Monosodium urate-crystal-stimulated phospholipase D in human neutrophils

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Protein kinase Cα (PKCα) and small GTPases of the Rho and ADP-ribosylation factor (Arf) family are implicated in the regulation of phospholipase D1 (PLD1) activity. Although they are involved in fMet-Leu-Phe (fMLP)-mediated PLD activation, their role in monosodium urate (MSU)-stimulated PLD1 activity in human neutrophils is not clear. The translocation of PKCα, RhoA and Arf from the cytosol to the membranes was monitored. fMLP induced a cytochalasin B (CB)-dependent recruitment of Arf, RhoA and PKCα to neutrophil membranes. CB also increased the activation of PLD 10-fold. In contrast with fMLP, MSU stimulated a sustained and time-dependent relocalization of Arf and PKCα, but not of RhoA, to the membrane fraction. MSU-stimulated PLD was activated with a time course preceding membrane recruitment of Arf and PKCα in the absence of CB. Furthermore, MSU-induced PLD activation and the membrane recruitment of PKCα, but not that of Arf, were inhibited by CB.

An anti-FcγRIIIB antibody, VIFcRIII, prevented the membrane relocalization of Arf and PKCα and the stimulation of the levels of tyrosine phosphorylation and of PLD activity induced by MSU. Erbstatin and ST-638, two inhibitors of tyrosine kinases, inhibited the MSU-induced translocation of Arf and PKCα but not MSU-induced tyrosine phosphorylation and PLD activation. Furthermore MSU crystals did not cause the tyrosine phosphorylation of PLD1. The present study indicates that soluble and particulate agonists show selectivity in inducing the translocation of RhoA in neutrophils and that the ability of MSU to increase PLD activation was independent of the membrane relocalization of Arf and PKCα.

Key words: ADP-ribosylation factor, gouty arthritis, inflammation, protein kinase C, RhoA, tyrosine kinase.

INTRODUCTION

The contribution of neutrophils to the pathogenesis of acute gouty inflammation is believed to be caused by events that follow the physical interaction of monosodium urate (MSU) crystals and neutrophils. The stimulation of neutrophils by MSU crystals leads to the production and release of several inflammatory mediators such as lysosomal enzymes [1,2], oxygen-derived free radicals [1,2] and products of the 5-lipoxygenase pathway of arachidonic acid metabolism [3,4], and the synthesis of a phospholipase A_2-activating protein [5] and proinflammatory cytokines such as interleukin 1 [6] and interleukin 8 [7]. The intracellular signalling events initiated by the physical interaction between MSU crystals and neutrophils rely to a critical degree on the activation of specific tyrosine protein kinase activities [8,9]. MSU crystals have also been reported to increase the concentration of intracellular free Ca^{2+} ions [4,10], the levels of Ins(1,4,5)P_3 after the hydrolysis of PtdIns(4,5)P_2 by a specific phospholipase C [11,12], and the activation of phosphatidyl-inositol 3-kinase [13]. Furthermore MSU crystals also potently stimulate a phosphatidylcholine-specific phospholipase D (PLD) [14].

Several mechanisms have been proposed for the activation of PLD in human neutrophils, including the participation of protein kinase C (PKC), GTP-binding proteins and protein tyrosine phosphorylation [15]. Neutrophil PLD activity is stimulated by conventional PKC isoforms such as PKCα and PKCβII [16]. Although the PKC-regulated PLD can be activated by phosphorylation-independent mechanisms [17], a conventional ATP-dependent mechanism contributes to the phorbol ester-stimulated PLD activity in granulocytes [16]. The activation of the membrane-associated PLD activity by non-hydrolysable GTP analogues is dependent on cytosolic small GTPases. Both ADP-ribosylation factor (Arf) and Rho proteins have been identified as potent PLD activators [18–20]. On stimulation of cells by physiological agonists, cytosolic RhoA is recruited to membranes, which in turn stimulate PLD activity [21]. Furthermore, several bacterial toxins that inactivate Rho proteins also decrease the stimulation of PLD activity by various agonists [22,23]. A type I phosphatidylinositol 4-phosphate 5-kinase is also activated by RhoA to generate PtdIns(4,5)P_2 [24], a required lipid cofactor for PLD activity [18]. The resynthesis of PtdIns(4,5)P_2 is associated with the enhanced stimulation of the Arf-dependent PLD by Arf1 [25]. Two mammalian PLDs, PLD1 [26] and PLD2 [27,28], have recently been cloned. PLD1 and its spliced PLD1b variant are activated in a similar manner by Arf [29]. The Arf-dependent PLD1 can be individually or synergistically stimulated by RhoA and PKCα [30]. In contrast, the PLD2 isoform is not activated by PKC, RhoA or Arf but requires PtdIns(4,5)P_2 for activity [27]. Unlike other PLD isoenzymes, PLD2 shows high basal activity *in vitro* [27,28].

The mechanism of PLD activation by MSU is as yet unclear. MSU-induced PLD activation, in contrast with neutrophil activation by chemotactic peptides, is largely insensitive to pertussis toxin [14]. The interaction of MSU with neutrophils has recently been shown to be mediated, at least in part, by their ability to interact with FcγRIIIB (CD16), a glycosyl-phosphatidylinositol-linked receptor [31]. The mechanisms of neutrophil activation by

microcrystals differ from those triggered by soluble chemotactic factors [8,9,14]. We hypothesized that urate crystals and fMet-Leu-Phe (fMLP) activate neutrophil PLD activity by specific and characteristic signalling pathways. In this study we have examined the roles of small GTPases and protein tyrosine phosphorylation in the stimulation of PLD activity by MSU crystals. The results provide evidence that both Arf and PKCα, but not RhoA, are recruited to membranes in response to MSU. However, the cytosol-membrane translocation of Arf and PKCα was not temporally correlated with PLD activation. Furthermore, the membrane recruitment of both Arf and PKCα was abolished by several inhibitors of tyrosine kinases, whereas PLD activity and tyrosine phosphorylation remained largely unaffected. Taken together, these results indicate that the Arf-regulated PLD1 is not the major PLD isoform activated by MSU crystals.

MATERIALS AND METHODS

Antibodies

The VIFcRIII anti-FcγRIII antibodies were purchased from Accurate Antibodies (Westbury, NY, U.S.A.) and the 3G8 anti-FcγRIII antibodies were purified from the ascitic fluids of mice inoculated with hybridoma 3G8 generously provided by Dr. J. Unkeless (Mount Sinai School of Medicine, New York, NY, U.S.A.). The anti-RhoA and the anti-PKCα antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.); the anti-phosphotyrosine antibody (UBI 05-321, clone 4G10) was from Upstate Biotechnology (Lake Placid, NY, U.S.A.). Goat anti-mouse IgG–FITC was obtained from Jackson Immune Research (West Grove, PA, U.S.A.). The polyclonal anti-Arf1 and the PLD1 antipeptide antibodies were generated in house and were described in previous studies [21,32].

Reagents

Di-isopropyl fluorophosphate and fMLP were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Triclinic MSU crystals were kindly provided by Dr. R. de Médicis and Dr. A. Lussier (University of Sherbrooke, Sherbrooke, Québec, Canada) and prepared as described previously [9]. The crystals used in this study were characterized by X-ray diffraction (Geigerflex D/max; Rigaku, MA, U.S.A.) and examination under phase and polarization microscopy and by scanning electron microscopy. Several distinct lots of crystals (sizes between 10 and 20 μm, specific areas between 0.7 and 2.4 cm²/g) were used with identical results (results not shown). Dextran T-500, Ficoll Paque and Sephadex G-10 were purchased from Pharmacia Biotech (Dorval, Québec, Canada).

Neutrophil purification

Venous blood was collected from healthy adult volunteers in isocitrate anticoagulant solution. Neutrophils were purified in a sterile manner as described previously [9] and resuspended in Hanks balanced salt solution (HBSS), pH 7.4, containing 0.8 mM Ca^{2+} ions but no Mg^{2+} ions.

Cell culture

HL-60 cells were obtained from ATCC (Rockville, MD, U.S.A.). They were maintained in RPMI 1640 supplemented with 10 % (v/v) heat-inactivated fetal calf serum, 2 mM glutamine, 100 i.u./ml penicillin and 100 μg/ml streptomycin. Differentiation towards granulocytic phenotype was started by the addition of 1.25 % (v/v) DMSO, as described previously [32].

Translocation assays

Neutrophils (4×10^7 cells/ml in HBSS) were treated with 1.1 mM di-isopropyl fluorophosphate for 30 min at 24 °C. The cell suspension was centrifuged and resuspended in HBSS at 10^7 cells/ml in HBSS. The cells were preheated for 5 min at 37 °C, then preincubated at 37 °C for 1 min with VIFcRIII (10 μg/ml), isotype-matched antibodies (IgM) or an equal volume of HBSS. Cells were stimulated with 3 mg/ml MSU for the indicated periods. In some experiments, cells were stimulated with 100 nM fMLP in the presence of 10 μM cytochalasin B (CB). The incubations were stopped by diluting the cells 1:5 with ice-cold RPMI 1640 and membranes were prepared. In brief, cell suspensions were centrifuged as indicated and resuspended at 1.6×10^7 cells/ml in ice-cold KCl/Hepes relaxation buffer [100 mM KCl/50 mM Hepes/5 mM NaCl/1 mM $MgCl_2$/0.5 mM EGTA/2.5 μg/ml aprotinin/2.5 μg/ml leupeptin/2.5 mM PMSF (pH 7.2)]. The suspensions were sonicated twice for 20 s each, then centrifuged for 7 min at 700 g. Unbroken cells and nuclei were discarded and the supernatants were ultra-centrifuged at 180000 g for 45 min in a Beckman TL-100 ultracentrifuge with a TL-100.4 rotor (65000 rev./min, 4 °C). Membrane pellets were washed once and resuspended in a small volume of buffer A containing 0.25 M Na_2HPO_4, 0.3 M NaCl, 2.5 % (w/v) SDS, 2.5 μg/ml aprotinin, 2.5 μg/ml leupeptin and 2.5 mM PMSF; samples were assayed for protein content with the Pierce Coomassie Brilliant Blue protein assay. Protein samples (30–60 μg) were resolved by SDS/PAGE [12 % (w/v) gel] and transferred to Immobilon PVDF membranes (Millipore Corporation, Bedford, MA, U.S.A.). Immunoblotting was performed with the anti-Arf1 (1:3500 dilution), anti-RhoA (1:1000 dilution) or anti-PKCα (1:5000 dilution) antibodies and proteins were revealed by using the enhanced chemiluminescence detection system, as described previously [21].

Tyrosine phosphorylation

Neutrophil suspensions (1.5×10^7 cells/ml) were transferred to 37 °C for 5 min and preincubated for 1 min with VIFcRIII (10 μg/ml), isotype-matched antibodies (IgM) or an equal volume of HBSS. The neutrophils were then stimulated with 3 mg/ml of MSU or 100 nM fMLP. At selected times, 100 μl of cell suspension was added to an equal volume of boiling 2 × Laemmli sample buffer [1 × Laemmli is 62.5 mM Tris/HCl (pH 6.8)/4 % (w/v) SDS/5 % (v/v) 2-mercaptoethanol/8.5 % (v/v) glycerol/2.5 mM orthovanadate/10 mM p-nitrophenyl phosphate/10 μg/ml leupeptin/10 μg/ml aprotinin/0.025 % Bromophenol Blue] and boiled for 7 min. Samples were then subjected to SDS/PAGE [7.5–20 % (w/v) gel] and transferred to Immobilon PVDF membranes. Immunoblotting was performed with the 4G10 anti-phosphotyrosine antibody at a final dilution of 1:4000 and revealed with the enhanced chemiluminescence detection system, as described previously [9].

Immunoprecipitation of PLD1

Neutrophils were preincubated as described above and stimulated at 4×10^7 cells/ml at 37 °C with 3 mg/ml of MSU for 5 min. Aliquots (500 μl) of the cells were lysed in an equal volume of boiling lysis buffer [62.5 mM Tris/HCl (pH 6.8)/3 % (w/v) SDS/1.5 % (v/v) 2-mercaptoethanol/8.5 % (v/v) glycerol/2.5 mM orthovanadate/10 μg/ml leupeptin/10 μg/ml aprotinin/0.025 % Bromophenol Blue] and boiled for 7 min. Immunoprecipitation was performed as described previously [32]. In brief, the lysates were filtered through Sephadex G-10 columns to remove the denaturing agents. The filtered

lysates were precleared with Protein A–Sepharose at 4 °C for 30 min in the presence of 1 % (v/v) Nonidet P40, 0.05 % BSA, 2 mM orthovanadate, 10 μg/ml leupeptin and 10 μg/ml aprotinin. The samples were then immunoprecipitated with 7 μl of the anti-PLD1 antibody. Protein A–Sepharose beads (50 μl, from a 30 % slurry) were then added and the samples were incubated for 1 h at 4 °C. The agarose beads were collected and washed four times with lysis buffer containing 1 % (v/v) Nonidet P40 but no SDS or 2-mercaptoethanol. Sample buffer (70 μl, 2 × concentration) was added to the beads, which were boiled for 7 min. The samples were then subjected to electrophoresis as described above. The membranes were first blotted with the anti-phosphotyrosine antibodies and then incubated for 30 min at 56 °C in stripping buffer [2 % (w/v) SDS/100 mM 2-mercaptoethanol/62.5 mM Tris base (pH 6.7)]. The membranes were then immunoblotted with anti-PLD1 (final dilution 1 : 2000) antibodies as described previously [32].

PLD measurements

Neutrophils were prelabelled with 1-*O*-[³H]alkyl-2-lysophosphatidylcholine (2 μCi per 10^7 cells) for 90 min. The cells were then washed and resuspended at 8×10^6 cells/ml. Samples of the cell suspensions (0.5 ml) were preincubated at 37 °C for 1 min with the indicated antibodies (10 μg/ml) or an equal volume of HBSS. Ethanol (final concentration 1.0 %, v/v) was added immediately before MSU crystals (3 mg/ml). The reactions proceeded for the indicated durations and were stopped by adding 1.8 ml of cold chloroform/methanol/HCl (50 : 100 : 1, by vol.) to 0.5 ml of the cell suspensions. The lipids were extracted, dried under nitrogen and resuspended in 50 μl of chloroform/methanol (2 : 1, v/v). The lipids were spotted on prewashed silica gel 60 TLC plates and phosphatidylethanol (PtdEt) was separated from the other lipids by using the solvent mixture chloroform/methanol/acetic acid (65 : 15 : 2, by vol.), as described previously [14]. The different lipids were revealed by Coomassie Brilliant Blue staining [0.03 % dye/35 % (v/v) methanol/200 mM NaCl]. The different lipid classes were scraped off the plates. Radioactivity was monitored by liquid-scintillation counting and the results were corrected for background radioactivity and quenching.

RESULTS

Crystal-induced cytosol-membrane translocation of Arf proteins and PKCα

Neutrophils were incubated with 3 mg/ml MSU crystals for 2.5, 5, 10 and 15 min and the levels of PtdEt formed were determined. As described previously [14] and as shown in Figure 1, the addition of MSU stimulated the formation of PtdEt in a time-dependent manner. PLD-mediated PtdEt formation was evident after 2.5 min of stimulation and the accumulation was progressive during the period analysed. In parallel samples of 1 % (v/v) ethanol-treated but otherwise unstimulated cells, there was no significant increase in PtdEt accumulation.

The amounts of Arf in neutrophil membranes from control or MSU-stimulated cells were then analysed by immunoblotting with anti-Arf antibodies. The results shown are representative of three experiments (Figure 2). MSU crystals induced a time-dependent recruitment of Arf to neutrophil membranes. Increases in membrane-associated Arf were barely detectable 5 min after the addition of MSU but a progressive increase was apparent after 10 and 15 min, the last time point tested. When expressed as a percentage of the unstimulated levels, the amounts of membrane-associated Arf averaged 119 ± 9.5 %, 121 ± 12.8 %,
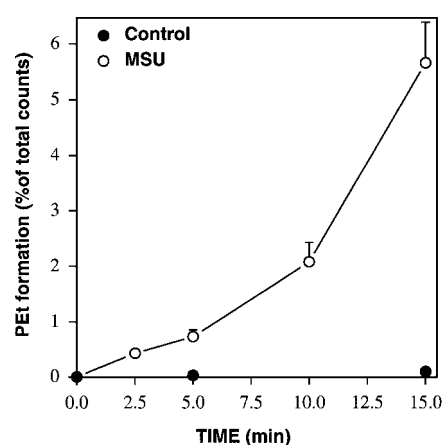

Figure 1   Time dependence of the formation of [³H]PtdEt in MSU crystals-stimulated neutrophils

Cells (8×10^6/ml) were labelled with 1-*O*-[³H]alkyl-2-lysophosphatidylcholine as described in the Materials and methods section and were stimulated with 3 mg/ml MSU for up to 15 min at 37 °C in the presence of 1% (v/v) ethanol. PtdEt from 4×10^6 cells was separated by TLC and located by staining with Coomassie Brilliant Blue. The plates were scraped in bands and [³H]PtdEt radioactivity was assayed. Results are means $\pm$ S.E.M. for three independent experiments. Abbreviation: PEt, phosphatidylethanol.

228.2 ± 39 % and 369 ± 89 % (means $\pm$ S.E.M., $n = 3$) at 2.5, 5, 10 and 15 min respectively. In unstimulated cells, the level of membrane-associated Arf remained constant during the period analysed.

The level of membrane-associated PKCα in the same samples was also determined by immunoblotting with anti-PKCα antibodies. As illustrated in Figure 2, PKCα was not present in membranes before stimulation with MSU. However, a significant cytosol-membrane translocation of PKCα was apparent 10 min after the addition of MSU, and the amounts of membrane-associated PKCα were larger at 15 min. The time dependence of PKCα translocation was virtually identical with that described for Arf.

In contrast with the effect of MSU on the translocation of Arf and PKCα, the level of membrane-associated RhoA was consistently slightly decreased at all the time points tested in MSU-
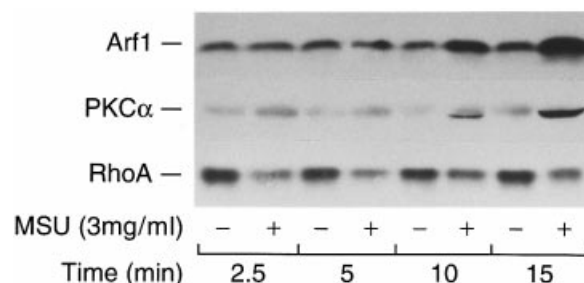

Figure 2   Time dependence of MSU-induced Arf, PKCα and RhoA translocation

Neutrophils were incubated in the absence or the presence of 3 mg/ml MSU at 37 °C. At 2.5, 5, 10 and 15 min, cell suspensions were processed and cellular membranes were purified as described in the Materials and methods section. Subsequently, neutrophil membranes were analysed for Arf, PKCα and RhoA content. The results are from one experiment representative of four similar experiments.
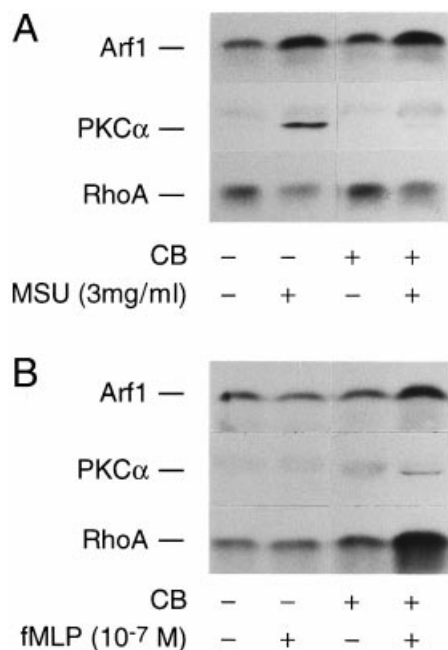
Figure 3   Comparison of the effects of CB on MSU and fMLP-induced responses

Where indicated, the neutrophils were preincubated with 10 μM CB for 5 min at 37 °C. (**A**) Cell suspensions were incubated in the absence or the presence of 3 mg/ml MSU for 15 min at 37 °C. (**B**) Cell suspensions were incubated in the absence or the presence of 100 nM fMLP for 2 min at 37 °C. Cellular membranes were purified and analysed for Arf, PKCα and RhoA content as described in the Materials and methods section. The results are from one experiment representative of four similar experiments.

Figure 4   Comparison of the effect of CB on fMLP- and MSU-induced PLD activation

Labelled neutrophils were incubated in the absence or the presence of 10 μM CB for 5 min at 37 °C and stimulated for 10 min with 100 nM fMLP or with 3 mg/ml MSU for 15 min at 37 °C. Ethanol [1 % (v/v)] was included in the assay. [^{3}H]PtdEt was separated and quantified as above. Results are means $\pm$ S.E.M. for three independent experiments. Abbreviation: PEt, phosphatidylethanol.

stimulated cells (Figure 2). Moreover, RhoA was not recovered in the first 700 **g** pellet containing crystals, cellular debris and nuclei. RhoA was found to be mainly cytosolic in neutrophils disrupted by sonication. The amount of RhoA in the cytosol derived from control and MSU-treated neutrophils was similar, as estimated by immunoblotting (results not shown).

A comparison of MSU-induced effects (Figures 1 and 2) indicates that the translocations of Arf and PKCα are not temporally correlated with the stimulation of PLD activity. Although PtdEt formation could be detected after 2.5 and 5 min of stimulation with MSU, there was no detectable increase in the amounts of membrane-associated Arf and PKCα. Thus at the early time points PLD activation can be dissociated from the translocation and activation of Arf and PKCα.

Effect of CB on MSU-induced small GTPase and PKCα translocation

CB has been shown to increase the fMLP-stimulated content of membrane-associated Arf in HL-60 granulocytes, an event closely associated with enhanced PLD activity [21]. We first verified that CB had a similar effect on the fMLP-induced responses in neutrophils. As shown in Figure 3(B), in the presence of CB detectable increases in the amounts of Arf, RhoA and PKCα were observed in membranes derived from fMLP-stimulated neutrophils.

We also conducted experiments to ascertain whether or not CB primes neutrophils for enhanced Arf translocation in response to MSU crystals. The results in Figure 3(A) show that, by itself, CB has no effect on the basal membrane level of Arf, and that it
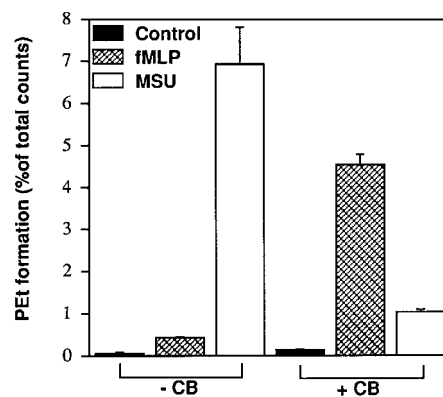
Table 1   Inhibitory effects of CB and VIFcRIII on the PLD response when added at selected times before or after the MSU crystals

Neutrophils were stimulated with 3 mg/ml MSU for 15 min at 37 °C in the presence of 1 % (v/v) ethanol. CB or VIFcRIII was added at the indicated times before or after the MSU crystals (zero time). The results are expressed as a percentage of the levels of PtdEt formed in the absence of CB or VIFcRIII respectively, and are means $\pm$ S.E.M. for three independent experiments. *Significantly different ($P \leqslant 0.05$) from the results obtained in the absence of CB or VIFcRIII (Student's paired t test, two-tailed). Abbreviation: n.d., not determined.

Time of addition (min)	PLD activity (% of control)	
	CB (10 μM)	VIFcRIII (10 μg/ml)
−5.0	15.1 ± 2.2*	n.d.
−1.0	n.d.	28.5 ± 1.6*
0	16.3 ± 2.7*	30.4 ± 1.4*
+0.5	21.2 ± 4.6*	30.1 ± 1.9*
+1.0	24.1 ± 4.4*	33.8 ± 2.5*
+2.5	32.7 ± 3.4*	43.9 ± 6.0*
+5.0	56.7 ± 3.3*	93.4 ± 8.9
+7.5	84.1 ± 5.6	95.7 ± 8.4
+10.0	94.1 ± 9.9	108 ± 1.7

did not significantly alter the amounts of membrane-associated Arf and RhoA in response to stimulation with MSU for 15 min. In contrast, the MSU-induced recruitment of PKCα to membranes was abolished by a pretreatment of cell suspensions with 10 μM CB (Figure 3A). Taken together the results indicate that the sensitivity of the pattern of Arf, RhoA and PKCα translocation induced by MSU crystals to CB differed from that elicited by the chemotactic peptide fMLP.

A similar dissociation between the effects of CB on the responses of neutrophils to MSU and fMLP was observed at the level of the activity of PLD (Figure 4). Pretreatment of the cells with CB enhanced the PLD response to fMLP 10.5 ± 0.9-fold, whereas it decreased the MSU-induced response by 84.9 ± 1.1 % ($n = 3$). CB inhibited MSU-induced PLD activity when added to the neutrophils up to 5 min after the agonist (Table 1). As indicated in Table 1, the addition of CB 0, 0.5, 1, 2.5 and 5 min
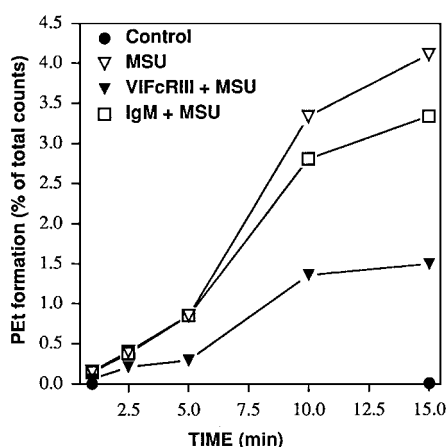
Figure 5   Effect of VIFcRIII on the PLD response induced by MSU crystals in human neutrophils

The cells were labelled and were incubated with or without 3 mg/ml MSU for 15 min at 37 °C in the presence of 1% (v/v) ethanol. At 1, 2.5, 5, 10 and 15 min, cell suspensions were processed for the determination of PLD activity as described in the Materials and methods section. The antibodies (VIFcRIII or control IgM) were added 1 min before MSU crystals. The results are from one experiment representative of two performed in duplicate. Abbreviation: PEt, phosphatidylethanol.
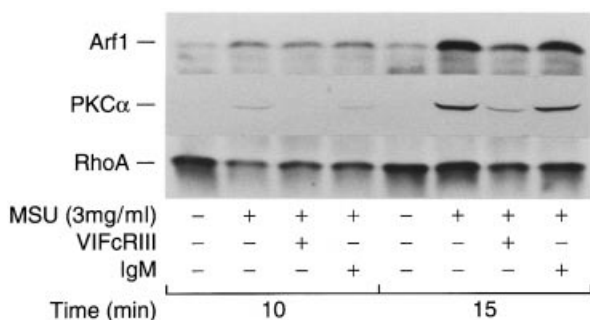

Figure 6   Inhibition by VIFcRIII of the stimulation of cytosol-membrane Arf and PKCα relocalization induced by MSU crystals in human neutrophils

The cells were incubated with VIFcRIII or IgM control antibodies 1 min before the addition of MSU crystals. At selected times the samples were processed and analysed for Arf, PKCα and RhoA membrane content as described in the Materials and methods section. The results are from one experiment representative of three similar experiments.

after the MSU crystals decreased the PLD response by $83.7 \pm 2.7\%$, $78.8 \pm 4.6\%$, $75.9 \pm 4.4\%$, $67.3 \pm 3.4\%$ and $43.3 \pm 3.3\%$ ($n = 3$) respectively. No significant decrease in the level of PtdEt formed was observed when CB was added to the cell suspensions 7.5 min after the MSU crystals.

Effects of anti-FcγRIII antibodies on cytosol-membrane PKCα and Arf translocation induced by MSU

We have previously observed that VIFcRIII, an antibody directed against CD16 (FcγRIII), selectively inhibited neutrophil activation by MSU crystals. The effects include a dose-dependent inhibition of tyrosine phosphorylation of proteins [31]. The effect of preincubation of neutrophils with 10 μg/ml VIFcRIII on the PLD activity induced by MSU crystals in human neutrophils is shown in Figure 5. VIFcRIII decreased MSU-induced responses

by up to 70%, whereas the isotope-matched IgM control showed only a marginal inhibitory effect on stimulated PLD activity. Similar inhibitory effects were observed when the VIFcRIII antibody was added 0, 0.5, 1 and 2.5 min after the MSU crystals (Table 1). The level of PtdEt formed was not decreased when the VIFcRIII antibody was added to the cell suspension 5 min after the MSU crystals. To examine the mechanisms of action of the VIFcRIII antibody on the PLD activation induced by MSU crystals, neutrophils were incubated with either 10 μg/ml of VIFcRIII or control IgM antibodies at 37 °C for 1 min and then stimulated with MSU crystals (3 mg/ml) for 10 or 15 min respectively. The amount of Arf in neutrophil membranes was determined by immonublotting with anti-Arf antibodies. The addition of MSU to neutrophil suspensions stimulated the cytosol-membrane translocation of Arf in a time-dependent manner, as described in Figure 2. Preincubation of the cells with VIFcRIII before stimulation with MSU led to a nearly complete inhibition of Arf translocation at 15 min (Figure 6). The isotype-matched IgM had no significant effect on the translocation of Arf induced by MSU. The basal level of membrane-associated Arf was not altered by preincubation of the cells with the isotype-matched IgM or with VIFcRIII (results not shown).

The effect of VIFcRIII on the MSU-induced translocation of PKCα was investigated next. The addition of VIFcRIII but not of IgM control antibodies significantly decreased the cytosol membrane translocation of PKCα stimulated by MSU crystals (Figure 6). The basal levels of membrane-associated PKCα were unchanged in IgM control or VIFcRIII-preincubated, but otherwise unstimulated, granulocytes (results not shown). Furthermore VIFcRIII was without effect on the RhoA content of membranes derived from control (results not shown) or MSU-stimulated neutrophils (Figure 6).

Role of tyrosine phosphorylation of proteins on MSU-induced PLD activation

As described previously [31], pretreatment of the cells with VIFcRIII (10 μg/ml, 1 min), but not the control-matched IgM antibodies, decreased the characteristic patterns of tyrosine phosphorylation induced by MSU crystals. Inhibition of stimulated tyrosine phosphorylation by VIFcRIII was observed at 5 and 15 min after exposure to MSU crystals (results not shown). The antibody 3G8, directed against CD16 (FcγRIII), had no effect on the MSU-stimulated tyrosine phosphorylation of proteins. We sought to determine whether the inhibitory effects of VIFcRIII on the MSU-induced PLD activation was secondary to inhibition of tyrosine phosphorylation of proteins. This relationship was examined by using two well-characterized and unrelated inhibitors of tyrosine kinases, erbstatin [33] and ST-638 [34]. In these experiments 1-O-[^{3}H]alkyl-2-acylphosphatidylcholine-prelabelled neutrophils were preincubated with 10 μg/ml erbstatin, 50 μM ST-638 or the vehicle DMSO for 30 min before stimulation with MSU crystals. These optimal concentrations of inhibitors have only marginal effects on MSU-stimulated PLD activation, as erbstatin and ST-638 decreased the rate of PtdEt synthesis in response to MSU (3 mg/ml, 15 min) by only $19.2 \pm 14.5\%$ and $10.5 \pm 10.9\%$ ($n = 3$), respectively. Next the effect of erbstatin and ST-638 on the MSU-stimulated tyrosine phosphorylation of proteins was examined. Preincubation of the cells with erbstatin or ST-638 had little effect on the tyrosine phosphorylation patterns induced by MSU crystals (3 mg/ml, 1 min). No decrease in tyrosine phosphorylation was observed when cell samples were analysed after 5 and 15 min of stimulation with the crystals respectively (results not shown).
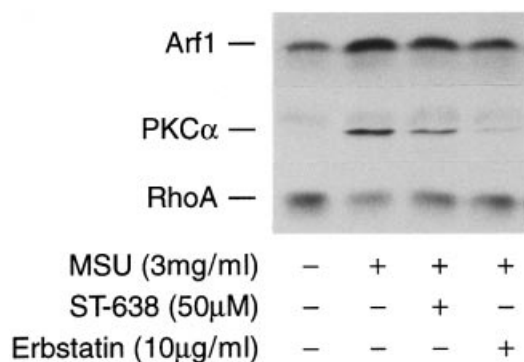
Figure 7   Effect of tyrosine kinase inhibitors on the stimulation of cytosol-membrane Arf and PKCα relocalization induced by MSU crystals in human neutrophils

The cells were preincubated for 30 min with 10 μg/ml erbstatin or 50 μM ST-638 at 37 °C, then stimulated for 15 min with 3 mg/ml MSU at 37 °C. The samples were processed and analysed for Arf, PKCα and RhoA membrane content as described in the Materials and methods section. The results are from one experiment representative of three similar experiments.

The activation of the neutrophil PLD in response to fMLP has been shown to be dependent on tyrosine kinase activities [35]. Therefore we verified the ability of erbstatin (10 μg/ml, 30 min) and ST-638 (50 μM, 30 min) to inhibit fMLP-induced PLD activation. Erbstatin and ST-638 inhibited the rate of PtdEt formation elicited by fMLP (100 nM, 10 min) by $88.1 \pm 4.5\%$ and $89 \pm 5.3\%$ ($n = 3$) respectively. Furthermore preincubation of the cells with erbstatin or ST-638 for 30 min decreased the intensity of tyrosine phosphorylation of most, if not all, the substrates whose phosphorylation was increased on stimulation by fMLP, indicating that the tyrosine kinase inhibitors were active (results not shown). Thus both PLD activation and tyrosine phosphorylation elicited by MSU were resistant to inhibition by erbstatin or ST-638.

Effect of tyrosine kinase and PKC inhibitors on MSU-induced cytosol-membrane PKCα and Arf translocation

The effects of tyrosine kinase inhibitors on the translocation of Arf, RhoA and PKCα induced by MSU crystals was examined next. Although the general tyrosine phosphorylation patterns were not significantly affected by erbstatin or ST-638, the two inhibitors decreased the cytosol-membrane translocation of Arf induced by MSU crystals (Figure 7). The cytosol-membrane translocation of PKCα induced by MSU crystals was also inhibited by a preincubation of the cells with the tyrosine kinase inhibitors. Erbstatin was slightly more potent than ST-638 as an inhibitor of MSU-induced responses (Arf and PKCα translocation). The effects of erbstatin or ST-638 were not reproduced by the specific PKC inhibitor GF-109203X, which potentiated the cytosol-membrane recruitment of PKCα in response to MSU crystals by 5 ± 1.4-fold without affecting the level of [³H]PtdEt formed in the presence or the absence of 1 μM GF-109203X [$1.282 \pm 0.213\%$ and $0.971 \pm 0.212\%$ ($n = 3$) of the total counts respectively]. Similar results were obtained with Ro-318220, another specific PKC inhibitor (results not shown).

Effect of MSU on tyrosine phosphorylation of granulocyte PLD1

The resistance to inhibition by the tyrosine kinase inhibitors of the tyrosine phosphorylation induced by MSU crystals prompted us to examine whether or not PLD1 was tyrosine phosphorylated on stimulation with MSU crystals in neutrophils. In these experiments, neutrophils were stimulated for 5 min with MSU crystals and PLD1 was immunoprecipitated under reducing conditions by using selective PLD1 antibodies [32]. The immunoprecipitates were sequentially immunoblotted with anti-phosphotyrosine and PLD1 antibodies. No tyrosine-phosphorylated protein was detected in the 120 kDa region in response to MSU crystals (results not shown). We recently demonstrated in DMSO-differentiated HL-60 cells that PLD1 could be tyrosine phosphorylated in response to stimulation with peroxides of vanadate (V^{4+}-OOH) [32]. Next we determined whether neutrophil PLD1 was similarly tyrosine phosphorylated in response to V^{4+}-OOH stimulation. The results of these experiments demonstrate that, in contrast with DMSO-differentiated HL-60 cells, neutrophil PLD1 was not tyrosine phosphorylated in response to V^{4+}-OOH treatment for 10 min (results not shown). Although the minor spliced PLD1 variant (PLD1b) found in HL-60 cells was not detected in neutrophils (results not shown), equal amounts of PLD1a were immunoprecipitated from neutrophils and HL-60 granulocytes as assessed by immunoblotting. Taken together, the results of the above experiments indicate that MSU-induced PLD activity (and fMLP as well) does not involve a direct tyrosine phosphorylation of the PLD1 isoenzyme in human neutrophils.

DISCUSSION

The results of the present study indicate that soluble and particulate agonists induce distinct patterns of translocation of Arf, PKCα and RhoA in human neutrophils. This represents the first documented evidence that stimulation in neutrophils of the activity of PLD by MSU crystals is accompanied by a translocation of Arf and PKCα, but not RhoA, from a cytosolic to a membrane fraction. However, the effect of MSU crystals on Arf and PKCα translocation seems not to be related to activation of PLD1. This conclusion is supported by several lines of evidence. First, the accumulation of PtdEt preceded the membrane recruitment of Arf and PKCα by several minutes. Secondly, the tyrosine kinase inhibitors erbstatin and ST-638 inhibited the membrane translocation of Arf and PKCα but had no significant impact on the stimulated levels of PLD activity. Thirdly, the stimulation of the activity of PLD by MSU crystals was inhibited by CB, whereas the translocation of Arf was not affected.

The PLD family of proteins is represented by three biochemically distinct enzymes: the oleate-dependent PLD, the Arf-regulated PLD (PLD1), and PLD2 [27,28,30]. Evidence from studies in vitro (with cell-free systems) indicates that PLD1, but not PLD2, is regulated by small GTPases of the Rho and Arf family [15,17]. Several toxins inactivating Rho proteins have been used to demonstrate a role for RhoA in receptor-mediated PLD activation [22,23]. We reported a membrane relocalization of Arf1, in the HL-60 human promyelocytic cell line, on stimulation of a G-protein-coupled receptor, the fMLP receptor [21]. Furthermore the increased amounts of membrane-associated Arf (detected by immunoblots) was tightly correlated with an increase in the levels of guanosine 5′-[γ-thio]triphosphate-stimulated PLD activity [21]. The present study indicates that both Arf and RhoA were recruited to neutrophil membranes on stimulation with fMLP, indicating that this G-protein-coupled receptor was functionally linked to the activation of small GTPases. However, the results of the present study dissociate the stimulation of the activity of PLD by MSU crystals from that of the small GTPases RhoA and Arf. The addition of MSU crystals to neutrophil suspensions did not stimulate a membrane trans-

location of RhoA, indicating that RhoA was not activated on stimulation with MSU crystals. The recruitment of other members of the Rho family of small GTPases, Rac1 and Cdc42, was not studied in the present report. However, although a role for Rac1 and Cdc42 in activating PLD cannot be excluded, studies *in vitro* have shown that granulocyte PLD1 was highly sensitive to stimulation by RhoA but less sensitive to activation by Cdc42 and Rac1 [36,37]. In contrast, the treatment of human neutrophils with MSU crystals recruited Arf to the membrane fraction. However, the activation of PLD by MSU crystals preceded the membrane recruitment of Arf by several minutes.

Cytoskeletal proteins can control PLD activity in human neutrophils. This is indicated by the ability of CB to potentiate the chemoattractant-induced stimulation of PLD activity. CB also greatly increased the membrane translocation of RhoA and Arf in response to fMLP. In contrast, CB inhibited the MSU crystal-induced translocation of PKCα without affecting that of Arf. The downstream effectors that connect the actin-based cytoskeleton reorganization and the activity of PLD in response to MSU crystals are unknown. There is evidence for a requirement for both RhoA, Rac1 and Cdc42 in various cytoskeletal responses including membrane ruffling and phagocytosis in leucocytes [38,39]. The absence of a subcellular redistribution of RhoA, even after disruption of the actin filament network, indicates that the Arf relocalization induced by MSU crystals is selective. These results also exclude a generalized non-specific effect of CB on the relocalization of small GTPases and suggest a role for RhoA in PLD activity induced by fMLP but not in that induced by MSU crystals. It is noteworthy that the cytosol-membrane translocation of Arf induced by MSU crystals was observed in the absence of CB and that neither the amount of membrane-associated Arf nor its time-dependent relocalization was altered by CB. In contrast, pretreatment of cells with CB abrogated the stimulation of PLD activity induced by MSU crystals by 85%. These results therefore provide additional evidence for a dissociation between the levels of leucocyte PLD activity and of membrane-associated Arf in response to MSU crystals.

The ability of MSU crystals to stimulate the membrane recruitment of PKC has not been reported previously. Specific PKC isoforms such as PKCα have been intimately associated with the mechanism of PLD1 activation in studies *in vitro* [16]. We demonstrated a time-dependent cytosol-membrane re-localization of PKCα and PKC δ (results not shown) in response to MSU. In addition we observed that, similarly to the activation of PLD, the membrane recruitment of PKCα by MSU crystals is not dependent on CB, and that in contrast with the responses induced by chemotactic factors, it is inhibited by actin filament disassembly. Therefore MSU-induced PLD activation could conceivably be attributed to activation and membrane recruitment of PKCα. However, three lines of evidence argue against this possibility. First, detailed comparative analyses of the time courses of the two events indicated that substantial PtdEt formation preceded the membrane recruitment of PKCα. Secondly, the addition of inhibitors of PKC had no effect on MSU crystal-induced PLD activity but markedly potentiated the membrane recruitment of PKCα. Thirdly, two inhibitors of tyrosine kinases, erbstatin and ST-638, decreased the translocation of PKCα but were without effect on MSU crystal-induced PLD activity.

The most striking consequence of the disruption of the actin filament network by CB was the impairment of the activation of PLD by MSU crystals. The lack of effect of CB on the stimulation of Arf translocation and its only partial effect on the mobilization of Ca^{2+} ions [4] induced by MSU crystals argue against an

inhibition of the physical interaction of the crystals with neutrophils by the fungal metabolite. A more likely explanation is that CB alters the downstream signalling pathways thereby activated, including the stimulation of PLD. It has previously been reported that cytochalasins inhibit the internalization of opsonized zymosan and the phagocytosis of immune complexes, but not their binding to leucocyte surfaces [40]. Alternatively, inhibition of PLD-derived phosphatidic acid formation by CB might itself be at least partly responsible for the inhibition of MSU crystal internalization but not membrane binding, as has previously been shown for the phagocytosis of *Mycobacterium tuberculosis* or opsonized zymosan by human macrophages [41]. Further studies to define the relationship between PLD activation and the formation of phagosomes are required to clarify this point.

Evidence for a role of FcγRIIIB (CD16) in MSU-induced PLD activity is also presented. The anti-FcγRIII antibody VIFcRIII, but not the isotype-matched control, inhibited the membrane recruitment of Arf and PKCα. Previous observations have indicated that the stimulation of tyrosine phosphorylation induced by MSU crystals was similarly inhibited by VIFcRIII [31]. These inhibitory effects were not observed with 3G8 (an anti-FcγRIII antibody), which recognizes a different epitope from that recognized by VIFcRIII. FcγRIIIB is a glycosyl-phosphatidylinositol-anchored protein that, in contrast with the other FcγR, lacks transmembrane and cytoplasmic domains. Although FcγRIIIB-induced signalling is still poorly understood, the engagement of FcγRIIIB triggers neutrophil activation through interaction with complement receptors such as CD18/CD11b [42–45]. Furthermore the anti-CD11b antibody VIM12, which interferes with the lectin-like interactions between CD16 and CD11b [46], also inhibited the activation of neutrophil by MSU crystals [31]. In this context it is noteworthy that the cross-linking of CD11b or CD18 stimulated PLD activity in human neutrophils [47]. Whether FcγRIIIB and CR3 clustering or co-capping is involved in MSU-induced signalling leading to the activation of PLD requires further investigation.

Inhibitor studies have provided evidence for a role for tyrosine phosphorylation in PLD activation. For example, erbstatin and ST-638 inhibited the stimulation of PLD activity of human granulocytes in response to chemotactic peptides [33] and V^{4+}-OOH (inhibitors of tyrosine phosphatases) [34,35] respectively. The observation that the MSU crystals increased tyrosine phosphorylation [8,9,31] and a concomitant PLD activation ([14], and the present paper) was consistent with this hypothesis. However, MSU-induced PLD activity is, similarly to the response of immune complexes [48] and in contrast with that induced by fMLP [33], not sensitive to inhibition by erbstatin and ST-638. The inhibitor results are consistent with the lack of effect of MSU crystals on the tyrosine phosphorylation of PLD1 in human neutrophils. The present results do not allow us to examine this apparent ambiguity rigorously. However, the two inhibitors tested, erbstatin and ST-638, had little effect on the degree of protein phosphorylation induced by MSU crystals. Furthermore, Vossebeld et al. [49] reported that the commonly used tyrosine kinase inhibitors prevented Fcγ receptor-mediated tyrosine phosphorylation only poorly.

In summary, the above results indicate that the cytoskeleton and other signalling requirements that underlie the activation of PLD by MSU crystals and chemotactic peptides are agonist-specific and distinct. It remains to be clarified whether soluble or particulate agonists stimulate a unique PLD isoform, with different subcellular localizations and sets of cofactor requirements, or whether they act on distinct PLD isozymes. However, the distinct patterns of membrane recruitments of Arf, RhoA and PKC and their requirements in PLD activation are

not consistent with a major role for the Arf-regulated PLD (PLD1) in the mediation of the responses by MSU crystals. More detailed conclusions await the characterization of the relevant neutrophil PLD isoforms, their subcellular distribution and their respective activation mechanisms to address their functions further.

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