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PHOSPHATIDIC ACID AND NOT DIACYLGLYCEROL GENERATED BY PHOSPHOLIPASE D IS FUNCTIONALLY LINKED TO THE ACTIVATION OF THE NADPH OXIDASE BY FMLP IN HUMAN NEUTROPHILS

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SUMMARY. It is widely accepted that the activation of the NADPH oxidase of phagocytes is linked to the stimulation of protein kinase C by diacylglycerol formed by hydrolysis of phospholipids. The main source would be choline containing phospholipid via phospholipase D and phosphatidate phosphohydrolase. This paper presents a condition where the activation of the respiratory burst by FMLP correlates with the formation of phosphatidic acid, via phospholipase D, and not with that of diacylglycerol. In fact: 1) in neutrophils treated with propranolol, an inhibitor of phosphatidate phosphohydrolase, FMLP plus cytochalasin B induces a respiratory burst associated with a stimulation of phospholipase D, formation of phosphatidic acid and complete inhibition of that of diacylglycerol. 2) The respiratory burst by FMLP plus cytochalasin B lasts a few minutes and may be restimulated by propranolol which induces an accumulation of phosphatidic acid. 3) In neutrophils stimulated by FMLP in the absence of cytochalasin B propranolol causes an accumulation of phosphatidic acid and a marked enhancement of the respiratory burst without formation of diacylglycerol. 4) The inhibition of the formation of phosphatidic acid via phospholipase D by butanol inhibits the respiratory burst by FMLP.

The mechanisms of the activation of the NADPH oxidase, the enzyme responsible of the respiratory burst associated with production of oxygen free radicals in polymorphonuclear and

Abbreviations used: PC, phosphatidylcholine; PA, phosphatidic acid; alkyl-lyso-PC, (1-O-alkyl)lysophosphatidylcholine; DAG, diacylglycerol; AAG, alkyl-acyl-glycerol; DG, diglyceride.

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mononuclear phagocytes, is still not clear. In spite of contrasting results (1,2,3) it is diffuse opinion (for ref. see 1, 4-6) that the activation by formyl-methionyl-leucyl-phenylalanine (FMLP) is linked to the increase in $[Ca^{2+}]_i$ and activation of protein kinase C (PKC), due to the formation of the second messengers, inositol phosphates and diacylglycerol (DAG). According to this view the increase in the production of DG by hydrolysis of phospholipids would be the essential process in the transmembrane signal for NADPH oxidase activation triggered by chemotactic peptides and other agonists (7). The increased production of DG occurs in two phases. The early is due to hydrolysis of phosphoinositides by phospholipase C, while the late is linked to hydrolysis of other phospholipids (8,9).

Recently it has been demonstrated that with FMLP a phospholipase D (PLD) is activated to selectively hydrolyze choline containing phospholipids (PC) generating phosphatidic acid (PA), which then forms DG by PA-phosphohydrolase (10-14). The activation of this sequence would be the main source of PA and DG produced during neutrophil stimulation. In fact the formation of DG by FMLP in cytochalasin B treated neutrophils is almost completely prevented by propranolol (13), an inhibitor of PA-phosphohydrolase (15). This finding prompted us to investigate the effect of this inhibition of formation of DG on the activation of the NADPH oxidase in neutrophils treated with FMLP.

MATERIALS AND METHODS

Materials. 1-O-[^{3}H]-octadecyl-sn-glycero-3-phosphocholine (110Ci/mmol), [γ-^{32}P]ATP (3000Ci/mmol) were purchased from Amersham. Escherichia coli DG kinase was from Lipidex, Inc. All other reagents were obtained from Sigma.

Methods. Human neutrophils were prepared as in (2). The respiratory burst was measured as stimulation of O_2 consumption by using a Clark oxygen electrode and using 1ml of cell suspension (2×10^7/ml) in the conditions of incubation described in (2). The NADPH oxidase was assayed as O_2^- formation (SOD-sensitive reduction of cytochrome c according to 23) on homogenate of neutrophils obtained by sonication of samples withdrawn at 1 and 3 minutes during the measurement of O_2 consumption. The labeling of neutrophils with [^{3}H]alkyl-lyso-PC, the lipids extraction, the separation of [^{3}H]alkyl-PA, [^{3}H]alkyl-DG and other lipids by TLC were performed according to Billah et al (13). DG mass was determined by enzymatic conversion to [^{32}P]PA according to Preiss (16). The conditions of incubation for the experiments on the changes of phospholipid metabolism were exactly those used for the measurement of O_2 consumption.

RESULTS AND DISCUSSION

The results reported in fig.1 show that in neutrophils, labeled in alkyl-PC by incubating with [^{3}H]alkyl-lysoPC, the stimulation with FMLP in presence of cytochalasin B induces a marked formation of [^{3}H]alkyl-PA and [^{3}H]alkyl-DG. The accumulation of [^{3}H]alkyl-PA is very rapid, reaching a maximum within 30-60 seconds and then decreases, while that of [^{3}H]alkyl-DG occurs later and continues even after 60 seconds due to its derivation from dephosphorylation of [^{3}H]alkyl-PA. In agreement with Billah et.al (13), 250 µM propranolol, a PA-phosphohydrolase inhibitor (15), almost completely prevents the accumulation of [^{3}H]alkyl-DG, and increases that of [^{3}H]alkyl-PA. The data reported in fig.1 also show that propranolol almost completely inhibits the total formation of DG, determined as mass by enzymatic conversion to [^{32}P]PA.

These findings agree with those presented by others (10-14) and demonstrates that FMLP induces the activation of the hydrolysis of

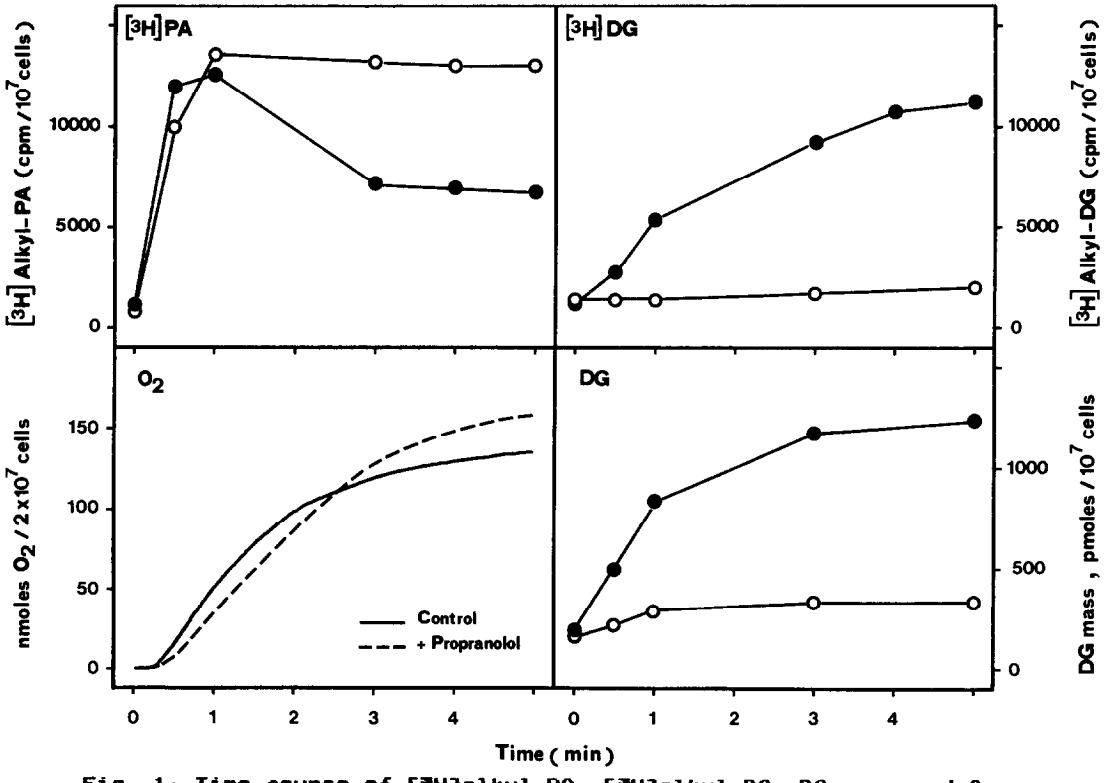

Fig. 1; Time course of [^{3}H]alkyl-PA, [^{3}H]alkyl-DG, DG mass and O_2 consumption by FMLP-stimulated neutrophils in the presence of cytochalasin B (5µg/ml). 250µM propranolol was added to neutrophils suspension 5 min. before 100nM FMLP. ●—● control, ○—○ plus propranolol. Data are of one experiment representative of four.

phosphatidylcholine by PLD and that this reaction is the predominant route of DG formation.

It is known that the DG formed by this route includes either diacylglycerol (DAG) and alkyl-acylglycerol (AAG) since PLD hydrolyses both alkyl- and diacyl- linked PC (10,13). The role of DAGs as activators of PKC (17) is well established, while the role of AAGs is unclear, because they are not effective cofactors of PKC (18), may inhibit PKC-activity (19) and prime the respiratory response to FMLP (20). In any case, if DAGs are essential messengers for the NADPH oxidase activation through the stimulation of PKC, then the inhibition of their formation by propranolol, should inhibit the respiratory burst by FMLP. The results reported in fig. 1 show that this is not the case, because the respiratory response to FMLP is not inhibited by propranolol in spite of the almost complete suppression of DG formation, either as mass and as [^{3}H]alkyl-DG. Fig. 1 reports the recording of the respiratory burst by FMLP. It can be seen that in the presence of the propranolol the O_2 consumption is depressed by about 25% in the early phase and then becomes similar and lasts longer than that in the absence of the drug. Propranolol alone does not modify the basal respiration of neutrophils.

This finding demonstrates that the concept of the essential role of DAG in the activation of NADPH oxidase in neutrophils is questionable.

It is worth pointing out that in the presence of propranolol, FMLP-stimulated neutrophils accumulate PA, the first product of the activated PLD. As shown in fig. 1 the time course of the accumulation of [^{3}H]alkyl-PA parallels that of O_2 consumption. This might be an indication for a role of this acidic phospholipid in the signal(s) formation for NADPH oxidase activation. In addition to the data presented in fig. 1 three other findings agree with this role of PA.

1. The first is the effect of propranolol when added at the end of the respiratory burst by FMLP in the presence of cytochalasin B. In this condition (fig. 2) propranolol induces a further stimulation of O_2 consumption and this recovery of the respiratory burst is associated with a further increase in the accumulation of [^{3}H]alkyl-PA and decrease in the formation of [^{3}H]alkyl-DG. The measurement of the NADPH oxidase on neutrophil homogenates has shown that the recovery of the respiratory burst is accompanied by the activation of the oxidase (data not shown).

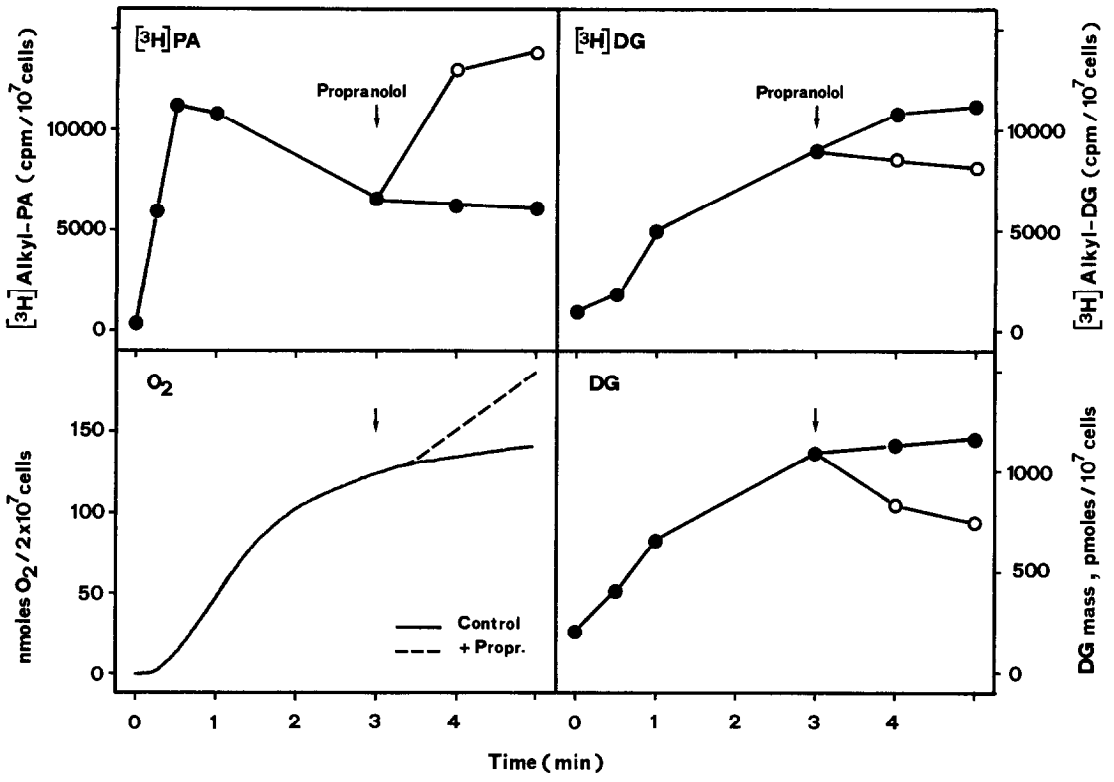

Fig. 2: Effect of 250μM propranolol added at the end of the respiratory burst (arrow) on the formation of [^{3}H]alkyl-PA, [^{3}H]alkyl-DG, DG mass and O_2 consumption in neutrophils stimulated by FMLP in the presence of cytochalasin B. ●—● control, ○—○ plus propranolol. Data are of one experiment representative of four.

2. The second finding is the effect of propranolol on the respiratory burst by FMLP in the absence of cytochalasin B. Fig. 3 shows that in this condition the stimulation of the hydrolysis of phosphatidylcholine through PLD by FMLP is very low. In fact the formation of DG as mass, of [^{3}H]alkyl-PA and [^{3}H]alkyl-DG is very small. Furthermore, in the absence of cytochalasin B, FMLP causes a respiratory burst which is much lower than that in the presence of cytochalasin B. When neutrophils are pretreated with propranolol the respiratory burst by FMLP is greatly potentiated, and this potentation is associated with an increase in the accumulation of [^{3}H]alkyl-PA, due to inhibition of its dephosphorylation to [^{3}H]alkyl-DG. The measurement of the NADPH oxidase on neutrophil homogenates has shown that the potentiation of the respiratory burst by propranolol is due to a greater activation of the oxidase (data not shown).

3. Very recently it has been shown (21) that the inhibition of DG formation by butanol or ethanol, due to the formation of

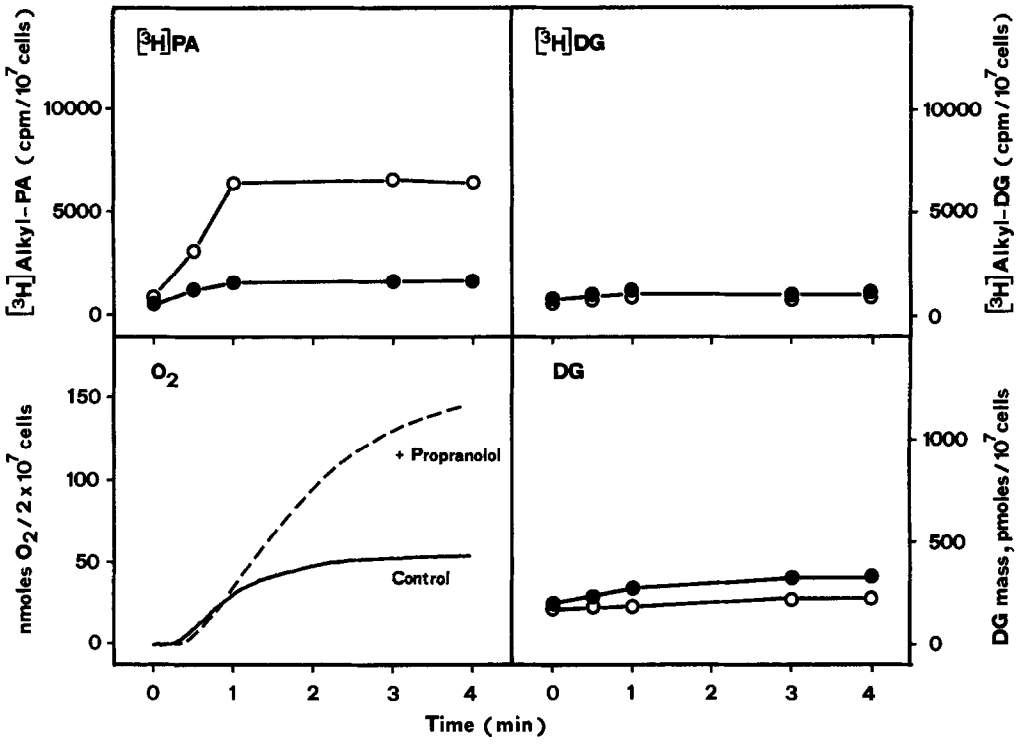

Fig. 3; Time course of [^{3}H]alkyl-PA, [^{3}H]alkyl-DG, DG mass and O_2 consumption by FMLP in the absence of cytochalasin B. 250µM propranolol was added to neutrophils suspension 5 min. before of 100nM FMLP. ●—● control, ○—○ plus propranolol. Data are of one experiment representative of four.

phosphatidylalcohols by PLD, causes an inhibition of the respiratory burst by FMLP and this finding has been interpreted as a demonstration that the activation of phospholipase D is functionally linked to the activation of the NADPH oxidase in neutrophils. We have confirmed these data and the fig. 4 shows that the inhibition of the respiratory burst by butanol is associated with the depression of the formation not only of [^{3}H]alkyl-DG and DG mass but also of [^{3}H]alkyl-PA. The comparison of the data obtained with propranolol and butanol clearly shows that the presence of the propranolol is associated with a persistent and increased formation of PA, while the inhibition of the respiratory burst by butanol is associated with a very marked depression of the PA formation. This is a clear indication that the respiratory burst correlates with the formation of PA.

All these findings 1) do not agree with the concept that in the case of the respiratory burst by FMLP, DAG is the essential messenger causally related with the activation of the NADPH oxidase; 2) are a clear indication that the formation of PA plays a

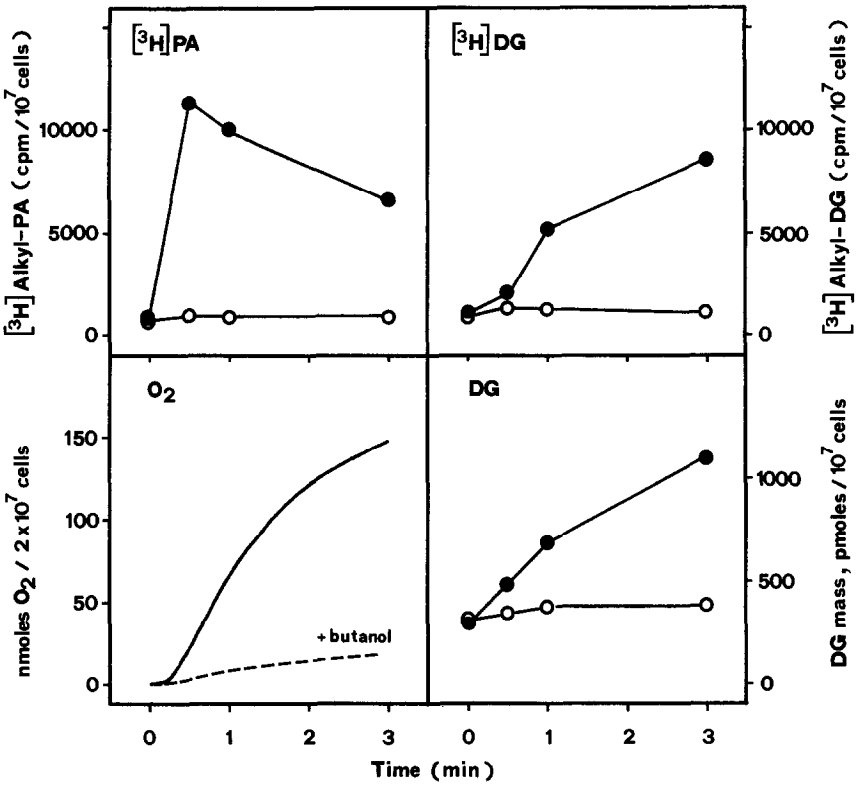

Fig. 4: Effect of 40mM butanol on the respiratory burst and formation of [^{3}H]alkyl-PA, [^{3}H]alkyl-DG and DG mass by FMLP in the presence of cytochalasin B. Butanol was added 5 min. before 100nM FMLP. ●—● control, ○—○ plus butanol. Data are of one experiment representative of three.

role in the formation of the signal(s) for the activation of the NADPH oxidase. Data showing that PA is capable of stimulating the NADPH oxidase activity in cell-free system of pig neutrophils have been previously obtained in our laboratory (22).

Researches is in progress in order to understand the mechanisms by which PA is involved in the activation of NADPH oxidase and on this function of PA in other conditions of neutrophils stimulation, in addition to those presented here. Furthermore,the possibility that the presence of propranolol, a cationic amphiphilic drug, is important for the activity of PA (15), is also under investigation in our laboratory.

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