The actin cytoskeleton regulates exocytosis of all neutrophil granule subsets

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Jog NR, Rane MJ, Lominadze G, Luerman GC, Ward RA, McLeish KR. The actin cytoskeleton regulates exocytosis of all neutrophil granule subsets. Am J Physiol Cell Physiol 292: C1690–C1700, 2007. First published January 3, 2007; doi:10.1152/ajpcell.00384.2006.—A comprehensive analysis of the role of the actin cytoskeleton in exocytosis of the four different neutrophil granule subsets had not been performed previously. Immunoblot analysis showed that, compared with plasma membrane, there was less actin associated with secretory vesicles (SV, 75%), gelatinase granules (GG, 40%), specific granules (SG, 10%), and azurophil granules (AG, 5%). Exocytosis of SV, SG, and AG was measured as increased plasma membrane expression of CD35, CD66b, and CD63, respectively, with flow cytometry, and GG exocytosis was measured as gelatinase release with an ELISA. N-formylmethionyl-leucyl-phenylalanine (FMLP) stimulated exocytosis of SV, GG, and SG with an EC50 of 15, 31, and 28 nM, respectively, with maximal response at 10−7 M FMLP by 5 min, while no exocytosis of AG was detected. Disruption of the actin cytoskeleton by latrunculin A and cytochalasin D induced a decrease in FMLP-stimulated CD35 expression after an initial increase. Both drugs enhanced the rate and extent of FMLP-stimulated GG, SG, and AG exocytosis, while the EC50 for FMLP was not altered. We conclude that the actin cytoskeleton controls access of neutrophil granules to the plasma membrane, thereby limiting the rate and extent of exocytosis of all granule subsets. Differential association of actin with the four granule subsets was not associated with graded exocytosis.

human; cell activation

NEUTROPHIL PARTICIPATION in inflammatory responses requires stimulus-coupled exocytosis of intracellular granules. Human neutrophils contain four major subsets of granules: secretory vesicles, gelatinase or tertiary granules, specific or secondary granules, and azurophil or primary granules. Secretory vesicles are created by endocytosis of plasma membrane, while gelatinase, specific, and azurophil granules are formed from the trans-Golgi network during neutrophil maturation. A hierarchical mobilization of these granule subsets, termed graded exocytosis, was demonstrated in vitro and in vivo, with secretory vesicles most easily mobilized, followed by gelatinase, then specific, and finally azurophil granules (3, 25, 37, 38). Exocytosis of these granule subsets results in release of luminal proteins extracellularly or into phagosomes and incorporation of granule membrane proteins into target membranes. Identification of luminal and membrane components of the different granule subsets suggested that graded exocytosis produced a stepwise transformation of neutrophils from passive circulating cells into cells capable of adhesion to vascular endothelium, diapedesis across vascular basement membranes, chemotaxis to a site of infection, and finally, enhanced phagocytosis and bactericidal activity. The molecular basis for the hierarchical exocytosis of neutrophil granule subsets is poorly defined.

Previous studies identified actin to be both a negative and a positive regulator of exocytosis. The cortical actin network was postulated to act as a physical barrier, preventing granule access to the plasma membrane (7). Evidence in favor of this hypothesis included demonstration that active rearrangement of cortical actin accompanied exocytosis (24, 32) and that pharmacological disruption of F-actin led to enhanced basal and stimulus-coupled exocytosis (14, 29, 36, 39, 42, 48). A second inhibitory role of the actin cytoskeleton could result from the binding of granules to F-actin, thereby preventing translocation of granules to their target membranes (30). A third mechanism of inhibition of exocytosis could be produced by downregulation of signal transduction pathways that lead to exocytosis (15, 42). Alternatively, actin may facilitate exocytosis, as shown by reduced exocytosis during pharmacological inhibition of actin reorganization in some cell types (14, 27, 29, 34). Actin reorganization may facilitate exocytosis by mediating granule translocation to a target membrane, facilitating transit through the cortical actin network, or providing energy for membrane fusion between granules and target membranes (10, 12, 17, 32).

A proteomic analysis of granule proteins recently performed by our laboratory (26) identified differential association of actin with granule subsets. These findings suggested that the actin cytoskeleton might differentially regulate neutrophil granule exocytosis, as has been recently reported in platelets (14). A comprehensive analysis of the role of the actin cytoskeleton in exocytosis of the different neutrophil granule subsets, however, has not been performed previously. To define the role of actin reorganization in neutrophil granule exocytosis and to determine the contribution of actin to graded exocytosis, we examined the effect of pharmacological disruption of the actin cytoskeleton on exocytosis of each granule subset in human neutrophils.

METHODS

Neutrophil isolation. Neutrophils were isolated from healthy human donors with plasma-Percoll gradients, as previously described (18). After isolation, neutrophils were washed and resuspended in LPS-free Krebs-Ringer phosphate buffer (pH 7.2) containing 0.2% dextrose (Krebs). Microscopic evaluation of isolated cells showed that >97% of cells were neutrophils. Trypan blue exclusion indicated that >97% cells were viable. The Human Studies Committee of the University of Louisville approved the use of human donors.

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Determination of F-actin content in neutrophils. Neutrophils were resuspended in Krebs supplemented with 0.54 mM Ca\(^{2+}\) and 1.2 mM Mg\(^{2+}\) (Krebs +) and incubated for 30 min at 37°C with specified concentrations of f-met-leu-phe (Sigma, St. Louis, MO). Cells were then incubated with or without 100 nM N-formylmethionyl-leucyl-phenylalanine (fMLP) for 45 s at 37°C, pelleted, and fixed and permeabilized with 3.7% paraformaldehyde and 2% saponin, respectively. Cells were stained for F-actin with fluorescein-phalloidin (Molecular Probes). The fluorescence intensity was measured by flow cytometry (Coulter Epics XL flow cytometer, Miami, FL).

Exocytosis. Exocytosis of secretory vesicles, specific granules, and azurophilic granules was determined by measuring plasma membrane expression of CD35, CD66b, and CD63, respectively, with flow cytometry as previously described (47). After incubation of neutrophils for the indicated times, exocytosis was terminated by addition of an ice-cold solution containing antibody and immediately placing cells on ice. For determination of CD35 and CD66b, neutrophils were incubated at 4°C for 30 min with FITC-conjugated monoclonal anti-CD35 (Pharmingen, San Diego, CA) or FITC-conjugated monoclonal anti-CD66b (Accurate Chemical, Westbury, NY). FITC-conjugated mouse IgG1 (Pharmingen) was used as an isotype control. For determination of CD63, cells were incubated with human IgG before addition of FITC-conjugated monoclonal anti-CD63. Cells were washed with 0.1% sodium azide, resuspended in 1% paraformaldehyde, and analyzed for fluorescence intensity by flow cytometry. Exocytosis of gelatinase granules was determined by measuring gelatinase release with a commercially available ELISA for matrix metalloproteinase 9 (R&D Systems, Minneapolis, MN) according to manufacturer’s instructions.

Granule fractionation. Neutrophil gelatinase, specific, and azurophilic granules were enriched by centrifugation on a three-layer Percoll density gradient as described by Kjeldsen et al. (22). Isolated neutrophils were resuspended in Krebs at 4 × 10\(^7\) cells/ml and incubated for 10 min on ice in 5 mM disopropyl fluorophosphate (Aldrich, Milwaukee, WI). After centrifugation, cells were resuspended at 4 × 10\(^7\) cells/ml in disruption buffer (mM: 100 KCl, 1 NaCl, 1 ATP-Na\(_2\), 3.5 MgCl\(_2\), 10 PIPES, and 0.5 phenylmethylsulfonyl fluoride (PMSF)), and were lysed by nitrogen cavitation at 380 psig and 4°C. The cavitate was collected dropwise into EGTA (final concentration 1.5 mM). Nuclei and unbroken cells were pelleted by centrifugation at 4°C at 400 g for 15 min. The postnuclear supernatant was layered onto a discontinuous Percoll gradient formed from three 9-ml layers of Percoll prepared in a buffer containing (mM) 100 KCl, 9-ml layers of Percoll prepared in a buffer containing (mM) 100 KCl, 3 NaCl, 1 ATP-Na\(_2\), 3.5 MgCl\(_2\), 1.25 EGTA, 10 PIPES, and 0.5 PMSF, to achieve a final density of 1.050, 1.090, and 1.120 g/ml. The gradient was centrifuged at 37,000 g for 30 min in an SS-34 fixed-angle rotor in a Sorvall RC-5B centrifuge. The separated granule fractions were recovered from the gradient interfaces by aspiration, and Percoll was removed by ultracentrifugation of each granule subset at 100,000g for 90 min. The relative purity of granule fractions was analyzed by immunoblotting for CD66b (a marker of specific granules), lactoferrin (a putative marker for specific granules), and gelatinase and by an ELISA for matrix metalloproteinase 9 (R&D Systems, Minneapolis, MN) according to manufacturer’s instructions. Neutrophil gelatinase, specific, and azurophilic granule fractions separated by centrifugation on Percoll gradients were analyzed for the content of CD66b (specific granule marker), gelatinase (gelatinase granule marker), and lactoferrin (putative marker for specific granules) by Western blotting and analyzed for myeloperoxidase (azurophil granule marker) by ELISA. Results of the ELISA are presented as % of total myeloperoxidase activity in each fraction (bottom), B: results of the latent alkaline phosphatase assay to identify plasma membrane and secretory vesicle membrane fractions. Latent alkaline phosphatase activity for 12 fractions obtained by centrifugation of the membrane fraction on a 2-layer Percoll density gradient, as described in METHODS, is shown. Fractions combined for plasma membrane and for secretory vesicle membrane are indicated.

![Fig. 1. Assessment of purity of granule fractions. A: results of analysis of relative purity of granule fractions. Plasma membrane and gelatinase, specific, and azurophilic granule fractions separated by centrifugation on Percoll gradients were analyzed for the content of CD66b (specific granule marker), gelatinase (gelatinase granule marker), and lactoferrin (putative marker for specific granules) by Western blotting and analyzed for myeloperoxidase (azurophil granule marker) by ELISA. Results of the ELISA are presented as % of total myeloperoxidase activity in each fraction (bottom). B: results of the latent alkaline phosphatase assay to identify plasma membrane and secretory vesicle membrane fractions. Latent alkaline phosphatase activity for 12 fractions obtained by centrifugation of the membrane fraction on a 2-layer Percoll density gradient, as described in METHODS, is shown. Fractions combined for plasma membrane and for secretory vesicle membrane are indicated.](http://ajpcell.physiology.org/)

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**ACTIN AND NEUTROPHIL EXOCYTOSIS**

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**Determinates of F-actin content in neutrophils.** Neutrophils were resuspended in Krebs supplemented with 0.54 mM Ca\(^{2+}\) and 1.2 mM Mg\(^{2+}\) (Krebs +) and incubated for 30 min at 37°C with specified concentrations of f-met-leu-phe (Sigma, St. Louis, MO). Cells were then incubated with or without 100 nM N-formylmethionyl-leucyl-phenylalanine (fMLP) for 45 s at 37°C, pelleted, and fixed and permeabilized with 3.7% paraformaldehyde and 2% saponin, respectively. Cells were stained for F-actin with fluorescein-phalloidin (Molecular Probes). The fluorescence intensity was measured by flow cytometry (Coulter Epics XL flow cytometer, Miami, FL).

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provide the secretory vesicle fraction, and fractions with minimal latent alkaline phosphatase activity were pooled to provide the plasma membrane fraction (Fig. 1B).

Western blot analysis. Whole granule fractions were resuspended in 2 ml of disruption buffer and centrifuged at 100,000 g for 10 min to obtain a solid pellet. Buffer was removed by aspiration, and the pellets were resuspended in 150 μl of disruption buffer. Sample volume was brought up to 1 ml by water and preincubated at 37°C. To quantitate phospholipid bilayer in the different fractions, granules and plasma membrane were washed in disruption buffer containing (mM) 10 10 PIPES, 10 ATP-γ-S, 100 KCl, 3 NaCl, 3.5 MgCl2, and 5 EGTA with 100 μg/ml 4-(2-aminoethyl)benzenesulfonyl fluoride hydrochloride (AEBSF), 5 μg/ml leupeptin, and 10 μg/ml aprotinin. After centrifugation at 50,000 g for 20 min at 4°C, samples were resuspended in disruption buffer containing 100 nM N,N,N-trimethyl-4-(6-phenyl-1,3,5-hexatrien-1-yl)phenylammonium p-toluenesulfonate (TMA-DPH; Molecular Probes) at 37°C. Fluorescence intensity of the samples was measured at excitation of 350 nm and emission of 430 nm after 20 s with an Hitachi 4500 fluorescence spectrometer set to zero on a 100 nM solution of TMA-DPH dye in disruption buffer. Fluorescence reading was multiplied by sample dilution to obtain total lipid per sample. All samples from one experiment were normalized to each other by assigning one sample a standard volume of 1.0 and dividing the fluorescence values for the other samples into the chosen sample’s total fluorescence to obtain a volume ratio. Proteins associated with equal amounts of phospholipid bilayer from each granule subset were subjected to 10% SDS-PAGE, transferred to nitrocellulose membrane, and probed with an anti-actin antibody (Chemicon, Temecula, CA).

To immunoblot for phosphorylated and total p38 MAPK and ERK, neutrophils were lysed in buffer containing 1% (vol/vol) Nonidet P-40, 10% (vol/vol) glycerol, 137 mM NaCl, 20 mM Tris-HCl, pH 7.4, 1 μg/ml aprotinin, 1 μg/ml leupeptin, 5 mM PMSF, 20 mM NaF, 1 mM sodium pyrophosphate, 1 mM sodium orthovanadate, and 1% (vol/vol) Triton X-100. Polyclonal antibodies to total and phosphorylated kinase (Cell Signaling Technology, Beverly, MA) were used at a dilution of 1:1,000 in 5% bovine serum albumin or 5% milk Tween 20-Tris-buffered saline to quantify total and activated kinase.

Results. Neutrophil granule subsets are associated with differential amounts of actin. To determine whether quantitative differences in the amount of actin associated with neutrophil granules might contribute to graded exocytosis, the relative amounts of actin associated with neutrophil plasma membrane and with the four granule subsets were determined by immunoblot analysis. To allow this comparison, proteins from equivalent amounts of membrane from each granule subset were loaded onto gels. Figure 2A shows that the amount of associated actin decreased significantly from plasma membrane to secretory vesicle (75%) to gelatinase granules (40%) to specific granules (10%) to azurophil granules (5%). Figure 2B shows a representative immunoblot from which these percentages were calculated. The relative amount of actin associated with each granule subset paralleled the extent to which that subset was reported to undergo exocytosis (3, 37). For example, secretory vesicles had the greatest amount of associated actin and un-
dergo exocytosis most easily, while azurophil granules had the least amount of associated actin and are the most resistant to exocytosis (3).

The differential association of actin with neutrophil granule subsets suggested that actin may differentially regulate the exocytosis of each granule subset, as recently reported for platelet granules (14). To examine the role of the actin cytoskeleton in neutrophil granule exocytosis, the impact of disrupting actin polymerization on FMLP-stimulated exocytosis was examined. Actin polymerization was inhibited by cytochalasin D, which caps the barbed ends of F-actin, and by latrunculin A, which sequesters G-actin (8, 9, 40, 44). Figure 3 shows the concentration-dependent effect of cytochalasin D and latrunculin A on basal and FMLP-stimulated cellular F-actin, as determined by phalloidin binding. Stimulation of neutrophils with $1 \times 10^{-7}$ M FMLP in the absence of actin disruption significantly increased total cellular F-actin. Figure 3A shows that pretreatment with cytochalasin D resulted in a dose-dependent increase in F-actin ($P < 0.001$). The ability of FMLP to stimulate an increase in F-actin content was inhibited in a dose-dependent manner, with complete inhibition at $1 \times 10^{-5}$ M and $3 \times 10^{-5}$ M cytochalasin D. Pretreatment with latrunculin A significantly reduced the basal level of F-actin ($E_{50} = 4.0 \pm 1.1 \times 10^{-7}$ M; $P < 0.001$), and the ability of FMLP to stimulate actin polymerization was completely inhibited at concentrations of $1 \times 10^{-6}$ M or higher (Fig. 3B). Thus, although both latrunculin A and cytochalasin D block FMLP-stimulated actin polymerization, they produce different effects on basal neutrophil F-actin levels.

The localization of F-actin and the different granule subsets before and after stimulation with FMLP in intact cells, and the effect of disruption of the actin cytoskeleton with latrunculin A on this localization, were examined by confocal microscopy. Figure 4 shows merged images of neutrophils stained with rhodamine-labeled phalloidin and FITC-labeled antibodies to CD35, CD66b, and CD63 and images of unstimulated cells stained only with antibodies to granule markers. In unstimulated cells the secretory vesicle marker CD35 demonstrated a granular cytoplasmic distribution that colocalized with cortical F-actin (Fig. 4, A and B). Stimulation with FMLP for 3 min resulted in enhanced F-actin formation in a polarized distribution, and CD35 redistributed to the area of enhanced F-actin formation (Fig. 4C). Pretreatment with latrunculin A resulted in a loss of cortical F-actin in unstimulated cells (Fig. 4E), while stimulation with FMLP induced some F-actin formation or redistribution in the cytoplasm that was not polarized (Fig. 4F). CD35 staining demonstrated reduced granularity, but remained distributed throughout the cytosol, in unstimulated, latrunculin A-pretreated cells (Fig. 4D) and redistributed to the center of the cell with F-actin after FMLP stimulation (Fig. 4F). The cytosolic distribution of the specific granule marker CD66b was less obviously granular in unstimulated cells than seen for CD35 (Fig. 4, G and H). Colocalization with F-actin was seen, but this colocalization was substantially less than that seen with CD35. Similar to the results with CD35, FMLP stimulation resulted in redistribution of CD66b to sites of polarized F-actin formation (Fig. 4I). Pretreatment with latrunculin A did not alter the distribution of CD66b in unstimulated cells (Fig. 4J), while FMLP stimulation resulted in colocalization with a cytosolic accumulation of F-actin (Fig. 4L). On the other hand, CD63 was present in a cytosolic granular distribution with no colocalization with F-actin in unstimulated or FMLP-stimulated neutrophils (Fig. 4, M–O). After latrunculin A pretreatment CD63 and F-actin remained in distinct locations in both unstimulated (Fig. 4, P and Q) and FMLP-stimulated (Fig. 4R) neutrophils.

**Disruption of actin polymerization regulates exocytosis in a granule-specific manner.** To determine whether disruption of the actin cytoskeleton by latrunculin A or cytochalasin D impacts the ability of FMLP to stimulate exocytosis, neutrophils were pretreated with each of these actin-modifying drugs before addition of FMLP. In the absence of actin-modifying drugs, FMLP significantly increased expression of CD35, a marker of secretory vesicles, as shown in Figs. 5A and 6A. Figure 5A shows that cytochalasin D alone caused a dose-dependent increase in CD35 expression ($P = 0.001$), with a maximal effect at $1 \times 10^{-5}$ M. Pretreatment with cytochalasin D up to concentrations of $3 \times 10^{-6}$ M did not alter the ability...
of FMLP to increase surface expression of CD35. At concentrations greater than $3 \times 10^{-6}$ M, however, cytochalasin D pretreatment blocked the FMLP-stimulated increase in CD35 expression ($P < 0.001$). These same concentrations of cytochalasin D inhibited FMLP-stimulated actin polymerization (Fig. 3A). Pretreatment with latrunculin A alone also increased CD35 surface expression, although this increase did not reach statistical significance in this group of experiments ($P = 0.078$) (Fig. 6A). In contrast to cytochalasin D, pretreatment with latrunculin A at concentrations of $3 \times 10^{-7}$ M and above resulted in a dose-dependent decrease in CD35 expression after FMLP stimulation ($P < 0.001$). These same concentrations of latrunculin A reduced total cellular F-actin and inhibited FMLP-stimulated actin polymerization (Fig. 3B).

Figures 5B and 6B show that stimulation of neutrophils with FMLP in the absence of actin-modifying drugs resulted in a significant release of gelatinase ($P = 0.004$). While pretreatment with cytochalasin D was associated with a small, non-significant increase in gelatinase release, FMLP-stimulated gelatinase release was significantly enhanced by cytochalasin D in a dose-dependent manner (Fig. 5B). Figure 6B shows that pretreatment with latrunculin A alone induced a small, dose-dependent increase in gelatinase release, which was significant at $10^{-6}$ M, and significantly enhanced FMLP-stimulated gelatinase release ($P = 0.01$). The cytochalasin D and latrunculin A dose-response curves for FMLP-stimulated gelatinase release paralleled those for inhibition of FMLP-stimulated F-actin formation.

CD66b expression and release of lactoferrin have been proposed as markers of specific granule exocytosis (1, 4, 11, 13, 31, 37, 41). The use of CD66b expression to measure exocytosis of these granules, however, is controversial. We recently reported (26) that immunoblot analysis found CD66b in specific granule fractions, but it was not detected in either gelatinase or azurophil granules. Additionally, our proteomic analysis of isolated human neutrophil granule subsets identified lactoferrin in gelatinase, specific, and azurophil granules (26). Thus we used increased expression of CD66b to quantify exocytosis of specific granules. Stimulation of neutrophils with FMLP, in the absence of actin-modifying drugs, significantly
increased surface expression of CD66b ($P < 0.001$). Pretreatment of neutrophils with cytochalasin D alone resulted in a dose-dependent increase in CD66b surface expression, with a maximal effect at $1 \times 10^{-5}$ M (Fig. 5C). Pretreatment with cytochalasin D at concentrations of $1 \times 10^{-5}$ M and higher significantly enhanced the FMLP-stimulated increase in CD66b expression ($P < 0.001$), while concentrations of cytochalasin D lower than $1 \times 10^{-6}$ M did not alter FMLP-stimulated specific granule exocytosis. Similarly, latrunculin A alone induced a dose-dependent increase in CD66b expression, with a maximal effect at $3 \times 10^{-7}$ M (Fig. 6C), and latrunculin A enhanced FMLP-stimulated CD66b expression at concentrations of $1 \times 10^{-7}$ M and above.

Unlike other granule subtypes, stimulation of neutrophils with FMLP in the absence of actin-disrupting drugs did not induce azurophil granule exocytosis, as measured by CD63 expression. As shown in Figs. 5D and 6D, neither cytochalasin D nor latrunculin A alone increased CD63 expression. However, pretreatment with latrunculin A at concentrations of $1 \times 10^{-7}$ M or higher and cytochalasin D at concentrations of $1 \times 10^{-5}$ M or higher resulted in a significant increase in CD63 expression following FMLP stimulation ($P < 0.015$).

**Disruption of actin cytoskeleton alters kinetics of FMLP-stimulated exocytosis.** To determine whether disruption of the actin cytoskeleton affects the kinetics of exocytosis, the time course of FMLP-stimulated exocytosis of all four granule
subsets was determined in the presence or absence of cytochalasin D and latrunculin A (Figs. 7 and 8). Pretreatment with cytochalasin D enhanced the initial rate of increase in CD35 expression (3 ± 1 vs. 13 ± 4 min to achieve 90% of maximal expression), the rate of increase in CD66b expression (9 ± 1 vs. 15 ± 3 min to achieve 90% of maximal expression), and the rate of release of gelatinase (8 ± 2 vs. 15 ± 2 min to achieve 90% of maximal release). FMLP-stimulated CD63 expression was maximally increased at 1 min in the presence of cytochalasin D (Fig. 7). Pretreatment with cytochalasin D also increased the maximal level of gelatinase release and CD66b expression, while the level of CD35 expression fell after 5 min. Latrunculin A pretreatment resulted in a time-dependent decrease in CD35 expression after FMLP stimulation (Fig. 8A). On the other hand, latrunculin A increased the rate of FMLP-stimulated gelatinase release (<1 vs. 12 ± 3 min to achieve 90% of maximal release) and the rate of expression of CD66b (2 ± 1 vs. 12 ± 3 min to achieve 90% of maximal expression) (Fig. 8, B and C). Pretreatment with latrunculin A increased the maximal level of CD66b expression and gelatinase release following FMLP stimulation. Similar to cytochalasin D, latrunculin A pretreatment allowed FMLP to maximally stimulate CD63 expression by 1 min (Fig. 8D).

To determine whether the actin cytoskeleton regulates the potency of FMLP, the effect of cytochalasin D and latrunculin A on the FMLP dose-response curves for exocytosis of each of the granule subsets was determined (Figs. 9 and 10). FMLP stimulated a dose-dependent increase in CD35 (P < 0.001, EC50 = 15 ± 2 nM), gelatinase release (P < 0.001, EC50 = 31 ± 4 nM), and CD66b (P < 0.001, EC50 = 28 ± 5 nM). The EC50 for CD35 expression was significantly less than that for gelatinase release or CD66b expression (P < 0.05). As before, FMLP alone had no effect on CD63 expression. Pretreatment with cytochalasin D significantly increased CD35 expression in the absence of FMLP but prevented an FMLP-stimulated increase in CD35 (Fig. 9A). Pretreatment with cytochalasin D had no significant effect on the EC50 for FMLP-stimulated exocytosis of gelatinase and specific granules (Fig. 9, B and C). However, the magnitude of gelatinase release (P = 0.004) and CD66b expression (P = 0.001) at concentrations of FMLP of 1 × 10−7 M and higher was significantly increased by cytochalasin D pretreatment. Cytochalasin D pretreatment also resulted in a concentration-dependent increase in FMLP-stimulated CD63 expression (EC50 = 60 ± 20 nM) (Fig. 9D). Figure 10A shows that pretreatment with latrunculin A prevented the FMLP-stimulated increase in CD35 expression. Latrunculin A pretreatment also increased the basal release of gelatinase and the basal expression of CD66b (Fig. 10, B and C). Pretreatment with latrunculin A had no effect on the EC50 for FMLP-stimulated exocytosis of gelatinase and specific granules. Similar to cytochalasin D, pretreatment with latrunculin A allowed FMLP to stimulate an increase in CD63 expression (EC50 = 44 ± 7 nM) (Fig. 10D). Thus disruption of the actin cytoskeleton did not alter the potency of FMLP-stimulated exocytosis for gelatinase and specific granules.

**Effect of disruption of actin cytoskeleton on signal transduction.** Previous reports demonstrated that disruption of the actin cytoskeleton resulted in enhanced receptor-mediated activation of signal transduction pathways (15, 42). To determine whether the enhanced exocytosis induced by inhibition of actin polymerization might be due to increased activity of signal transduction pathways, the effect of latrunculin A and cytochalasin D pretreatment on FMLP-stimulated p38 MAPK and ERK phosphorylation was examined. Figure 11 shows that while pretreatment with cytochalasin D and latrunculin A increased basal kinase phosphorylation, FMLP-stimulated kinase activation was not enhanced. Thus regulation of signal transduction pathway activation by the actin cytoskeleton is unlikely to be an explanation for the effect of disruption of actin reorganization on exocytosis.

**DISCUSSION**

The results of the present study suggest that a major role of the actin cytoskeleton is to limit access of neutrophil granules...
to the plasma membrane under basal conditions. Additionally, the actin cytoskeleton acts to limit the rate and extent of granule exocytosis for secretory vesicles, gelatinase granules, and specific granules. Finally, the actin cytoskeleton blocks fusion of azurophil granules with the plasma membrane under both basal and stimulated conditions. These activities can be viewed as protective mechanisms that prevent release of destructive enzymes unless neutrophils are appropriately stimulated by proinflammatory mediators. Our demonstration that minimal actin was associated with azurophil granules suggests that an actin network is capable of inhibiting neutrophil exocytosis independently of granule-associated actin.

Published reports over the past three decades showed that disruption of the actin cytoskeleton in various mammalian cells can enhance or inhibit exocytosis (14, 19, 29, 33, 37, 39, 42, 43, 46, 48). The mechanisms proposed for actin regulation of exocytosis include restricting access of granules to the plasma membrane, assisting granule mobilization, facilitating granule transit through the cortical actin network, facilitating membrane fusion, and downregulating signal transduction pathway activation (15, 45). Disruption of the actin cytoskeleton would enhance exocytosis if actin restricted access to the plasma membrane or inhibited signal transduction pathways. On the other hand, disruption of the actin cytoskeleton would inhibit exocytosis if actin reorganization contributed to granule translocation, facilitated membrane fusion, or facilitated granule transit through the cortical actin network. To examine the basis for enhanced FMLP-stimulated exocytosis, we determined the

**Fig. 8.** Latrunculin A pretreatment alters the kinetics of FMLP-stimulated exocytosis. Disruption of the actin cytoskeleton with latrunculin A enhanced FMLP-stimulated exocytosis of gelatinase granules (B) and specific granules (C) and enabled FMLP stimulation of azurophil granules (D), but caused a decrease in CD35 expression following FMLP stimulation (A). Neutrophils (4 x 10^6/ml) were incubated with (■) or without (●) 1 μM latrunculin A for 30 min and then stimulated with 10^-7 M FMLP or buffer for the indicated time. Exocytosis of secretory vesicles, specific granules, and azurophil granules was determined by using flow cytometry to measure mcf for CD35, CD66b, and CD63, respectively. Exocytosis of gelatinase granules was determined by measuring gelatinase release. Data are presented as means ± SE for 4 experiments for each granule subset.

**Fig. 9.** FMLP dose response in presence and absence of cytochalasin D. Disruption of the actin cytoskeleton with cytochalasin D had no effect on the EC50 for FMLP-stimulated exocytosis of secretory vesicles (A), gelatinase granules (B), and specific granules (C) but decreased the magnitude of FMLP-stimulated secretory vesicle exocytosis and increased the magnitude of FMLP-stimulated gelatinase and specific granule exocytosis, at concentrations of FMLP of 10^-7 M and above. Incubation with cytochalasin D enabled an FMLP dose-dependent increase in azurophil granule exocytosis (D). Neutrophils (4 x 10^6/ml) were incubated with (■) or without (●) 30 μM cytochalasin D for 30 min and then stimulated for 3 min with the indicated concentration of FMLP or buffer. Exocytosis of secretory vesicles, specific granules, and azurophil granules was determined by using flow cytometry to measure mcf for CD35, CD66b, and CD63, respectively. Exocytosis of gelatinase granules was determined by measuring gelatinase release. Data are presented as means ± SE for 5 (CD35 and gelatinase), 7 (CD66b), or 6 (CD63) experiments.
effect of pretreatment with cytochalasin D and latrunculin A on the kinetics of FMLP-stimulated exocytosis. Cytochalasin D binds to the barbed end of filamentous actin (F-actin), capping the actin filament and preventing its elongation (44). This capping property of cytochalasin D is consistent with the dose-dependent decrease in FMLP-stimulated actin reorganization shown in Fig. 2A. The contradictory finding that cytochalasin D alone increases phalloidin binding, suggesting increased actin polymerization, has been reported previously (21, 35). The mechanism for this increased actin polymerization is unknown, although it has been reported that cytochalasin D binds to the lateral edge of actin filaments, leading to their cleavage (44). Latrunculin A binds to globular actin (G-actin), thereby preventing elongation of actin filaments on stimulation and leading to a decrease in F-actin as basal actin turnover takes place (9, 40). Thus increasing concentrations of latrunculin A reduced basal levels of F-actin and prevented FMLP-stimulated actin polymerization. In the present study disruption of the actin cytoskeleton resulted in a significantly more rapid rate of exocytosis of specific and gelatinase granules, and the magnitude of exocytosis of each of the two granule subsets was increased. Disruption of the cytoskeleton was necessary for exocytosis of azurophil granules, with the maximal response to FMLP by 1 min. These findings are most consistent with the actin cytoskeleton acting to restrict granule access to the plasma membrane. Because of its location, the cortical actin network has been assumed to serve as the barrier to granule access to the plasma membrane in other cell types.

Previous studies found that disruption of the actin cytoskeleton resulted in enhanced receptor-mediated activation of signal transduction pathways (15, 42). To determine whether actin regulation of neutrophil exocytosis results from a general inhibition of signal transduction pathways, we chose to examine FMLP-stimulated p38 MAPK and ERK activation. Although latrunculin A and cytochalasin D pretreatment enhanced basal activity of both kinases, neither drug significantly altered FMLP-stimulated kinase activity, suggesting that regulation of signal transduction pathways does not contribute to actin regulation of exocytosis. These findings do not eliminate actin regulation of one or more specific signaling pathways that mediate exocytosis, but they do suggest that the role of the actin cytoskeleton is not through global regulation of signal transduction.

The effect of disrupting the actin cytoskeleton on secretory vesicle exocytosis was more complex than for the other granule subsets. Both cytochalasin D and latrunculin A induced secretory vesicle exocytosis in the absence of FMLP stimulation, suggesting that the actin cytoskeleton acts to inhibit access of

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**Fig. 10.** FMLP dose response in presence and absence of latrunculin A. Disruption of the actin cytoskeleton with latrunculin A prevented FMLP-induced exocytosis of secretory vesicles (A) but had no effect on the EC50 for FMLP-stimulated exocytosis of gelatinase (B) and specific (C) granules. Incubation with latrunculin A enabled an FMLP dose-dependent increase in azurophil granule exocytosis (D). Neutrophils (4 x 10⁹/ml) were incubated with (●) or without (○) 1 μM latrunculin A for 30 min and then stimulated for 3 min with the indicated concentration of FMLP or buffer. Exocytosis of secretory vesicles, specific granules, and azurophil granules was determined by using flow cytometry to measure mcf for CD35, CD66b, and CD63, respectively. Exocytosis of gelatinase granules was determined by measuring gelatinase release. Data are presented as means ± SE for 5 (CD35), 4 (gelatinase and CD63), or 7 (CD66b) experiments.

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**Fig. 11.** Disruption of the actin cytoskeleton enhances basal, but not FMLP-stimulated, activation of p38 MAPK and ERK. Neutrophils (4 x 10⁹/ml) were incubated with or without 30 μM cytochalasin D or 1 μM latrunculin A for 30 min at 37°C to disrupt the actin cytoskeleton then incubated with 3 x 10⁻⁷ M FMLP or buffer for 1 min. The incubation was stopped by addition of lysis buffer. Proteins were separated by 10% SDS-PAGE and immunoblotted for phosphorylated p38 MAPK (pp38) or phosphorylated ERK (pERK). The immunoblots were then stripped and reprobed for total p38 MAPK or ERK. The blots show that latrunculin A and cytochalasin D alone enhanced basal ERK and p38 MAPK phosphorylation, while FMLP-stimulated activation was not affected.
secretory vesicles to the plasma membrane in unstimulated cells. On the other hand, disruption of the cytoskeleton resulted in a biphasic response to FMLP stimulation, in which an initial rapid increase in CD35 expression was followed by reduced expression. The enhanced initial rate of exocytosis in the presence of latrunculin A and cytochalasin D argues against a role of actin reorganization in secretory vesicle translocation, fusion with the plasma membrane, or transit through the cortical actin network. Possible explanations for these observations include shedding of CD35, as had been reported for selectins (20), or internalization by a process of nonphagocytic endocytosis (10). The observation that CD35 was not detected in the supernatant of neutrophils stimulated by FMLP in the presence of latrunculin A suggests that disruption of the actin cytoskeleton does not lead to CD35 shedding. Berger et al. (2) reported previously that CD35 (also termed the type 1 complement receptor) was internalized into multilamellar vesicles after FMLP stimulation. Thus endocytosis may be responsible for the reduced CD35 expression in FMLP-stimulated, latrunculin A-pre-treated cells.

The EC50 for FMLP-stimulated exocytosis of secretory vesicles was significantly less than that for gelatinase and specific granules, which demonstrated similar EC50 values. An EC50 for FMLP-stimulated exocytosis of azurophil granules could not be calculated. These findings are consistent with the concept of graded exocytosis, in which mobilization of individual granule subsets depends on stimulus intensity. The graded response to FMLP observed in the present study differs, however, from previous reports that measured percent granule released on a fixed level of stimulation. Sengelov and colleagues found that in vitro stimulation of human neutrophils with 10 nM FMLP led to exocytosis of 65% of secretory vesicles, 30% of gelatinase granules, 10% of specific granules, and <5% of azurophil granules (38), and exocytosis of gelatinase, specific, and azurophil granules was 40%, 22%, and 7%, respectively, during in vivo exudation of neutrophils (37). Our data indicate that maximal exocytosis of specific and gelatinase granules occurs at similar levels of stimulus intensity, although the percentage of each granule subset capable of exocytosis at maximal stimulation was not determined. Pretreatment with cytochalasin D and latrunculin A did not induce a significant alteration in the EC50 for FMLP-stimulated gelatinase granule or specific granule exocytosis, indicating that disruption of the actin cytoskeleton did not alter the potency of FMLP.

The immunoblot analysis indicated that there was differential actin association with plasma membrane and each granule subset. These findings were supported by confocal microscopy demonstrating greater colocalization of F-actin with secretory vesicles than specific granules and the absence of colocalization of F-actin with azurophil granules. Confocal microscopy also suggested that a population of secretory vesicles and specific granules may exist at the plasma membrane or within cortical actin. Localization of some neutrophil granules at the plasma membrane by electron microscopy has been reported previously (6, 28). The relative amounts of actin associated with each granule subset only partially paralleled the ability of that subset to undergo stimulated exocytosis. Secretory vesicles were associated with more actin than the other granule subsets and were most easily stimulated to undergo exocytosis, and azurophil granules had little associated actin and failed to exhibit exocytosis on FMLP stimulation. On the other hand, the EC50 values for gelatinase and specific granules did not differ, while specific granules were associated with significantly less actin. Similarly, specific and azurophil granules were associated with similar amounts of actin, although stimulated exocytosis of azurophil granules did not occur in absence of actin disruption. Thus, despite differences in the amount of actin associated with the different granule subsets, our data do not support a role for the actin cytoskeleton in graded exocytosis.

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