The Receptor for Urokinase-Type Plasminogen Activator and Urokinase Is Translocated From Two Distinct Intracellular Compartments to the Plasma Membrane on Stimulation of Human Neutrophils

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The cellular receptor for urokinase-type plasminogen activator (uPAR) binds pro-urokinase (pro-uPA) and facilitates its conversion to enzymatically active urokinase (uPA). uPA in turn activates surface-bound plasminogen to plasmin, a process of presumed importance for a number of biologic processes including cell migration and resolution of thrombi. We have previously shown that uPAR is expressed on the plasma membrane of circulating neutrophils, and we now report that stimulation with phorbol myristate acetate (PMA), FMLP, or tumor necrosis factor-α results in a rapid increase in the expression of uPAR. This process is accompanied by an increased cell-associated plasminogen activation after preincubation of neutrophils with pro-uPA in vitro. By subcellular fractionation of unstimulated neutrophils, 50% of uPAR is recovered in fractions containing latent alkaline phosphatase, corresponding to an intracellular compartment of easily mobilizable secretory vesicles distinct from both primary and specific granules, whereas the remaining 50% of uPAR is associated with a compartment eluting close to the specific granules. In contrast, the ligand pro-uPA is primarily (≈80%) found in the specific granules, but small amounts of pro-uPA/uPA (≈20%) coelute with latent alkaline phosphatase. Stimulation of neutrophils with FMLP results in translocation of uPAR as well as of pro-uPA from the secretory vesicles, whereas stimulation with PMA is required to translocate material from specific granules. Flow cytometry of neutrophils saturated with exogenous diisopropyl flurophosphate-uPA shows a large excess (≈90%) of unoccupied uPAR on resting as well as FMLP- and PMA-stimulated neutrophils, suggesting a possible role for exogenous pro-uPA in providing neutrophils with a potential for plasminogen activation. These processes may be important for neutrophil extravasation and migration through extracellular matrix and for the contribution of neutrophils to resolution of thrombi.

THE CELLULAR RECEPTOR for urokinase-type plasminogen activator (uPAR) is a membrane protein, which binds urokinase (uPA) with high specificity and affinity (kd = 0.1 to 1 nmol/L).[1] On peripheral blood cells uPAR is found on neutrophils and monocytes, whereas it is absent from erythrocytes, platelets and resting lymphocytes.[2-4] The uPAR protein is a highly glycosylated membrane protein of molecular weight 55 to 60 kD that is attached to the plasma membrane by a glycosyl-phosphatidyl-inositol anchor.[5,6] The uPAR-specific mRNA encodes a nascent single-chain protein of 313 amino acids, which is proteolytically processed at the COOH-terminus during biosynthesis.[6,7] The mature uPAR protein is composed of three homologous domains of which the NH_2-terminal possesses the ligand-binding properties.[8] For recent reviews of the protein structure of uPAR see Plough et al[9] and Moller.[10] Primary functions of uPAR appear to be potentiation of the uPA-catalyzed plasminogen activation system and localization and confinement of this proteolytic activity to the cell surface.[11,12] Pro-uPA has been found in the specific granules of neutrophils, and, after neutrophil activation, the enzyme is released and translocated to the cell surface.[13] We now report that in resting neutrophils uPAR molecules are stored in two distinct intracellular compartments. One of these is the secretory vesicles that rapidly translocates on neutrophil activation[14]; therefore, uPAR and the associated potential for cell-associated plasmin generation appears on the cell surface. By subcellular fractionation of neutrophils, the other uPAR-containing compartment sediments close to B_{12}-binding protein, a marker of specific granules, and requires more intense stimulation for translocation to occur.

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MATERIALS AND METHODS

Isolation of Peripheral Blood Neutrophils

Neutrophils were isolated from venous blood of healthy individuals. Informed consent and approval from the local ethics committee was obtained. The blood was collected by venipuncture during the day and anticoagulated with 130 μmol/L trisodium citrate or 10 U/mL heparin (final concentrations). Erythrocytes were removed by sedimentation for 45 minutes at room temperature after a 1 + 1 dilution of the blood sample with isotonic saline containing 2% (wt/vol) dextran T-500 (Pharmacia, Uppsala, Sweden). The neutrophil-rich supernatant was harvested and processed for further studies.[15]

Stimulation of Neutrophils for Flow Cytometry

Neutrophils were incubated at 2×10^6 cells/mL in buffer A (10 mmol/L Na-phosphate, 130 mmol/L NaCl, 5.0 mmol/L KCl, 1.27 mmol/L $MgSO_4$, 0.95 mmol/L $CaCl_2$, 5.0 mmol/L glucose, pH 7.4) and stimulated at 37°C with (1) 8.1 μmol/L phorbol myristate acetate (PMA; Sigma, St Louis, MO) prepared and stored at −20°C as a 1.62 mmol/L stock solution in dimethyl sulfoxide (DMSO)[16]; (2) 10 nmol/L FMLP (Sigma) prepared and stored at −20°C as a 1

mmol/L stock solution in DMSO[17]; or (3) 25 U/mL recombinant human tumor necrosis factor-α (TNF-α; Roche, Basel, Switzerland).[18]

Flow Cytometry

Neutrophils were washed at 4°C in buffer B (12 mmol/L sodium phosphate, 137 mmol/L sodium chloride, 2.7 mmol/L potassium chloride, pH 7.4) supplemented with 10 mmol/L EDTA, 10 mmol/L sodium azide, and 1% (wt/vol) bovine serum albumin (BSA; Sigma), labeled with monoclonal antibodies (MoAbs), and processed for flow cytometry as described.[19] The following MoAbs were used in excess as measured in titration experiments: anti-uPAR (clone R4, which recognizes all uPAR irrespective of any bound ligand)[4,20] and anti-uPA (clone 16, which reacts with the catalytic B-chain of uPA; (G. Høyer-Hansen, personal communication, June 1993, and Grøndahl-Hansen et al[21]). After incubation with these antibodies followed by appropriate washing, the cells were labeled with phycoerythrin (PE)-conjugated goat antimouse Ig (Medac, Hamburg, Germany). Erythrocytes were removed by treatment with Lysis Buffer (Becton Dickinson, San José, CA), and the specific fluorescence was estimated quantitatively by flow cytometry on FACScan (Becton Dickinson) using PE calibration beads (Flow Cytometry Standards Corp, Eugene, OR) as described.[19] The median fluorescence intensity of a single symmetric peak in the fluorescence histogram was used for quantitation of each individual antigen. In some experiments, unstimulated neutrophils and neutrophils stimulated with 10 nmol/L FMLP or 8.1 μmol/L PMA were preincubated with 20 nmol/L diisopropyl flurophosphate (DFP)-uPA for 1 hour at 4°C in buffer B with 10 g/L BSA, washed, and subsequently labeled with MoAb anti-uPA and PE-conjugated goat antimouse Ig.

Measurement of uPAR-Dependent Plasminogen Activation on the Surface of Neutrophils

The potential of FMLP-stimulated and nonstimulated neutrophils to support uPAR-dependent plasminogen activation was determined using an adaption of the method previously described.[4] The deviations from this method were made to ensure minimal activation of the cells before assay. Neutrophils, isolated as described above, were either stimulated with FMLP (10 nmol/L) at 37°C or incubated at 4°C or 37°C. Cells were incubated with pro-uPA (1.0 nmol/L) for 10 minutes at 4°C (in control experiments this procedure was found to result in 60% saturation of uPAR compared with that using incubation at 37°C). The cells were then washed 3 times in buffer A at 4°C and, finally, were incubated at a density of 2×10^6 cells/mL in 50 mmol/L Tris-HCl, pH 7.4, 100 mmol/L NaCl with 50 mg/L Glu-plasminogen[12] and 0.2 nmol/L of the plasmin-specific fluorogenic peptide substrate H-D-Val-Leu-Lys-7-amido-4-methylcoumarin (a kind gift from Bachem AG, Bubendorf, Switzerland) at 37°C. Plasmin generation was measured continuously as previously described.

The relative amounts of bound pro-uPA on the variously treated cells were calculated by fitting of the plasmin generation curves to a set of equations describing the generation of plasmin in mixtures of pro-uPA and plasminogen,[22] using kinetic constants determined for the individual reactions on the monocytoid cell line U937.[11,12]

Subcellular Fractionation

Approximately 3×10^8 neutrophils prepared as buffy coat were purified further by gradient centrifugation on Lymphoprep (Nycomed, Oslo, Norway), cleared of erythrocytes by hypotonic lysis, resuspended, washed, and adjusted to 3×10^7 cell/mL in buffer A.[14] The cell suspension was treated for 10 minutes at 0°C with 5 mmol/L DFP (Sigma) to inhibit serine proteinases.[13] Unstimulated cells and cells stimulated at 3×10^7 cells/mL in buffer A for 15 minutes at 37°C with either 10 nmol/L FMLP or 8.1 μmol/L PMA were pelleted, resuspended in 10 mL buffer C (10 mmol/L 1,4-piperazine-diethanesulfonic acid, 100 mmol/L KCl, 3 mmol/L NaCl, 1 mmol/L disodium adenosine triphosphate, 3.5 mmol/L magnesium chloride, pH 7.3) with 0.5 mmol/L phenylmethylsulfonyl fluoride and 10 μg/mL aprotinin, and were then disrupted by nitrogen cavitation into a test tube with 1.25 mmol/L EGTA (final concentration).[14] Nuclei were removed by centrifugation (400*g* for 15 minutes), and the supernatant (approximately 10 mL from 3×10^8 neutrophils) was separated on 28 mL of a two-layer Percoll density gradient (14 mL of density 1.05 g/mL, 14 mL of density 1.12 g/mL) by centrifugation at 48,000*g* for 20 minutes at 4°C.[16] Fractions of 1.5 mL were collected from the bottom of the tube and assayed by enzyme-linked immunosorbant assay (ELISA) for the HLA class I/β_2-microglobulin complex as a plasma membrane marker,[23] for B_{12}-binding protein as a marker of specific granules using a ^{57}Co-labeled cyanocobalamin (Amersham, Buckinghamshire, UK) binding assay,[16] and for myeloperoxidase as a marker of primary granules by ELISA with rabbit antihuman myeloperoxidase (DAKO, Copenhagen, Denmark) (Birgens et al, unpublished method). Alkaline phosphatase activity was assayed before and after addition of 150 μL of a 2% (vol/vol) Triton X-100 solution to each fraction to measure latent alkaline phosphatase as a marker of rapidly mobilizable secretory vesicles.[14]

Detection of uPAR and uPA/pro-uPA in Samples From Subcellular Fractionation

Fractions from subcellular fractionation of neutrophils prepared as described above were cleared of Percoll by ultracentrifugation at 180,000*g* for 120 minutes, after adjusting the volume by topping up with an approximately equal volume of Percoll 1.12 g/mL to avoid trapping of biologic material.[16] Any biologic material overlaying the hard-packed pellet of Percoll was recovered and resuspended in the supernatant.

Estimation of uPAR by cross-linking. uPAR was measured semiquantitatively by chemical cross-linking to ^{125}I-labeled ATF (amino terminal fragment of the ligand uPA) as described previously.[4] Purified ATF was a kind gift from Dr A. Mazar (Abbott Laboratories, Chicago, IL).

Estimation of uPAR by ELISA. The content of uPAR was measured by an ELISA based on two MoAbs recognizing different epitopes on uPAR[20] (Rønne et al, *J Immunol Methods,* in press). The assay measures free uPAR as well as uPAR occupied by uPA.

Estimation of pro-uPA/uPA by enzyme activity. Pro-uPA/uPA was determined quantitatively using a specific plasminogen activation assay, after activation of pro-uPA. Twenty-microliter aliquots from the subcellular fractionation were incubated with 0.2 mg/L plasmin in 800 μL of 50 mmol/L Tris-HCl, pH 7.4, 100 mmol/L NaCl, 0.01% (vol/vol) Tween 80 for 5 minutes at 37°C, before the addition of 20 mg/L Glu-plasminogen and 0.2 nmol/L H-D-Val-Leu-Lys-7-amido-4-methylcoumarin. The rate of plasmin generation was measured continuously and quantified by comparison with standard curves made using purified pro-uPA.

Estimation of pro-uPA/uPA by ELISA. The concentration of pro-uPA/uPA in the different fractions was measured by a modification of the uPA-ELISA described in Nielsen et al.[24] An MoAb recognizing the aminoterminal part of uPA (clone 6) was used as the catching antibody.[25] The detecting antibody was a biotinylated MoAb recognizing the carboxyterminal part of uPA (clone 5).[24] The assay was improved by using 1% skimmed milk powder in phosphate buffer as blocking agent, instead of BSA, and by substituting 0.1% Triton X-100 with 0.05% Tween 20.[21,26]

Recovery of uPAR and pro-uPA/uPA in Cell Extracts and Supernatants of Unstimulated, FMLP-Stimulated and PMA-Stimulated Cells

DFP-treated neutrophils were stimulated at 3×10^7 cells in 1 mL buffer A at 37°C with 10 nmol/L FMLP or 8.1 μmol/L PMA and harvested at 5, 10, and 15 minutes. Unstimulated cells served as controls. The supernatant was saved for uPAR and pro-uPA/uPA ELISA. After 2 washings the cells were solubilized in 1 mL buffer B supplemented with 1% (vol/vol) Triton X-100, cleared of cell debris by centrifugation, and assayed for uPAR and pro-uPA/uPA by ELISA.

RESULTS

Flow Cytometry of uPAR and pro-uPA/uPA on PMA-, FMLP-, and TNF-α–Stimulated Neutrophils

Expression of uPAR and uPA on the plasma membrane of neutrophils was measured by flow cytometry of unstimulated neutrophils and after stimulation with 8.1 μmol/L PMA, 10 nmol/L FMLP, or 25 U/mL TNF-α. PMA caused a rapid fourfold increase in the surface expression of uPAR and pro-uPA/uPA, reaching a plateau at 6 minutes (Fig 1A and B, respectively). The response to FMLP and TNF-α occurred more slowly and did not reach the same maximum level as obtained by PMA stimulation. However, even the slow response to TNF-α was significantly different from that for the unstimulated control ($P < .01$), except for the value for uPA at 6 minutes ($P > .10$; N = 10; Rank Sum test). Prolonged stimulation of neutrophils with FMLP from 15 to 60 minutes did not cause further increase of uPAR expression on the plasma membrane.

The pro-uPA/uPA binding capacity of unstimulated, PMA-, and FMLP-stimulated neutrophils was assayed by flow cytometry with monoclonal anti-uPA antibodies after saturation by preincubation with 20 nmol/L DFP-uPA for 1 hour at 4°C. Mock-treated cells served as the control. The receptor was 7% saturated on fMLP-stimulated cells and 12% saturated on PMA-stimulated cells (Fig 2).

The "arbitrary fluorescence units" in these experiments refer only to relative quantitation of each individual antigen and cannot be used for comparisons of one antigen to the other.

Increased Potential for Plasmin Generation on FMLP-Stimulated Neutrophils

We have previously shown that the presence of cell-surface uPAR is essential for uPA-catalyzed plasminogen activation on peripheral blood leukocytes.[4] Therefore, we investigated whether the mobilization of uPAR to the cell surface on FMLP stimulation of neutrophils had a functionally significant effect, resulting from an increased potential for plasminogen activation. The degree of uPAR occupancy on both stimulated and nonstimulated cells was too low to be reliably detected in the assay used to determine plasminogen activation; therefore, the cells were preincubated with pro-uPA before the assay. Pro-uPA was used here, rather than active uPA as used previously,[4] because of the necessity for the presence of plasma during this preincubation to minimize nonspecific neutrophil activation.[15] Under these con-

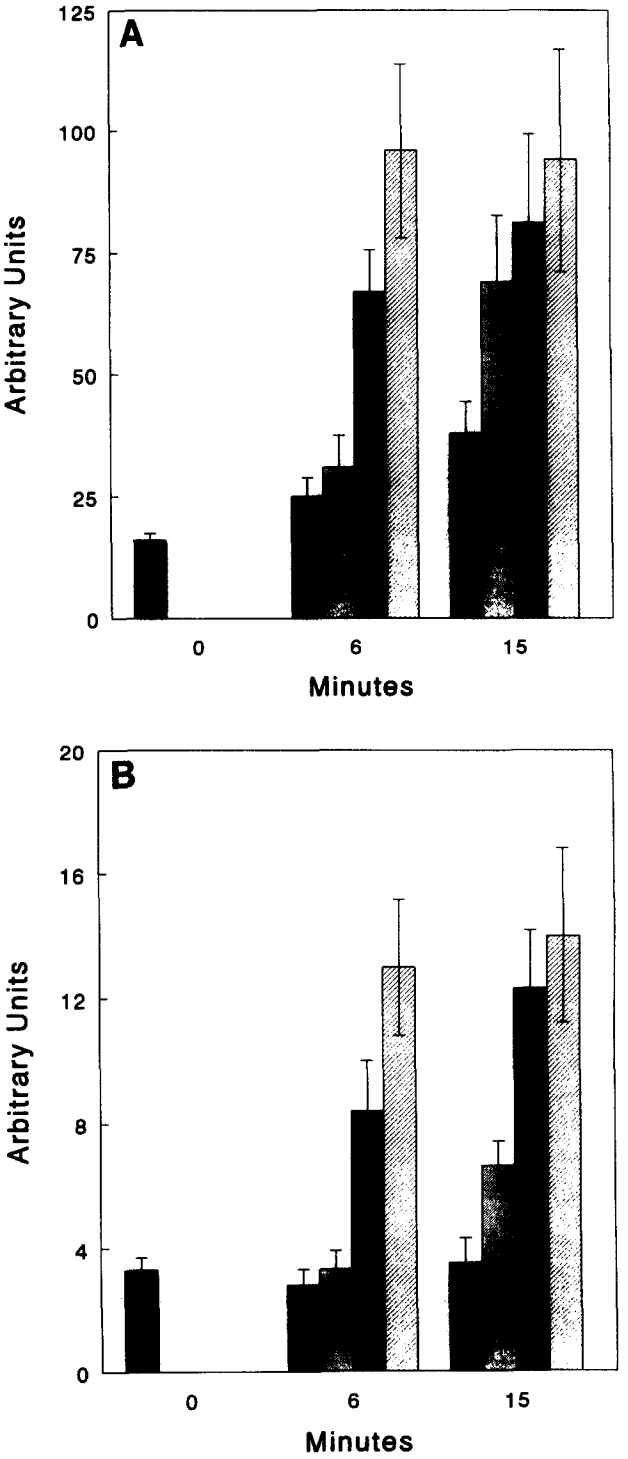

Fig 1. Flow cytometry of PMA-, FMLP-, and TNF-α–stimulated increase of uPAR and pro-uPA/uPA expression on neutrophils. Isolated neutrophils were stimulated at 2 × 10^6 cells/mL in buffer A with 8.1 μmol/L PMA (▨), 10 nmol/L FMLP (■), or 25 U/mL TNF-α (▨) for 6 and 15 minutes at 37°C. Stimulated neutrophils and mock-treated controls (■) were harvested and processed for flow cytometry of uPAR (A) and pro-uPA/uPA (B) as described in Materials and Methods. The amount of surface-exposed uPAR and pro-uPA/uPA was determined with calibration standards as described in Plesner et al[19] and expressed as mean of 10 estimations ±1 SD.

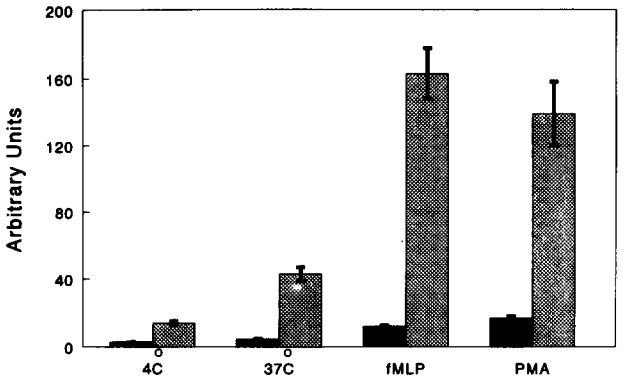

Fig 2. Flow cytometry of uPA-binding to unstimulated, FMLP-, and PMA-stimulated neutrophils is shown. Neutrophils were isolated and treated with DFP as described in Materials and Methods, washed in buffer A, adjusted to 2 × 10^6 cells/mL, stimulated with 8.1 μmol/L PMA or 10 nmol/L FMLP for 15 minutes at 37°C, washed, and incubated in buffer B (supplemented with 10 g/L BSA) at 2 × 10^6 cells/mL for 1 hour at 4°C with 20 nmol/L DFP-uPA. Mock-treated cells were included as control. Finally, the cells were washed in buffer B supplemented with 10 mmol/L EDTA, 10 mmol/L sodium azide, and 1% (wt/vol) BSA and labeled with monoclonal anti-uPA (clone 16) followed by PE-conjugated goat antimouse Ig. Cells incubated with exogenous DFP-uPA (⊠) showed an approximately 10-fold higher fluorescence compared with that for cells expressing only endogenous pro-uPA/uPA (■) (mean ± 1 SD; n = 10). DFP-inactivated uPA was used to avoid unintentional interaction with cell-associated plasminogen activator inhibitor.[1]

ditions, plasminogen activation is characterized by an initial lag-phase followed by an increasing rate of plasmin generation (Fig 3) because of reciprocal activation of pro-uPA and plasminogen on the cell surface, as we have previously described using U937 cells.[11] Figure 3 shows plasmin generation to be much faster on the FMLP-stimulated cells than on either of the unstimulated controls. These plasmin-generation curves were used to estimate the relative concentrations of receptor-bound pro-uPA, which were 100%, 225%, and 690% for unstimulated cells at 4°C, unstimulated cells at 37°C, and FMLP-stimulated cells, respectively.

Detection of uPAR and uPA/pro-uPA After Subcellular Fractionation of Neutrophils

Nonstimulated neutrophils were disrupted by nitrogen cavitation and subjected to subcellular fractionation by discontinuous Percoll gradient centrifugation.[14] The sedimentation profile of primary granules, specific granules, secretory vesicles, and plasma membranes was shown by measurements of myeloperoxidase, B_{12}-binding proteins, latent alkaline phosphatase, and the HLA class I/β_2-microglobulin complex, respectively (Fig 4A). The localization of uPAR was determined by its capacity to bind uPA in a semiquantitative, chemical, cross-linking assay using ^{125}I-labeled ATF as ligand and by ELISA using two monoclonal anti-uPAR antibodies.[4] Both methods show two peaks of uPAR, one corresponding to specific granules and the other to easily mobilizable secretory vesicles. In two independent experiments, uPAR was found to be evenly distributed between the two compartments (Fig 4B). Pro-uPA/uPA was measured in the same fractions by enzyme activity and by ELISA using two monoclonal anti-uPA antibodies.[4,21,26] Both methods showed a major peak of pro-uPA/uPA corresponding to specific granules and smaller amounts in fractions corresponding to secretory vesicles. By ELISA, the ratio was approximately 80/20 in two independent experiments (Fig 4C).

After stimulation of neutrophils with 10 nmol/L FMLP, latent alkaline phosphatase disappeared because secretory vesicles translocated to the plasma membrane and total alkaline phosphatase coeluted with plasma membrane (Fig 5A).[14] No translocation of B_{12}-binding protein and myeloperoxidase was observed on FMLP-stimulation (Fig 5A). uPAR associated with the secretory vesicles was translocated by FMLP-stimulation and appeared in the plasma membrane containing fractions, whereas the rest coeluted with specific granules as it did in unstimulated neutrophils (Fig 5B). Most of the pro-uPA/uPA in FMLP-stimulated neutrophils coeluted with specific granules, whereas a minor amount was translocated and coeluted with plasma membrane (Fig 5C). Stimulation of neutrophils with 8.1 μmol/L PMA caused translocation of alkaline phosphatase to the plasma membrane, partial translocation of myeloperoxidase, and secretion of approximately 50% of B_{12}-binding protein; the rest eluted in a similar position as that for unstimulated cells (Fig 6A, arrow). Compared with unstimulated cells, ≈70% of uPAR and ≈40% of pro-uPA/uPA was recovered in cell extracts; ≈30% of uPAR and ≈20% of pro-uPA/uPA was found in the supernatant of PMA-stimulated cells (mean of two experiments). Thus, the total recovery (cells + supernatant) of uPAR was 100%, whereas the

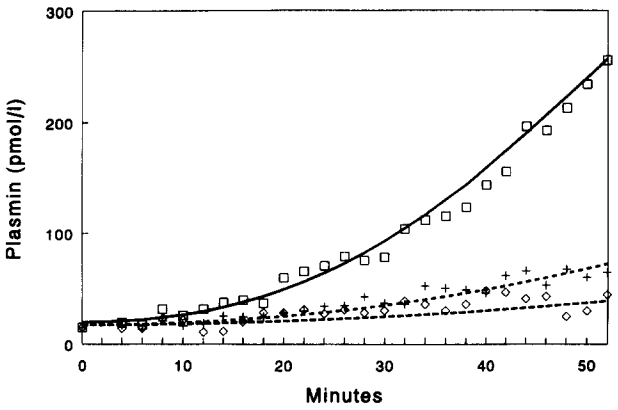

Fig 3. uPAR-dependent plasminogen activation on the surface of neutrophils is shown. The data shown are from a single experiment and are representative of data obtained in experiments with cells from three individual donors. Data are shown for unstimulated cells maintained at 4°C (– – ◇ – –) and 37°C (– – + – –), and for FMLP-stimulated cells at 37°C (— □ —). Plasmin-generation curves were converted to concentrations of receptor-bound pro-uPA as described in Materials and Methods. The initial pro-uPA concentration in this experiment were calculated to be 0.29 pmol/L, 0.65 pmol/L, and 2.00 pmol/L, respectively. It was shown in a control experiment that addition of FMLP to nonstimulated cells subsequent to incubation with pro-uPA, but before assay, had no effect on plasmin generation.

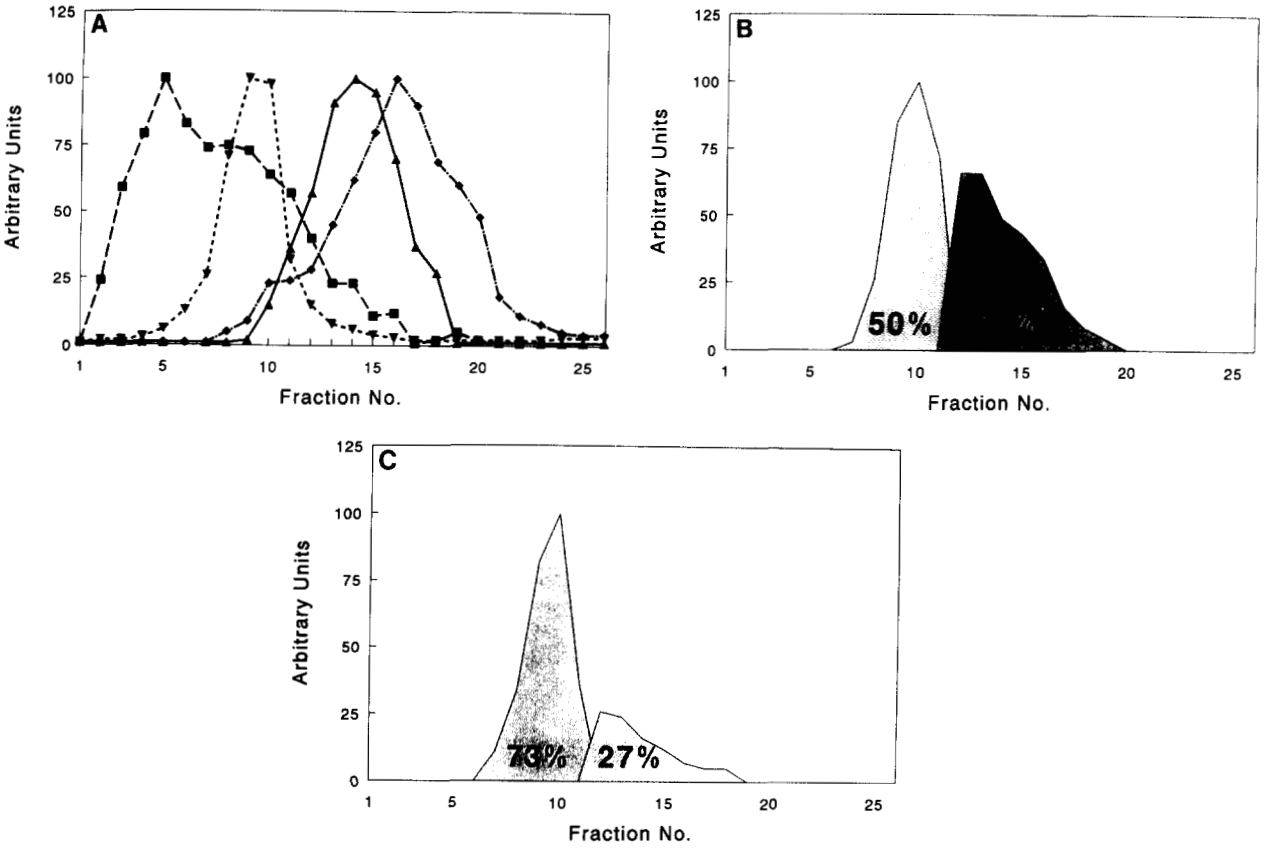

Fig 4. Subcellular fractionation of unstimulated neutrophils. Neutrophils (3×10^8) were isolated and processed as described in Materials and Methods. The cells were suspended in 10 mL buffer A, treated with 5 mmol/L DFP for 10 minutes at 0°C, washed, resuspended in 10 mL buffer C, and subsequently disrupted by nitrogen cavitation in the presence of protease inhibitors as described in Material and Methods. The cavitate was separated by gradient centrifugation on Percoll, and the fractionation was monitored for HLA class 1/β_2-microglobulin (plasma membrane) (—·♦—·—), latent alkaline phosphatase (easily mobilizable intracellular vesicles) (—▲—), B_{12}-binding proteins (specific granules) (– –▼– –), and myeloperoxidase (primary granules) (—■— –) (A). The fractions were cleared of Percoll by centrifugation at 180,000*g* for 120 minutes, and the supernatants assayed for uPAR by ELISA and by cross-linking to ^{125}I-labeled ATF-uPA followed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The gels were exposed for autoradiography and subsequently cut and counted in a γ-counter to give an estimate of uPAR in the granulocyte fractions. Pro-uPA/uPA was measured by ELISA and by a plasminogen activation assay as described in Materials and Methods. After fractionation of unstimulated neutrophils, uPAR is evenly distributed between fractions containing B_{12}-binding protein corresponding to specific granules and fractions containing latent alkaline phosphatase corresponding to easily mobilizable secretory vesicles (B). In two independent experiments the distribution of uPAR in specific granules to uPAR in secretory vesicles measured by ELISA was 50/50 and 53/47, respectively. Pro-uPA/uPA is primarily found in fractions coisolating with specific granules, but small amounts coisolate with latent alkaline phosphatase (C). By ELISA, the ratio of pro-uPA/uPA in these two compartments was 73/27 and 89/11, respectively.

recovery of pro-uPA/uPA was only ≈60%. Most of the remaining cell-associated uPAR and pro-uPA/uPA coeluted with plasma membrane (Fig 6B and C, respectively). Neither uPAR nor pro-uPA/uPA was released from unstimulated or FMLP-stimulated neutrophils. Both proteins were quantitatively recovered in cell extracts. The pertubation caused by FMLP and PMA stimulation results in a minor shift to a lower density of the myeloperoxidase-containing granules (Figs 5A and 6A, respectively).

DISCUSSION

Early events in neutrophil activation are known to be associated with translocation of a specialized intracellular compartment of easily mobilizable vesicles.[14] We have now shown that the expression of uPAR on neutrophils is increased immediately after neutrophil activation under conditions that cause translocation of these easily mobilizable vesicles but do not cause the release of specific granules. We have also shown that the enhanced expression of uPAR is correlated with an increased potential for pericellular plasminogen activation. Using two different methods, we have also found that a significant fraction (≈50%) of uPAR coisolates with latent alkaline phosphatase (a marker of these vesicles), during subcellular fractionation of unstimulated neutrophils, and that FMLP-stimulation causes translocation of uPAR from this compartment to the plasma membrane. uPAR (≈50%) is also found in fractions containing most of the pro-uPA/uPA. Although it was not directly shown in our study, it is possible that pro-uPA/uPA is receptor-bound in this compartment. However, the methods

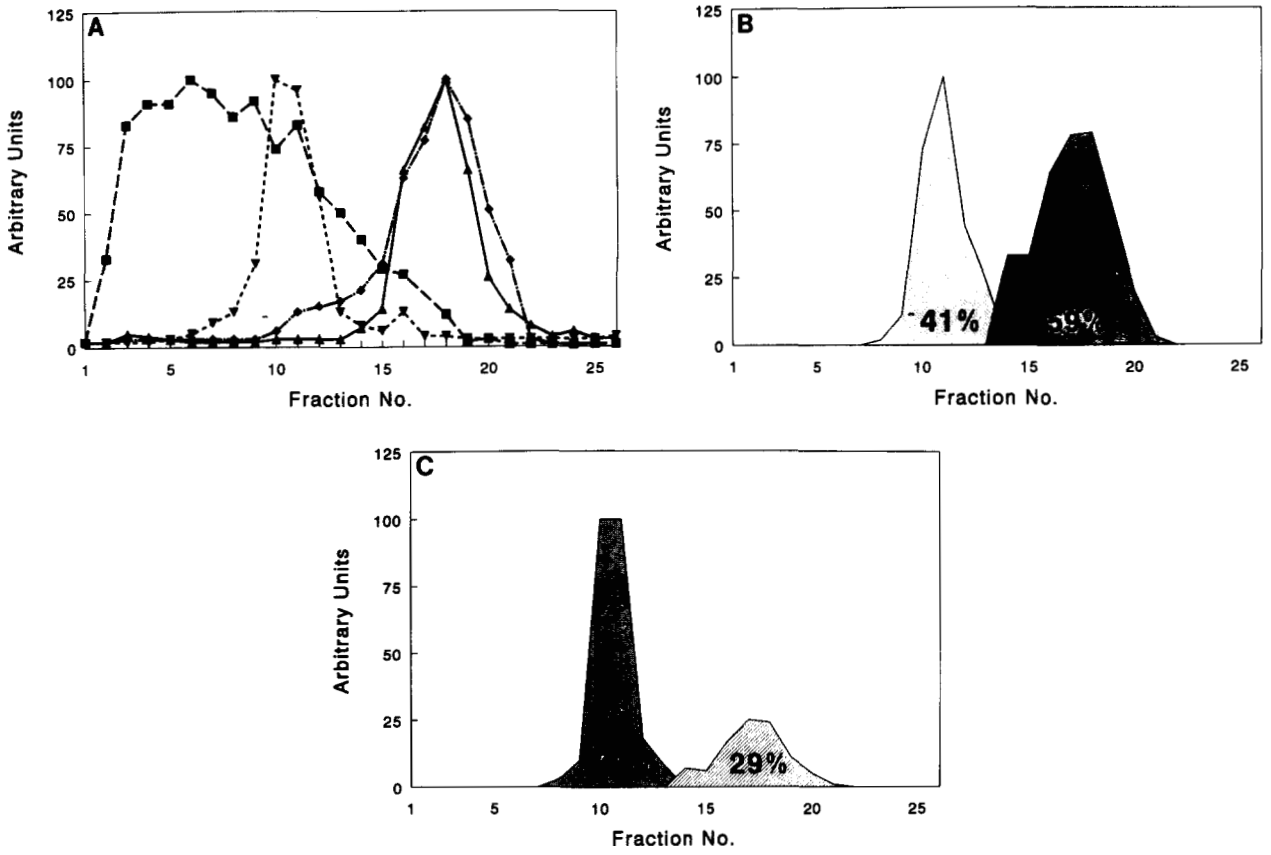

Fig 5. Subcellular fractionation of FMLP-stimulated neutrophils. Neutrophils were isolated and treated with DFP as described in Materials and Methods and in the legend to Fig 4, adjusted to 3 × 10^7 cells/mL in buffer A, stimulated with 10 nmol/L FMLP for 15 minutes at 37°C, and processed by nitrogen cavitation and gradient centrifugation as described. Alkaline phosphatase lost its latency and coeluted with the plasma membrane marker HLA class 1/β_2-microglobulin (A). B_{12}-binding protein and myeloperoxidase was recovered quantitatively in a similar position as that from unstimulated cells. Symbols typifying each marker of a subcellular compartment are similar to Fig 4. Approximately 50% of uPAR translocated and coeluted with alkaline phosphatase and HLA class 1/β_2-microglobulin; the rest coeluted with B_{12}-binding protein as it did in unstimulated cells (B). In two independent experiments, the ratio of uPAR in specific granules to uPAR translocated to plasma membrane was 40/60 and 41/59, respectively, as determined by ELISA. Most of the pro-uPA/uPA coeluted with B_{12}-binding protein, whereas a minor fraction, in some experiments, translocated with uPAR and alkaline phosphatase and was found in the HLA class 1/β_2-microglobulin–containing fractions (C). In two experiments, the respective ratios determined by ELISA were 71/29 and 100/0.

used do not allow us to conclude that pro-uPA/uPA is associated with uPAR in neutrophil granules in vivo, because redistribution and binding may take place during the fractionation procedure. To clarify this problem double-labeling immunoelectron microscopy should be applied.

It has previously been shown that pro-uPA/uPA is present in specific granules of neutrophils and that neutrophil activation by PMA, resulting in exocytosis of specific granules, causes increased amounts of plasma membrane-associated uPA activity but not secretion of pro-uPA/uPA.[13] The finding that pro-uPA/uPA, which does not possess a transmembrane domain or anchor, remains associated with the plasma membrane rather than being secreted after neutrophil activation points to the possibility that pro-uPA/uPA may exist in a receptor-bound form in the specific granules or may be engaged by receptor binding during the process of activation.

In our experiments, we found some release of uPAR and pro-uPA/uPA from PMA-stimulated neutrophils and only a 60% total recovery of uPA, but this may be an artefact because of proteolysis. pro-uPA/uPA has been shown to be particularly sensitive to proteolysis from neutrophil-derived enzymes and can only be found after DFP-treatment of neutrophils to irreversibly inactivate serine proteinases.[13] Also, uPAR is vulnerable to proteolytic enzymes and can in fact be cleaved by uPA to release the ligand-binding domain.[27] When unstimulated or FMLP-stimulated neutrophils were studied, both uPAR and pro-uPA/uPA were recovered quantitatively in cell extracts, and neither uPAR nor pro-uPA/uPA was secreted to the supernatant. Although uPAR and pro-uPA/uPA may be shed from neutrophils under some conditions, we believe that the primary function of these molecules is to provide a potential for cell-associated plasminogen activation and to focus this process at the cell surface.[28]

Small amounts of pro-uPA/uPA colocalized with uPAR and latent alkaline phosphatase by subcellular fractionation of unstimulated neutrophils and is presumably present in

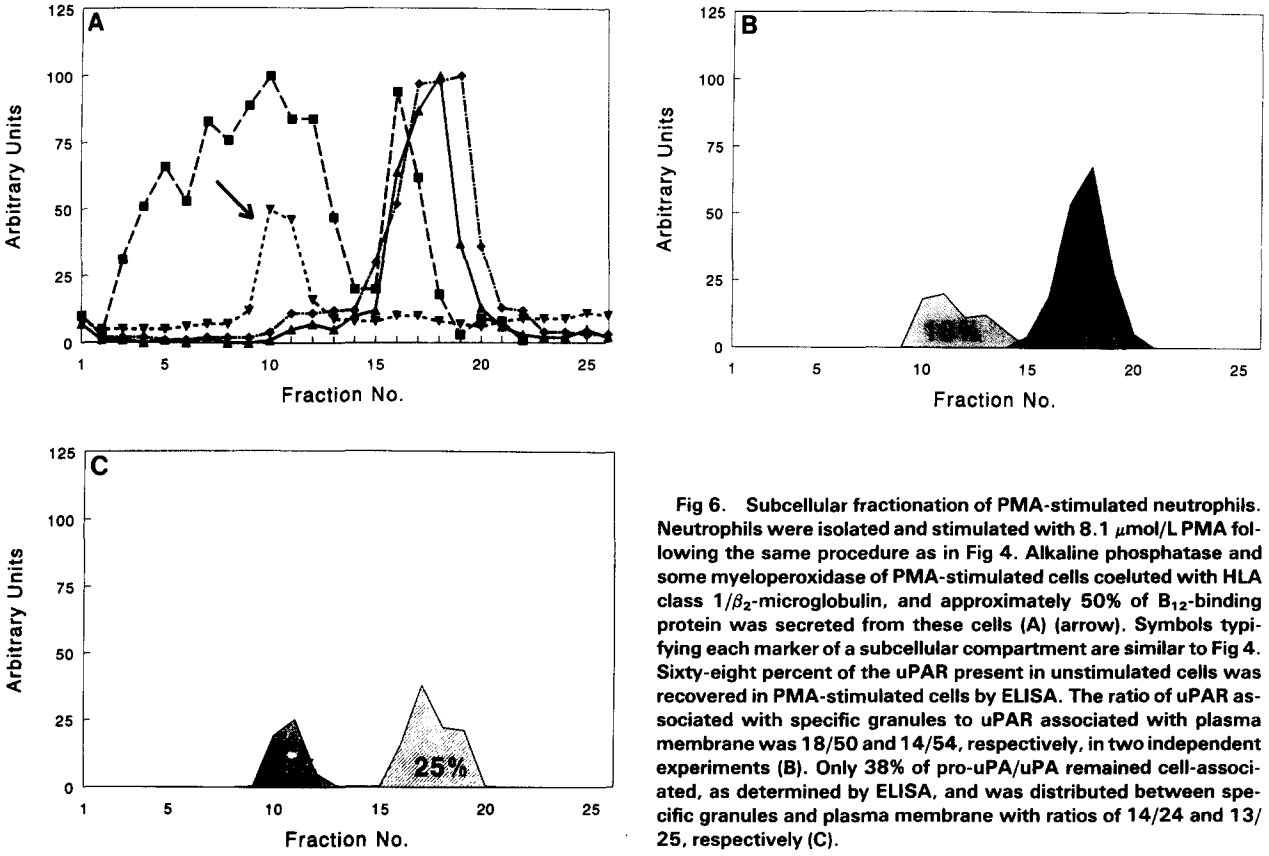

Fig 6. Subcellular fractionation of PMA-stimulated neutrophils. Neutrophils were isolated and stimulated with 8.1 μmol/L PMA following the same procedure as in Fig 4. Alkaline phosphatase and some myeloperoxidase of PMA-stimulated cells coeluted with HLA class 1/β_2-microglobulin, and approximately 50% of B_{12}-binding protein was secreted from these cells (A) (arrow). Symbols typifying each marker of a subcellular compartment are similar to Fig 4. Sixty-eight percent of the uPAR present in unstimulated cells was recovered in PMA-stimulated cells by ELISA. The ratio of uPAR associated with specific granules to uPAR associated with plasma membrane was 18/50 and 14/54, respectively, in two independent experiments (B). Only 38% of pro-uPA/uPA remained cell-associated, as determined by ELISA, and was distributed between specific granules and plasma membrane with ratios of 14/24 and 13/25, respectively (C).

the previously described secretory vesicles,[14] possibly in association with uPAR. After stimulation of neutrophils with FMLP, translocation of pro-uPA/uPA to the plasma membrane could be shown by ELISA after subcellular fractionation and by flow cytometry. The translocated pro-uPA/uPA could not reproducibly be shown by enzyme assay, possibly because of lower sensitivity of this assay compared with ELISA or because of interference from inhibitors of plasminogen activation or plasmin.

Pro-uPA/uPA was measured by two different methods after subcellular fractionation of neutrophils, an ELISA based on two MoAbs and an enzyme activity assay. Both methods showed a major peak of pro-uPA/uPA eluting in exactly the same fractions as one of the peaks of uPAR, but, by close analysis, these fractions peak at a slightly lower density than B_{12}-binding protein as a marker of specific granules. The difference between the elution profile of pro-uPA/uPA and uPAR, on one side, and B_{12}-binding protein, on the other, was marginal ($\approx$1 fraction) but reproducible and may be caused by a heterogeneity of specific granules, such as has been shown recently in a study of gelatinase-containing granules.[29] Further studies using double-labeling techniques and immunoelectron microscopy will be needed to show directly whether pro-uPA/uPA and uPAR are present in the same granules and to study the relationship of these to granules containing gelatinase, B_{12}-binding protein, and other markers.

Flow cytometry with monoclonal anti-uPA antibody of FMLP- and PMA-stimulated neutrophils showed a very substantial increase in fluorescence after saturation with exogenous DFP-uPA, suggesting that only approximately 10% of the receptor molecules may be occupied by endogenous pro-uPA/uPA. Thus, neutrophils may be partly dependent on pro-uPA/uPA from exogenous sources to achieve a maximum potential for plasminogen activation. Endothelial cells have been shown to produce pro-uPA/uPA during inflammation and in response to treatment with TNF-α in vitro and may serve as a source of exogenous pro-uPA/uPA for neutrophils before or during extravasation.[30,31] The tendency for higher total uPA-binding capacity on FMLP-stimulated compared with that for PMA-stimulated neutrophils seen in Fig 2 may be explained by release of more proteolytic enzymes after PMA-stimulation, with subsequent partial proteolytic degradation of uPAR.

On the basis of our findings, we propose a model for enhanced pericellular plasminogen activation after neutrophil stimulation. Early events will cause translocation of uPAR and of small amounts of pro-uPA/uPA from an intracellular compartment of secretory vesicles to the plasma membrane. An excess of receptor-binding sites will be available for binding of pro-uPA/uPA, possibly provided by neighboring cells, eg, activated endothelial cells.[30,31] More intense events of activation will cause the subsequent release of specific granules, including uPAR and endogenous pro-uPA/uPA, that may further increase the cell surface-associated potential for plasminogen activation. These processes may be of

potential importance for extravasation of neutrophils, migration through extracellular matrix, tissue remodelling, and resolution of thrombi.[4,32,33]

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