Translocation of annexin I to plasma membranes and phagosomes in human neutrophils upon stimulation with opsonized zymosan: possible role in phagosome function

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Annexin I in the cytosol of resting neutrophils was translocated to the plasma membranes upon addition of opsonized zymosan (OZ). Maximum translocation could be detected 1 min after stimulation with OZ, and decreased thereafter. Subcellular fractionation studies demonstrated that annexin I could not be detected in the granule fractions in either resting or activated cells, but was found in association with the phagosome fraction. The marked translocation of annexin I was unique to OZ, since formyl-Met-Leu-Phe induced only slight translocation of annexin I to the plasma membranes, and phorbol 12-myristate 13-acetate had no effect at all. The mechanism regulating the translocation of annexin I is not clear. Annexin I is not phosphorylated in resting or stimulated cells. The correlation between the elevation in the intracellular calcium ion concentration ($[Ca^{2+}]_i$) and the degree of translocation of annexin I to the plasma membranes induced by the different stimuli, together with the inhibition of these processes by the addition of EGTA, indicate that the translocation of annexin I can probably be attributed to the rise in $[Ca^{2+}]_i$. However, this cannot be the sole mechanism since ionomycin, which caused an increase in $[Ca^{2+}]_i$ similar to that induced by OZ, was less efficient than OZ in inducing translocation of annexin I. The induction of annexin I translocation to the plasma membrane by OZ, which was the only agent that induced phagosome formation, and the detection of annexin I in the phagosome fraction, suggest that annexin I participates in phagosome function.

INTRODUCTION

The annexins (lipocortins) are a family of calcium-dependent phospholipid-binding proteins present in various tissues that have been classified into several distinct groups according to their amino acid sequences [1–3]. Annexin I has been isolated, cloned and sequenced [4]; large amounts were found to be present in several cell types, including phagocytes [5]. Several cellular functions have been suggested for the annexins: inhibition of phospholipase A_2 (PLA_2), attachment of cytoskeletal elements to plasma membranes, initiation of membrane fusion in exocytosis, and inhibition of cell migration [6–8]. Annexin I was originally identified as a secreted inhibitor of pancreatic PLA_2 [9]. The inhibitory effect appeared to be due to the binding of substrate phospholipids by annexin I rather than to a specific interaction with the enzyme [10]. Addition of extracellular annexin I to macrophages inhibited PLA_2 activity and superoxide generation [11]. Annexin I has previously been shown to be the major mediator of Ca^{2+}-dependent vesicular aggregation in neutrophil cytosol and of the Ca^{2+}-dependent fusion of liposomes with other liposomes or with the cytoplasmic face of neutrophil plasma membrane vesicles in cell-free experiments [12–15]. However, a well defined biological function has not yet been determined.

In the present study, the distribution of annexin I in neutrophils stimulated by different agents was monitored *in vitro* by immunoblotting analysis of the fractionated neutrophils, in order to determine a possible role for annexin I in these cells.

MATERIALS AND METHODS

Neutrophil isolation

Neutrophils were separated by Ficoll/Hypaque centrifugation, dextran sedimentation, and hypotonic lysis of erythrocytes [16].

Superoxide anion measurement

The production of superoxide anions by intact cells was measured as the superoxide dismutase-inhibitable reduction of ferricytochrome *c* [17] by a modification of the microtitre plate technique of Pick and Mitzell [18]. Cells were suspended (5×10^5 cells/well) in 100 μl of Hanks' Balanced Salts Solution (HBSS) containing 150 μM ferricytochrome *c*. Cells were stimulated by the addition of the appropriate agent, and the reduction of ferricytochrome *c* was followed by the change in absorbance at 550 nm at 2 min intervals on a Thermomax Microplate Reader (Menlo Park, CA, U.S.A.). The maximal rates of superoxide generation were determined using the molar absorption coefficient $\epsilon_{550} = 21\ mM^{-1} \cdot cm^{-1}$.

Isolation of membrane and cytosol fractions

Membrane and cytosol fractions were prepared as described previously [17]. Cells (5×10^7 cells in 10 ml) in HBSS were treated with di-isopropyl fluorophosphate (DFP) for 20 min at 18 °C, washed twice with HBSS, suspended in HBSS at a concentration of 5×10^6 cells/ml and stimulated with the different agents for

Abbreviations used: $[Ca^{2+}]_i$, intracellular calcium ion concentration; DFP, di-isopropyl fluorophosphate; FMLP, formyl-Met-Leu-Phe; HBSS, Hanks' balanced salts solution; OZ, opsonized zymosan; PLA_2, phospholipase A_2; PMA, phorbol 12-myristate 13-acetate.

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the desired times. The reaction was stopped by addition of 20 ml of cold HBSS and immediate centrifugation at 4 °C. Cells were suspended at 10^8 cells/ml in relaxation buffer (100 mM KCl, 3 mM NaCl, 3.5 mM $MgCl_2$, 0.25 mM EGTA, 1 mM ATP, 10 mM Pipes, pH 7.4) containing 1 mM PMSF and 100 μM leupeptin at 4 °C and were then sonicated for 3×10 s, resulting in about 95 % cell breakage. Higher concentrations of EGTA in the lysis buffer affected the translocation of annexin I to the membranes. After centrifugation (2 min; 15600 ***g***) to remove granules, nuclei and unbroken cells, the supernatants were centrifuged in a Beckman Airfuge (30 min; 134000 ***g***) to obtain a cell membrane pellet and a cytosol supernatant. Membranes were suspended at 10^9 cell equivalents/ml in 0.34 M sucrose/0.5 × relaxation buffer containing 1 mM dithiothreitol. Solubilized membranes and cytosol were stored at −70 °C.

In experiments in which neutrophils were not pretreated with DFP, annexin I detected in the membranes had been degraded into smaller proteolytic fragments, in accordance with the results of a recent study [19].

Preparation of antibodies against annexin I

A full-length annexin I cDNA was selected from a Lambda-ZAP (Stratagene) library derived from RNA of the human promyelocytic leukaemia cell line HL60, which was induced to differentiate with dibutyryl cAMP. Recombinant human annexin I was purified as described previously [20]. Host bacteria (JM 109) were infected with the phagemids to yield transformants containing cDNA inserts in the Bluescript plasmid (Stratagene). Recombinant human annexin I expressed in *Escherichia coli* was present in the insoluble inclusion body fraction and not in the soluble form, as shown by SDS/PAGE and Western blotting performed with antibodies against annexin I (provided by Dr. B. Pepinsky, Biogen, Cambridge, MA, U.S.A.). The recombinant human annexin I protein was purified from 10 % gels. The bands containing recombinant human annexin I were cut out and electroeluted (Bio-Rad). A clean band of recombinant human annexin I (35 kDa) was verified by electrophoresis and Western blotting. This was used to immunize a goat by injection in Freund's complete adjuvant. The polyclonal antibodies were specific in detecting annexin I, confirmed by a comparison with immunoblot analysis performed with antibodies against annexin I (provided by Dr. B. Pepinsky). The goat antiserum against recombinant human annexin I did not react with other annexins (provided by Dr. B. Pepinsky).

Immunoblot analysis

Samples were solubilized in 2 × sample buffer (12 % SDS, 8 M urea, 250 mM Tris, 8 mM EDTA, 0.2 mM leupeptin, 2 mM PMSF, pH 6.9) [17]. The amount of protein in each sample was quantified with the Pierce BCA protein assay (Pierce Chemical Co., Rockford, IL, U.S.A.), using BSA standards to confirm the exact number of cell equivalents. Cytosol or membrane samples were analysed by electrophoresis on polyacrylamide gels. The resolved proteins were electrophoretically transferred to nitrocellulose, which was stained with Fast Green to detect protein banding, and then blocked in 5 % non-fat dried milk in Tris-buffered saline. The blots were incubated overnight in Tris-buffered saline/1 % gelatin containing goat antiserum against recombinant human annexin (diluted 1:1000). Immunoblots were incubated with 1 mg/ml horseradish peroxidase-conjugated rabbit anti-goat IgG (Biomakor) and developed with 4-chloro-1-naphthol and H_2O_2.

The relative changes in annexin I content were quantified using densitometry in a reflectance mode (Hoefer Scientific Instruments, San Francisco, CA, U.S.A.). Neutrophil cytosol and membrane samples were serially diluted to give total amounts of 0.25–60 mg and immunoblotted. The detection of annexin I was linear in the ranges 0.25–30 μg of cytosol and 0.25–15 μg of membrane protein, which correspond to 1×10^5–1.2×10^7 cell equivalents.

Subcellular fractionation

Subcellular fractionation was performed according to the method of Borregaard et al. [21], with some modifications. Neutrophils (3×10^8) were treated and sonicated as described above. Nuclei and unbroken cells were pelleted by centrifugation of the sonicated neutrophils at 500 ***g*** for 10 min at 4 °C. The supernatant was decanted and loaded on to a precooled discontinuous density gradient. Percoll, 10 × concentrated relaxation buffer and distilled water were mixed to give solutions of densities 1.05 and 1.12 g/ml. A portion of the denser solution (14 ml) was underlayered with 14 ml of the lighter solution, and the cell material was layered on top. The gradients were centrifuged at 32800 ***g*** for 35 min (4 °C) using a fixed-angle Beckman JA20 rotor. Three bands were visible, and were collected using a Pasteur pipette. The bands were assayed for myeloperoxidase [21], cytochrome *b* and Na^+/K^+-ATPase [22], which are markers for azurophil granules (α), specific granules (β) and plasma membranes (γ) respectively.

Assay of myeloperoxidase activity

Myeloperoxidase activity was assayed [22] by adding 50 μl of 0.1 M phosphate buffer, pH 6.8, and 25 μl of 0.2 M citrate phosphate buffer, pH 5, followed by 25 μl of *o*-phenylenediamine dihydrochloride (4 mg/ml) and 0.03 % H_2O_2. Colour development was stopped after 10 min at room temperature by addition of 3 M HCl, and the absorbance was measured at 490 nm. The colour that developed over this time period was directly proportional to the amount of enzyme. Total enzyme values in neutrophils were measured after lysis of cells in control tubes with 10 μl of 10 % Triton X-100 and vigorous vortexing.

Cytochrome b_{558} determination

Cytochrome b_{558} was determined as described previously [23]. A 250 μl volume of fraction material was placed in a cuvette and brought to a total volume of 0.75 ml with relaxation buffer and Triton X-100 (final concentration of Triton was 0.2 %, v/v). The unreduced spectrum of the sample between 600 and 400 nm (Uvikon 810 spectrophotometer; Kontron Analytical, Zurich, Switzerland) was computer-subtracted from the spectrum obtained following reduction of the sample by a few grains of crystalline sodium dithionite. Cytochrome *b* was quantified using molar absorption coefficients of 10^6 $mM^{-1} \cdot cm^{-1}$ at 425 nm and 21.7 $mM^{-1} \cdot cm^{-1}$ at 558 nm.

Ouabain-sensitive Na^+/K^+-ATPase

Ouabain-sensitive Na^+/K^+-ATPase was analysed with ATP as substrate, as described previously [24].

Subcellular isolation of phagosomes

Phagosomes were formed in neutrophils by internalization of blue dyed latex beads (0.8 μM) or opsonized zymosan (OZ) according to Pizon et al. [24]. Cells (2×10^8 in 50 ml of HBSS)

were exposed to latex beads diluted 1:200 or to OZ (1 mg/ml) for 15 min at 37 °C. The cells were washed with cold PBS (500 ***g*** for 5 min) and processed for phagosome isolation at 4 °C. They were then divided into two samples, each of which was centrifuged, and the cell pellet was suspended in 1 ml of homogenization buffer (250 mM sucrose, 3 mM imidazole, pH 7.4) The cells were disrupted by homogenization in a 2 ml Dounce homogenizer with 20 strokes of a tight-fitting pestle at 4 °C. Disruption of the cells was monitored in the light microscope and stopped before major breakage of the cell nucleus had occurred. The homogenate was then centrifuged at 500 ***g*** for 5 min to pellet the unbroken cells and nuclei. The supernatant containing the phagosomes (1 ml) was brought to 40 % sucrose by adding an equal volume of 62 % sucrose (all sucrose solutions are w/w, and contain 3 mM imidazole, pH 7.4). This was laid on top of a 1 ml 62 % sucrose cushion. After addition of 2 ml portions of 35 %, 25 % and 10 % sucrose solutions, the gradients were centrifuged for 60 min at 4 °C in an SW40 rotor at 10000 ***g***. The phagosomes containing latex beads at the interface of the 10 % and 25 % sucrose, and those containing OZ at the interface of the 25 % and 35 % sucrose, were collected using a capillary pipette and peristaltic pump and resuspended in 10 ml of cold PBS. Phagosomes were finally isolated by pelleting at 40000 ***g*** for 15 min at 4 °C, and were quickly frozen in liquid nitrogen.

Immunoprecipitation, electrophoresis and autoradiography

Loading of $^{32}PO_4$, immunoprecipitation, electrophoresis and autoradiography were performed as described previously [25]. Neutrophils were treated with DFP as described above, washed once, suspended at 10^8 cells/ml in loading buffer (137 mM NaCl, 0.8 mM $MgCl_2$, 5.4 mM KCl, 5.6 mM glucose, 10 mM Hepes, pH 7.4) supplemented with 1 mCi/ml $^{32}PO_4$, and incubated with intermittent mixing for 90 min at 30 °C. The loaded neutrophils were pelleted by centrifugation, and resuspended at 5×10^5 cells/ml in the same buffer at 37 °C supplemented with 1 mM $MgCl_2$ and 0.5 mM $CaCl_2$. The stimulants were added for 2 min. The reaction was terminated by 5-fold dilution with ice-cold loading buffer and the solution centrifuged at 500 ***g*** for 5 min. Cell were sonicated and centrifuged to remove granules, nuclei and unbroken cells as described above. A 250 μl sample of cell equivalents of the neutrophil lysates were added to 250 μl of solubilization buffer (150 mM NaCl, 5 mM EDTA, 10 mM Tris, pH 7.5, 1 % sodium deoxycholate, 1 % Nonidet P-40). Goat antiserum raised against recombinant annexin I was added (5 μl) followed by incubation on ice overnight. The extracts were brought to a volume of 0.4 ml in solubilization buffer containing 30 μl of a 50 % (w/v) slurry of recombinant Protein A–Sepharose. The samples were tumbled end-over-end for 1 h, and washed twice with 1 ml of solubilization buffer containing 20 % (w/v) sucrose and 0.15 % (w/v) BSA and twice with 1 ml of solubilization buffer containing 20 % (w/v) sucrose. The samples were boiled in SDS sample buffer, and electrophoresed on an SDS/10 %-polyacrylamide gel. Gels were stained with Coomassie Blue, dried and used for autoradiography with intensifying screens at −70 °C.

Measurement of intracellular free Ca^{2+} concentration ($[Ca^{2+}]_i$)

$[Ca^{2+}]_i$ was measured as previously described [24] by using the fluorescent Ca^{2+} indicator fura-2/AM. Purified neutrophils (1×10^7 cells/ml) were suspended in RPMI 1640 medium (Bet-Haemek) and loaded with fura-2/AM at a final concentration of 1 μM. Following a 20 min incubation at 37 °C, the cells were diluted 10-fold and incubated for an additional 40 min. After loading and washing, neutrophils were resuspended in a simplified saline buffer (145 mM NaCl, 5 mM KCl, 1 mM Na_2HPO_4, 1 mM $CaCl_2$, 0.5 mM $MgSO_4$, 5 mM glucose, 10 mM Hepes, pH 7.4). Fluorescence intensity was recorded with a spectrofluorimeter (Perkin Elmer LS-5). Excitation wavelengths were 339 nm (2.5 nm slit) and 380 nm (2.5 nm slit), and the emission wavelength was 550 nm (2.5 nm slit). The $[Ca^{2+}]_i$ corresponding to fluorescence emitted by trapped fura-2 was calculated by the equation: $[Ca^{2+}]_i = 224\ nM \times (R - R_{min})/(R_{max} - R)$ (where 224 nM is the K_d value for fura-2). R_{max} was obtained after dye release with 4 % Triton X-100 and the addition of 2 mM $CaCl_2$. R_{min} was determined by setting $[Ca^{2+}]$ to 1 nM by addition of 2 mM EGTA and 20 mM Tris base to lysed cells.

Analysis of data

All densitometry and autoradiographic observations presented were confirmed in at least three or four separate experiments performed on different preparations of cells. Differences between means were analysed by Student's *t* test. Plots were drawn as least-squares regression lines and tested by analysis of variance.

RESULTS

In order to investigate the role of annexin I during neutrophil activation, its distribution after stimulation with 1 mg/ml OZ, 50 ng/ml phorbol 12-myristate 13-acetate (PMA) or 0.1 μM formyl-Met-Leu-Phe (FMLP) was determined by immunoblot analysis. The concentrations of the stimuli used were defined according to their similar effects in inducing superoxide production, as detected by cytochrome *c* reduction. The amounts of superoxide generated were 11.2 ± 1.6, 13.1 ± 2.5 and 9.8 ± 1.1 nmol of O_2/min per 10^6 cells for OZ, PMA and FMLP respectively (means ± S.E.M.). The distribution of annexin I in the cytosol and membrane fractions during a period of 15 min after OZ stimulation is presented in Figure 1. The same number of cell equivalents was used for both membrane and cytosol fractions. In resting cells, annexin I was found only in the cytosol. Upon stimulation, annexin I was translocated to the

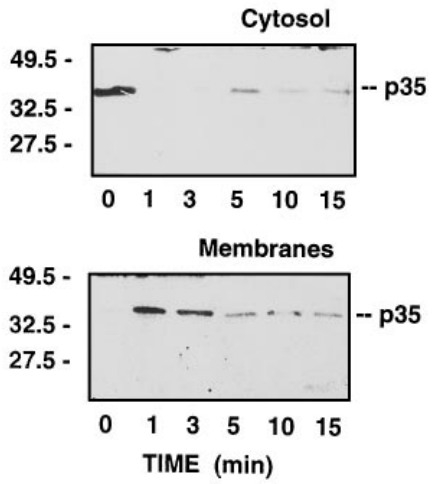

Figure 1 Kinetics of translocation of annexin I in OZ-stimulated neutrophils

Translocation of annexin I to the membranes was analysed by SDS/PAGE immunoblotting in neutrophils stimulated with 1 mg/ml OZ. Samples of 2×10^6 cell equivalents of cytosol or membranes were applied per lane. Molecular masses (kDa) are indicated on the left; p35 denotes annexin I. Shown is a representative experiment; five others gave similar results.

Table 1 Distribution of annexin I in cytosol and membrane fractions of neutrophils stimulated with 1 mg/ml OZ

The determination by densitometry of the relative amounts of annexin I in neutrophil cytosol or membranes are means ± S.E.M. from four experiments. Units are arbitrary units of density, with higher numbers representing darker bands on the immunoblot.

	Annexin I (units)	
Time after OZ stimulation (min)	Cytosol	Membranes
0 (resting cells)	195 ± 5	20 ± 2
1	26 ± 5	190 ± 15
3	28 ± 9	172 ± 11
5	66 ± 10	56 ± 12
10	51 ± 7	58 ± 10
15	60 ± 12	58 ± 15

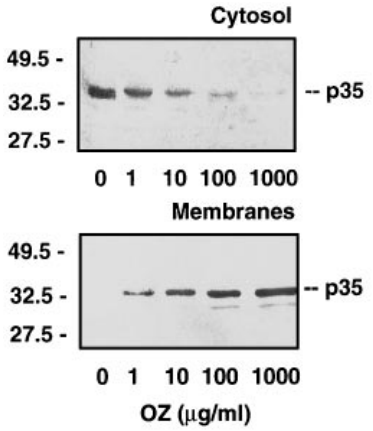

Figure 2 Annexin I translocation in neutrophils stimulated with OZ, as a function of concentration

OZ was added to the cells at 1–1000 μg/ml for 2 min. Samples of 2×10^6 cell equivalents of cytosol or membranes were applied per lane. Molecular masses (kDa) are indicated on the left; p35 denotes annexin I. Shown is a representative experiment; five others gave similar results.

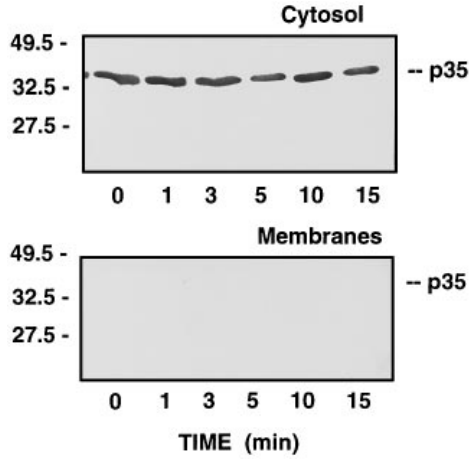

Figure 3 Effect of PMA on annexin I localization in neutrophils

Neutrophils were stimulated with 50 ng/ml PMA, and annexin I localization was analysed by SDS/PAGE immunoblotting. Samples of 10^7 cell equivalents of cytosol or membranes were applied per lane. Molecular masses (kDa) are indicated on the left; p35 denotes annexin I. The arbitrary units of density were similar at all time points. Shown is a representative experiment; five others gave similar results.

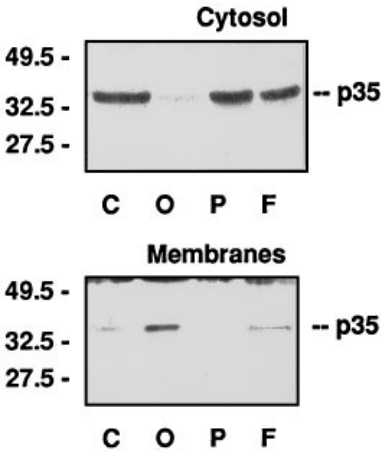

Figure 4 Annexin I in cytosol and membrane fractions from neutrophils stimulated with different agents for 2 min

A representative SDS/PAGE immunoblot analysis is shown. Lane C, resting cells; lane O, 1 mg/ml OZ-stimulated cells; lane P, 50 ng/ml PMA-stimulated cells; lane F, 0.1 μM-FMLP stimulated cells. Samples of 2×10^6 cell equivalents of cytosol or membranes were applied per lane. Molecular masses (kDa) are indicated on the left; p35 denotes annexin I. Shown is a representative experiment; five others gave similar results.

membranes. Maximal translocation of annexin I to the membranes was achieved 1 min after stimulation with OZ; at 5–15 min of stimulation, translocation was lower than that detected at 1 or 3 min after stimulation. At 5–15 min of stimulation small amounts of annexin I were detected in the cytosol fraction. The relative amounts of annexin I in membrane and cytosol fractions were measured by densitometry (Table 1). The total amount of annexin I in the membranes plus cytosol, as estimated by densitometry, after 5–15 min was lower than that estimated either in the cytosol of resting cells or in the membranes of OZ-stimulated cells at 1 or 3 min. Annexin I could not be detected in the extracellular medium after neutrophil stimulation (results not shown), indicating that it is not secreted from the neutrophils during activation. The OZ-induced translocation of annexin I from the cytosol to the membranes was dose-dependent in the concentration range 1 μg/ml–1 mg/ml (Figure 2).

In contrast to OZ, PMA did not stimulate the translocation of annexin I (Figure 3). There was no change in the amount of annexin I found in the cytosol at 15 min following PMA stimulation, and it was not detected in the membrane fraction. FMLP induced only a slight translocation of annexin I to the membranes (results not shown). Figure 4 shows the effects of 2 min of treatment with OZ, PMA or FMLP on the translocation of annexin I to membranes. OZ stimulated a massive translocation of annexin I from the cytosol to the membranes; PMA did not induce any translocation and FMLP induced only a slight translocation. A change could not be detected in the amount of annexin I in the cytosol 2 min after stimulation with FMLP, as shown in Figure 4, or at 15 min following stimulation (results not shown), confirming the low degree of translocation induced by this agent.

In order to determine the nature of the membranes which are the target for annexin I translocation, the neutrophils were

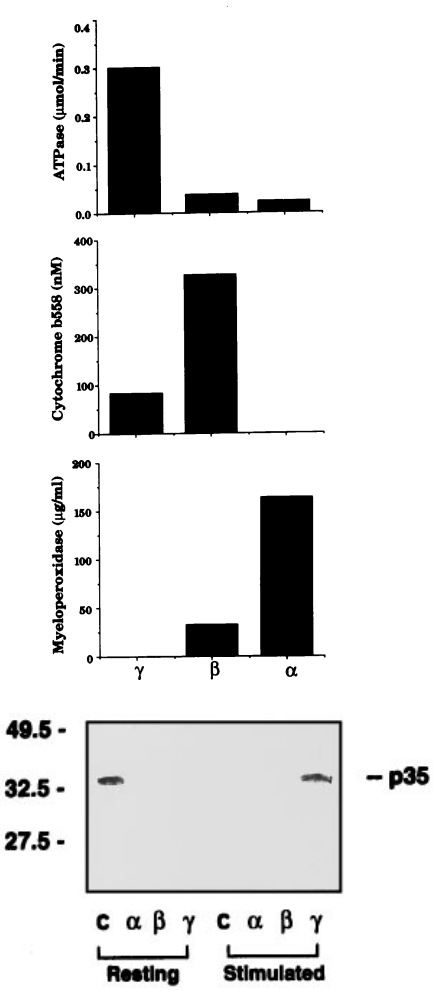

Figure 5 Distribution of annexin I in subcellular fractions in resting neutrophils and after activation with OZ

Top panels: profile of discontinuous density Percoll gradient of the postnuclear supernatant of disrupted resting neutrophils. After centrifugation three bands were visible, denoted α, β and γ in order of decreasing density. Fractions were analysed for myeloperoxidase, cytochrome b_{558} and Na^+/K^+-ATPase as markers of azurophil granules, specific granules and plasma membranes respectively. Bottom panel: SDS/PAGE immunoblot analysis of annexin I in cytosol (C) and α, β and γ fractions of resting cells and after stimulation with OZ for 2 min. Molecular masses (kDa) are indicated on the left; p35 denotes annexin I. The results are representative of five experiments.

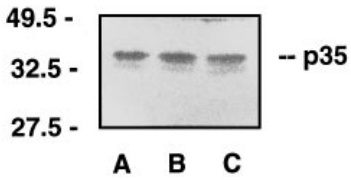

Figure 6 SDS/PAGE immunoblot analysis of annexin I in the phagosome fraction isolated from neutrophils

Samples of 10^8 cell equivalents of phagosomes formed by internalization of OZ (lane A) or latex beads (lane B), and of cytosol from resting neutrophils (lane C), were applied. Molecular masses (kDa) are indicated on the left; p35 denotes annexin I. The results are representative of three experiments.

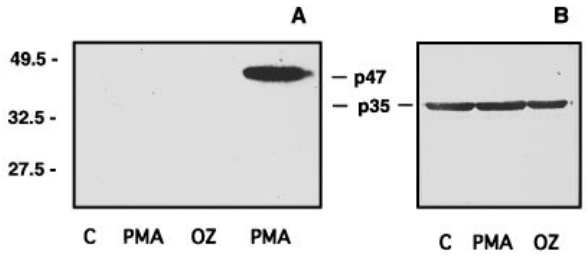

Figure 7 Autoradiogram (A) and immunoblot analysis (B) of annexin I immunoprecipitated from neutrophil lysates

Lysates were prepared from resting neutrophils (lane C) or from cells stimulated with either 50 ng/ml PMA or 1 mg/ml OZ for 2 min. The phosphorylation of the oxidase cytosolic factor p47 immunoprecipitated from lysate of the PMA-stimulated neutrophils is demonstrated (**A**). Molecular masses (kDa) are indicated on the left; p35 denotes annexin I. The results are representative of three experiments.

separated into subcellular fractions after activation with OZ. Figure 5 (top panels) shows a typical separation profile of resting neutrophils on a two-layer Percoll gradient. The α band contains the azurophil granule marker myeloperoxidase, the β band contains the specific granule marker cytochrome b_{558} and the γ band contains the plasma membrane marker Na^+/K^+-ATPase. The cytosol is located on top of the gradient. Immunoblot analysis of the different fractions before and after activation with OZ (Figure 5, bottom panel) demonstrates that annexin I was detected in the cytosol of resting cells and in the plasma membranes of OZ-stimulated neutrophils. Annexin I could not be detected in the azurophil or specific granule fractions in either resting or OZ-stimulated neutrophils. Similarly, the slight translocation induced by FMLP was targeted to the plasma membranes and not to the granule fractions (results not shown).

At 15 min after exposure of neutrophils to OZ, only trace amounts of annexin I could be detected in the plasma membranes or in the cytosol (Figure 1). Since annexin I could not be detected in the extracellular milieu, the possibility was raised that annexin I is found in the phagosome fraction. Neutrophils were exposed to OZ or to latex beads for 15 min at 37 °C and phagosomes were isolated as described in the Materials and methods section. As shown in Figure 6, annexin I was indeed located in the phagosome fraction.

The mechanism by which the translocation of annexin I to the plasma membrane is stimulated by OZ is not known. The involvement of phosphorylation in the regulation of annexin I translocation was studied. As shown in Figure 7(A), stimulation of neutrophils for 2 min with either PMA or OZ did not induce phosphorylation of annexin I. Large amounts of immunoprecipitated annexin I could be detected by Western blot analysis under these experimental conditions (Figure 7B). The phosphorylation of the oxidase cytosolic factor p47 immunoprecipitated from a lysate of neutrophils stimulated with PMA is shown as a positive control (Figure 7A).

Next, we studied whether the differences between the effects of the various stimuli on the translocation of annexin I to the membranes could be attributed to differences in the increases in $[Ca^{2+}]_i$ induced by these agents. Table 2 presents the peak levels of $[Ca^{2+}]_i$ induced by the different agents and the relative translocation of annexin I at 2 min after stimulation. There was a strong correlation between these two parameters. Since OZ caused a greater increase in $[Ca^{2+}]_i$ and induced greater translocation of annexin I, the effect of the Ca^{2+} chelator EGTA on neutrophils stimulated by OZ was analysed. EGTA at two

Table 2 Correlation between $[Ca^{2+}]_i$ and the translocation of annexin I to neutrophil membranes

The determination by densitometry of the relative amounts of annexin I (in arbitrary units) in neutrophil membranes are means ± S.E.M. from four experiments. The levels of $[Ca^{2+}]_i$ are the peak levels induced by the various agents.

	$[Ca^{2+}]_i$ (nM)	Annexin I in neutrophil membranes
Control	65 ± 6	40 ± 8
OZ (1 mg/ml)	330 ± 12	200 ± 12
FMLP (0.1 μM)	127 ± 17	75 ± 8
PMA (50 ng/ml)	67 ± 8	26 ± 8

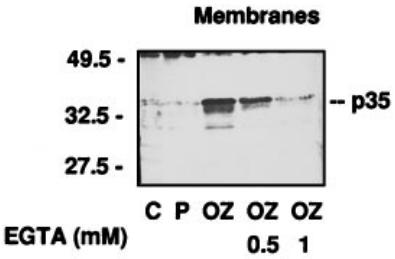

Figure 8 Effect of EGTA on annexin I translocation induced by 1 mg/ml OZ

A representative SDS/PAGE immunoblot analysis is shown. Samples of 5×10^6 cell equivalents of membranes were applied per lane. Lanes: C, resting cells; P, 50 ng/ml PMA; OZ, 1 mg/ml OZ. Molecular masses (kDa) are indicated on the left; p35 denotes annexin I. Shown is a representative experiment; five others gave similar results. In the same experiment the effect of EGTA on $[Ca^{2+}]_i$ was detected. $[Ca^{2+}]_i$ in resting cells was 62 nM; in OZ-stimulated cells it was 360 nM in the absence of EGTA, and values of 178 nM and 95 nM were measured in the presence of 0.5 mM and 1 mM EGTA respectively.

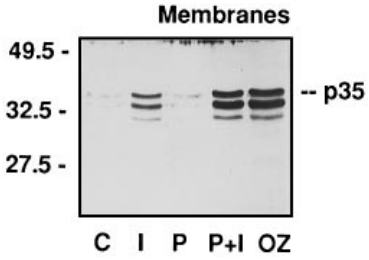

Figure 9 Effect of ionomycin on annexin translocation to neutrophil membranes induced by 50 ng/ml PMA

A representative SDS/PAGE immunoblot analysis is shown. Samples of 2×10^6 cell equivalents of membranes were applied per lane. Lanes: C, resting cells; I, 250 nM ionomycin; P, 50 ng/ml PMA; P + I, PMA + ionomycin; OZ, 1 mg/ml OZ. Molecular masses (kDa) are indicated on the left; p35 denotes annexin I. Shown is a representative experiment; five others gave similar results.

different concentrations prevented the elevation in $[Ca^{2+}]_i$, and also inhibited the OZ-induced translocation of annexin I to the membranes (Figure 8). Likewise, the effect of the calcium ionophore ionomycin on annexin I translocation was studied (Figure 9). Annexin I was barely detected in the membranes of resting or PMA-stimulated cells. Ionomycin (250 nM) by itself, which caused an increase in $[Ca^{2+}]_i$ (from 60 ± 11 nM in resting cells to 380 ± 31 nM), induced significant translocation of annexin

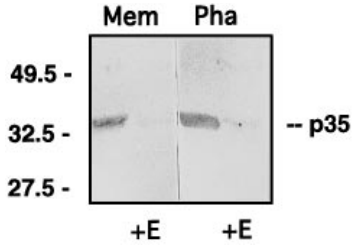

Figure 10 Effect of EGTA on the binding of annexin I to the plasma membrane and phagosome fractions of cells exposed to OZ

A representative SDS/PAGE immunoblot analysis is shown. Samples of 2×10^6 cell equivalents of membranes were applied per lane; phagosomes were isolated from 10^8 cell equivalents. Mem, plasma membranes; Pha, phagosomes, +E, +2mM EGTA. No EGTA was in the samples in the first lane of each pair. Molecular masses (kDa) are indicated on the left; p35 denotes annexin I. Shown is a representative experiment; two others gave similar results.

I to the membranes. When neutrophils were stimulated with PMA in the presence of 250 nM ionomycin, a greater translocation of annexin I to the membranes was observed, similar to that induced by OZ.

In order to study whether binding of annexin I to the plasma membranes and the phagosome fraction is reversible in the absence of Ca^{2+}, membranes and phagosomes of neutrophils exposed to OZ, as described above, were separated and treated with EGTA: 2 mM EGTA was added to the fractions for 5 min, which were then pelleted and analysed. As shown by immunobloting analysis (Figure 10), plasma membranes and phagosomes from neutrophils stimulated with OZ contained annexin I. The translocation of annexin I to either plasma membranes or the phagosome fraction was reversed by addition of the calcium chelator EGTA.

DISCUSSION

The present study demonstrates that OZ specifically induces translocation of annexin I from the cytosol to the plasma membrane in human neutrophils, as detected by immunoblot analysis. This effect was found to be specific for OZ, since FMLP caused only slight translocation of annexin I to the neutrophil plasma membrane and PMA had no effect whatsoever. The massive translocation of annexin I to the plasma membrane only after exposure to OZ (the only agent that induced formation of phagosomes), together with the observation of its localization in the phagosome fraction, suggest that annexin I may be involved in phagosome function. The presence of annexin I in the phagosome fraction (Figure 6) may explain why the sum of the amounts of annexin I in the membranes plus cytosol 10 or 15 min after stimulation with OZ was less than the original amount of annexin I found either in the cytosol of resting cells or in the plasma membranes 1 or 3 min after stimulation (Figure 1; Table 1). The 'missing' annexin I was probably pelleted in the phagosome fraction, together with the unbroken cells and the nuclei, during centrifugation of the sonicated cells. In experiments in which phagosomes were separated, the cells were disrupted by homogenization and a very-low-speed centrifugation was performed. A role for annexins in phagosome formation had been suggested previously for another member of the annexin family, namely annexin III. Annexin III was found in the periphagosomal region upon neutrophil ingestion of OP or opsonized yeast [26,27].

The presence of annexin I in the cytosol of resting neutrophils

shown here is in accordance with other studies on neutrophils [28–30], keratinocytes and the adrenal medulla [31,32]. Annexin II was found in association with the membranes in epithelial MDCK cells [33]. In that study PMA was found not to affect the distribution of annexin II, whereas an alteration in $[Ca^{2+}]_i$ did, similar to the effects of these agents in our study. In contrast, it has recently been reported [26] that annexin III is translocated to the plasma membrane upon stimulation with either PMA or OZ in neutrophils and monocytes. The discrepancy in results may be attributed to the different annexins studied.

The mechanism by which annexin I is translocated to the plasma membrane is not clear. Although annexin I has been described as a major substrate for both protein kinase C [34,35] and the epidermal growth factor receptor protein tyrosine kinase [36] *in vitro*, it does not undergo phosphorylation in stimulated neutrophils (Figure 7). These results suggest that phosphorylation is not involved in regulating the translocation of annexin I to the plasma membrane in neutrophils. In accordance with our results, it was reported [37] that annexin I is not phosphorylated in macrophages stimulated with PMA. In addition, another study [38] has shown that, although annexin I is phosphorylated by purified protein kinase C *in vitro*, it is not phosphorylated in human endothelial cells stimulated by either PMA or A23187.

The correlation between the elevation in $[Ca^{2+}]_i$ and the degree of translocation induced by each of the stimuli (Table 2), together with the dependence of translocation on a rise in $[Ca^{2+}]_i$ (Figures 8 and 9), suggest that translocation may be attributed to an increase in $[Ca^{2+}]_i$. In accordance with our results, a previous study has shown that treatment of HL-60 cells with A23187, which resulted in a rise in $[Ca^{2+}]_i$, caused annexin I to associate with the membrane-enriched particulate fractions [28]. Although the translocation of annexin I to the plasma membrane is dependent on an elevation in $[Ca^{2+}]_i$, an increase in $[Ca^{2+}]_i$ alone (caused by ionomycin) was less efficient in inducing translocation (Figure 8). When PMA was added together with ionomycin, a large degree of translocation was observed, indicating a role for other intracellular processes in the translocation of annexin I to the membranes. It has also been reported [39] that the Ca^{2+} requirement for different members of the annexin family was lowest when the annexins were bound to vesicles composed of phosphatidic acid, as compared with phosphatidylserine or phosphatidylinositol. These results suggest that phosphatidic acid may facilitate annexin I translocation. In addition, another study [38] has shown that annexin V promoted dose-dependent inhibition of annexin I phosphorylation *in vitro*, which could be overcome by increasing the concentration of phosphatidylserine. Since annexin I remained refractory to protein kinase C-dependent phosphorylation in intact endothelial cells, these authors suggested that annexin V might exert its inhibitory effect towards protein kinase C *in vivo*, provided that the binding of annexin I to phospholipids can occur at physiological concentrations of Ca^{2+}. Although calcium ions are not solely responsible for the translocation of annexin I, they are essential for maintaining the binding of annexin I to the plasma membranes or phagosomes, since this binding is reversed by the addition of the calcium chelator EGTA (Figure 10). Similar to our results, it has been shown that the binding *in vitro* of annexin I to plasma membranes or granules from neutrophils is reversed by EGTA [30].

Cell fractionation studies provide evidence that annexin I is not associated with granule fractions of either resting or stimulated neutrophils. Our results are in contrast with studies that demonstrate the translocation of annexins to separated granules *in vitro*. Borregaard et al. [29] have shown that subcellular fractions enriched with azurophil granules, specific granules or plasma membranes bind annexins, including annexin I, with no qualitative differences when incubated with cytosol in the presence of Ca^{2+} at high concentrations. They concluded that annexins are not likely to control the selectivity of neutrophil granule exocytosis by preferential binding to granule subsets. Sjolin et al. [30] have shown that annexin I binds, *in vitro*, to separated specific granules and plasma membranes, but not to azurophil granules, and thus may participate in the degranulation of the specific granules. One of their earlier studies [40], however, has shown, similar to our findings, that annexins are translocated only to plasma membranes and not to subcellular granules *in situ* as a result of a rise in $[Ca^{2+}]_i$. In both *in vitro* studies [29,30], when the $CaCl_2$ concentration was lowered from 0.5 mM to 50 μM, which is also far above the physiological concentration, the binding of annexins to the granules or plasma membranes was prevented. The Ca^{2+} concentrations used in these experiments are far in excess of those found in stimulated cells. Thus the results may not be relevant to the behaviour of intracellular annexin I *in vivo*.

One of the suggested roles for annexin I is the inhibition of PLA_2 activity [6]. The necessity of PLA_2 activity for NADPH oxidase stimulation has been reported by us and others [41–45]. In a previous study [41] we showed that PMA and OZ induce similar amounts of superoxide generation, which are correlated with the activation of PLA_2 induced by the two stimuli. Thus it was expected that annexin I would behave similarly when treated with the various stimuli if indeed it plays a role in the regulation of PLA_2 activity. The differences between the effects of the various inducers on the translocation of annexin I questions the role of annexin I as a regulator of PLA_2 activity in stimulated neutrophils. However, we cannot rule out the possibility that different types of PLA_2 [46] are activated by the various stimuli and that annexin I regulates only one of them.

In conclusion, annexin I is found in the cytosol of resting neutrophils, and is translocated to the plasma membranes only after exposure to OZ. Annexin I is not associated with the granules of either resting or activated neutrophils, but is found in the phagosome fraction. These results suggest that annexin I plays a role in the function of phagosomes in human neutrophils.

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