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Evidence of ζ Protein Kinase C Involvement in Polymorphonuclear Neutrophil Integrin-dependent Adhesion and Chemotaxis*

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Classical chemoattractants and chemokines trigger integrin-dependent adhesion of blood leukocytes to vascular endothelium and also direct subsequent extravasation and migration into tissues. In studies of human polymorphonuclear neutrophil responses to formyl peptides and to interleukin 8, we show evidence of involvement of the atypical ζ protein kinase C in the signaling pathway leading to chemoattractant-triggered actin assembly, integrin-dependent adhesion, and chemotaxis. Selective inhibitors of classical and novel protein kinase C isozymes do not prevent chemoattractant-induced neutrophil adhesion and chemotaxis. In contrast, chelerythrine chloride and synthetic myristoylated peptides with sequences based on the endogenous ζ protein kinase C pseudosubstrate region block agonist-induced adhesion to fibrinogen, chemotaxis and F-actin accumulation. Biochemical analysis shows that chemoattractants trigger rapid translocation of ζ protein kinase C to the plasma membrane accompanied by rapid but transient increase of the kinase activity. Moreover, pretreatment with C3 transferase, a specific inhibitor of Rho small GTPases, blocks ζ but not α protein kinase C plasma membrane translocation. Synthetic peptides from ζ protein kinase C also inhibit phorbol ester-induced integrin-dependent adhesion but not NADPH-oxidase activation, and C3 transferase pretreatment blocks phorbol ester-triggered translocation of ζ but not α protein kinase C. These data suggest the involvement of ζ protein kinase C in chemoattractant-induced leukocyte integrin-dependent adhesion and chemotaxis. Moreover, they highlight a potential link between atypical protein kinase C isozymes and Rho signaling pathways leading to integrin-activation.

Leukocyte extravasation is crucial for an appropriate and effective immune response. Interaction with vascular endothelium is a carefully regulated multistep process leading to selective migration of various leukocyte subtypes. Adhesion molecules and activating factors control different steps of this process. Selectins and α_4-integrins both mediate the initial interaction and the subsequent rolling along the endothelium, whereas only integrins mediate firm adhesion (1). The transition from rolling to firm adhesion requires intracellular biochemical changes, since integrins do not recognize the ligand unless activated. Several factors have been reported to trigger activation of integrin-dependent leukocyte adhesion and motility, including phorbol esters, cytokines, and chemoattractants (2–4). Pertussis toxin-induced inhibition of lymphocyte homing highlighted the role of $G\alpha_i$-protein linked receptors and their ligands as physiological activators of integrin-dependent lymphocyte adhesion *in vivo* (5). Classical chemoattractants, such as formyl-Met-Leu-Phe (fMLP),[1] and chemokines, such as interleukin 8 (IL-8), trigger $\alpha_4\beta_1$-integrin-dependent lymphocyte adhesion to vascular cell adhesion molecule-1 (VCAM-1) and $\alpha_M\beta_2$-integrin-dependent polymorphonuclear neutrophil adhesion to fibrinogen through a Pertussis toxin-sensitive signaling pathway previously shown to involve Rho small GTP-binding proteins (6).

A well known signaling cascade triggered by chemoattractants involves activation of phosopholipase C, leading to diacylglycerol (DAG) and inositol triphosphate accumulation, intracellular calcium increase, and activation of the serine-threonine protein kinase C (PKC) (7). PKC is a family of closely related proteins with serine-threonine kinase activity subdivided in subfamilies according to their sensitivity to Ca^{2+} and DAG. These include the classical, Ca^{2+}-DAG sensitive, isozymes (α, β_I, β_{II}, and γ PKC); the novel, Ca^{2+}-independent but DAG-sensitive, isozymes (δ, ϵ, η, and θ PKC); and the atypical, Ca^{2+}- and DAG-independent, isozymes (ζ and λ/ι PKC). Involvement of PKC in integrin activation has been originally suggested by the capability of the DAG-synthetic analog phorbol myristate acetate (PMA), a direct activator of DAG-sensitive PKCs, to activate integrin-dependent leukocyte aggregation and adhesion (2). However, PMA is not a physiological agonist, and in studies reported earlier (6), we showed that chemoattractant activation of rapid integrin-dependent leukocyte adhesion was not affected by calphostin C, an inhibitor of DAG-sensitive PKC isozymes that interferes with DAG binding to the C1 regulatory domain (8).

To clarify the involvement of PKC in integrin activation and chemotaxis triggering by chemoattractants in human polymorphonuclear neutrophils, we used PKC inhibitors able to discern among different subfamilies of isozymes. Particularly, we exploited the highly selective inhibitory activity of synthetic peptides with sequence derived from the N-terminal pseudosub-

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[1] The abbreviations used are: fMLP, formyl-Met-Leu-Phe; IL-8, interleukin-8; DAG, diacylglycerol; PKC, protein kinase C; PMA, phorbol myristate acetate; PBS, phosphate-buffered saline; FCS, fetal calf serum.

strate region of PKC (9). We show evidence suggesting that Ca^{2+}-DAG-sensitive PKC isoforms are not involved in the chemoattractant-triggered signaling pathway leading to integrin-dependent neutrophil adhesion and chemotaxis. In contrast, inhibitors of the atypical ζ PKC block adhesion and chemotaxis induced by chemoattractants as well as adhesion triggered by phorbol esters. Inhibitors of the atypical ζ PKC also block chemoattractant-induced G-actin assembly. Moreover, chemoattractant and PMA-induced plasma membrane translocation of ζ PKC is selectively blocked by C3 transferase, a specific inhibitor of Rho small GTP-binding protein. Thus, our data suggest a role of ζ PKC as downstream effector of signaling pathways leading to integrin activation and movement in human neutrophils. Furthermore, a potential functional link with Rho small GTPases is proposed.

EXPERIMENTAL PROCEDURES

Isolation of Human Polymorphonuclear Cells—Human blood polymorphonuclear neutrophils were isolated by dextran sedimentation and centrifugation over Ficoll-Hypaque (Amersham Pharmacia Biotech) as described previously (6). Contaminating erythrocytes were lysed by hypotonic saline, and then neutrophils were washed with PBS and finally resuspended in RPMI 1640 containing 10% FCS. All of the above procedures were done using reagents prepared in endotoxin-free water for clinical use.

Peptides and Other Reagents—All peptides (synthesized at the Stanford University Protein and Nucleic Acid Facility) were solubilized immediately before use at a 1 mM concentration in PBS, pH 7.2. In some cases (myristoylated ζ PKC short and long) peptides were heated to 37–40 °C to achieve complete solubility. Reported below are peptide sequences from the pseudosubstrate region of human PKC isozymes: α PKC long, RFARKGALRQKNVHEVK (positions 19–35); α PKC short, RFARKGALRQKNV (positions 19–31); α PKC scramble short, LVFQGKERVARRNHKA; α PKC scramble long, LFQGKRVARRNA; ζ PKC long, SIYRRGARRWRKLYRAN (positions 113–129); ζ PKC short, SIYRRGARRWRKL (positions 113–125); ζ PKC scramble long, RLRYRNKRIWRSAYAGR; ζ PKC scramble short, RLYRKRIWRSAGR.

PBS, fMLP, PMA, leupeptin, pepstatin, aprotinin, soybean trypsin inhibitor, phenylmethylsulfonyl fluoride, and human fibrinogen were purchased from Sigma; diisopropyl fluorophosphate was from Aldrich; RPMI 1640 and FCS were from Irvine; antibodies against PKC isozymes were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA) and Transduction Laboratories; calphostin C, Gö6976, Gö6850 (bisindolylmaleimide I), and chelerythrine chloride were from Alexis Biochemicals (San Diego, CA); recombinant C3 transferase was from Upstate Biotechnology Inc. (Lake Placid, NY); dihydrorhodamine and nitrobenzoxadiazole/phallacidin were from Molecular Probes (Eugene, OR); unlabeled phallacidin was from Sigma; horseradish peroxidase was from Sigma; trisacryl protein A was from Pierce; and myelin basic protein was from Sigma.

Adhesion Assay—Eighteen-well glass slides were coated for 120 min at 37 °C with human fibrinogen (Sigma) (20 μg/well in endotoxin-free PBS). Neutrophils (5×10^4/well; 2.5×10^6/ml in RPMI 1640, containing 10% heat-inactivated FCS and 20 mM HEPES, pH 7.3) were added, incubated for 10 min at 37 °C, and then stimulated by the addition of the agonists before washing, fixation on ice in 1.5% glutaraldehyde for 60 min, and computer-assisted enumeration of cells bound in 0.2 mm^2, as described (6).

Chemotaxis Assay—Neutrophils migration was assessed using 1-μm pore size transwells (Bio-Coat, Becton Dickinson). Neutrophil were in RPMI 1640, containing 10% heat-inactivated FCS and 20 mM HEPES, pH 7.3, at 2×10^6/ml. 100 μl of cell suspension were added to the top well, and 600 μl of medium, containing agonists and inhibitors, were added to the bottom well. After fixation with 1.5% glutaraldehyde, migrated cells were counted by fluorescence-activated cell sorting using polystyrene beads (Polyscience) as an internal standard (10)

NADPH-oxidase Activation—Activation of neutrophil NADPH-oxidase was evaluated by measuring reduction of dihydrorhodamine induced by superoxide anion-derived hydrogen peroxide, as previously reported (11, 12). Neutrophils, resuspended at 2×10^4/ml in PBS, pH 7.3, containing 1 mM $CaCl_2$, 1 mM $MgCl_2$, 20 μg/ml horseradish peroxidase, 0.5 μM dihydrorhodamine (reaction buffer), were stimulated under stirring at 37 °C. The time course of hydrogen peroxide-induced dihydrorhodamine reduction was evaluated with a spectrofluorimeter with 505 of excitation wavelength and 534 of emission wavelength. Calibration was done using as a standard a defined amount of hydrogen peroxide.

Quantification of F-actin Content—F-actin was quantified by methanol extraction of nitrobenzoxadiazole/phallacidin-stained cells, as described previously (66). Neutrophils (5×10^6) were pretreated on ice for 30 min with 30 μg/ml IB4 mouse monoclonal antibody anti-human β_2-integrins (Fab_2 fragment) to prevent agonist-triggered integrin-mediated neutrophil aggregation and signaling. The cells were then washed; resuspended in PBS, pH 7.3, containing 1 mM $CaCl_2$, 1 mM $MgCl_2$; stimulated under stirring at 37 °C; fixed with 2% paraformaldehyde; and stained for 30 min at room temperature in PBS containing 0.1% Triton X-100 and 0.4 μM nitrobenzoxadiazole/phallacidin. After sedimentation at 16,000 × *g*, the pellet was overlaid with 0.5 ml of absolute methanol and extracted for 6 h in the dark at 4 °C. After cell sedimentation, the supernatant was collected, and the fluorescence intensity was determined with a spectrofluorimeter (excitation at 465 nm; emission at 535 nm). To determine the level of nonspecific binding, a 10-fold molar excess of unlabeled phallacidin was included. The value of nonspecific binding was subtracted to yield specific F-actin staining and quantification.

Measurement of ζ PKC Activity—Neutrophils were pretreated with diisopropyl fluorophosphate (5.8 mM for 20 min on ice) and subsequently pretreated on ice for 30 min with 30 μg/ml IB4 mouse monoclonal antibody anti-human β_2-integrins (Fab_2 fragment) to prevent agonist-triggered integrin-mediated neutrophil aggregation and signaling. Neutrophils were then washed, resuspended at 4×10^7/ml in PBS containing 1 mM Ca^{2+}/Mg^{2+}, and stimulated with agonists under stirring at 37 °C. Stimulation was stopped with lysis buffer containing 50 mM Tris-HCl, pH 7.5; 1% Triton X-100; 0.01% SDS; 150 mM NaCl; 50 mM NaF; 10 mM sodium pyrophosphate; 2 mM EDTA; 1 mM EGTA; 1 μM dithiothreitol; 1 μM phenylarsine oxide; 1 mM phenylmethylsulfonyl fluoride; 2.5 mM benzamidine; and 20 μg/ml of leupeptin, pepstatin, aprotinin, and soybean trypsin inhibitor. After 30 min on ice, lysates were centrifuged at 16,000 × *g* for 1 min to remove cell debris. Rabbit polyclonal anti-ζ PKC (1 μg) or control rabbit serum was added to an equal amount of cell lysates, followed by immunoprecipitation with trisacryl protein A. Equal amounts of ζ PKC were immunoprecipitated as confirmed by Western blot analysis (data not shown). After four washings, immunoprecipitates were subjected to the kinase reaction for 30 min at 30 °C in 50 μl of kinase buffer containing 0.5 mM EGTA, 10 mM $MgCl_2$, 20 mM HEPES, pH 7.4, 50 μM ATP, 5 μCi of [γ-^{32}P]ATP, and 2 μg of myelin basic protein as a substrate. The reaction was stopped by the addition of 5% trichloroacetic acid, and the reaction mixture was filtered through phosphocellulose paper. After four rinses with 1% phosphoric acid radioactivity on the filter was determined with a scintillation counter.

PKC Translocation Assay—Neutrophils were pretreated with diisopropyl fluorophosphate (5.8 mM for 20 min on ice) and subsequently electroporated in the presence of 25 μg/ml of recombinant C3 transferase, as previously reported (6). The effectiveness of the treatment was tested every time by evaluating C3 transferase-induced inhibition of triggered adhesion, as previously reported (6) (data not shown). After C3 treatment, neutrophils were pretreated on ice for 30 min with 30 μg/ml of IB4 mouse monoclonal antibody anti-human β_2-integrins (Fab_2 fragment) to prevent agonist-triggered integrin-mediated neutrophil aggregation and signaling. Neutrophils were then washed, resuspended at 3×10^7/ml in PBS containing 1 mM Ca^{2+}/Mg^{2+}, and stimulated under stirring with agonists at 37 °C. Stimulation was stopped by diluting the cells in a 10 times larger volume of ice-cold PBS. Cells, resuspended in 1 ml of ice-cold PBS containing 8% sucrose, 1 mM phenylmethylsulfonyl fluoride, and 20 μg/ml leupeptin, pepstatin, aprotinin, soybean trypsin inhibitor were sonicated, and the homogenates were centrifuged at 800 × *g* for 10 min to remove nuclei and unbroken cells. The postnuclear supernatant was loaded on a discontinuous sucrose gradient (50% sucrose, 30% sucrose) and centrifuged for 90 min at 100,000 × *g*. The light membrane fraction (plasma membrane) was collected in the 30% layer (13). Following SDS-polyacrylamide gel electrophoresis on 9% acrylamide, proteins (10 μg/lane) were electroblotted on 0.2-μm pore size nitrocellulose filters, probed with rabbit anti-α or -ζ PKC polyclonal antibodies (Santa Cruz Biotechnology), followed by goat polyclonal anti-rabbit horseradish peroxidase conjugated (Sigma) and developed using ECL (Amersham Pharmacia Biotech). The specificity of the anti-ζ PKC antibodies was confirmed by the capability to recognize a unique band of approximately 72 kDa on blotted nitrocellulose filters of either rat brain lysates or baculovirus-purified human recombinant ζ PKC (Panvera) (data not shown).

RESULTS

Inhibitors of Classical and Novel PKCs Do Not Block Chemoattractant-induced Integrin-dependent Neutrophil Adhesion and Chemotaxis—The role of PKC in neutrophil integrin activation and chemotaxis was initially investigated using various PKC inhibitors. As previously reported, calphostin C did not block fMLP or IL-8-triggered neutrophil adhesion to fibrinogen. Gö6976, a structurally unrelated PKC inhibitor with selectivity for classical (α, β, and γ) isozymes of PKC (14), did not interfere with chemoattractant-induced adhesion. Gö6850 (bisindolylmaleimide I), a third structurally unrelated inhibitor of classical and novel (δ, ϵ, η, and θ) PKC (14), had no detectable effect on chemoattractant-induced neutrophil adhesion. (Fig. 1*A*). In contrast, adhesion stimulated by the phorbol ester PMA, a direct activator of DAG-dependent PKC isozymes, was effectively blocked by all these inhibitors (Fig. 1*A*). Staurosporine, a potent but relatively nonselective kinase inhibitor, also inhibited PMA but not chemoattractant-induced neutrophil adhesion (data not shown). Moreover, analysis of neutrophil chemotaxis showed no influence of Gö6976 and Gö6850 on neutrophil migration toward the chemoattractants fMLP and IL-8 (Fig. 1*B*); staurosporine was also ineffective (data not shown). Because of its reported light dependence (15), the effect of calphostin C on chemotaxis was not evaluated. These data clearly suggest that chemoattractant activation of neutrophil integrin-dependent adhesion and chemotaxis does not involve DAG-sensitive PKC isozymes.

Chelerythrine Chloride Blocks Chemoattractant-induced Integrin-dependent Neutrophil Adhesion and Chemotaxis—To verify the previous data, we used Chelerythrine chloride, a specific (but not isozyme-selective) PKC inhibitor that represents a unique class of PKC inhibitors that competitively interfere with the phosphate acceptor site and noncompetitively inhibits the ATP binding site (16). Surprisingly, pretreatment of neutrophils with chelerythrine chloride efficiently blocked fMLP, IL-8, and PMA-induced adhesion in a dose-dependent manner (Fig. 2*A*). Moreover, chelerythrine chloride also inhibited chemotaxis to both agonists in a dose-dependent manner (Fig. 2*B*). We conclude that a chelerythrine chloride-sensitive but calphostin C-, Gö6976-, Gö6850-, and staurosporine-insensitive PKC isoform is involved in chemoattractant triggering of integrin-dependent neutrophil adhesion and chemotaxis. Importantly, none of the inhibitors affected expression of β_2-integrins, cell viability as assessed by trypan blue exclusion, or intracellular Ca^{2+} elevation stimulated by fMLP or IL-8 (data not shown).

Myristoylated Pseudosubstrate Peptides from α PKC Do Not Block Chemoattractant-induced Integrin-dependent Neutrophil Adhesion and Chemotaxis—To further address these findings, we took advantage of the ability of synthetic pseudosubstrate peptides, with sequences based on the endogenous PKC pseudosubstrate region (9, 17), to inhibit PKC kinase activity. Peptides were synthesized with an N-terminal myristic acid to facilitate their diffusion through the plasma membrane, as previously reported. (18). As shown in Fig. 3*A*, myristoylated peptides from α PKC, "short," encompassing amino acids 19–31 (reported to be a more potent inhibitor (9)) and "long," amino acids 19–35 (encompassing the entire pseudosubstrate region, which is identical in both α and β classical PKC isozymes) had no effect on adhesion stimulated by fMLP and IL-8. In contrast, PMA-induced adhesion was blocked in a dose-dependent manner, whereas nonmyristoylated peptides with the same α PKC sequence and myristoylated peptides with a "scrambled" sequence were ineffective, even at the highest concentrations. Both short and long myristoylated peptides from α PKC were also unable to block neutrophil chemotaxis to fMLP and IL-8 (Fig. 3*B*). These data confirm the previous observations and show that myristoylated peptides from PKC pseudosubstrate region are suitable tools to study PKC function in human neutrophils.

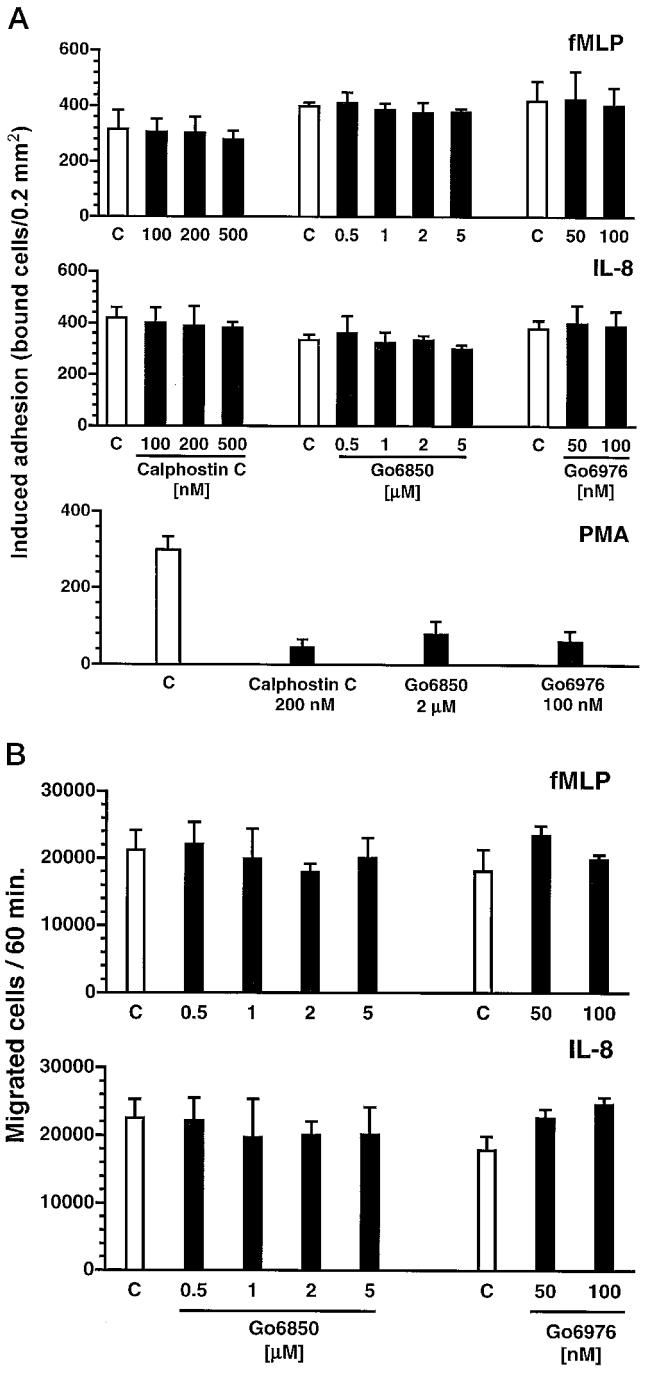

FIG. 1. **Effect of PKC inhibitors on agonist-induced neutrophil adhesion and chemotaxis.** Human polymorphonuclear neutrophils were treated with dimethyl sulfoxide (*C*, control) or with the indicated concentration of calphostin C, Gö6850, or Gö6976 at 37 °C for 30 min. Because of its light dependence (13), the calphostin C pretreatment and adhesion assay were carried out under cool white fluorescent light. *A*, adhesion was stimulated at 37 °C for 3 min with 100 nM fMLP or 10 nM IL-8 or for 15 min with 150 nM PMA. Values are the mean counts of bound cells in 0.2 mm^2 in 3–7 experiments; *error bars* represent S.D. values. Background binding in the absence of agonist was minimal (39 ± 11 cells/0.2 mm^2) and was subtracted to yield induced adhesion. *B*, chemotaxis toward 10 nM fMLP or 1 nM IL-8 was carried out at 37 °C for 60 min. Values are the mean counts of migrated cells in 4–6 experiments; *error bars* show S.D. values. Background migration in the absence of agonist was minimal (<0.2% of agonist-induced migration) and was subtracted.

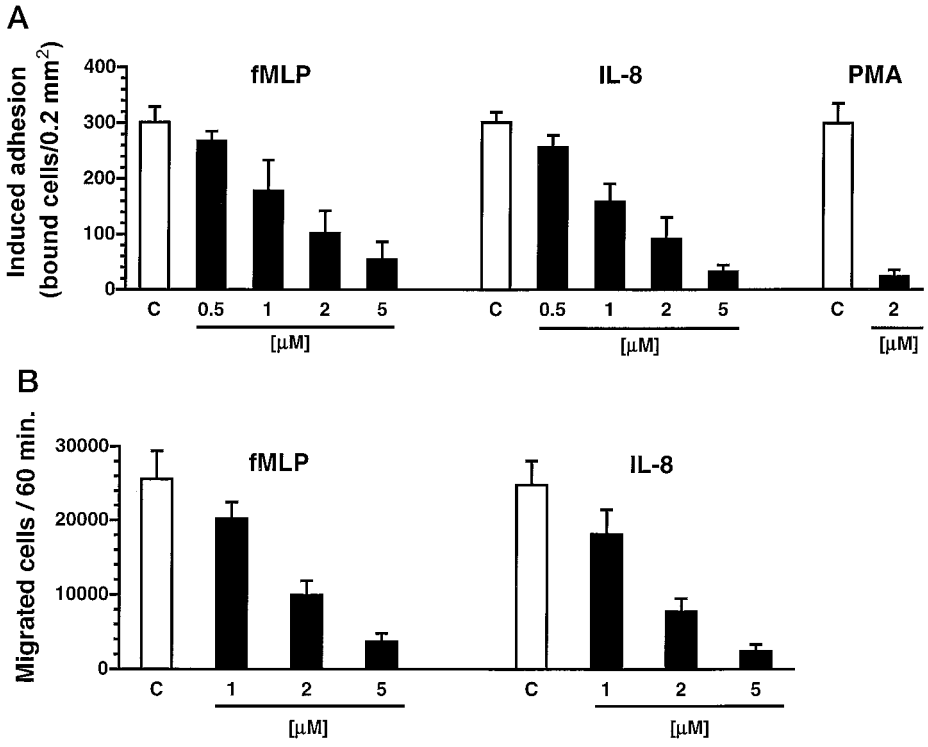

FIG. 2. **Effect of chelerythrine chloride on agonist-induced neutrophil adhesion and chemotaxis.** Human polymorphonuclear neutrophils were treated with dimethyl sulfoxide (*C*, control) or with the indicated concentration of chelerythrine chloride. *A*, adhesion was stimulated at 37 °C for 3 min with 100 nM fMLP or 10 nM IL-8 or for 15 min with 150 nM PMA. Values are the mean counts of bound cells in 0.2 mm^2 in 3–7 experiments; *error bars* show S.D. values. Background binding in the absence of agonist was minimal (39 ± 11 cells/0.2 mm^2) and was subtracted to yield induced adhesion. *B*, chemotaxis toward 10 nM fMLP or 1 nM IL-8 was carried out at 37 °C for 60 min. Values are the mean counts of migrated cells in 4–6 experiments; *error bars* show S.D. values. Background migration in the absence of agonist was minimal (<0.2% of agonist-induced migration) and was subtracted.

Myristoylated Pseudosubstrate Peptides from ζ PKC Block Chemoattractant-induced Integrin-dependent Neutrophil Adhesion and Chemotaxis—The previous data show that a DAG-independent PKC isozyme, sensitive to chelerythrine chloride but not to other PKC inhibitors and peptides, is involved in chemoattractant-induced neutrophil adhesion and chemotaxis. Western blot analysis revealed that human neutrophils express PKC isozymes of the classical (α, β_I, and β_{II}), novel (δ), and atypical (ζ) subfamilies of PKC, also consistent with previous a report (19), whereas in control rat brain lysates ϵ, η, and λ/ι PKCs were also expressed (data not shown). Thus, the ζ isozyme was the only atypical PKC expressed in human neutrophils. Since the atypical ζ PKC isozyme has been previously shown to be chelerythrine chloride-sensitive (20) but insensitive to staurosporine (21–23), we hypothesized that ζ PKC might be a target of chelerythrine chloride inhibition in our model and thus an effector of chemoattractant-triggered signaling pathways leading to integrin-dependent adhesion and chemotaxis. We tested the effect of peptides derived from the pseudosubstrate region of ζ PKC. Two myristoylated peptides from ζ PKC (short, encompassing amino acids 113–125, and long, amino acids 113–129, encompassing the entire pseudosubstrate region) inhibited in a dose-dependent manner fMLP- and IL-8-stimulated adhesion (Fig. 4*A*). The long peptide was the most efficient inhibitor, with more than 90% inhibition at 50 μM. Nonmyristoylated peptides with the same ζ PKC sequence and myristoylated peptides with a scrambled sequence were ineffective, even at the highest concentrations (Fig. 4*A*). We then asked whether ζ PKC pseudosubstrate peptides might affect chemoattractant-induced chemotaxis. Myristoylated ζ PKC peptides inhibited in a dose-dependent manner fMLP- and IL-8-induced chemotaxis (Fig. 4*B*). As shown for the adhesion, the long peptide was the most efficient inhibitor, whereas peptides that were nonmyristoylated or myristoylated with a scrambled sequence were ineffective (Fig. 4*B*). Again, cell viability, expression of β_2-integrins, and intracellular Ca^{2+} elevation induced by fMLP and IL-8 were not affected by pretreatment with any of the peptides (data not shown).

Myristoylated Pseudosubstrate Peptides from ζ PKC Block PMA-induced Integrin-dependent Neutrophil Adhesion—To verify the specificity of the ζ PKC peptides, we tested their effect on PMA-induced adhesion. Surprisingly, myristoylated ζ PKC pseudosubstrate peptides also blocked in dose-dependent manner PMA-triggered adhesion; again the long peptide was the most efficient inhibitor, with more than 85% inhibition at 50 μM. Peptides that were nonmyristoylated or myristoylated with a scrambled sequence were ineffective (Fig. 5*A*). This finding was rather unexpected, since the pseudosubstrate regions of α and ζ PKCs are quite different (23% of homology), and ζ PKC is not activated by PMA. We then evaluated the activation of the superoxide-generating NADPH-oxidase system of neutrophils, another function triggered by PMA. Myristoylated ζ PKC pseudosubstrate peptides did not interfere with PMA-triggered NADPH-oxidase activation, even at low doses of PMA and high doses of peptides (Fig. 5*B*), whereas myristoylated α PKC pseudosubstrate peptides effectively blocked NADPH-oxidase activation. These data support the selectivity of ζ PKC peptides, suggesting that atypical ζ PKC may be an effector of PMA-triggered signaling pathways leading to integrin activation.

Inhibitors of ζ PKC Block Chemoattractant-induced Actin Assembly—Chemoattractants stimulate in neutrophils rapid cytosolic G-actin polymerization leading to accumulation of filamentous actin (F-actin). Although F-actin accumulation does not seem not to be necessary for leukocyte integrin activation, as suggested by the incapacity of cytochalasin B to block lymphocyte function antigen type 1-mediated lymphocyte aggregation (27), actin polymerization is likely to be required for leukocyte shape change, such as polarization and directional movement during chemotaxis. Thus, we asked whether ζ PKC could be involved in signaling events leading to G-actin polymerization triggered by chemoattractants. As shown in Fig. 6, fMLP and IL-8 induced rapid increase of F-actin content. Neutrophil pretreatment with chelerythrine chloride or with myristoylated ζ PKC pseudosubstrate peptides almost completely abolished the increase of F-actin. In contrast, peptide myristoylated with a scrambled sequence did not block F-actin accumulation. Thus, ζ PKC seems to be involved in signaling cas-

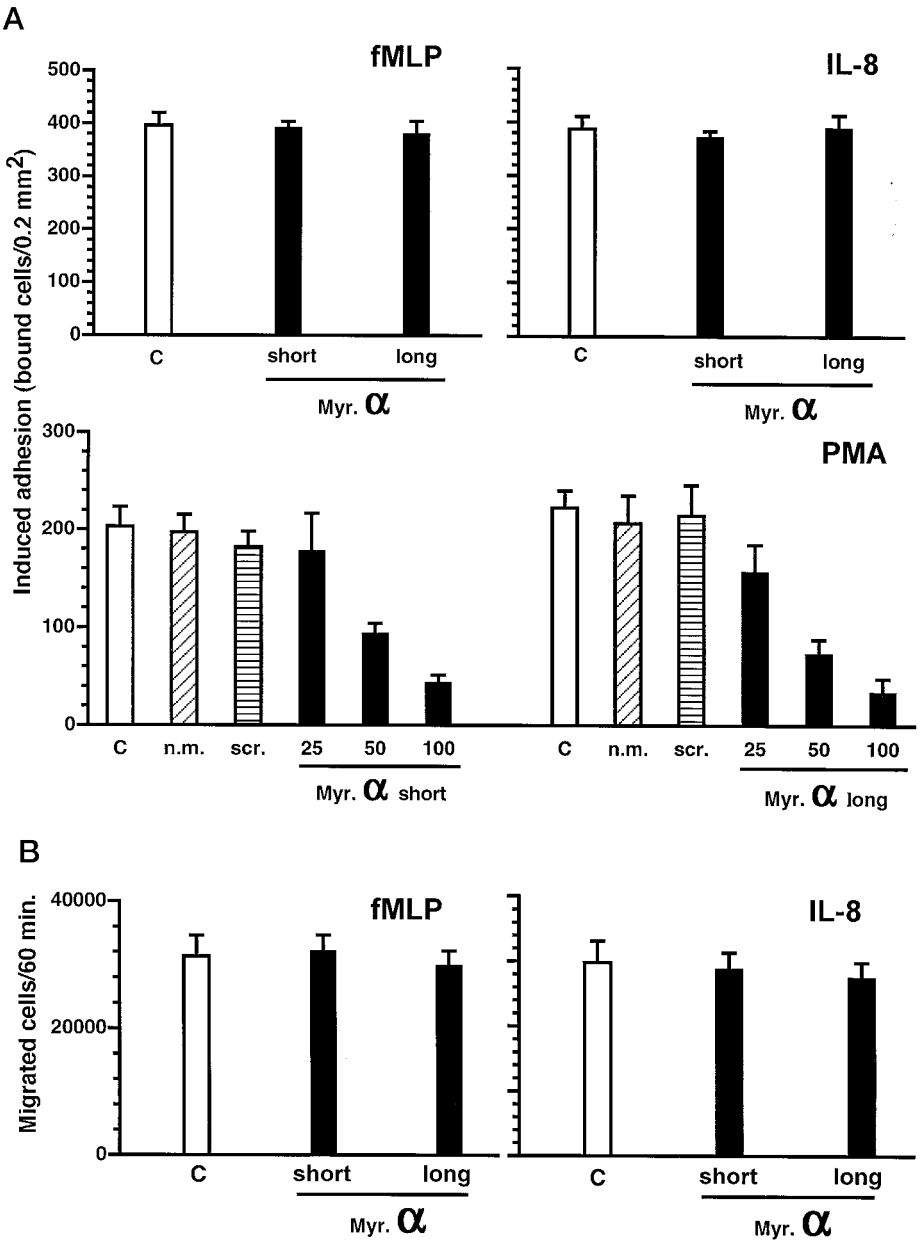

FIG. 3. **Effect of α PKC pseudosubstrate peptides on agonist-induced neutrophil adhesion and chemotaxis.** Human polymorphonuclear neutrophils were treated at 37 °C for 30–60 min with buffer (*C*, control); in the case of fMLP and IL-8 with 100 μM myristoylated peptides from the pseudosubstrate region of α PKC (*Myr. α short* and *long*); in the case of PMA, with the indicated concentrations of myristoylated peptides from the pseudosubstrate region of α PKC (*Myr. α short* and *long*); or with 100 μM of identical nonmyristoylated peptides (*n.m.*) and myristoylated scrambled peptide (*scr.*). *A*, adhesion was stimulated at 37 °C for 3 min with 100 nM fMLP or 10 nM IL-8 or for 15 min with 35 nM PMA. Values are the mean counts of bound cells in 0.2 mm^2 in 4–6 experiments; *error bars* show S.D. values. Background binding in the absence of agonist was minimal (41 ± 8 cells/0.2 mm^2) and was subtracted to yield induced adhesion. *B*, chemotaxis toward 10 nM fMLP or 1 nM IL-8 was carried out at 37 °C for 60 min. Values are the mean counts of migrated cells in three experiments; *error bars* show S.D. values. Background migration in the absence of agonist was minimal (<0.2% of agonist-induced migration) and was subtracted.

cade leading to G-actin polymerization.

Chemoattractants but Not PMA Trigger ζ PKC Activity—To characterize further ζ PKC involvement in proadhesive signaling pathways, we measured its kinase activity. In nonstimulated neutrophils, ζ PKC showed constitutive kinase activity (Fig. 7), as also previously reported (25). fMLP and IL-8 induced a 4.3- and 2.4-fold increase, respectively, of ζ PKC activity. Consistent with the rapid kinetics of chemoattractant-induced cell adhesion, fMLP- and IL-8-induced increase of ζ PKC activity was very rapid, occurring within 10 s, the earliest time point measurable (Fig. 7). However, increase of ζ PKC activity was transient, with a decrease of about 63% (fMLP) and 51% (IL-8) within 30 s. In contrast, PMA did not induce any significant increase of ζ PKC activity even after 5 min of stimulation (Fig. 7). fMLP and IL-8 but not PMA also triggered an increase of ζ PKC autophosphorylating activity, as measured in a kinase assay carried out in the absence of myelin basic protein (data not shown).

Chemoattractants as Well as PMA Trigger Plasma Membrane Translocation of ζ PKC—Translocation of PKC isozymes to different cellular compartments is a hallmark of selective PKC activation (24), and it is thought to be as important as or more important than altered kinase activity in regulating PKC functions within the cell (24); translocation is likely to be particularly important in regulating ζ PKC function because, unlike classical PKC isoforms, ζ PKC displays a high level of constitutive kinase activity in *in vitro* assays (Refs. 25 and 26, and see above). We therefore evaluated ζ PKC distribution in cytosol, light membrane, and particulate (insoluble) fractions in neutrophils stimulated with proadhesive agonists. In nonstimulated neutrophils, ζ PKC was mainly detected in the particulate (insoluble) fraction, whereas reactivity in the cytosol was very weak (Fig. 8). Following fMLP or IL-8 stimulation, ζ PKC was also found in the light membrane fraction (Fig. 8). Consistent with the rapid kinetics of chemoattractant-induced cell adhesion, fMLP and IL-8-induced translocation of ζ PKC was very rapid, occurring within 10 s, the earliest time point measurable. In contrast to increased kinase activity, ζ PKC translocation was higher after 30 s of stimulation. Although PMA is not a direct activator of ζ PKC, it also stimulated translocation of ζ PKC to the light membrane fraction (Fig. 8), albeit with slower kinetics. These last findings, to-

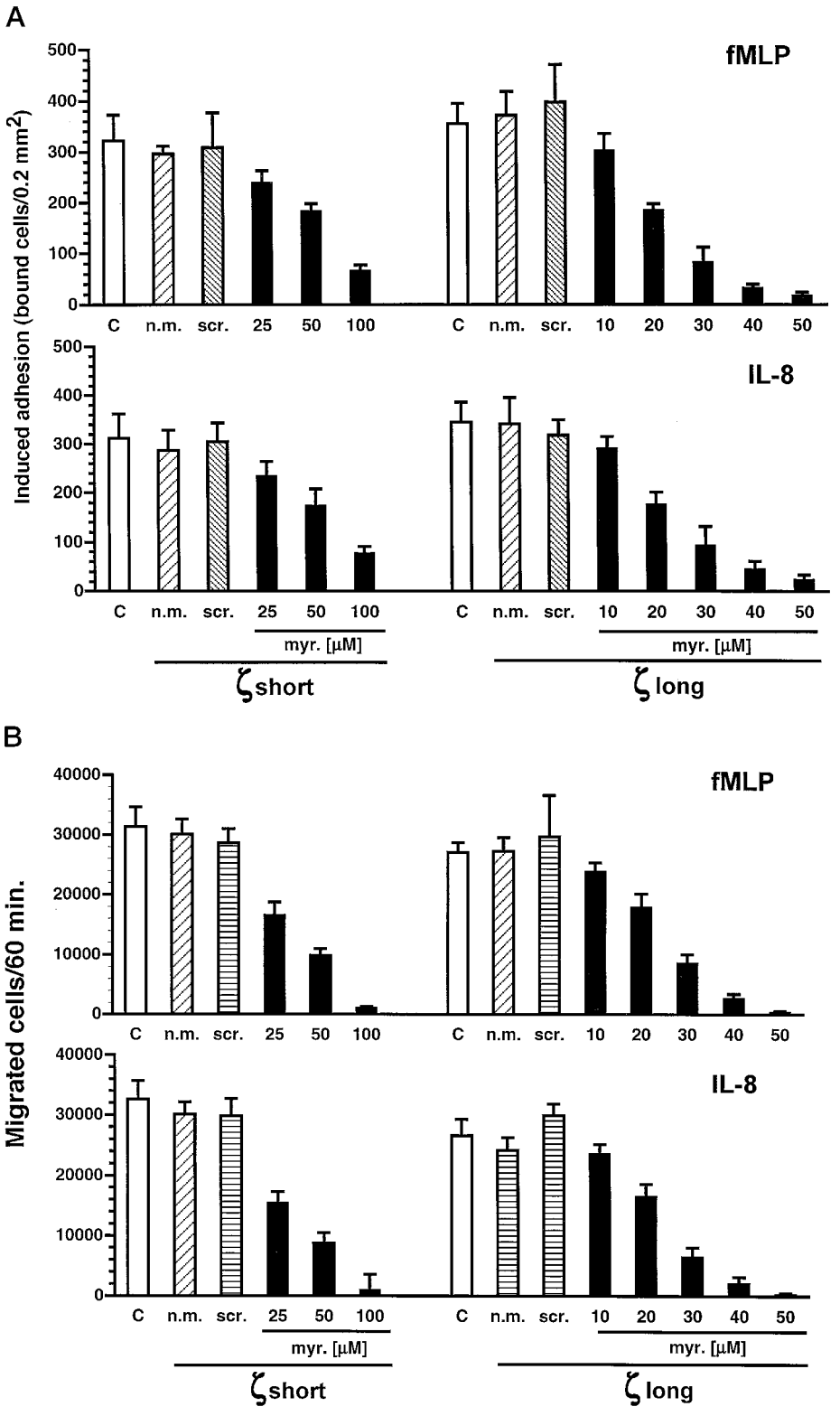

FIG. 4. **Effect of ζ PKC pseudosubstrate peptides on chemoattractant-induced neutrophil adhesion and chemotaxis.** Human polymorphonuclear neutrophils were treated at 37 °C for 30–60 min with buffer (*C*, control), with the indicated concentrations of myristoylated peptides from the pseudosubstrate region of ζ PKC (*Myr. ζ short* and *long*), or with 100 μM (ζ "short") or 50 μM (ζ "long") of identical nonmyristoylated peptides (*n.m.*) and myristoylated scrambled peptide (*scr.*). *A*, adhesion was stimulated at 37 °C for 3 min with 100 nM fMLP or 10 nM IL-8. Values are the mean counts of bound cells in 0.2 mm^2 in 4–6 experiments; *error bars* show S.D. values. Background binding in the absence of agonist was minimal (41 ± 8 cells/0.2 mm^2) and was subtracted to yield induced adhesion. *B*, chemotaxis toward 10 nM fMLP or 1 nM IL-8 was carried out at 37 °C for 60 min. Values are the mean counts of migrated cells in three experiments; *error bars* show S.D. values. Background migration in the absence of agonist was minimal (<0.2% of agonist-induced migration) and was subtracted.

gether with the capability of ζ PKC pseudosubstrate peptides to inhibit PMA-induced adhesion but not superoxide anion release, further suggest that ζ PKC can be an effector of PMA-triggered signaling pathways leading to integrin activation.

C3 Transferase Blocks Chemoattractant as Well as PMA-induced Plasma Membrane Translocation of ζ PKC—The previous data raise questions about the capability of DAG analogues to trigger translocation of atypical PKCs. The small GTP-binding protein Rho has been previously shown to mediate both chemoattractant and PMA-induced integrin activation in leukocytes (6, 27). Chemoattractants as well as PMA have been also shown to activate RhoA by increasing its guanine nucleotide exchange activity (6, 28). Thus, both Rho and ζ PKC appear to be effectors of chemoattractant- and PMA-induced signaling pathways leading to integrin activation and motility. Therefore, we hypothesized a functional relationship between Rho and ζ PKC. To verify this hypothesis, we evaluated ζ PKC light membrane translocation in neutrophils pretreated with *Clostridium botulinum* C3 transferase, which specifically ADP-ribosylates Rho (A, B, and C much more effectively than Rac or

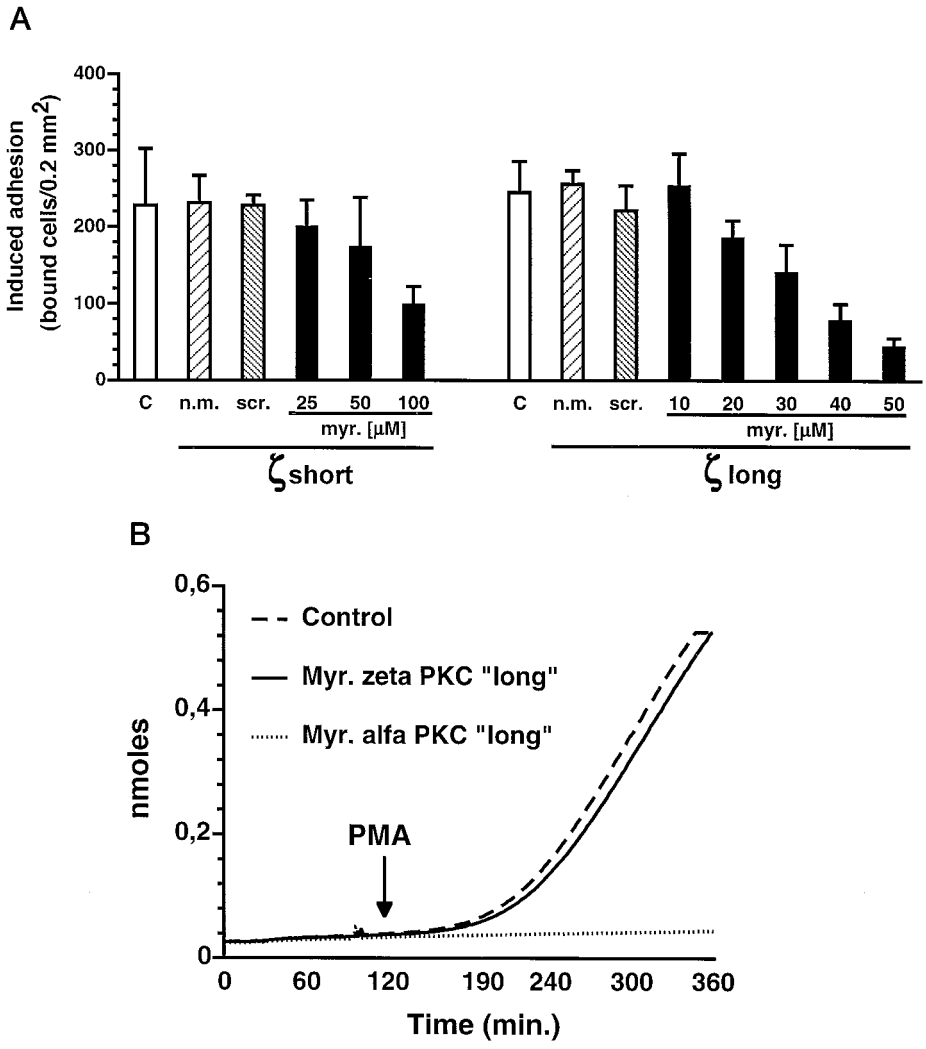

FIG. 5. **Effect of ζ PKC pseudosubstrate peptides on PMA-induced neutrophil adhesion and NADPH-oxidase activation.** *A*, human polymorphonuclear neutrophils were treated at 37 °C for 30–60 min with buffer (*C*, control), with the indicated concentrations of myristoylated peptides from the pseudosubstrate region of ζ PKC (*Myr. ζ short* and *long*), or with 100 μM (*ζ short*) or 50 μM (*ζ long*) of identical non-myristoylated peptides (*n.m.*) and myristoylated scrambled peptide (*scr.*). Adhesion was stimulated at 37 °C for 15 min with 35 nM PMA. Values are the mean counts of bound cells in 0.2 mm^2 in 4–6 experiments; *error bars* are S.D. values. Background binding in the absence of agonist was minimal (41 ± 8 cells/0.2 mm^2) and was subtracted to yield induced adhesion. *B*, human polymorphonuclear neutrophils were treated at 37 °C for 30 min with 50 μM of myristoylated peptide from the pseudosubstrate region of ζ PKC (*ζ long*) or 100 μM of myristoylated peptide from the pseudosubstrate region of α PKC (*α long*). Stimulation was under stirring at 37 °C with 35 nM PMA. Shown are time courses of released hydrogen peroxide.

CDC42) on asparagine 41 in the effector region of the GTPase (29). C3 transferase blocked chemoattractant as well as PMA-stimulated neutrophil adhesion (data not shown), as we previously reported (6). C3 transferase almost completely abolished fMLP-, IL-8-, and PMA-induced ζ PKC translocation to the light membrane fraction (Fig. 9). In contrast, translocation of α PKC triggered by chemoattractants or PMA was completely unaffected (Fig. 9). We conclude that ζ PKC plasma membrane targeting in response to chemoattractants or to phorbol esters is dependent on signaling through C3 transferase-sensitive Rho GTPases.

DISCUSSION

Chemoattractants stimulate a variety of polymorphonuclear neutrophil responses, including integrin activation, movement, exocytosis, superoxide anion release, and gene expression. The seven-transmembrane domain of chemoattractant receptors activates an amplified and branching cascade of second messengers through either α or βγ subunits of heterotrimeric GTP-binding proteins (30, 31). The aim of our study was to analyze the involvement of the serine-threonine protein kinase C in chemoattractant-induced signaling pathways leading to integrin-dependent leukocyte adhesion and chemotaxis. We focused on human polymorphonuclear neutrophils as they represent a model with direct pathophysiological implications. The following conclusions can be drawn from our data: (*a*) DAG-activable PKC isoforms are not involved in integrin-dependent neutrophil adhesion and motility triggered by chemoattractants; (*b*) ζ PKC, the only atypical, DAG-insensitive PKC isoform expressed in human neutrophils, is involved in signaling pathways triggered by proadhesive agonists and leading to adhesion and motility; (*c*) ζ PKC is involved in the signaling cascade controlling chemoattractant-induced actin polymerization; (*d*) a functional link between ζ PKC and Rho small GTPases involved in integrin triggering seems to exist.

Involvement of DAG-sensitive PKC as downstream effector to integrin activation has been previously suggested. Phorbol esters, at nanomolar concentrations, mimic in T cells the antigen receptor-induced adhesion strengthening process, and this latter is blocked by specific PKC inhibition. Moreover, either α and β subunits of lymphocyte function antigen type 1 (CD11a/CD18) have been shown to be phosphorylated upon agonist stimulation (32, 33) and the time course of phosphorylation correlates to the extent of cell-cell adhesion. Although data obtained by site-directed mutagenesis of the heterodimer (34) have raised doubts about the importance of PKC-induced integrin phosphorylation in adhesion triggering, PKC can phosphorylate cytoskeletal proteins known to associate with activated integrins, such as α-actinin (35) and talin (36), and this association is thought to affect the integrin avidity state. Note, however, that integrin triggering by T-cell antigen receptor as well as by PMA are very slow phenomena when compared with chemoattractant-induced integrin activation. Calphostin C, Gö6976, Gö6850, staurosporine, and α PKC myristoylated pseudosubstrate peptides, all inhibitors of DAG-sensitive

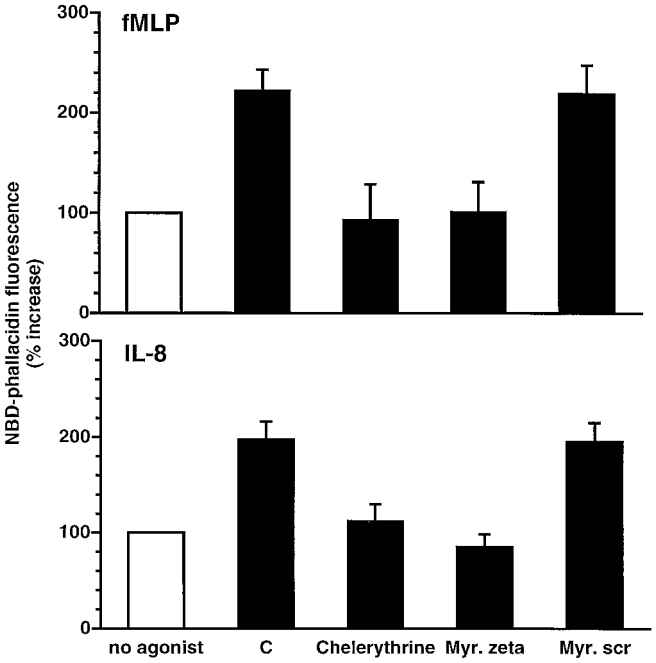

FIG. 6. **Effect of ζ PKC inhibition on chemoattractant-induced actin assembly.** Human polymorphonuclear neutrophils were treated at 37 °C for 30 min with buffer (*no agonist* and control (*C*)), with 5 μM chelerythrine chloride, or with 25 μM of myristoylated long peptide from the pseudosubstrate region of ζ PKC (*Myr. zeta*) or 25 μM of myristoylated scrambled peptide (*Myr. scr.*). Actin assembly was stimulated under stirring at 37 °C for 45 s with 10 nM fMLP or 1 nM IL-8. Values are the average of percentage increase over control in the absence of agonist in three experiments; *error bars* show S.D. values.

PKCs, were completely unable to block rapid integrin-dependent adhesion and chemotaxis induced by chemoattractants. Interestingly, Rho activation by chemoattractants, an essential signaling event to integrin activation, is independent of DAG-sensitive PKCs (6). Moreover, activation of these PKC isozymes by chemoattractant does not compensate cAMP-induced inhibition of chemoattractant-triggered Rho guanine nucleotide exchange (28). Thus, it seems that DAG-sensitive PKCs, although activated, *de facto* are neither necessary nor sufficient in the signaling machinery leading to rapid integrin activation and movement triggered in polymorphonuclear neutrophils by physiological stimuli such as chemoattractants.

Involvement of the atypical ζ PKC in signaling pathways leading to neutrophil adhesion and chemotaxis is suggested by three different lines of evidence. Chelerythrine chloride, previously shown to inhibit ζ PKC kinase activity, was the only inhibitor to effectively block chemoattractant-induced integrin activation and chemotaxis. Second, the data obtained with chelerythrine chloride were confirmed by the inhibitory activity of synthetic peptides derived from ζ PKC regulatory regions. An isozyme-selective sequence of 17 amino acids, mimicking the substrate but with a serine-threonine to alanine substitution that prevents phosphorylation, is present at the N terminus of the C1 domain of the regulatory region of PKC; in the absence of allosteric activators, this endogenous pseudosubstrate region maintains PKC in nonactive form. Synthetic peptides derived from this region, either microinjected or myristoylated, have been widely used in different cell types as potent and selective inhibitors of PKC phosphorylating activity (17, 37–41). Moreover, synthetic peptides from the pseudosubstrate region of ζ PKC have been successfully used, by microinjection, to block ζ PKC-dependent signal transduction in *Xenopus* oocytes and in mouse fibroblasts (42–44). Nonmyristoylated ζ PKC peptides, which presumably do not pass the plasma membrane, and myristoylated peptides with scrambled sequence did not block adhesion and chemotaxis triggering in polymorphonuclear neutrophils. Furthermore, myristoylated pseudosubstrate peptides from ζ PKC blocked PMA-induced integrin-dependent adhesion but not NADPH-oxidase activation. These data support the specificity of ζ PKC pseudosubstrate peptides and show that myristoylated peptides from ζ PKC can be a useful tool to investigate ζ PKC involvement in leukocyte functions. Finally, involvement of ζ PKC in signaling pathways leading to adhesion and chemotaxis correlates with the capability of fMLP and IL-8 to trigger an increase of ζ PKC activity as well as light membrane translocation. Notably, this is the first evidence of ζ PKC activation and translocation by a chemokine. These data are in agreement with recent studies showing that fMLP induces translocation of ζ PKC to the light membrane compartment in human neutrophils (45, 46). Moreover, analysis of intracellular distribution of ζ PKC after agonist stimulation confirmed that also PMA triggers light membrane translocation of ζ PKC. Together with the effect of ζ PKC peptides, this suggests that DAG-sensitive PKCs can activate atypical PKC-dependent signaling pathways. Interestingly, PMA did not increase ζ PKC activity, and chemoattractant-stimulated increase of ζ PKC activity was transient. Thus, ζ PKC light membrane translocation does not systematically correlate with the increase of its kinase activity. This suggests that light membrane targeting could be sufficient to allow ζ PKC constitutive kinase activity to trigger downstream effects. The light membrane compartment is normally identified with the cell plasma membrane (13). Plasma membrane translocation of proteins is often a conditioning step to enzyme activation. For instance, cytosolic components of the superoxide-generating NADPH-oxidase system in human neutrophils, $p47^{phox}$, $p67^{phox}$, and the small G-protein Rac translocate to the plasma membrane upon agonist stimulation, and this correlates with superoxide anion release (47). Thus, ζ PKC targeting to plasma membrane may represent, apart from increased kinase activity, an essential step in integrin and movement triggering by chemoattractants.

The capability of C3 transferase to block chemoattractant as well as PMA-triggered ζ PKC translocation suggests a functional interaction between the small GTP-binding protein Rho and ζ PKC. As for ζ PKC, signaling through the small GTP-binding protein Rho is also required for chemoattractant-induced leukocyte adhesion and chemotaxis (48) as well as for PMA-triggered adhesion. Rho and ζ PKC could participate in two independent pathways, each contributing to the final phenomenon. However, by analogy with the previously shown capability of the small GTPase Ras to interact with and to activate ζ PKC (49), we hypothesized a functional relationship between ζ PKC and Rho. This hypothesis was confirmed by C3 transferase-induced inhibition of ζ but not α PKC translocation triggered either by chemoattractants or PMA, suggesting that a functional Rho effector domain is critical to translocation of atypical but not classical PKC isozymes. Even if these observations need to be further investigated using different approaches, such as, for example, *in vitro* interaction between recombinant Rho and ζ PKC or cell line transfection with dominant negative Rho, our data suggest for the first time that ζ PKC could be a downstream effector of Rho signaling pathways leading to neutrophil integrin activation and movement triggered by G-protein linked chemoattractant receptors. Moreover, they suggest that Rho small GTPases link DAG-sensitive PKC to translocation of atypical isozymes and that PMA-triggered integrin-dependent adhesion requires Rho-mediated ζ PKC translocation.

The inhibitory effect of chelerythrine chloride and ζ PKC myristoylated pseudosubstrate peptides on chemoattractant-triggered F-actin accumulation suggests the involvement of this atypical PKC isozyme in the cascade of intracellular events controlling globular actin assembly. The small GTPase Rho has

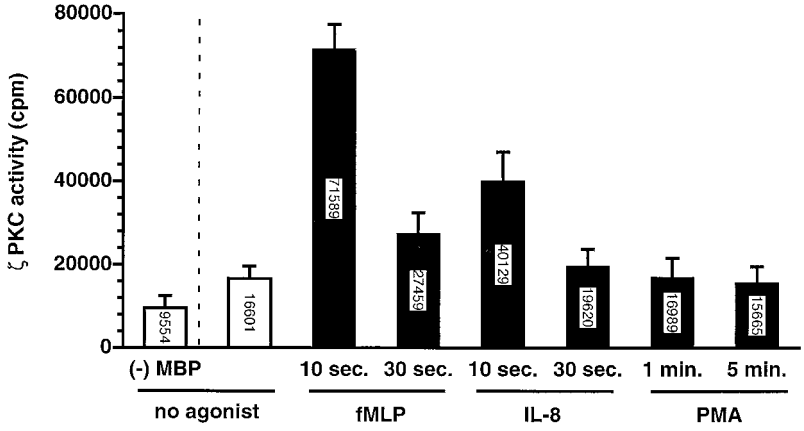

FIG. 7. **Chemoattractant-induced increase of ζ PKC activity.** Human polymorphonuclear neutrophils were stimulated with buffer (no agonist) or with fMLP (100 nM), IL-8 (10 nM), or PMA (150 nM) for the indicated times. *No agonist* (−) myelin basic protein (*MBP*) represents radioactivity in the absence of exogenous substrate and is a measurement of ζ PKC autophosphorylating activity in nonstimulated neutrophils. Values are the mean counts of three experiments. *Error bars* show S.D. values. Background radioactivity, in the absence of immunoprecipitated ζ PKC, was 7467 ± 880 and was subtracted.

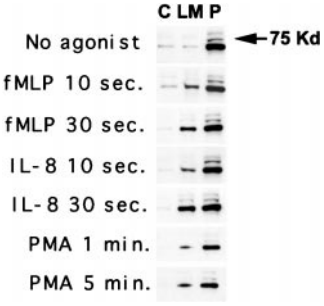

FIG. 8. **Translocation of ζ PKC to the plasma membrane.** Human polymorphonuclear neutrophils were stimulated with buffer (*no agonist*) or with fMLP (100 nM), IL-8 (10 nM), or PMA (150 nM) for the indicated times. Shown are protein immunoblots of cytosolic (*C*), light membrane (*LM*), and particulate (*P*) fractions separated on sucrose gradient and probed with anti-ζ PKC antibody.

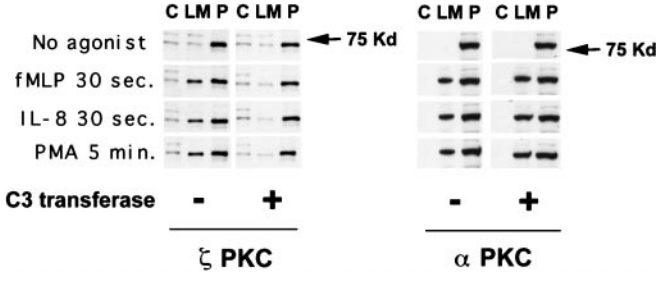

FIG. 9. **Effect of C3 transferase on ζ PKC translocation to the plasma membrane.** Human polymorphonuclear neutrophils were treated with control medium (−) or C3 transferase (+) and stimulated with buffer (*no agonist*) or with fMLP (100 nM) or IL-8 (10 nM) for 30 s or with PMA (150 nM) for 5 min. Shown are protein immunoblots of cytosolic (*C*), light membrane (*LM*), and particulate (*P*) fractions probed with anti-ζ PKC or anti-α PKC antibodies.

been suggested to control basal more than agonist-triggered actin polymerization in HL-60 cells (50). Moreover, in Swiss 3T3 fibroblasts, Rho has been shown to trigger stress fiber assembly by the bundling of actin filaments (51), and in the same cell line, Rho-kinase, a Rho downstream effector, induces actin polymerization to a very small extent (52). Finally, in cell-free system, Rho seems unable to activate F-actin formation (53). However, other reports showed that Rho is responsible for *de novo* actin polymerization in neutrophils (48), Vero cells (54), and mast cells (55). Thus, it is possible that Rho controls actin polymerization in a cell-dependent manner, and as for adhesion triggering, it is tempting to speculate that ζ PKC could mediate Rho-induced actin polymerization. Moreover, ζ PKC is likely to cooperate with other signaling molecules, such as, for example, the small GTPase CDC42, recently shown to be able to trigger actin assembly in cell free system (53).

The ζ isozyme of PKC has previously been implicated in mitogenic signal transduction (42–44). More recently, it has been shown to associate with the actin cytoskeleton (56) and has been hypothesized to be involved in cytoskeleton rearrangement induced by cytokines (57). Thus, ζ PKC involvement in signaling pathways leading to cytoskeleton rearrangement and cell motility is not totally unexpected. Interestingly, in resting neutrophils, ζ as well as α PKCs are mainly detected in the insoluble cellular fraction. This could suggest that in neutrophils α and ζ PKC are constitutively associated with some cytoskeletal elements, perhaps intermediate filaments, as also previously suggested for β PKC (58, 59). The ability of C3 transferase to block ζ PKC but not α PKC translocation to the membrane compartment suggests an isozyme-specific interaction between Rho and PKC signaling pathways. ζ PKC kinase activity, although constitutively high (25, 26), can be increased by lipidic second messengers, such as phosphatidylinositol 3,4,5-phosphate (60) a product of phosphatidylinositol 3-OH kinase. Moreover, wortmannin, a specific phosphatidylinositol 3-kinase inhibitor (61), has been previously reported to inhibit Rho-dependent ζ PKC activation induced by interleukin-2 (57) as well as ζ PKC nuclear translocation during ischemia (62). Thus, it is possible that phosphatidylinositol 3-kinase activity, previously shown to be activated also by C3 transferase-sensitive Rho GTPases (63, 64), mediates Rho-dependent ζ PKC translocation induced by proadhesive agonists. However, preliminary results show that wortmannin as well as LY294002, another specific phosphatidylinositol 3-kinase inhibitor (65), do not block chemoattractant as well as PMA-induced ζ PKC translocation.[2] This suggests that phosphatidylinositol 3-kinase activity is not essential to ζ PKC translocation triggered by chemoattractants or PMA.

In conclusion, we provide evidence suggesting the involvement of ζ PKC in the regulation of neutrophil cytoskeleton, integrin-dependent adhesion, and chemotaxis and the possibility that ζ PKC could be a novel downstream effector of Rho signaling pathways leading to leukocyte integrin activation and motility.

[2] C. Laudanna, G. Constantin, and E. C. Butcher, manuscript in preparation.

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