Protein Phosphatase Inhibitors Okadaic Acid and Calyculin A Alter Cell Shape and F-Actin Distribution and Inhibit Stimulus-Dependent Increases in Cytoskeletal Actin of Human Neutrophils

By Peter Kreienbühl, Hansuli Keller, and Verena Niggli

PHOSPHORYLATION and dephosphorylation reactions play a major role in regulating biologic responses. This applies also to leukocyte activation. Activators and inhibitors of protein kinases, particularly protein kinase C (PKC), have the capacity to elicit or modify various leukocyte responses, including leukocyte motility. These modifications of neutrophil functions are associated with increased actin polymerization in response to activators or inhibitors of PKC and accumulation of F-actin in newly formed pseudopods. Cytoskeletal proteins have been shown to be targets of protein kinases, especially PKC. Smooth muscle myosin light chain kinase, as well as myosin light chain from human platelets are phosphorylated by PKC and the reaction can be induced by phorbol esters. Other cytoskeletal protein kinase substrates include myotube intermediate filaments such as desmin and vimentin, vinculin, talin, and profilin.

Chemoattractants were also found to increase myosin phosphorylation in Dictyostelium and phosphorylation changes correlated temporally with cyclic adenosine monophosphate (cAMP)-induced cell shape changes. A 47,000-d protein derived from human platelets may modulate actin polymerization through phosphorylation, as shown in vitro, and PKC-mediated phosphorylation of tau was found to reduce its ability to promote tubulin polymerization and to cross-link actin filaments. Shape changes, motility, and actin polymerization may thus be controlled by protein kinases via regulation of different cytoskeletal proteins that are involved in actin filament contraction, membrane linkage, and control of polymerization.

Little is known regarding the signals involved in eliciting increased actin polymerization in neutrophils upon activation with chemotactic peptide. Direct activation of PKC by phorbol ester has been shown to stimulate actin polymerization. We have recently found that the potent but unspecific protein kinase inhibitor staurosporine increases the level of actin associated with the Triton X-100-insoluble cytoskeleton in neutrophils, similar to the reaction caused by phorbol ester, whereas a derivative more specific for PKC was inactive. The latter inhibitor did not prevent the chemotactic peptide-induced increase in cytoskeletal actin. In summary, the results available so far suggest that increased actin polymerization can be elicited via at least three different pathways: (1) activation of neutrophils by chemotactic peptides via an unknown PKC-independent pathway; (2) direct activation of PKC by phorbol ester; and (3) inhibition of the basal activity of an as yet unidentified staurosporine-sensitive enzyme not identical with PKC.

Okadaic acid has been suggested as a new probe for the study of cellular regulation. Okadaic acid is a polyether fatty acid isolated from the marine sponges Halichondra Okadaia and Halichondria melanodocia. Okadaic acid inhibits preferentially protein phosphatase 2A at lower concentrations and also, at higher concentrations, phosphatase 1. It thus blocks the dephosphorylation of protein kinase substrates, thereby increasing the amount of actual phosphate bound to protein. Thus, okadaic acid may become a useful tool in analyzing the role of protein phosphorylation in relation to biologic activity. We show here that okadaic acid alone has a number of biologic effects on leukocytes and is also capable of modifying the effects of leukocyte activators such as chemotactic peptides or phorbol esters.

To complete our studies, we have in addition used another phosphatase inhibitor, calyculin A (structurally unrelated to okadaic acid), which inhibits in vitro both phosphatase 1 and 2A at nanomolar concentrations with equal potency. Calyculin A affected neutrophil functions in the same way as okadaic acid, but at 50-fold lower concentrations. A control substance, methyl okadaate, which is inactive on localization of resting cells. Inhibitors alone had no significant effect on the amount of cytoskeleton-associated actin. The increase in cytoskeletal actin observed at 30 minutes of stimulation with phorbol ester or 5 to 30 minutes of stimulation with chemotactic peptide, however, was abolished by okadaic acid or calyculin A, suggesting an important role of a phosphatase. In contrast, the early increase in cytoskeleton-associated actin observed at 1 minute of stimulation with peptide was not affected. This finding indicates that the increased association of actin with the cytoskeleton in the early and the later stages of neutrophil activation may be mediated by different signalling pathways.
phosphatases in vitro, had, in contrast, no significant effects at 1 μmol/L.

**MATERIALS AND METHODS**

Reagents and suppliers were as follows: N-formyl-L-norleucyl-L-leucyl-L-phenylalaninyl-L-norleucyl-L-tyrosyl-L-lysine (fNLPNTL; Bachem, Bubendorf, Switzerland); human serum albumin (HSA; Behringwerke, Marburg, Germany); Metrizoate (Nycomed AS, Oslo, Norway); Methocel (Pro Chem AG, Zürich, Switzerland); neutrophil isolation medium (Los Alamitos Diagnostics, Los Alamitos, NM); okadaic acid (Moana Bioproducts, Honolulu, Hawaii); calyculin A and methyl okadaate (LC Services Corporation, Woburn, MA); phorbol 12-myristate 13-acetate (PMA), fluorescein isothiocyanate-dextran (FITC-dextran, FD-70), paraformaldehyde and lyssolecithin (L-α-hydrophosphatidylcholine) (Sigma Chemical Corp, St Louis, MO); N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl) phallacidin (NBD-phallacidin; Molecular Probes Inc, Junction City, OR); HEPES and glutaraldehyde (Serva Feinbiochemica, Heidelberg, Germany); dimethylsulfoxide (DMSO) and diisopropylfluorophosphate (in aqueous solution, HC1-free, carrier-free) (Amersham, Buckinghamshire, UK); Triton X-100 (a 10% solution in H2O, stored under nitrogen) (Pierce, Rockford, IL). Water-insoluble compounds were dissolved in DMSO. The corresponding amount of DMSO was always included in the controls. Concentrations of DMSO up to 0.4%, the maximal amount used, did not affect neutrophil functions. Moreover, calyculin A had the same effects on neutrophils that okadaic acid had, but at a 50-fold lower concentration, in the presence of only 0.02% to 0.04% DMSO. The basic medium (pH 7.2) was prepared as follows: 138 mmol/L NaCl, 6 mmol/L KCl, 20 mmol/L HEPES, pH 7.4, 10 mmol/L NaHCO3, 5 mmol/L NaHPO4, 5.5 mmol/L glucose, 20 mmol/L HEPES, 2% (wt/vol) HSA. EGTA was included to minimize cell aggregation and adhesion. The omission of EGTA did not affect our results.

**Cell preparation.** Human blood was obtained by venepuncture from healthy volunteers and heparinized (10 U/mL). Neutrophil granulocytes were separated in a first step with Metrizoate-Methocel 29 and in a second step with neutrophil isolation medium.30 The cells (97% to 100% neutrophils) were then washed and resuspended in basic medium.

**Shape change and fluid pinocytosis assays.** The cell suspension (10⁶ cells/mL in basic medium) was preincubated in a reciprocating water bath in plastic tubes with FITC-dextran (5 mg FITC-dextran/mL cell suspension) and with 0.4% DMSO or okadaic acid at the concentrations indicated at 37°C for 10 minutes. Incubation was then continued with or without additional stimuli at uniform concentrations for 30 minutes. Under these conditions, cell adhesion to the tubes was minimal, as 93% ± 8% (mean ± SD of 3 experiments) of the cells were recovered from the plastic tubes after 40 minutes at 37°C in the shaking water bath. The reaction was terminated by fixation in 1% glutaraldehyde (vol/vol; final concentration) at 37°C for 30 minutes. Fixed cells were washed five times and resuspended in phosphate-buffered saline (PBS) containing 1 mg NaN₃/mL. The net uptake of FITC-dextran was assessed by means of flow cytometry (Epics Profile II; Coulter Corporation, Hialeah, FL). The mean channel was determined.

Cell shape of the fixed cells was determined by Sykes-Morse chambers using a Zeiss IM 35 microscope (X 100 oil immersion objective) and differential interference contrast microscopy (Nornaski Optics) (Carl Zeiss, Oberkochen, Germany). Photographs were taken using a Kodak T Max 400 film (Eastman Kodak, Rochester, NY).

The criteria for the classification of neutrophil shape (200 cells/sample) have been described previously. Briefly, we distinguished between the following types of shapes. (1) Spherical neutrophils were unstimulated control cells characterized by a circular contour and a smooth surface. (2) Spherical neutrophils with unifocal projections were basically spherical cells with a smooth surface and a circular outline, except for an unifocal projection on one side. (3) Polarized cells were elongated cells showing front-tail polarity. (4) Nonpolar cells with surface projections were cells without morphologic front-tail polarity, but with numerous major projections distributed all over the cell surface. In the present study two subtypes have been distinguished. One is the type observed after stimulation with PMA or diacylglycerols characterized by multiple surface lamella, which often show a pointed end. The other shape type, the shape produced by okadaic acid or calyculin A, is new. The surface projections have a more wavy appearance. Shape changes in living cells after stimulation were recorded by videomicroscopy within the first 50 minutes.

**The localization of F-actin by staining with NBD-phallacidin.** The intracellular localization of F-actin was determined by NBD-phallacidin staining of paraformaldehyde-fixed cells, as previously described by Howard and Meyer,31 with modifications as detailed in Roos et al.3

**Determination of cytoskeleton-associated actin.** Cells were resuspended in basic medium without HSA or EGTA, pH 7.4. To block endogenous proteases, the isolated neutrophils were preincubated with diisopropylfluorophosphatase32 before the assays, as previously described.30 The amount of cytoskeletal actin, as the percentage of actin insoluble in 1% Triton X-100, was determined as described previously,33 with small modifications.3 Briefly, aliquots (450 μL) of the cell suspension were incubated in the presence of 10 mmol/L EDTA and with 0.4% DMSO or phosphatase inhibitors (stock solutions in DMSO). Stimuli were added in 10 μL medium to the cell suspension of 450 μL. EDTA was added to minimize cell aggregation and adhesion and to block proteolysis after solubilization. The omission of EDTA did not alter the results. The adhesion of cells to Eppendorf tubes was minimal, as 94% ± 6% (mean ± SD, n = 3) of cells could be recovered from the tubes after 40 minutes of incubation at 37°C in the presence of 10⁻⁹ mol/L INLNPNTL. For the quantification of cytoskeletal actin, the reaction was stopped by the addition of 500 μL of a twofold-concentrated ice-cold lysis buffer containing 2% Triton X-100, 160 mmol/L KCl, 20 mmol/L Tris, pH 7.5, 20 mmol/L EDTA, and 0.02% NaN₃. The tubes were incubated on ice for 10 minutes, followed by centrifugation and washing of the pellets as described.3 The amount of actin in pellets and supernatants was analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) as detailed in Niggli and Keller.30 Differences between data were analyzed with the Student's t-test for paired data, with a P value of <.05 considered significant.

**Protein phosphorylation in intact neutrophils.** Neutrophils were isolated, treated with diisopropylfluorophosphate (see previous section) and washed several times with a phosphate-free buffer (138 mmol/L NaCl, 6 mmol/L KCl, 20 mmol/L HEPES, pH 7.4, 5.5 mmol/L glucose). The cells were resuspended in this buffer (20 × 10⁶ cells/mL) and were incubated with 0.5 mCi of [32P]orthophosphate/mL for 1 hour at 37°C in a shaking water bath. At the end of the incubation period, the cells were washed twice with a 0.5-fold volume of the above buffer, and then resuspended in this buffer containing, in addition, 10 mmol/L EDTA (3 × 10⁶ cells/mL). The omission of EDTA did not alter our results. The cells were subsequently exposed to 0.4% DMSO or phosphatase inhibitors and stimuli as described in the previous section. The reaction was stopped by the precipitation of protein with trichloroacetic acid as described.30 The precipitates were electrophoresed through 7.5% to 20% gradient gels, followed by autoradiography.
RESULTS

Effect of phosphatase inhibitors on protein phosphorylation in intact neutrophils. We were interested in obtaining information on the role of phosphatases in neutrophil functions. It was important to know whether okadaic acid, which inhibits phosphatases 2A and 1 in vitro, is also active in intact neutrophils. Therefore, we have analyzed the effect of okadaic acid on protein phosphorylation in neutrophils preloaded with $^{32}$PO$_4$. Incubation of cells with $10^{-8}$ mol/L PMA for 5 minutes resulted in increased incorporation of $^{32}$PO$_4$ into several proteins, among them bands of 47 Kd and 70 Kd (Fig 1, lane 2). PMA also induced dephosphorylation of a 20-Kd band. These findings are comparable to results previously obtained in human neutrophils. Incubation of the cells with $10^{-9}$ mol/L fNLPNTL for 5 minutes resulted mainly in increased phosphorylation of the 70-Kd band and dephosphorylation of the 20-Kd band (Fig 1, lane 3). Preincubation of cells for 30 minutes with 1 μmol/L okadaic acid, followed by a further incubation either with medium or PMA or fNLPNTL, resulted in a marked increase in incorporation of $^{32}$PO$_4$ into at least 24 bands (Fig 1, lanes 4 through 6). For example, in the experiment shown in Fig 1, incorporation of $^{32}$PO$_4$ into major phosphoproteins of 80 to 90 Kd was stimulated twofold by okadaic acid, and into bands of 52 to 54 Kd fivefold. Comparable results have been found recently for electroporpermeabilized human neutrophils equilibrated with [32P]ATP and 2 μmol/L okadaic acid. Apparently, kinases are constitutively active in resting cells, and phosphatases control phosphorylation of a variety of substrates. We have also studied the time course of the effects of okadaic acid on protein phosphorylation in resting cells. Increases in phosphorylation occurred slowly. The first effects were observed 15 minutes after the addition of the inhibitor (1.3-±0.2-fold increase in phosphorylation of the 52 to 54-Kd band). Phosphorylation of this band was 1.7-±0.3-fold increased after 20 minutes, 2.6-±0.5-fold after 30 minutes, and 3.8-±0.7-fold after 40 minutes (mean ± SD, n=3 to 4). Phosphorylation was still increasing after 40 minutes. This slow time course may at least partly be due to slow penetration of okadaic acid into the cells, as 2 μmol/L okadaic acid has been shown to induce in electroporpermeabilized human neutrophils a 1.5-fold increase in phosphorylation of a 59-Kd band within 3 minutes. Calyculin A at 0.02 μmol/L markedly increased phosphorylation of the same bands, just as okadaic acid did (6- to 7-fold increase in phosphorylation of the 52- to 54-Kd band after 40 minutes).

Figure 1 thus shows that okadaic acid can enter the neutrophils and is active on cellular phosphatases. Questions on specific protein phosphatase substrates possibly involved in the effects of okadaic acid on neutrophils described below will be addressed in future studies.

Shape changes induced by okadaic acid and calyculin A. Okadaic acid produced dose-dependent shape changes at concentrations of 0.5 μmol/L and higher. Almost 100% of the cells showed a nonpolar configuration at 1 μmol/L. Nonpolar cells with surface projections induced by okadaic acid were different from those observed in nonpolar cells induced by PMA (Fig 2). Okadaic acid-induced projections were less pointed and more wavelike. This morphologic difference is so characteristic that one can tell on the basis of the morphologic alterations whether okadaic acid was present in active concentrations. The dose-response curve is shown in Fig 3A. Furthermore, okadaic acid was found to suppress front-tail polarity induced by $10^{-7}$ mol/L fNLPNTL in a dose-dependent fashion (Fig 3B). Cells treated with $10^{-9}$ mol/L fNLPNTL and 1 or 2 μmol/L okadaic acid showed a similar morphology as cells treated with the respective concentrations of okadaic acid alone (Fig 2B and B+). The cells consisted almost completely of nonpolar cells with surface projections. Neutrophils stimulated with $10^{-9}$ mol/L PMA plus increasing concentrations of okadaic acid were mainly nonpolar with surface projections (Fig 3C). However, in the absence or at low concentrations of okadaic acid (<0.5 μmol/L), the cells showed the morphology characteristic for PMA, whereas at high concentrations (1 and 2 μmol/L), the morphology was very similar.

[Image of autoradiogram]
Fig 2. Effect of okadaic acid on neutrophil morphology. Shape changes of human neutrophils after stimulation in suspension with 1 μmol/L okadaic acid, 10^{-8} mol/L fNLPNTL, or 10^{-9} mol/L PMA. Neutrophils were incubated in basic medium (containing 1.1 mmol/L EGTA and 2% HSA) with the following additions: (A) control in basic medium with 0.4% DMSO; (A+) 1 μmol/L okadaic acid; (B) 0.4% DMSO + 10^{-9} mol/L fNLPNTL; (B+) 1 μmol/L okadaic acid + 10^{-9} mol/L fNLPNTL; (C) 0.4% DMSO + 10^{-9} mol/L PMA; (C+) 1 μmol/L okadaic acid + 10^{-9} mol/L PMA. Cells were preincubated at 37°C for 10 minutes with 0.4% DMSO or with 1 μmol/L okadaic acid, followed by incubation for another 30 minutes with or without 10^{-9} mol/L fNLPNTL or 10^{-9} mol/L PMA. The total incubation time for all samples was 40 minutes. The cells were subsequently fixed for 30 minutes with glutaraldehyde and washed. Photographs were taken using Nomarski Optics. Scale bar, 10 μm.

to that observed with okadaic acid alone (Fig 2C and C+). Calyculin A had effects on cell morphology comparable to those of okadaic acid, but at 50- to 100-fold lower concentrations (Fig 4F). Half-maximal effects of calyculin A on cell shape occurred at 0.010 ± 0.003 μmol/L (mean ± SD, n = 5). Up to 100% of the cells showed a nonpolar morphology similar to that induced by okadaic acid, at 0.02 μmol/L calyculin A. As shown for okadaic acid (Fig 2), preincubation of cells with calyculin A before the addition of PMA or fNLPNTL also induced the morphology typical for calyculin A alone. The control substance methyl okadaate, which does not inhibit phosphatases in vitro,28 did not significantly affect cell morphology at 1 μmol/L. At high concentrations of okadaic acid (>2 μmol/L) or calyculin A (>0.02 μmol/L), neutrophils assumed a multilobed appearance, because of the formation of multiple small and large bleb-like protrusions (not shown).

The time course of the response has been studied by continuous videorecording of neutrophils stimulated with 1 μmol/L okadaic acid. The typical morphologic changes developed after about 15 minutes of stimulation. Comparable results were obtained with cells incubated in plastic tubes and fixed after different time points; half-maximal effects were observed at 15 to 20 minutes and maximal effects were observed at 20 to 25 minutes of incubation with okadaic acid. The shape changes performed by individual cells were so slow that they could not be detected without time-lapse recording. Thus, the shape changes induced by okadaic acid are much slower than those induced by chemotactic peptides or PMA. The time course of appearance of shape changes correlates with the slow increase in

Fig 3. Concentration-dependent effects of okadaic acid on neutrophil shape changes. (○–○) Spherical cells; (■–■) nonpolar cells with surface projections; (▲–▲) polarized cells. Neutrophils were incubated in basic medium (containing 1.1 mmol/L EGTA and 2% HSA) with increasing concentrations of okadaic acid alone at 37°C for 40 minutes (A), or with okadaic acid for 10 minutes and then incubation was continued for another 30 minutes after the addition of 10^{-8} mol/L fNLPNTL (B), or with okadaic acid for 10 minutes and then incubation was continued for another 30 minutes after the addition of 10^{-9} mol/L PMA (C). DMSO was always 0.4% (final concentration). Controls without okadaic acid were incubated for 40 minutes with 0.4% DMSO, without or with subsequent addition of PMA or fNLPNTL. At the end of the incubation period of a total of 40 minutes, cells were fixed with glutaraldehyde and examined using Nomarski Optics. Values are the mean ± SD of five experiments.
protein phosphorylation observed in the presence of okadaic acid (see above).

Reorganization of F-actin induced by okadaic acid and calyculin A. Unstimulated neutrophils show a diffuse cytoplasmic distribution of F-actin. After stimulation with 1 or 2 μmol/L okadaic acid, or with 0.01 to 0.02 μmol/L calyculin A, for 40 minutes, neutrophils showed a shift of F-actin, which now appeared to be associated with the cell outline (Fig 4B, b, F, and f). Close examination of the cells showed that occasional fluorescence over the cell body was actually associated with plasma membrane projections appearing in the focal plane (Fig 4B and b). This contrasts with the pictures obtained with PMA in which F-actin accumulated in distinct thin surface lamella (Fig 4C and c). Combined stimulation of neutrophils with PMA and phosphatase inhibitors, or fNLPNTL and phosphatase inhibitors, resulted in an F-actin distribution similar to that observed with inhibitors alone (Fig 4D, d, E, and e). Incubation of the cells for 30 to 40 minutes with 1 μmol/L okadaic acid or 0.02 μmol/L calyculin A, concentrations that had maximal
effects on cell morphology (Fig 3), did not significantly affect the level of cytoskeleton-associated actin (Tables 1 and 2).

Effect of okadaic acid on fluid pinocytosis (net uptake of FITC-dextran). PMA (10^(-9) mol/L) alone produces a marked stimulation of uptake of FITC-dextran. Okadaic acid alone had relatively little effect. It produced a marginal dose-dependent increase in fluid pinocytosis up to almost twofold the activity of the control cells. The stimulating activity of fNLPNTL or 10^-9 mol/L PMA on pinocytosis was not significantly modified by increasing concentrations of okadaic acid up to 2 μmol/L (Fig 5).

Effect of okadaic acid and calyculin A on the stimulus-dependent association of actin with the cytoskeleton. fNLPNTL (10^-4 mol/L) induced a rapid transient increase in cytoskeletal actin, followed by a decrease (Tables 1 and 2). This time course is comparable with previously published data obtained with the same technique.33 The chemotactic peptide-dependent increase in cytoskeletal actin also correlates with a decrease in G-actin, and an increase in F-actin as determined with an NBD-phallacidin-binding assay.11,36-38

After 30 minutes, the percentage of cytoskeletal actin was lower than after 1 minute, but it was still significantly increased above controls (Tables 1 and 2, P < 0.005; see also Niggli and Keller10 Fig 4). A comparable small increase in F-actin persisting for at least 20 minutes after the addition of stimulus has also been reported by investigators using the NBD-phallacidin assay.36,38 This persisting increase is not due to activation of cells by adhesion to plastic tubes as a result of long-term incubation, for the following reasons. Adhesion is certainly not a major event under our conditions, as 94% ± 6% (mean ± SD, n = 3) of the cells could be recovered from the tubes after 40 minutes of incubation in plastic tubes. Moreover, we analyzed in separate experi-

Table 1. Effect of Okadaic Acid on the Association of Actin With the Neutrophil Cytoskeleton

<table>
<thead>
<tr>
<th>First Addition</th>
<th>Second Addition</th>
<th>Cytoskeletal Actin (relative to control)</th>
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<tbody>
<tr>
<td>Experiment 1 (10-min preincubation)</td>
<td></td>
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<tr>
<td>None</td>
<td>None (30 min)</td>
<td>1.00 ± 0.19</td>
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<tr>
<td>1 μmol/L OA</td>
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<tr>
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<tr>
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<td>3.12 ± 0.86</td>
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<tr>
<td>1 μmol/L OA</td>
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<td>0.89 ± 0.21</td>
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<tr>
<td>None</td>
<td>10^-4 mol/L PMA (30 min)</td>
<td>2.19 ± 0.61</td>
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<tr>
<td>1 μmol/L OA</td>
<td>10^-4 mol/L PMA (30 min)</td>
<td>0.99 ± 0.21</td>
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<tr>
<td>Experiment 2 (30-min preincubation)</td>
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<tr>
<td>None</td>
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<tr>
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<tr>
<td>1 μmol/L OA</td>
<td>10^-4 mol/L fNLPNTL (5 min)</td>
<td>1.02 ± 0.30</td>
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</table>

Neutrophils were preincubated for 5 minutes with EDTA (10 mmol/L, final concentration), followed by a further preincubation with DMSO (0.4%, final concentration) or okadaic acid (first addition) for 10 (experiment 1) or 30 minutes (experiment 2). Subsequently, basic medium, fNLPNTL, or PMA was added (second addition) and the incubation was continued for the time specified. Cytoskeletal actin was then determined as described in Materials and Methods (mean ± SD of three independent experiments). In control cells incubated with DMSO, 7% to 15% of the total cellular actin was associated with the cytoskeleton.

Abbreviation: OA, okadaic acid.

Table 2. Effects of Calyculin A and Methyl Okadaate on the Association of Actin With the Neutrophil Cytoskeleton

<table>
<thead>
<tr>
<th>First Addition</th>
<th>Second Addition</th>
<th>Cytoskeletal Actin (relative to control)</th>
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</thead>
<tbody>
<tr>
<td>None</td>
<td>None (30 min)</td>
<td>1.00 ± 0.44 (n = 5)</td>
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<tr>
<td>0.02 μmol/L CA</td>
<td>None (30 min)</td>
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<tr>
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<td>0.02 μmol/L CA</td>
<td>10^-6 mol/L fNLPNTL (30 min)</td>
<td>1.01 ± 0.25 (n = 5)</td>
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<td>10^-6 mol/L PMA (30 min)</td>
<td>2.29 ± 0.83 (n = 4)</td>
</tr>
<tr>
<td>0.02 μmol/L CA</td>
<td>10^-6 mol/L PMA (30 min)</td>
<td>0.94 ± 0.23 (n = 4)</td>
</tr>
<tr>
<td>1 μmol/L MOA</td>
<td>10^-6 mol/L PMA (30 min)</td>
<td>2.25 ± 0.61 (n = 4)</td>
</tr>
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</table>

Neutrophils were preincubated in the presence of EDTA (10 mmol/L) with either DMSO (0.2%, final concentration) or CA, or MOA (first addition). Preincubation time was 10 minutes for all samples, except for the samples subsequently incubated for 1 minute with fNLPNTL. The latter two samples were preincubated with CA or DMSO for 30 minutes. Subsequently, basic medium, fNLPNTL, or PMA was added (second addition) and the incubation was continued for the time specified. Cytoskeletal actin was then determined as described in Materials and Methods (mean ± SD). In control cells incubated with DMSO, 6% to 17% of the total cellular actin was associated with the cytoskeleton.

Abbreviations: CA, calyculin A; MOA, methyl okadaate.

Fig 5. Effect of okadaic acid on fluid pinocytosis. Fluid pinocytosis was determined by flow cytometry. Cells were stimulated with increasing concentrations of okadaic acid alone (8), okadaic acid and 10^-5 mol/L fNLPNTL (A), or okadaic acid and 10^-5 mol/L PMA (B). Neutrophils were incubated with 0.4% DMSO or with increasing concentrations of okadaic acid (first stimulus) at 37°C for a total of 40 minutes. DMSO was always 0.4% (final concentration). After 10 minutes of incubation with DMSO or with okadaic acid, the second stimulus was added and incubation was continued for another 30 minutes. Cells were fixed in glutaraldehyde and the relative fluorescence intensity was determined using flow cytometry. Values are the mean ± SDM of five experiments.
ments the amount of cytoskeletal actin in only those cells, which were removable from the tubes after 40 minutes of incubation at 37°C. These cells, incubated for 30 minutes with 10⁻⁹ mol/L fNLPNTL, still showed a 1.7-±0.4-fold (mean ± SD, n = 3) increase in cytoskeletal actin compared with controls (P < .05). The latter experiment has been performed in the presence of less than 0.001% DMSO. These results show that this persistent increase in cytoskeletal actin is due neither to adhesion nor to the presence of DMSO.

The effect of 1 μmol/L okadaic acid on cytoskeletal actin was studied using either 10 or 30 minutes of preincubation with the inhibitor before the addition of stimulus. The latter conditions were used especially for subsequent short-time incubation with stimuli, to ensure maximal entry of okadaic acid into the cells. Preincubation of cells for 10 or 30 minutes with okadaic acid had no significant effect on the peptide-induced rapid initial increase in cytoskeletal actin (1 minute), but inhibited the increase obtained at 5 and 30 minutes after addition of fNLPNTL by at least 80% (Table 1, P < .05). Preincubation of cells with okadaic acid also completely prevented the PMA-induced increase in cytoskeletal actin (Table 1, P < .025). Comparable results were obtained with 0.02 μmol/L calyculin A (Table 2). Calyculin A inhibited the increase in cytoskeletal actin induced by 30 minutes of incubation with fNLPNTL by at least 75% (P < .0025) and that by PMA by at least 88% (P < .0125). In contrast, the initial increase at 1 minute was not significantly affected (0% to 14% inhibition). Longer preincubation times up to 40 minutes with calyculin A or okadaic acid did not result in inhibition of the early event (not shown). The inactive control substance methyl okadaate did not significantly affect the increase induced, for instance, by PMA (Table 2).

Thus, okadaic acid and calyculin A counteract the increases in cytoskeletal actin induced in neutrophils by either 30 minutes of incubation with PMA or long-time (5 to 30 minutes) incubation with fNLPNTL. However, the peptide-dependent rapid initial increase in cytoskeletal actin appears to be refractory to the action of phosphatase inhibitors. The concentrations used had maximal effects on cell morphology of resting cells, and completely prevented fNLPNTL-induced development of cell polarity (Figs 2 through 4).

DISCUSSION

Incubation of human neutrophils with micromolar concentrations of okadaic acid or nanomolar concentrations of calyculin A alone results in characteristic shape change and shift of F-actin to the plasma membrane, whereas the level of cytoskeletal actin is not significantly altered. This is in contrast to findings with chemotactic peptide or PMA, in which altered shape is associated with a marked increase in cytoskeletal actin and F-actin. Shape changes and membrane association of F-actin may thus occur without an increase in cytoskeleton-associated actin. However, it is important to note that the shape changes induced by okadaic acid are very slow and are much slower than those induced by fNLPNTL or PMA. Furthermore, we cannot exclude that okadaic acid acts by increasing the turnover of actin filaments without increasing the level of cytoskeletal actin. Endotoxin has also been reported to induce changes in shape and F-actin localization in neutrophils without changing the level of F-actin.39

Okadaic acid and calyculin A are thought to act by inhibiting protein phosphatases, thereby increasing net phosphate incorporation into proteins. Indeed, both inhibitors have been shown to markedly stimulate overall levels of protein phosphorylation in intact cells and in permeabilized neutrophils.20,35,40 We found that micromolar concentrations of okadaic acid (Fig 1) or 0.02 μmol/L calyculin A induce a marked increase in protein phosphorylation of at least 25 bands in intact neutrophils. The present results, obtained using two phosphatase inhibitors with different structure and potency, show that phosphatases have an important regulatory function in neutrophils. Increased phosphorylation of one or several substrates as a result of phosphatase inhibition appears to lead to changes in cell morphology and F-actin location. A correlation between effects on phosphorylation and shape changes is confirmed by the comparably slow time course of both events. The finding that calyculin A acts on neutrophils at 50-fold lower concentrations than okadaic acid suggests that the effects of these inhibitors are due to inhibition of a phosphatase 1-type enzyme, which is inhibited by calyculin A in vitro at low nanomolar concentrations. However, these findings could also be explained by differences in the efficiency of penetration of these inhibitors into the neutrophils. In macrophages, okadaic acid was found to produce a dose-dependent increase in the phosphorylation of the 20-Kd light chain of myosin, with half-maximal effects occurring in the micromolar range.41

Interestingly, preincubation of neutrophils with micromolar concentrations of okadaic acid or 0.02 μmol/L calyculin A completely prevents increased association of actin with the cytoskeleton induced by PMA or 5 to 30 minutes of incubation with fNLPNTL (Tables 1 and 2). In a recent study, the effect of okadaic acid on the PMA-induced increase in F-actin has been tested in intact human neutrophils, using NBD-phallacidin binding.42 Downey et al42 found only a small partial inhibition induced by okadaic acid. The reason for the discrepancy between our data and theirs may be the short (5 minutes) preincubation time with the drug used by Downey et al,42 which in our hands would not be sufficient for maximal effects. Interestingly, it has been shown recently that pretreatment of neutrophils with okadaic acid produces a time-dependent inhibition of the phorbol ester-induced respiratory burst that becomes maximal after 30 minutes of preincubation with okadaic acid.35

Both phorbol ester and chemotactic peptide may activate PKC in neutrophils.34,42 Inhibition of phosphatases by the inhibitors would be expected to increase the phosphorylation of PKC substrates even further. Our findings suggest that overphosphorylation of a substrate(s) of PKC or other kinases inhibits the stimulus-dependent increase in cytoskeletal actin. Possibly, activation of a phosphatase, and the resulting dephosphorylation of a protein, plays a crucial role in phorbol ester-dependent signalling for increased
actin polymerization, implicating a novel mechanism. PMA may act on this phosphatase indirectly, via activation of PKC, or possibly even directly. A direct effect of PMA on a phosphatase would explain the finding that various PKC inhibitors do not prevent PMA-induced actin assembly. In our hands, the PKC-specific inhibitor CGP 41 251\textsuperscript{44} also does not inhibit the PMA-dependent increase in cytoskeletal actin (V. Niggli, unpublished data, April 1991). Moreover, our results suggest that a comparable signal is operative in the late stage of activation by fNLPNTL. In this context, our recent observation that staurosporine, a potent inhibitor of several protein kinases, stimulates actin association with the cytoskeleton at low (nanomolar) concentrations is of interest.\textsuperscript{10} A decrease in protein phosphorylation, induced either by activation of a phosphatase or inhibition of a kinase, may thus be able to trigger actin reorganization. How inhibition of a phosphatase leads to changes in actin localization and prevents stimulus-dependent increases in the level of cytoskeleton-associated actin is not known. A 82-Kd PKC substrate in neutrophils may be involved. This protein only associates with membranes in the dephosphorylated form and okadaic acid (1 \textmu mol/L) has been shown to prevent dephosphorylation and membrane association of this protein in chemotactic peptide-stimulated neutrophils.\textsuperscript{45} Thelen et al propose a role of this protein in reversible actin-membrane linkage.\textsuperscript{45} 

In contrast to the above findings, preincubation of cells with okadaic acid or calyculin A does not prevent the rapid increase in cytoskeletal actin occurring 1 minute after the addition of fNLPNTL. This suggests different signaling or regulatory pathways for increased association of actin with the cytoskeleton in the early and late stages of neutrophil activation. The effects of okadaic acid and calyculin A on the morphology and F-actin distribution of PMA- and fNLPNTL-stimulated cells are rather complex. On the one hand, these inhibitors prevent the association of actin with the cytoskeleton. On the other hand, they prevent fNLPNTL- or phorbol ester-induced shape changes (polarization or formation of pseudopods) and, instead, impose the typical morphology induced by the inhibitors alone. Therefore, they do not just restore the resting state of the neutrophils, but rather activate and disturb the typical morphology of cells activated by other stimuli. We have noted that the effect of PMA on cell morphology overrides that of fNLPNTL.\textsuperscript{5} Okadaic acid and calyculin A were now found to override the effects of both PMA and fNLPNTL. The increase in phosphorylation shows a corresponding hierarchy (fNLPNTL < PMA < okadaic acid) (Fig 1). This may indicate that the morphologic effects could be related to the degree of phosphorylation. Suppression of cell polarity (Fig 2) by okadaic acid is one, but not the only, explanation for inhibition of macrophage chemotaxis induced by micromolar concentrations of the drug.\textsuperscript{41} 

Okadaic acid had no effect on the marked increase in pinocytosis induced by fNLPNTL or PMA under conditions in which it has been shown to prevent stimulus-dependent increases in cytoskeletal actin (Fig 5). Thus, pinocytosis can occur in the absence of a significantly increased association of actin with the cytoskeleton. The two events appear to be dissociated. 

In summary, the results presented here suggest that the steady-state activity of a phosphatase (possibly type 1) is instrumental in maintaining cell shape and F-actin localization in resting human neutrophils. Moreover, we have evidence for a novel signalling or regulatory pathway involved in stimulus-dependent increases in cytoskeletal actin. This pathway, requiring the activity of a phosphatase sensitive to okadaic acid and calyculin A, appears to be operative in the later stage of neutrophil activation by chemotactic peptide and in activation by phorbol ester.

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Protein phosphatase inhibitors okadaic acid and calyculin A alter cell shape and F-actin distribution and inhibit stimulus-dependent increases in cytoskeletal actin of human neutrophils

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