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Staurosporine stimulates phospholipase D activation in human polymorphonuclear leukocytes

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Treatment of 1-*O*-[^{3}H]alkyl-2-acyl-phosphatidylcholine-prelabeled human polymorphonuclear leukocytes (PMNs) with staurosporine (50 nM to 1 μM) induced a time- and concentration-dependent generation of tritiated phosphatidic acid (PA), reaching approximately 225% of the control value at 15–20 min. In the presence of ethanol, staurosporine induced a production of phosphatidylethanol (PEt) reaching, 250% of control values, and partial inhibition of PA production, consistent with PLD activation. The amount of ether-linked acylglycerol (EAG) was weakly enhanced (29%) after 5 min of PMN treatment; longer treatment resulted in no significant EAG production, suggesting a possible late inhibition of PA hydrolase activity. Staurosporine concentrations that induced an elevation in PA completely depressed protein kinase C (PKC) activity in both soluble and particulate cell fractions, suggesting that PLD activation may occur independently from PKC activation. PLD may thus represent a potential cellular target for staurosporine action.

Staurosporine; Phospholipase D; Protein kinase C; Neutrophil

1. INTRODUCTION

Phospholipase D (PLD) catalyzes the hydrolysis of phosphatidylcholine (PC) to phosphatidic acid (PA), which is a precursor of diacylglycerol (DAG) and ether-linked-acylglycerol (EAG). Stimulation of the PLD pathway in various cell types has been proposed to be involved in cellular responses [1–4]. In human polymorphonuclear leukocytes (PMN) stimulated by formyl peptides, the PLD pathway represents a major source of PA and diglycerides [5,6]. In the presence of ethanol, PLD also catalyzes a transphosphatidylation reaction [7] which transforms PC into a non-physiological metabolite, phosphatidylethanol (PEt). The mechanism PLD activation is believed to occur through both protein kinase C (PKC)-dependent and independent processes.

Staurosporine, a microbial alkaloid from streptomyces species, is a potent PKC inhibitor [8] which interferes with the binding of ATP to the catalytic domain of PKC [9]. Staurosporine has been largely used to investigate the role of PKC in signal transduction pathways and has led to some conflicting data. In many cell types, the activation of PLD by phorbol esters is inhibited by kinase inhibitors including staurosporine [10–15], a finding consistent with the involvement of PKC in PLD activation. In lymphocytes [16] and mast cells [17], PLD activation by phorbol esters is not altered by staurosporine. A staurosporine-induced enhancement of PLD activation in phorbol ester-treated rabbit platelet membrane [18] and in formyl peptide-stimulated polymorphonuclear leukocytes [19] has also been reported. The present study shows that staurosporine concentrations that block PKC activation promotes stimulation of PLD in human PMNs. PLD appears as a potential cellular target for regulation by staurosporine.

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Abbreviations: PMN, polymorphonuclear leukocytes; PA, phosphatidic acid; PEt, phosphatidylethanol; EAG, ether-linked acylglycerol; PKC, protein kinase C; PLD, phospholipase D.

2. MATERIALS AND METHODS

2.1. *Reagents*

1-*O*-[^{3}H]octacedyl-sn-glycerophosphocholine (specific activity 169 Ci/mmol) was from Amersham (les Ullis, France), staurosporine from Kamiya Biomedical Co. (Thousands Oaks, CA), precoated silica gel G plates (0.25 mm thick) from Merck (Nogent, France) and other reagents from Sigma Co. (St Louis, MO).

2.2. *PMN preparation*

Human venous blood, heparinized at 10 units/ml, was obtained from healthy volunteers. PMNs were isolated by one-step centrifugation of whole blood on a cushion of a mixture of Ficoll and Hypaque (monopoly resolving medium, from Flow Laboratories, Puteaux, France), as previously described [20,21]. The purified PMNs (97%) were subjected to hypotonic lysis, washed and resuspended in HBSS (pH 7.4).

2.3. *Preparation of labeled PMNs and lipid analysis*

PMNs (2×10^7 cells/ml) were incubated in calcium-free HBSS con-

taining 10 mM HEPES, 0.1% BSA and 5 μCi/ml 1-*O*-[^{3}H]octadecyl-sn-glycero-3-phophocholine for one hour [11]. Lipids were extracted as described in [22] and aliquots of the chloroform extracts were dried under N_2 and spotted onto TLC silicagel plates. Analysis of lipids by TLC with a solvent system consisting of chloroform/methanol/acetic acid/H_2O (75:48:12.5:3.5) confirmed that approximately 85% of the cellular radioactivity was found in 1-*O*-alkyl-2-acyl-3-glycero-phosphocholine under these conditions [11]. Aliquots of 3×10^6 labeled PMNs in 400 μl of HBSS were prewarmed for 5 min at 37°C and treated in the presence or absence of staurosporine. In some experiments, 1% ethanol was added to the medium 3 min before the addition of staurosporine. Reactions were terminated by adding 1.5 ml of chloroform/methanol/acetic acid (100:200:2); lipids were extracted and separated by TLC. A solvent system consisting of chloroform/methanol/acetic acid (65:15:2) was used to resolve phosphatidic acid ($R_f = 0.42$) from PEt ($R_f = 0.62$), as described elsewhere [11]. 1-*O*-Alkyl-2-acyl-glycerol (EAG; $R_f = 0.62$) was separated using chloroform/methanol/acetic acid (98:2:1), as reported [23].

2.4. *Protein kinase C assay*

PMNs were treated in the absence or presence of 100 and 500 nM staurosporine for 15 min at 37 °C, then were washed and spun down. Pellets were resuspended in lysis buffer containing 20 mM Tris, pH 7.5, 0.25 mM sucrose, 2 mM PMSF, 0.01% leupeptine, 50 mM 2-mercaptoethanol, 2 mM EDTA and 5 mM EGTA and disrupted by sonication. Postnuclear supernatants were spun down at $100,000 \times g$ for one hour and the soluble fraction separated from pellets (particulate fraction). Pellets were homogeneized by sonication in lysis buffer containing 0.1% Triton and centrifuged at $100,000 \times g$ for 60 min at 4°C. Tthe supernatant, referred to as the particulate fraction. PKC activity was assayed by measuring the incorporation of ^{32}P into histone [24]. Briefly, the reaction mixture contained 10 mM $MgCl_2$, 1 mM $CaCl_2$, 2 μg of phosphatidylserine, 0.1 μg of 1,2-sn-diolein, 20 μg of histone, 10 μM ATP (containing 0.5 μCi of [^{32}P]ATP) and 2 μg of soluble or elutable protein in 100 μl of Tris-HCl 20 mM, pH 7.5. Reactions were started with ATP, allowed to run for 10 min at 30°C and stopped by the addition or 400 μl of ice-cold 20% trichloracetic acid followed by 100 μl of BSA (2.5 mg/ml) as carrier. Precipitates were washed after dissolution of the pellet in 0.5 N NaOH and quantified by scintillating counting. PKC activity is expressed in pmoles of ^{32}P incorporated per min per mg protein and was calculated by subtracting the activity measured in the absence of calcium, phosphatidylserine and diolein from that measured in their presence. No significant PKC activity was detected in the non eluable particulate fraction and the PKC activity of the particulate fraction is threrefore that found in the elutable fraction.

2.5. *Statistical analysis*

Statistically significant differences between experiments performed in the presence and absence of staurosporine were determined using the paired Student's *t*-test with a threshold of $P < 0.05$.

3. RESULTS AND DISCUSSION

PMNs were first labeled in 1-*O*-[^{3}H]octadecyl-2-acyl-sn-glycero-3-phosphocholine and then incubated in the presence of staurosporine (50 nM to 1 μM) for 15 min at 37°C. Under these conditions, staurosporine induced a concentration-dependent rise in the basal amount of tritiated PA, which reached a maximal measured value of approximately 225% ($P < 0.001$) of the control value (no staurosporine treatment), as shown in Fig. 1. The staurosporine-induced PA elevation was rapid, reaching 21% ($P < 0.05$) of control value at 5 min of cell treatment, and plateaued at 15–20 min (Fig. 2). The increase in PA levels may be interpreted as being due to

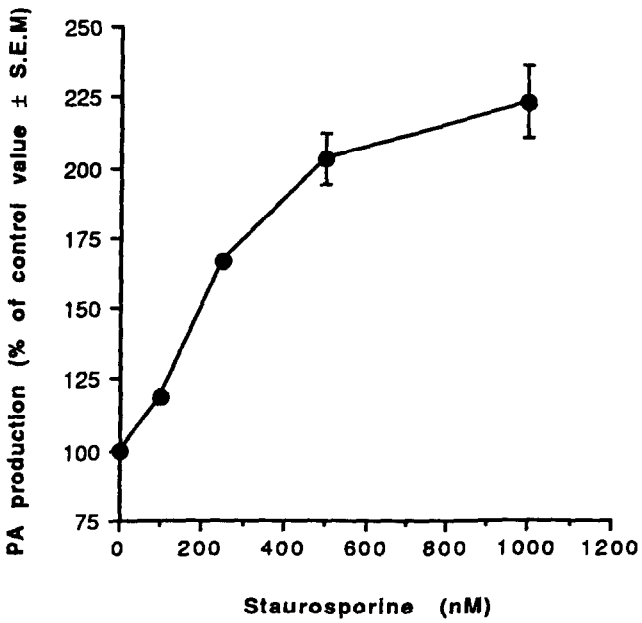

Fig. 1. Staurosporine stimulates phosphatidic acid (PA) production in PMNs. PMNs prelabeled in alkyl-PC were treated in the absence (control) or presence of various concentrations of staurosporine for 15 min. PA production was evaluated after extraction of lipids and separation by TLC. Results are the means of 3 experiments and are expressed as % PA production relative to controls (not treated by staurosporine; 4410 ± 444 dpm/3 × 10^6 PMNs).

activation of PLD and/or inhibition of PA degradation. Measurement of ether-linked acylglycerol (EAG), the dephosphorylated form of PA, showed that EAG production reached approximately 29% ($P < 0.05$) of control values by 5 min, whereas longer periods of PMN treatment resulted in no statistically significant EAG production (Fig. 2). This late decrease in EAG production relative to that in PA raises the possibility that staurosporine inhibited PA hydrolase activity. To determine whether the PA elevation was due to PLD activation, we measured phosphatidylethanol (PEt), a relatively inert metabolite widely accepted as marker of PLD activation. In the presence of 1% ethanol, staurosporine (0.1 to 1 μM) induced a concentration-dependent production of PEt, with partial inhibition of the PA elevation (Fig. 3). The maximal measured potentiation of the PEt level reached approximately 250% ($P < 0.001$) of control values. However, the enhancement of PEt and PA represented less than 1% of the labeling of the PC pool; no significant decrease in the labeled PC pool was thus observed (results not shown). These data are consistent with an activation of PLD in staurosporine-treated PMNs.

The activation of PLD by phorbol esters in various cell types is believed to occur through a PKC-dependent process since these agents bind to and activate PKC [25]. As staurosporine is a potent PKC inhibitor, we determined the degree of PKC inhibition when PMNs were treated with staurosporine. After incubation with

100 and 500 nM staurosporine for 15 min, PMNs were washed and fractionated into soluble and particulate fractions. The activation of PKC in both fractions, as assessed by histone phosphorylating activity, was strongly depressed (Table I), suggesting that PLD activation by staurosporine is not related to PKC activation.

The ability of staurosporine to elevate of PA in resting PMNs is a novel aspect of its biological activating properties. The PA elevation appeared to be mainly due to PLD activation, as evidenced by the use of ethanol, which permits the accumulation of PEt by inhibiting PA formation (Fig. 3). These data are consistent with the concept that the activated PLD in staurosporine-treated PMNs catalyzes both the hydrolysis of PC to PA and the transphosphatidylation reaction. The enhancement of PA level could also be explained, in part, by inhibition of PA phosphohydrolase, as suggested by the weak generation of EAG relative to that of PA (Fig. 2). Alternatively, the PA elevation could result from a potentiation of diglyceride kinase. Such a possibility has been reported with an endogenous PKC inhibitor, sphingosine [26].

Activation of PLD in many cell types has been suggested to be mediated through both PKC-dependent and independent processes, on the basis of the observation that kinase inhibitors such as staurosporine, K252a and H-7 only partially prevent PLD activation. With certain cells, the use of kinase inhibitors has led to conflicting data. For example, in lymphocytes, staurosporine failed to alter PMA-induced PLD activation [16]. In mast cells, differential alteration of PLD activation according to the stimulant used has been reported: antigen-induced PA production was inhibited, whereas PMA-stimulated PLD activation was unaffected [17]. Enhancement of PLD activation by staurosporine has been reported in platelet membranes stimulated with PMA [18] and in formyl peptide-stimulated human

Table I

Inhibition of PKC activity in soluble and particulate fractions derived from staurosporine-treated PMNs

	Histone phosphorylation (% of controlvalues ±S.D.)	
Staurosporine (nM)	Soluble fraction	Particulate fraction
0 (control)	100% (2425 ± 560)	100% (153 ± 45)
0	100%	14 ± 9
500	0	0

PMNs were treated in the absence (control) or presence of 100 and 500 nM staurosporine for 15 min at 37°C, then washed and sonicated. Soluble and particulate fractions were separated by ultracentrifugation. The calcium- and phospholipid-dependent PKC activity of both fractions was assessed in vitro by measuring the incorporation of phosphate into histone. Results are expressed as % of control values, which are given in brackets in pmol P/min/mg protein.

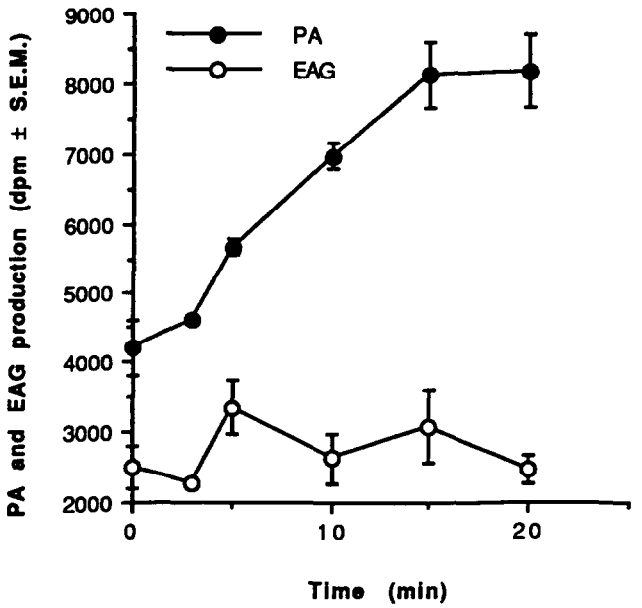

Fig. 2. Time course of phosphatidic acid (PA) and ether-linked acylglycerol (EAG) production by staurosporine-treated PMNs. PMNs labeled in alkyl-PC were treated in the absence (control) and presence of 500 nM staurosporine for various times. Lipids were extracted and separated by TLC. PA and EAG production were calculated by subtracting the production by control cells from that of cells treated with staurosporine. Results are the means of 3 experiments.

PMNs [19]. These contrasting effects of staurosporine would be explained if, in addition to its ability to depress PKC-mediated PLD activation, staurosporine promote PLD activation independently of PKC activation. This latter possibility is supported here by our observation that staurosporine concentrations that blocked PKC activation (Table I) induced PLD activation (Figs. 1–3). Both effects of staurosporine, i.e. stimulation and inhibition of PLD activation may operate to differing extents depending on the cell type, the stimulant and concentration of staurosporine. PKC-independent mechanisms of enhanced PA production in staurosporine-treated PMNs may involve direct activation of PLD by staurosporine or indirect effects via intermediate components. These possibilities remain to be investigated in cell-free systems. Alternatively, staurosporine may induce PLD activation through the activation of uncharacterized kinases [27] or through an elevation of cytosolic calcium [28]. PKC inhibitors such as sphingoid bases, including sphingosine, have been shown to activate of PLD in NG 108–15 cells through a mechanism not involving PKC activation [29]. Sphingosine also inhibits PA hydrolase activity [30]. Activation of PLD by sphingosine requires a long aliphatic chain and a 2-free amino group, structures not present in staurosporine, suggesting that staurosporine and sphingosine may enhance PLD activation through different mechanisms.

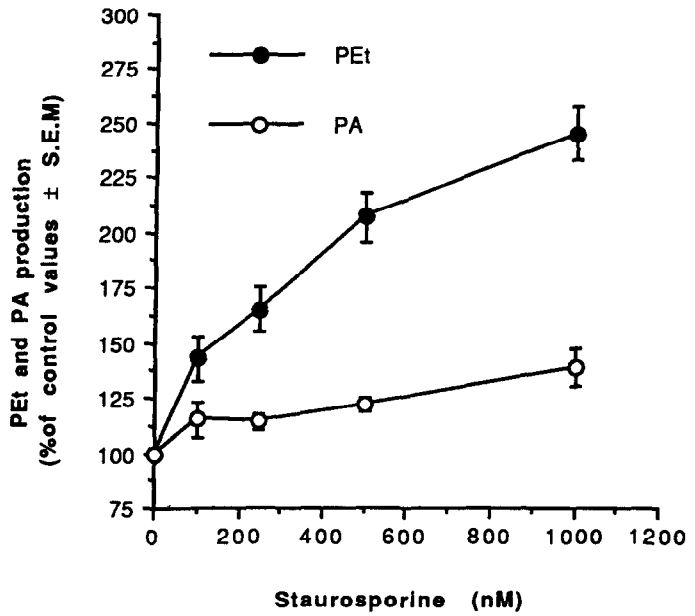

Fig. 3. Staurosporine stimulates phosphatidylethanol (PEt) production in PMNs. PMNs labeled in alkyl-PC were treated with 1% ethanol for 3 min and then incubated in the absence (control) or presence of various staurosporine concentrations for 15 min. PEt and PA productions were evaluated after extraction of lipids and separation by TLC. Results are the means of 3 experiments and are expressed as % of control values (927 ± 80 and 2318 ± 280 dpm/3 × 10^6 PMNs for PEt and PA, respectively).

The enhancement of PLD activity by staurosporine may have some pharmacological consequences. Although staurosporine is not an endogenous molecule, it may be useful for clarifying the mechanism of PLD activation and the functional involvement of the PLD pathway in cell functions. In human PMNs, PA elevation has been correlated [31] and linked [3,4] to simulation of the respiratory burst. In cell-free systems, PA stimulates the production of superoxide anion [32]. The significance of PA elevation in staurosporine-treated cells is not known, but staurosporine has various activating and priming properties. In PMNs, staurosporine induces exocytosis [33] and actin polymerization [34] and, under certain conditions, potentiates the respiratory burst mediated by chemoattractants [20,35]. In other cells, staurosporine promotes changes in morphology and differentiation similar to those induced by phorbol esters [36]. It also mimics nerve growth factor for the induction of neuropeptide gene expression [37]. Whether or not these cellular responses are due to the consequences of PLD activation remains to be examined. In addition to its enhancing effect on PLD activation, staurosporine may inhibit PA hydrolase, as suggested by the weak EAG production relative to that of PA (Fig. 2). This effect may reduce availability of DAG for PKC and thereby may depress PKC activation, in addition to its known interfering effect with the ATP binding site of PKC (Table I).

In conclusion, this study provides evidence that staurosporine concentrations that block PKC activation induces PLD activation in PMN. Phospholipase D may thus represent a potential target for staurosporine action. PLD activation may contribute to the reported stimulating properties of staurosporine in various cell types. Staurosporine does not appear a very appropriate probe for investigating the role of PKC in the mechanism of PLD activation but should become an interesting agent allowing to identify a new step of cell signalling.

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