Volume 279, number 2, 249–252 FEBS 09416 February 1991

ADONIS 001457939100134S

Activation of phospholipase D in rabbit neutrophils by fMet-Leu-Phe is mediated by a pertussis toxin-sensitive GTP-binding protein that may be distinct from a phospholipase C-regulating protein

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Received 27 November 1990

Stimulation by *N*-formyl-Met-Leu-Phe (fMLP) of rabbit peritoneal neutrophils, in which phosphatidylcholine was preferentially labeled with 1-*O*-[³H]octadecyl lyso platelet-activating factor, activated phospholipase D, resulting in the formation of [³H]PA from [³H]PC. A direct activator of GTP-binding proteins (G-proteins), NaF, also stimulated [³H]PA formation. fMLP-stimulated [³H]PA formation was inhibited by pertussis toxin (IAP) in a time- and dose-dependent manner. IAP also inhibited fMLP-stimulated IP_3 formation, but the inhibition of IP_3 formation was significantly greater than that of [³H]PA formation. These results indicate that activation of phospholipase D by fMLP in rabbit neutrophils is mediated by an IAP-sensitive G-protein that may be distinct from a phospholipase C-regulating protein.

Phospholipase D; Formylmethionylleucylphenylalanine, *N*-; GTP-binding protein; Pertussis toxin; Rabbit neutrophil

1. INTRODUCTION

In addition to classical effectors such as phosphoinositide-specific phospholipase C (PI-PLC) and phospholipase A_2 which generate phospholipid-related second messengers, phospholipase D (PLD) appears to couple with receptors in many types of mammalian cells [1]. PLD hydrolyzes phosphatidylcholine (PC), possibly other phospholipids too, to produce phosphatidic acid (PA) and choline. PLD also catalyzes the transphosphatidylation reaction in the presence of primary alcohols which are receptors of phosphatidyl moiety to generate a characteristic product phosphatidylalcohols. Taking advantage of the latter reaction, activation of PLD in neutrophils has been well examined. A chemotactic peptide *N*-formyl-Met-Leu-Phe (fMLP) and a complement C5a stimulate the PLD activity in human neutrophils [2,3]. The PLD activation by fMLP in human neutrophils seems to be mediated by a pertussis toxin (IAP)-sensitive GTP-binding protein (G-protein) [4]. In the present study, we demonstrated that an IAP-sensitive G-protein coupling to PLD in rabbit peritoneal neutrophils may be distinct from that regulating PI-PLC.

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Abbreviations: PLD, phospholipase D; PI-PLC, phosphoinositide-specific phospholipase C; PC, phosphatidylcholine; PA, phosphatidic acid; fMLP, *N*-formyl-Met-Leu-Phe; IAP, pertussis toxin; G-protein, GTP-binding protein; [³H]lyso PAF, 1-*O*-[³H]octadecyl lyso platelet-activating factor; PEt, phosphatidylethanol; BSA, bovine serum albumin

2. MATERIALS AND METHODS

2.1. *Materials*

Glycogen (from oyster, Type II), PA (from egg), PC (from egg), fMLP and cytochalasin B were purchased from Sigma, Medium 199 was from Gibco, IP_3 assay kit and 1-*O*-[³H]octadecyl lyso platelet-activating factor ([³H]lyso PAF) were from Amersham, and IAP was from Seikagaku Kogyo Co. Phosphatidylethanol (PEt) was produced from egg PC using crude cabbage PLD by the method of Yang and Freer [5].

2.2. *Preparation, labeling and IAP treatment of rabbit neutrophils*

Neutrophils were harvested from rabbit peritoneum 6–10 h after the injection of 500 ml of sterile 0.15% (w/v) glycogen in 150 mM NaCl. The cells were washed with Ca^{2+}-free Ringer buffer containing 1.2 mM $MgCl_2$, 0.09% (w/v) glucose, 0.1% (w/v) bovine serum albumin (BSA) and 10 mM Hepes, pH 7.4 (Buffer A). The washed neutrophils were suspended at 2×10^7 cells/ml in Medium 199 containing 0.1% BSA and incubated at 37°C for 3 h with [³H]lyso PAF (0.5 μCi/ml) under 5% CO_2/air atmosphere. IAP was added to the cell suspension during the indicated last times of 3 h labeling. After labeling, neutrophils were washed twice with and then suspended in Buffer A.

2.3. *[³H]PA and [³H]PEt formation in labeled neutrophils*

[³H]Lyso PAF-labeled neutrophils (2×10^7 cells/ml) were prewarmed at 37°C for 5 min in the presence of 5 μM cytochalasin B and 1 mM Ca^{2+} and, in some cases, 1% ethanol when appropriate. To 450 μl of the cell suspension was added 50 μl of fMLP or NaF. After incubation at 37°C for the indicated times, the reaction was terminated by adding 2 ml of chloroform/methanol (2:1, by volume).

Lipids were extracted by the method of Bligh and Dyer [6] after termination of the reaction, and then PA and PEt were added to samples as standards. Phospholipids were separated by two-dimensional thin-

layer chromatography as reported by Ciruchalla et al. [7]. Each spot of lipids visualized with iodine vapour was scraped off, the radioactivity was counted, and [^{3}H]PA and [^{3}H]PEt formed were calculated.

2.4. *IP_3 formation by fMLP stimulation*

Control and IAP-treated neutrophils (2×10^7 cells/ml) were prewarmed at 37°C for 5 min in the presence of 5 μM cytochalasin B and 1 mM Ca^{2+}. Stimulation was initiated by adding 30 μl of fMLP to 270 μl of the prewarmed neutrophil suspension. After 30 s, the reaction was terminated by adding 100 μl of 10% perchloric acid since the maximal level of IP_3 formation stimulated by fMLP was observed at 30 s (data not shown). Samples were titrated to pH 7.0 with 1.52 M KOH/75 mM Hepes and then centrifuged to remove precipitated $KClO_4$. IP_3 content in 100 μl of the resultant supernatant was determined using the IP_3 assay kit [8].

3. RESULTS

As already reported in human neutrophils [2], fMLP stimulated [^{3}H]PA or [^{3}H]PEt formation in the absence or presence of 1% ethanol in [^{3}H]lyso PAF-labeled neutrophils in a time-dependent manner (Fig. 1). In the presence of ethanol, [^{3}H]PEt was formed at the expense of [^{3}H]PA production. Of all [^{3}H]phospholipids, only [^{3}H]PC decreased accompanied by the increase of [^{3}H]PA or [^{3}H]PEt (data not shown). These results indicate that under these conditions [^{3}H]PA produced upon fMLP stimulation is profoundly derived from [^{3}H]PC by the action of PLD.

When neutrophils were treated with IAP for 3 h, [^{3}H]PA formation stimulated by 10^{-7} M fMLP was inhibited by IAP in a concentration-dependent fashion, reaching complete inhibition at 10–100 ng IAP/ml (Fig. 2). This result is consistent with the observation in human neutrophils [4] and provides evidence that PLD activation by fMLP is mediated by an IAP-sensitive G-protein. We found that [^{3}H]PA formation stimulated by platelet-activating factor and leukotriene B_4 was also inhibited by IAP (data not shown). The involvement of a G-protein in the PLD activation was further supported by the finding that a common G-protein activator NaF augmented [^{3}H]PA formation in a time- and concentration-dependent manner (Fig. 3). It is well established that in neutrophils an IAP-sensitive G-protein couples fMLP receptors to PI-PLC [9,10]. If a G-protein coupling to PLD is the same as that regulating PI-PLC, IAP could inhibit both PI-PLC and

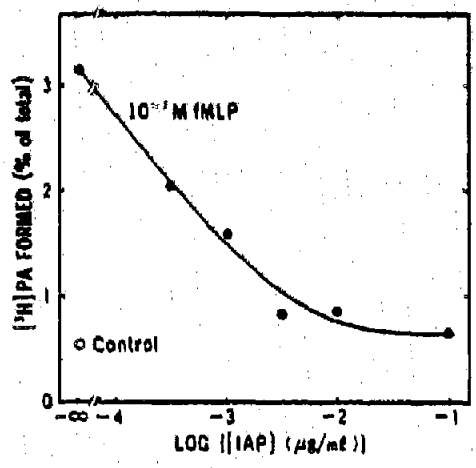

Fig. 2. Inhibition by IAP of fMLP-stimulated [^{3}H]PA formation. Rabbit neutrophils were treated with various concentrations of IAP during 3 h labeling. The IAP-treated, labeled cells were preincubated with 5 μM cytochalasin B and 1 mM Ca^{2+} at 37°C for 5 min, and then exposed to Buffer A (○) or 10^{-7} M of fMLP (●). After incubation at 37°C for 2 min, [^{3}H]PA was determined.

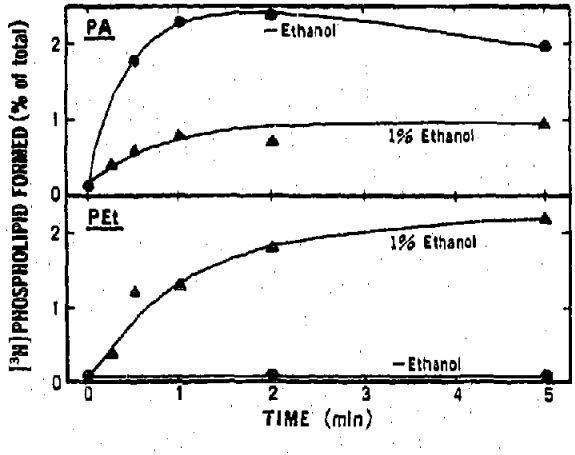

Fig. 1. [^{3}H]PA and [^{3}H]PEt formation in labeled rabbit neutrophils stimulated by fMLP. [^{3}H]Lyso PAF-labeled neutrophils were preincubated with 5 μM cytochalasin B and 1 mM Ca^{2+} in the absence (●) or presence (▲) of 1% ethanol at 37°C for 5 min, and then stimulated with 10^{-7} M of fMLP. After incubation at 37°C for the indicated times, [^{3}H]PA (upper panel) and [^{3}H]PEt (lower panel) were determined.

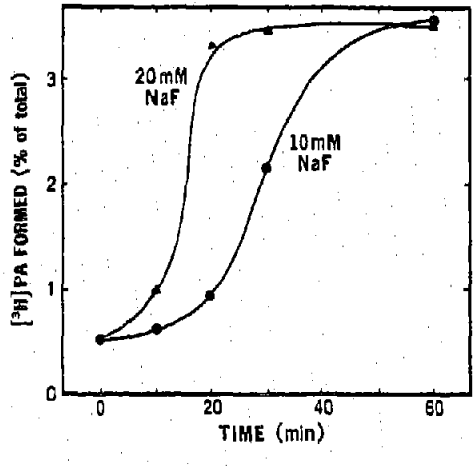

Fig. 3. [^{3}H]PA production by NaF in labeled neutrophils. [^{3}H]Lyso PAF-labeled neutrophils were preincubated with 5 μM cytochalasin B and 1 mM Ca^{2+} at 37°C for 5 min, and then incubated with 10 mM (●) or 20 mM NaF (▲). After incubation at 37°C for the indicated times, [^{3}H]PA formed was determined.

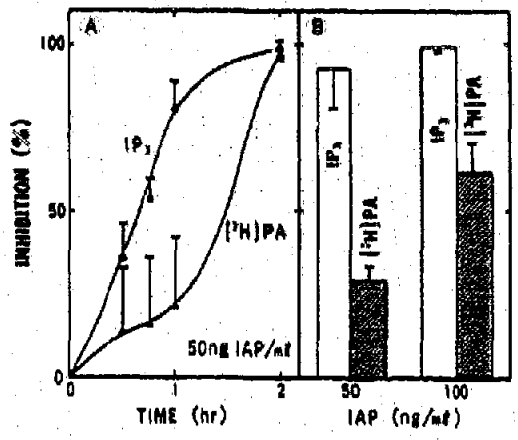

Fig. 4. Comparison of IAP inhibitions of IP_3 and [^{3}H]PA formation stimulated by fMLP. (A) Time-dependent inhibition by IAP of IP_3 (•) and [^{3}H]PA (▲) formation. Each set of rabbit neutrophils was incubated in Medium 199 at 37°C for 3 h in the absence or the presence of [^{3}H]lyso PAF for IP_3 or [^{3}H]PA assay. IAP (50 ng/ml) was added during the last 0.5–2 h of 3 h incubation. Preincubation and stimulation with 10^{-7} M fMLP of neutrophils were carried out as described in Fig. 2, and then IP_3 and [^{3}H]PA formed were determined. IP_3 level in control neutrophils (0.20 ± 0.12 pmol/10^6 cells) was increased by 10^{-7} M fMLP to 2.40 ± 1.13 pmol/10^6 cells. (B) Comparison of IAP inhibitions of IP_3 (open column) and [^{3}H]PA (hatched column) formation in the same set of neutrophils. Neutrophils were labeled with [^{3}H]lyso PAF at 37°C for 3 h. IAP (50 or 100 ng/ml) was added during the last 1 h of the labeling periods. After washing with Buffer A, neutrophils were divided into two portions and then IP_3 content and [^{3}H]PA level before and after stimulation with 10^{-7} M fMLP were determined.

PLD activity stimulated by fMLP with the same or similar kinetics. To test this, we compared time courses of IAP inhibitions of fMLP-stimulated PLD and PI-PLC activities, which were assessed by [^{3}H]PA and IP_3 formation, respectively (Fig. 4). When neutrophils were pretreated with 50 ng IAP/ml, the extent of inhibition in fMLP-stimulated IP_3 formation was significantly greater than that in [^{3}H]PA formation up to 1 h (Fig. 4A). To firmly confirm the result shown in Fig. 4A, the experiments were carried out in the same set of cells: IAP (50 or 100 ng/ml) was added during the last 1 h of the labeling period, the labeled and IAP-treated neutrophils were divided into two portions, and then fMLP-stimulated IP_3 and [^{3}H]PA levels were determined (Fig. 4B). Consistent with the results in Fig. 4A, IP_3 formation was greatly inhibited (80 and 100% inhibition by 50 and 100 ng IAP/ml, respectively) whereas [^{3}H]PA formation was only partially inhibited (30% and 60% inhibition by 50 and 100 ng IAP/ml, respectively).

4. DISCUSSION

Stimulation of PLD through receptors in some types of mammalian cells appears to be mediated by G-proteins [4,11,12]. The type of G-proteins differs from one type of cell to another. In human neutrophils, an IAP-sensitive G-protein seems to couple receptor to PLD since IAP blocks fMLP stimulation of PLD [4]. We confirmed this report in rabbit neutrophils as shown in Fig. 2. Furthermore, we found that NaF also stimulated [^{3}H]PA formation (Fig. 3) and that IAP had no effect of NaF stimulation of the enzyme activity (data not shown). Based on these observations, one can propose a mechanism for PLD activation that fMLP activates an IAP-sensitive G-protein through the receptor stimulation, and the activated G-protein, in turn, enhances PLD activity either directly or indirectly.

It is believed that in some types of cells protein kinase C, which is activated by diacylglycerol produced by PI-PLC, enhances PLD activity [1]. In neutrophils fMLP-stimulated PI-PLC activation is mediated by an IAP-sensitive G-protein [9,10]. These results together suggest a possibility that PLD activation is a consequence of the PI-PLC activation. However, PI-PLC was more sensitive to IAP inhibition than PLD (Fig. 4). From this observation, we hypothesize that a different subtype of IAP-sensitive G-proteins couples fMLP receptors to each of effector enzymes, PI-PLC and PLD.

Thus, it has to be considered that a single receptor interacts with two or more G-proteins and signal transduction through a single receptor may branch out between a receptor and G-proteins to regulate multiple effector systems. Rabbit neutrophils contain two IAP substrates; one is the predominant IAP substrate G_{i2} and the other is a small amount of G_{i3} [13]. Gierschik et al. have recently indicated that both G_{i2} and G_{i3} couple to the fMLP receptor by demonstrating that fMLP stimulation of membranes from myeloid differentiated HL-60 cells markedly enhances the cholera toxin-dependent [^{32}P]ADP-ribosylation of G_{i2} and G_{i3} [14]. These observations support the notion cited above. Alternatively, different states of G-proteins, e.g. the ADP-ribosylated and nonribosylated G-proteins, may regulate distinct effectors. At present, however, it is more difficult to elucidate which subtype of IAP substrates, G_{i2} or G_{i3}, or which state of G-proteins regulates which effector system, PI-PLC or PLD, in rabbit neutrophils.

Acknowledgement: This study was supported in part by a Grant-in-Aid for Scientific Research from the Ministry of Education, Science and Culture of Japan.

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