation of diacylglycerol and the subsequent activation of protein kinase C [2]. More recently, another source of diglycerides has emerged through the hydrolysis of alkylacyl-GPC by a specific phospholipase C [3,4]. Interestingly, AAG inhibits protein kinase C activation [5] and therefore could contribute to turning off protein kinase C-related transduction processes. PMN exhibit high levels of alkylacyl-GPC [6] and are activated by a large variety of stimuli during inflammation. In addition, a source of diglycerides different from phosphatidylinositol has been pointed out in human PMN [7,8]. Therefore, we focused our attention on the characterization of alkylacyl-GPC hydrolysis in human PMN upon stimulation by two different types of stimuli: fMLP and phorbol esters. The most recent developments in studies of phosphatidylcholine hydrolysis have emphasized the role of a PLD in several cell models [9–12]. In this respect, a recent work in human neutrophil suggests the activation of a PLD by various agonists [13]. In order to discriminate definitively between the two possible

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Abbreviations: PMN, polymorphonuclear leukocytes; PAF, platelet-activating factor; AAG, 1-*O*-alkyl-2-acyl-*sn*-glycerol; PA, 1-*O*-alkyl-2-acyl-*sn*-glycerophosphate; alkylacyl-GPC, 1-*O*-alkyl-2-acyl-*sn*-glycerophosphocholine; PLD, phospholipase D; PMA, phorbol myristate acetate; fMLP, *N*-formyl-Met-Leu-Phe; TLC, thin-layer chromatography

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routes for alkylacyl-GPC hydrolysis, i.e. through phospholipase C or phospholipase D activity, we have based the present work on the exclusive property of phospholipase D of transphosphatidylation [14]. We demonstrate here the differential activation of a PLD upon cell challenge with fMLP and PMA. Cytochalasin B appeared to enhance the activity of a phosphatidate phosphatase [15] since, in its absence, only PA were formed.

2. MATERIALS AND METHODS

[^{3}H]Concanavalin A (60 Ci/mmol), 1-*O*-[^{3}H]octadecyl-2-acetyl-*sn*-glycero-3-phosphocholine (132–179 Ci/mmol, [^{3}H]-PAF) were purchased from Amersham (England); Plasmagel from Laboratoires Roger Bellon (France); Percoll from Pharmacia (France); and fMLP, cytochalasin B, PMA, *p*-nitrophenyl phosphate, fatty-acid-free bovine serum albumin and PA from Sigma (France).

2.1. *Cell incubation*

Cells (10^7/ml in 137 mM NaCl, 2.7 mM KCl, 5.5 mM glucose, 20 mM Hepes, pH 7.4) were isolated on Percoll [16,17]. Cells were then incubated for 15 min at 37°C with [^{3}H]PAF (7–14 nM, 1 μCi/10^7 cells) and washed twice with Hepes buffer containing 2.5% (w/v) bovine serum albumin. After incubation, 53 ± 3.7% of the initial [^{3}H]PAF was incorporated into cells, with 80% of the total cell radioactivity in alkylacyl-GPC, 17% in unmetabolised PAF, and 3% in minor compounds.

Stimulation was performed at 37°C in 137 mM NaCl, 2.7 mM KCl, 5.5 mM glucose, 1.3 mM $CaCl_2$, 1.0 mM $MgCl_2$, buffered with 20 mM Hepes. Cells were first incubated for 10 min with 5 μM cytochalasin B. Thereafter, either fMLP (1 μM, unless otherwise stated) or PMA (100 nM) was added and incubation allowed to proceed up to 5 min. Parallel experiments were performed in the presence of ethanol (0.5%, v/v) which was added together with agonist.

2.2. *Phospholipid analysis*

Lipids were extracted according to the acidic procedure of Bligh and Dyer [18]. EDTA (2.6 mM final) and cold PA (10 μg/sample) were also added. Products of [^{3}H]alkylacyl-GPC hydrolysis were separated by TLC as described by Cohen et al. [19]. Plates were scanned for radioactivity using an automatic TLC linear analyzer (Berthold LB 2848). Radioactive spots were scraped off and counted for radioactivity using Picofluor as scintillation fluid.

2.3. *Intracellular localization of products from phospholipase D activity*

Stimulation was stopped by adding a large volume of cold Hepes buffer. Cells were pelleted and suspended in the lysis buffer, disrupted by cavitation and fractionated on a Percoll gradient as in [15,16]. Lipids were then directly extracted from gradient fractions: 7 ml isopropanol containing 2% acetic acid and cooled to −20°C were mixed with the 2 ml gradient fraction and centrifuged at 3000 × *g* for 15 min at 4°C. Lipids were extracted from the supernatant by addition of 8 ml methanol (with 2% acetic acid), 16 ml chloroform and 14 ml water. The organic phase was concentrated and the lipids separated as given above.

2.4. *Lipid standards*

[^{3}H]Alkylacyl-GPC was obtained by incubation of [^{3}H]PAF with cultures of HL-60 cells. The labeled phospholipid was submitted to the action of phospholipase C, yielding [^{3}H]AAG, or to PLD, forming [^{3}H]PA and [^{3}H]PE.

2.5. *Data presentation*

Results are expressed as means ± SE from 3 separate experiments. Curves were drawn using Sigmaplot software (from Sigma).

3. RESULTS

3.1. *[^{3}H]Alkylacyl-GPC hydrolysis upon cell stimulation with fMLP*

In resting labeled PMN, only trace amounts of [^{3}H]PA and [^{3}H]AAG were detected, as shown in fig.1A. Upon cell stimulation by fMLP, however, a net increase in both products was monitored (fig.1B). When ethanol was added together with fMLP, formation of PE was demonstrated as depicted in fig.1C. In all cases, triglycerides remained constant.

Time course experiments performed in the absence of ethanol demonstrated a very rapid rise in [^{3}H]PA at 30 s with concomitant generation of AAG upon cell challenge with fMLP (fig.2A,C). In the presence of ethanol, both products were similarly decreased, whereas PE was synthesized (fig.2B). Since AAG was affected to the same extent as [^{3}H]PA by the presence of ethanol, we assumed that AAG was formed via hydrolysis of the initial PA. Both PA and AAG remained almost constant after 60 s, indicating that enzyme activation was over at that time. Enzyme activity was noticeable at 10 nM fMLP, increasing continuously at least up to 10 μM effector (fig.3). When PE was formed (fig.3B), the amounts of both PA and DG were similarly diminished (fig.3A,C) as had also been observed in the time course studies (fig.2).

3.2. *[^{3}H]Alkylacyl-GPC hydrolysis upon PMN stimulation with phorbol ester*

At variance with fMLP, PA generation induced by PMA began only after 1 min (fig.4A), the rate increasing thereafter at least up to 5 min. Forma-

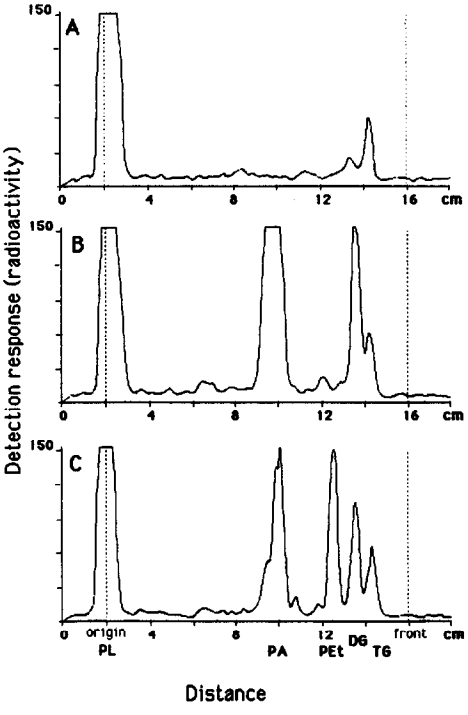

Fig.1. Representative TLC scans of radioactive products. PMN labeled with [^{3}H]PAF were incubated at 37°C for 1 min and processed for lipid analysis as described in section 2. (A) Resting neutrophils; (B) stimulation by fMLP; (C) as in (B), fMLP in the presence of ethanol (0.5%, v/v final). PL, major phospholipids; PA, alkylacylglycerophosphate; PEt, phosphatidylethanol; DG, alkylacylglycerol; TG, triglyceride.

tion of [^{3}H]AAG (fig.4C) was delayed as compared with PA (fig.4A). In addition, no effect of ethanol on AAG radioactivity was observed prior to 2 min, indicating that formation of AAG above the basal level did not take place during that period. Ethanol induced the formation of PE (fig.4B) with kinetics that parelleled quite well those of PA (fig.4A). Because of the metabolic deviation to PE, PA underwent a decrease (fig.4A) and, to a less extent, AAG. Thus, as compared to fMLP, PMA-induced alkylacyl-GPC hydrolysis was a slow process, since the same amounts of products (in the presence or absence of ethanol) were obtained at 45 s with fMLP and 5 min with PMA (cf. figs 2 and 4).

3.3. *Hydrolysis of [^{3}H]alkylacyl-GPC in the absence of cytochalasin B*

In the absence of cytochalasin B, a rise in PA

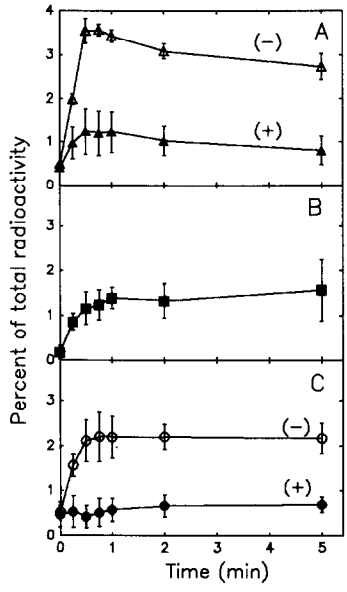

Fig.2. Time course of [^{3}H]PA and [^{3}H]AAG generation upon PMN stimulation with fMLP. Products derived from [^{3}H]alkylacyl-GPC hydrolysis with 1 μM fMLP were checked in the absence (−, open symbols) or presence (+, full symbols) of ethanol in the incubation medium: (A) [^{3}H]PA, (B) [^{3}H]PE, (C) [^{3}H]AAG. Results are expressed as percentages of total radioactivity per sample (6×10^5 dpm/10^7 cells).

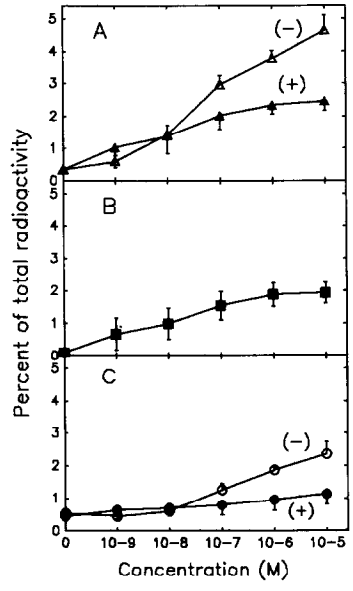

Fig.3. Concentration dependence of fMLP-induced [^{3}H]alkylacyl-GPC hydrolysis in human PMN. Stimulation was performed for 1 min at 37°C. Data shown as in fig.2.

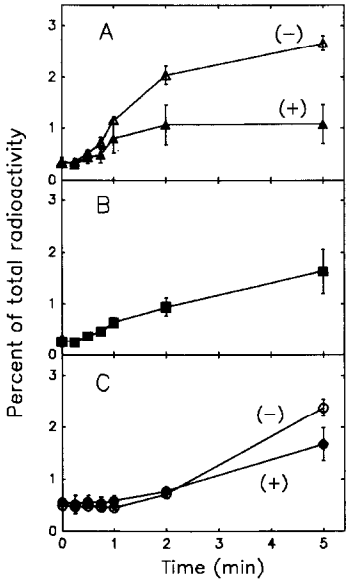

Fig.4. Time course of [^{3}H]PA and [^{3}H]AAG formation upon PMN stimulation with PMA. For details and data expression, see fig.2. (A) [^{3}H]PA, (B) [^{3}H]PE, (C) [^{3}H]AAG. [PMA]: 100 nM.

content was also detected within 1 min in the presence of fMLP (fig.5A). Instead AAG appeared constant as a function of incubation time.

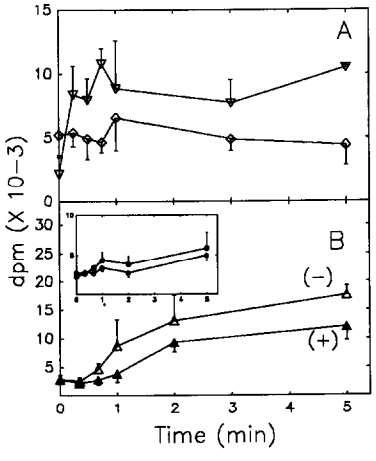

Fig.5. [^{3}H]Alkylacyl-GPC hydrolysis induced by fMLP and PMA in the absence of cytochalasin B. (A) Cells were labeled with [^{3}H]alkyl-lyso-GPC (80–120 Ci/mmol) and stimulated with 100 nM fMLP, in the absence of ethanol [PA (▽), AAG (◇)]. (B) Effect of PMA in cells labeled from [^{3}H]alkylacetyl-GPC in the absence (−, open symbol) or presence of ethanol (+, full symbol). Only PA (△, ▲) and AAG (inset: (○,●) have been plotted.

With PMA as an agonist (fig.5B), PA appeared at a rate similar to that observed in the presence of cytochalasin B (fig.4A). At variance with the results in fig.4C, AAG remained at basal level up to 5 min (fig.5B, inset) and consequently ethanol decreased only the amount of PA (fig.5B). Therefore, in the absence of cytochalasin B, PA was the only product formed from alkylacyl-GPC with both agonists.

3.4. *Intracellular localization of PLD products*

As shown in fig.6A, the distributions of products found in the plasma membrane and granules displayed opposite profiles across the gradient as reported earlier [16]. Whereas [^{3}H]alkylacyl-GPC labeling exhibited a bimodal distribution (fig.6B),

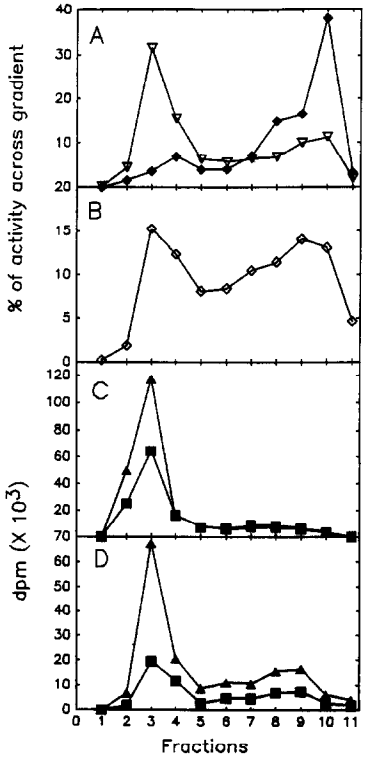

Fig.6. Subcellular distribution of phospholipase D activity. After stimulation (1 min for fMLP; 5 min for PMA), cells were fractionated as in [16] and phospholipids directly extracted from gradient fractions (see text). (A) Profiles of the various markers, i.e. [^{3}H]concanavalin A [plasma membrane (▽)] and *N*-acetyl *β*-D-glucosaminidase [granules (♦)]. (B) Total radioactivity of each gradient fraction. On stimulation of cells with either fMLP (C) or PMA (D) both [^{3}H]PA (▲) and [^{3}H]PE (■) were found in the plasma membrane.

the hydrolysis products [^{3}H]PA and [^{3}H]PE were found exclusively in the plasma membrane upon cell stimulation by fMLP (fig.6C) or PMA (fig.6D).

4. DISCUSSION

The present results provide evidence in support of alkyl-PA as being the first product formed from [^{3}H]alkylacyl-GPC hydrolysis, when human PMN are challenged with either fMLP or PMA. Based on data obtained on transphosphatidylation, it is concluded that PA formation occurs via a PLD acting on alkylacyl-GPC, AAG appearing as a secondary product formed through PA hydrolysis by phosphatidate phosphohydrolase. The PLD activity was monitored on a total cell basis, with the substrate being generated in situ by incorporation of [^{3}H]PAF. This procedure was chosen because it allows specific labeling of choline glycerophospholipid. However, we cannot exclude diacyl-GPC as being the probable substrate of the PLD described here [13]. Cytochalasin B has been revealed to be a useful tool to amplify PMN stimulation [20]. Indeed, the amounts of PA formed in fMLP-stimulated PMN increased from 1.8 to 2.6% upon cytochalasin B addition. However, the main effect of cytochalasin B was to promote the appearance of AAG, which was no longer concerned in the process of [^{3}H]alkylacyl-GPC hydrolysis induced by fMLP or PMA (fig.5A), at variance with a recent report [13].

The question then arises of the physiological significance of this new PLD activity, and whether PA can act as an intracellular second messenger. One of the PMN responses upon stimulation is the activation of the membrane-bound NADPH oxidase responsible for the 'respiratory burst'. There is a body of evidence indicating that diacylglycerol originating from either phosphatidylinositol or phosphatidylcholine cannot account for the entire activation process of the NADPH oxidase [8,21–24]. For instance, the kinetics of formation of total diglyceride are not correlated with the respiratory burst induced by PAF, opsonized particles, or fMLP (without cytochalasin B) [25]. Instead, a correlation between O_2 generation and PA has been pointed out [26] and a phosphatidylinositol-related PLD has been recently reported [27]. The kinetics of PA generation originating from alkylacyl-GPC reported in the present paper exhibit a striking similarity with the time course of the respiratory burst induced (without cytochalasin B) by fMLP and PMA [27]. Whereas the maximum rise in the case of fMLP is at 30–45 s, the PMA-induced burst commences increasing only after 1 min [28]. In addition, the localization of PLD activity in the plasma membrane (fig.6) provides further support for the direct activation by PA of NADPH oxidase in isolated plasma membranes of pig neutrophils [29]. Taken together, all of our data describe the stimulation of a phosphatidylcholine-related PLD in human PMN, which could be involved in a second type of transduction sequence for NADPH oxidase activation.

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