Calcium-induced translocation of annexins to subcellular organelles of human neutrophils

Carola SJÖLIN,*†, Olle STENDAHL† and Claes DAHLGREN*

*Phagocyte Research Laboratory, Department of Medical Microbiology and Immunology, University of Göteborg, S-413 46 Göteborg, Sweden and †Department of Medical Microbiology, University of Linköping, S-581 85 Linköping, Sweden

The annexins are Ca^{2+} -regulated, phospholipid-binding proteins which have been suggested to take part in cellular events such as exocytosis. The subcellular localization of annexins in human neutrophils was determined using monoclonal antibodies against annexins I, II, IV and VI and a polyclonal peptide antiserum against an annexin consensus sequence. Several annexins were translocated to the light membrane fraction enriched in plasma membranes and secretory vesicles. Annexins were associated also with the azurophil and specific granules. Whereas annexins I, IV and VI and one unidentified 35 kDa protein translocated to each of the isolated organelles, annexin II, a 66 kDa annexin IV-like

protein, and a 38 kDa annexin I-like protein exhibited organelle-related differences in their association with membranes. The 38 kDa annexin associated only with specific granules and the secretory vesicles/plasma membrane but not with azurophil granules. Annexin II and the 66 kDa annexin IV-like protein associated with each of the neutrophil organelles, but the binding to specific granules and secretory vesicles/plasma membrane showed a Ca^{2+} -dependency different from that of azurophil granules. This observation suggests that these proteins may contribute to the secretory process in neutrophils.

INTRODUCTION

The neutrophil granulocyte is of great importance for eliminating invading micro-organisms. The neutrophil contains at least three types of granules (azurophil and specific granules, and secretory vesicles) [1,2], which store a variety of antimicrobial proteins, enzymes and mobilizable cell-surface receptors. Recognition of foreign material as well as the antibacterial activity of neutrophils involves the fusion of membranes, e.g. the formation of phagolysosomes by fusion of the phagocytic vesicles with the granules, and fusion between the plasma membrane and intracellular vesicles during exocytosis.

Exocytosis in neutrophils is not a constitutive process, but occurs in response to external stimuli. Even though cells show a diversity in the regulation of exocytosis, available data suggest that a rise in the intracellular Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$) is one important signal [3]. The mechanism of neutrophil degranulation/secretion is only partially understood. The process is triggered by a variety of substances which, on coupling to extracellularly exposed receptors, generate signals across the plasma membrane that may generate a rise in $[\text{Ca}^{2+}]_i$ [4]. The contents of the three granule populations in the neutrophil are discharged at different $[\text{Ca}^{2+}]_i$ [5].

The fusion step of the secretory process is believed to require molecules that can establish contact between the membranes and reduce repulsive forces [3], thus allowing the formation of a fusion pore. Such molecules have not yet been identified, but proteins with a Ca^{2+} -dependent phospholipid-binding capacity have been suggested as candidates [6]. Proteins exhibiting these characteristics were originally given different names, e.g. calelectrins, calpactins, endonexins, lipocortins and synexins, but are presently designated as members of the annexin family [7]. The function of these molecules is not yet evident but, as they are ubiquitously distributed within the Animal Kingdom [8–11] and

also in plants [12], they are probably involved in fundamental cellular events, such as exocytosis [13].

In an approach to investigate this hypothesis, we have investigated the localization of annexins in resting and activated neutrophils. We found that the annexins are present in the cytosol, and that a selective translocation to the plasma membrane occurs when $[\text{Ca}^{2+}]_i$ is raised [14]. However, because the technique that we used to determine translocation involves a procedure that should cause annexins supposedly associated with the neutrophil granules to become dissociated, in this study we have used a well characterized technique [15] to determine annexin translocation. We found that a rise in Ca^{2+} caused translocation of several annexins from the cytosol to the plasma membrane and also to each of the isolated organelles.

MATERIALS AND METHODS

Isolation of granulocytes

Human polymorphonuclear leucocytes (neutrophil granulocytes) were isolated from fresh, heparinized blood or buffy coats as described by Bøyum [16]. Briefly, erythrocytes were sedimented in dextran and leucocytes were collected from the supernatant by centrifugation and purified from remaining erythrocytes by hypotonic lysis. Mononuclear leucocytes were removed by centrifugation on Ficoll-Hypaque. The purified neutrophils were washed twice in Krebs–Ringer medium (120 mM NaCl, 4.9 mM KCl, 1.2 mM MgSO_4 , 1.7 mM KH_2PO_4 , 8.3 mM Na_2HPO_4 , 10 mM glucose, pH 7.3) and then treated for 10 min with diisopropyl fluorophosphate (DFP; final concentration 5 mM) before being resuspended in cold relaxation buffer (see below).

Disruption of cells and subcellular fractionation

Neutrophils (from 4–5 buffy coats) in relaxation buffer [100 mM

Abbreviations used: $[\text{Ca}^{2+}]_i$, intracellular $[\text{Ca}^{2+}]$; DFP, di-isopropyl fluorophosphate; NBT, NitroBlue Tetrazolium; PMSF, phenylmethanesulphonyl fluoride; PVDF, poly(vinylidene difluoride).

† To whom correspondence should be addressed.

KCl, 3 mM NaCl, 3.5 mM $MgCl_2$, 10 mM Pipes, 1 mM $ATP(Na)_2$, 0.5 mM phenylmethanesulphonyl fluoride (PMSF), pH 7.4] were disrupted in a nitrogen bomb (2500 kPa, 5 min; Parr, Moline, IL, U.S.A.). The homogenate droplets were collected into a volume of 100 mM EGTA (pH 7.4) appropriate for a final concentration of 1.5 mM EGTA. The homogenates were centrifuged at 400 *g* for 15 min (4 °C) and the post-nuclear supernatants were layered onto precooled discontinuous density gradients [17]. Percoll, 15 mM EGTA in 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 heavy 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, denoted α , β and γ in order of decreasing density. They were collected using a Pasteur pipette and assayed for myeloperoxidase [18], vitamin B_{12} -binding protein [19] and alkaline phosphatase [20], which are marker molecules of azurophil granules, specific granules and plasma membrane/secretory vesicles respectively.

To study translocation of annexins, cytosol (S2) was prepared without EGTA, i.e. the cavitated preparation was collected without any Ca^{2+} -chelator and centrifuged for 60 min (100000 *g*, 4 °C) and the pellet was discarded. Subcellular organelles were prepared in the presence of EGTA as described above. The α , β and γ fractions were centrifuged (100000 *g*, 60 min), and the pelleted material was washed without EGTA and resuspended in the cytosol (cytosol made from 2×10^8 cells was mixed with the respective organelle, isolated from at least 1×10^9 cells). In order to allow annexins to associate with the membranes, $CaCl_2$ (final concentration 10, 100 or 1000 μM) was added to the S2/organelle mixtures, which were incubated for 15 min at 37 °C. Controls consisted of S2/organelle mixtures incubated with 2 mM EGTA or without Ca^{2+} chelator. Mixtures were centrifuged (100000 *g*, 60 min), the supernatants were discarded, and the pelleted membranes were washed once and thereafter resuspended in relaxation buffer supplemented with EGTA (2 mM). After incubation for 15 min at 37 °C, organelles were pelleted and the resulting supernatants analysed for content of annexins.

Annexin antibodies

An antiserum against an annexin consensus sequence was kindly provided by Dr. Thomas C. Südhof (Howard Hughes Medical Institute, Dallas, TX, U.S.A.). The antiserum was raised in a rabbit using a peptide containing an annexin consensus sequence (Cys-Met-Lys-Gly-Leu-Gly-Thr-Asp-Glu-Asp-Thr-Leu-Ile). The peptide was synthesized on an ABI peptide synthesizer, and the purity was verified by h.p.l.c. and m.s. The peptide was coupled to keyhole-limpet haemocyanin as described [21]. Of three rabbits injected with the peptide, one (K-304) showed strong immunoreactivity on blots against four bovine annexins, which were originally referred to as calelectrins [22,23].

Monoclonal antibodies against annexins I, II, IV and VI, which reacted strongly with the respective annexin in a lysate of fibroblasts, were purchased from Zymed Labs Inc., San Francisco, CA, U.S.A.

SDS/PAGE and Western blotting

SDS/PAGE was performed essentially according to Laemmli [24]. The samples were electrophoresed reduced in 10% (w/v) homogenous gels, which were silver stained or blotted onto poly(vinylidene difluoride) (PVDF) membranes using a Tris/glycine buffer system [25]. The consensus antibody was used

diluted 1:1000 in 1% (w/v) dried milk, 1% (w/v) BSA, 0.05% (v/v) Tween20 in PBS, and the annexin monoclonal antibodies were used diluted 1:5000 in the same solution. Alkaline phosphatase-conjugated swine anti(rabbit Ig) or goat anti(mouse Ig) was used as the secondary antibody and blots were developed in NitroBlue Tetrazolium (NBT; 0.3 mg/ml) and bromochloroindolyl phosphate (BCIP; 0.15 mg/ml) dissolved in 0.1 M $NaHCO_3$, 1.0 mM $MgCl_2$, pH 9.8.

Chemicals

Percoll and Ficoll-Hypaque were from Pharmacia, Uppsala, Sweden. Keyhole-limpet haemocyanin was purchased from Calbiochem Corp., La Jolla, CA, U.S.A. ATP, 5-bromo-4-chloro-3-indolyl phosphate, EGTA, NBT and PMSF were products from Sigma Chemical Co., IL., U.S.A. The PVDF membrane was from Millipore, Bedford, MA, U.S.A. Swine anti-(rabbit Ig) was from Dako A/S, Glostrup, Denmark and goat anti-(mouse Ig) from Jackson ImmunoResearch Laboratories, West Grove, PA, U.S.A.

RESULTS

Effect of an *in vitro* rise in Ca^{2+} on annexin localization

After fractionation of neutrophils on discontinuous Percoll gradients, four cellular fractions are readily identified: the cytosol (S2), the plasma membrane/secretory vesicle-enriched fraction (γ), specific granules (β) and azurophil granules (α) (Figure 1). In order to study Ca^{2+} -induced translocation of cytosolic proteins to neutrophil organelles, the α , β and γ fractions were isolated and separately suspended in cytosol and the concentration of Ca^{2+} was raised to 100 and 1000 μM respectively. Following removal of the cytosol, the organelles were suspended in an EGTA-containing buffer that allows translocated proteins to be released from the different organelles. The membranes were once again pelleted and the supernatants analysed for content of annexins. To detect translocated annexins, a polyclonal antiserum directed against an annexin consensus sequence was used. Immunoblotting revealed that annexins associated with each of the isolated organelles (Figure 2a). At the highest Ca^{2+} concentration, altogether five annexins were translocated to the γ fraction; the molecular masses of these were 66, 40, 39, 38 and 35 kDa. We assume that these annexins have been associated with either the secretory vesicle or the plasma membrane of the γ fraction, or with both. Annexins were translocated also to azurophil and specific granules (Figure 2a). Common to both types of granules (and to the γ fraction as well) were annexins of 66, 40 and 35 kDa molecular mass. A 38 kDa protein was associated with the specific granule and this annexin was also found in the γ fraction. All annexins recognized by the consensus antiserum were also represented in silver-stained gels (Figure 2b).

Identification of translocated annexins

To identify translocating annexins, the EGTA extracts obtained from the experiment with the highest Ca^{2+} concentration (1000 μM) were examined using commercially available monoclonal antibodies against annexins I, II, IV and VI. A fibroblast lysate served as a positive control for the annexins detected by these antibodies. Annexin I was translocated to all of the organelles (Figure 3a) and was detected as a 40 kDa protein (Figure 3e). The annexin I monoclonal also recognized a 38 kDa protein, which was associated with specific granules and plasma membrane/secretory vesicles but not with azurophil granules. This species was not present in the fibroblast lysate and may

Figure 1 Subcellular fractionation of neutrophils

A postnuclear supernatant of disrupted neutrophils was fractionated on a discontinuous Percoll density gradient. After centrifugation, three bands were visible (d), which were denoted α , β , and γ in order of decreasing density. Cytosol components were located in the upper portion of the gradient (S2). Fractions were analysed for (a) myeloperoxidase (a marker of azurophil granules), (b) vitamin B₁₂-binding protein (a marker of specific granules) and (c) alkaline phosphatase (present in the plasma membrane and secretory vesicles), which was assayed in the absence of Triton X-100 to measure the fraction of enzyme localized to the plasma membrane, and in the presence of Triton X-100 to measure the total of secretory vesicles plus plasma membrane.

therefore represent an annexin I-like protein unique to neutrophils. Annexins II, IV and VI, as identified in the fibroblast lysate, were all translocated to each of the isolated organelles (Figure 3b–3d); they were detected as proteins of molecular mass 39, 35 and 66 kDa respectively (Figure 3e). The monoclonal antibody against annexin IV recognized an additional protein of 66 kDa (Figure 3c), which was not found in the fibroblast lysate and which therefore cannot be identical to annexin VI.

Using the monoclonal antibodies, all annexins detected by the consensus antibody were identified (Figure 3e). However, the 35 kDa annexin of the consensus antibody was not represented in the fibroblast lysate and is therefore probably not identical to annexin IV (Figure 3c). This is also indicated by the observation that annexin IV was translocated to all granules in equal amounts

Figure 2 Annexin association to membranes of organelles in the presence of Ca²⁺

Isolated azurophil granules (α), specific granules (β) and plasma membrane/secretory vesicles (γ) were mixed respectively with cytosol in the presence of 2 mM EGTA (E) or CaCl₂ at 100 μ M (4) or 1000 μ M (3). Membranes were collected and washed, then resuspended in 2 mM EGTA to elute Ca²⁺-dependent proteins from the membranes, which were pelleted. The supernatants were assayed under reducing conditions in 10% (w/v) polyacrylamide gels, which were immunoblotted using the annexin consensus antiserum (a) or silver-stained (b).

but the 35 kDa annexin was translocated to all granules but in unequal amounts (Figures 3c and 3e). We therefore suggest that the consensus antibody reacts with a 35 kDa annexin that is not identical with annexin IV but is possibly identical with annexin III and/or annexin V.

The suggested identities of the translocating annexins are summarized in Table 1.

Ca²⁺ dependencies of annexin-binding to the organelles

To detect possible differences between the neutrophil organelles in the requirement of Ca²⁺ for translocation of specific annexins, binding was studied at four different Ca²⁺ concentrations (1, 10, 100 and 1000 μ M). When neither Ca²⁺ nor EGTA was added to the medium, we estimate the concentration of Ca²⁺ to be ~ 1 μ M, originating from trace contaminations of the chemicals included in the buffer. Organelles and cytosol were mixed and incubated at 37 °C. After removal of the cytosol, the translocated proteins were released from the membranes by EGTA and the samples were analysed using the annexin monoclonals. Translocation of any annexin did not occur at concentrations lower than 100 μ M Ca²⁺, as defined by comparison with the control (Figure 4). No differences between the annexin-binding capacities of the different organelles could be demonstrated for the translocation of annexins I (40 kDa), IV and VI (Figures 4a, 4c and 4d). The Ca²⁺ requirement for translocation of each of these annexins differed however. Translocation of annexin I occurred in the presence of 100 μ M Ca²⁺ and was increased by 1000 μ M whereas annexin VI reached maximal binding at the lower Ca²⁺ concentration (Figure 4a and 4d). Binding of annexin IV to organelles required 1000 μ M Ca²⁺ (Figure 4c).

Translocation of annexin II and the annexin IV-like 66 kDa protein exhibited Ca²⁺-dependent differences in the annexin-binding capacity of the organelles (Figures 4b and 4c). Whereas

Figure 3 Identification of annexins associated with membranes of neutrophil organelles

Isolated azurophilic granules (α), specific granules (β) and plasma membrane/secretory vesicles (γ) were mixed respectively with cytosol in the presence of 1000 μM CaCl_2 or 2 mM EGTA. Membranes were collected and washed, then resuspended in 2 mM EGTA to elute Ca^{2+} -dependent proteins from the membranes, which were pelleted. The supernatants were assayed under reducing conditions in 10% (w/v) polyacrylamide gels, which were immunoblotted with monoclonal antibodies against annexin I (a), annexin II (b), annexin IV (c), annexin VI (d) or with the annexin consensus antibody (e). A lysate of fibroblasts (f) was used as a positive control for the monoclonal antibodies. The molecular masses (kDa) of the bands visualized are shown.

Table 1 Suggested identities of translocated annexins

This table summarizes the translocated proteins detected by the commercial monoclonal antibodies (A) against annexins I, II, IV and VI and/or the consensus antibody (CS). The experimental procedure is described in Figure 3.

Molecular mass of the translocating annexin (kDa)	Specificity of the detecting antibody	Suggested identity
66	CS, A VI	Annexin VI
66	A IV	?
40	CS, A I	Annexin I
39	CS, A II	Annexin II
38	CS, A I	?
35	A IV	Annexin IV
35	CS	Annexin III/IV

Figure 4 Ca^{2+} dependence of the association of annexins to neutrophil organelles

Isolated azurophilic granules (α), specific granules (β) and plasma membrane/secretory vesicles (γ) were mixed respectively with cytosol in the presence of: 2 mM EGTA (E); 10, 100 or 1000 μM CaCl_2 (5, 4 and 3 respectively); or no additive (6). Membranes were collected and washed, then resuspended in 2 mM EGTA to elute Ca^{2+} -dependent proteins from the membranes, which were pelleted. The supernatants were assayed under reducing conditions in 10% (w/v) polyacrylamide gels, which were immunoblotted using the monoclonal antibodies against annexin I (a), annexin II (b), annexin IV (c) and annexin VI (d).

Table 2 Summarized presentation of translocating annexins determined by monoclonal antibodies

Monoclonal antibodies against annexins (A) I, II, IV and VI were used to determine the presence of annexins in EGTA extracts of Ca^{2+} -dependent organelle-associated neutrophil proteins. The experimental procedure is the same as in Figure 4. The symbol + indicates the presence of an annexin in EGTA extracts or fibroblasts (f).

Monoclonal antibody	Molecular mass of translocating annexin (kDa)	Annexin present				Granule-related difference observed
		f	α	β	γ	
A I	40	+	+	+	+	
A I	38			+	+	Yes*
A II	39	+	(+)	+	+	Yes†
A IV	66			(+)	+	Yes‡
A IV	35	+	+	+	+	
A VI	66	+	+	+	+	

* Binding is organelle specific.
† Maximal binding to β and γ reached at 100 μM Ca^{2+} ; binding to α induced by 1000 μM Ca^{2+} .
‡ Maximal binding to β and γ reached at 100 μM Ca^{2+} ; binding to α induced at 100 μM Ca^{2+} and increased by 1000 μM Ca^{2+} .

translocation to specific granules and secretory vesicles/plasma membrane of these annexins was maximal at 100 μM Ca^{2+} , binding to azurophil granules required or was increased by the 1000 μM concentration. The 38 kDa annexin I-like protein, which was selectively associated with specific granules and secretory vesicles/plasma membrane, did not exhibit differences between organelles in the requirement of Ca^{2+} (Figure 4a). The translocating properties of the different annexins are summarized in Table 2.

DISCUSSION

Previous work using different routes of cell activation has shown that secretion of each of the neutrophil granules is regulated independently. Because the neutrophil granules are discharged at different concentrations of intracellular Ca^{2+} [5], we aimed to investigate whether the involvement of annexins, which are Ca^{2+} -dependent and phospholipid-binding proteins, might explain the divergent regulation of secretion. The capacity of the neutrophil granules to bind annexins was therefore studied in order to detect granule-related differences. We used a well characterized assay system, which allowed soluble proteins from disintegrated cells to interact with membrane preparations in a buffer containing high amounts of Ca^{2+} , and allowed elution of the translocated proteins from the membranes with a Ca^{2+} -depleted medium. By using this technique, we found that annexins could bind to the plasma membrane/secretory vesicles as well as to the subcellular granules. We have shown in an earlier study that translocation of annexins can occur also *in situ*, as a result of a rise in $[\text{Ca}^{2+}]_i$ [14]. In those studies, annexins were only translocated to the plasma membrane and not to subcellular granules. The lack of translocation to granules was, however, interpreted as an artefact, as the granules were isolated in the presence of EGTA. Annexins supposedly associated with granules should then have become dissociated. On the other hand, the observed translocation to the plasma membrane could be explained if the plasma membrane upon disintegration reseals with an outside-out orientation of the membrane, so that molecules associated with the inner side of the membrane escape the action of EGTA. The present results, however, clearly show that annexins bind also to subcellular granules.

The use of the consensus antiserum revealed several annexins that were translocated to each of the neutrophil granules, but indicated also the existence of granule-related differences in the translocation of annexins. The four monoclonal antibodies against annexins I, II, IV and VI identified altogether six different annexins, the molecular masses of which corresponded to those detected by the consensus antiserum. Annexins I, II, IV and VI were detected as proteins of 40, 39, 35 and 66 kDa respectively. In addition, the annexin I monoclonal antibody recognized a 38 kDa protein and the annexin IV monoclonal a 66 kDa protein; neither of these were present in fibroblasts and they may thus represent neutrophil-specific annexins. The combination of the results of experiments with the consensus antibody and those with the annexin monoclonal antibodies revealed the existence of a seventh annexin, with a molecular mass of 35 kDa.

The results obtained with the consensus antiserum indicated granule-related differences in the translocation properties of the annexins. Some of the differences could be confirmed using the monospecific annexin antibodies. One of the differences related to the specificity of the binding to a membrane. The annexin I-like protein (38 kDa) was the only annexin able to discriminate between different neutrophil membranes as it did not associate with the membrane of azurophil granules. No difference in the binding of this annexin to specific granules and secretory

vesicles/plasma membrane could, however, be detected. A second type of granule-related difference refers to the Ca^{2+} -dependency of the association of annexins with the organelles. Annexin II and the annexin IV-related 66 kDa protein were maximally translocated to specific granules and secretory vesicles/plasma membrane at a 100 μM Ca^{2+} concentration, whereas association of these annexins to azurophil granules required the higher Ca^{2+} concentration, namely 1000 μM . No differences were detected regarding the membrane association of annexins I, IV and VI and the 35 kDa annexin detected by the consensus antibody. Differences might nevertheless emerge if experiments were performed to estimate the EC_{50} values of the Ca^{2+} -dependent association of specific annexins to the different types of organelles. Such estimations would however require that the free Ca^{2+} concentration be carefully controlled over a relatively narrow range of concentrations and that the method for measurement of translocation be quantitative, and not semi-quantitative as is immunoblotting.

Even if a fraction of the cellular content of the Golgi, the endoplasmic reticulum and the mitochondria copurifies with the γ fraction in Percoll gradients [26], these light membranes are scarce in the neutrophil [27], indicating that the major constituents of the γ fraction are secretory vesicles and plasma membrane vesicles.

Because the substrate for membrane-localized alkaline phosphatase also has full access to the enzyme (no latency) after disintegration of the cells [2], the plasma membrane fragments must either remain open or be resealed with an outside-out orientation. As annexins preferentially bind to the cytoplasmic face of membranes [28] and have been shown to associate both with the plasma membrane and the secretory vesicles [29], we assume that the annexins associated with the γ fraction were translocated both to the secretory vesicles and to non-closed plasma membrane fragments.

Annexins may participate in exocytosis as promoters of membrane fusion [6]. A function as initiators of fusion pores in neutrophil secretion is consistent with the fact that annexins translocate to the plasma membrane as well as to the granules. Azurophil granules and specific granules show different degranulation dynamics primarily because of the different Ca^{2+} requirements for their exocytosis; specific granules being more sensitive to a rise in intracellular Ca^{2+} and consequently are released before the azurophil granules [5]. The fact that the annexin I-like 38 kDa protein specifically recognized two granule populations but not a third, and that annexin II and the annexin IV-like 66 kDa protein translocated to different granules depending on the Ca^{2+} concentration, suggests that these proteins may contribute to the regulation of the secretory process in neutrophils.

This work was supported by grants from the Swedish Medical Research Council, the King Gustaf V 80-Year Foundation and the Swedish Society Against Rheumatism. C. S. is the recipient of a Ph.D. grant from the Medical Faculty, University of Göteborg.

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Received 23 September 1993/30 December 1993; accepted 12 January 1994