Vimentin Is Transiently Co-localized with and Phosphorylated by Cyclic GMP-dependent Protein Kinase in Formyl-peptide-stimulated Neutrophils*

(Received for publication, January 28, 1991)

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The effects of cGMP-dependent protein kinase (G-kinase), a major cellular receptor of cGMP, were investigated in activated human neutrophils. Immunocytochemistry demonstrated that G-kinase translocated from a diffuse localization in the cytoplasm to the cytoskeleton and nucleus after stimulation with N-formyl-methionyl-leucyl-phenylalanine (fMLP), and transiently co-localized with the intermediate filament protein, vimentin. During this time period, the most remarkable co-localization of G-kinase and vimentin was observed between 1–2.5 min stimulation with fMLP. At that time co-localization of G-kinase and vimentin was predominantly confined to filaments which extended from regions adjacent to the nucleus into the uropod. Distinctive localization for only G-kinase was observed at the microtubule organizing center and euchromatin of the nucleus. The filamentous staining pattern for G-kinase and vimentin was enhanced in the presence of 8-Br-cGMP. Coincident with co-localization of G-kinase and vimentin in adherent neutrophils was a transient increase in cGMP levels and an increase in the phosphorylation of vimentin in fMLP-stimulated cells. The increase in cGMP levels was dependent upon cell adherence, was enhanced by preincubating neutrophils with L-arginine (the precursor for nitric oxide synthesis), and attenuated with the nitric oxide synthase inhibitor, Nω-monomethyl-L-arginine. Phosphorylation of vimentin in the fMLP-stimulated neutrophil was observed in the presence or absence of exogenous cGMP, although in the presence of low concentrations of 8-Br-cGMP a more rapid phosphorylation of vimentin was observed that correlated with the enhanced co-localization of G-kinase and vimentin. Phosphorylation of vimentin was not observed in non-activated cells treated with 8-Br-cGMP, suggesting that phosphorylation only occurs when G-kinase is co-localized with vimentin. The presence of the protein kinase C inhibitors, staurosporine or H-7, did not inhibit vimentin phosphorylation during fMLP stimulation, while 8-Br-cGMP enhanced phosphorylation in fMLP-treated cells. This suggests that neither protein kinase C nor cAMP-dependent protein kinase catalyze the phosphorylation of vimentin in neutrophils activated by fMLP. These results indicate that vimentin and G-kinase are co-localized in neutrophils and that vimentin is phosphorylated by G-kinase in response to the co-localization of the two proteins. A model for the targeting of G-kinase and vimentin is presented which hypothesizes that the transient redistribution of G-kinase may regulate neutrophil activation.

Neutrophil activation in response to chemotactic agents, phagocytic stimuli, and other endogenous regulators is a complex process involving migration, phagocytosis, degranulation, and generation of toxic oxygen metabolites. This process is mediated and regulated by a wide variety of signal transduction pathways that allow the neutrophil to respond to its external environment. The second messenger concept and cyclic nucleotide signaling in living cells is basic to many regulatory pathways (Sutherland, 1972). A role for cGMP in neutrophil chemotaxis (Estensen et al., 1973) and degranulation (Ignarro and Cech, 1976; Weissmann et al., 1975) has been proposed, but the intracellular mechanism of cGMP action is not understood. In addition, not all biochemical studies of cGMP levels have correlated with specific neutrophil functions (Simchowitz et al., 1980; Smolen et al., 1980; Smolen and Weissmann, 1981). Thus, delineating the role of cGMP in the neutrophil requires further consideration and approaches, since cell activation is rapid and transient (Sklar, 1986; Sklar et al., 1984), subpopulations of neutrophils exist which vary in responsiveness to certain stimuli (Gallin, 1984; Seligmann et al., 1984), and the polarity of structure of the neutrophil during chemotaxis, phagocytosis, and degranulation suggests that activation takes place within precise intracellular compartments.

A major receptor protein for cGMP in biological systems is cGMP-dependent protein kinase (G-kinase). Since its discovery (Kuo and Greengard, 1970), G-kinase has been found in several cell types (Lincoln and Corbin, 1983; Walter, 1981), although its function is only now beginning to be understood.

* This research was supported by United States Public Health Service General Research Support Award 5-S01-FR-05406, a University of North Carolina Jr. Faculty Award, National Science Foundation Grant DGB-8018417, and National Institutes of Health Grant HL-34646. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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G-kinase has been implicated in the regulation of various cell functions, especially in smooth muscle relaxation (Lincoln and Johnson, 1984). However, minimal information exists regarding the regulatory role of G-kinase in neutrophils. Previously, we have shown that G-kinase is present in neutrophils at high concentrations and is transiently redistributed to specific targeting proteins or structures in neutrophils stimulated with the chemotactic peptide N-formyl-methionyl-leucyl-phenylalanine (fMLP) (Przywansky et al., 1990).

In the past two decades, an interest has emerged concerning the regulatory nature of intermediate filaments (IF) in signal transduction. IF, which represent the 10 nm, or intermediate structural fibrils of the cytoskeleton, are prominent in neutrophils (Malech, et al., 1977; Oliver, 1978; Stossel, 1977). Although vimentin has been identified as the IF protein in neutrophils for some time (Parysek and Eckert, 1984), very little is known about its regulation of neutrophil signal transduction. However, studies have shown that neutrophil vimentin is phosphorylated in vitro by cAMP-dependent protein kinase (O'Connor et al., 1981) and is phosphorylated in situ by an unidentified kinase in response to chemotactic factors such as formyl-peptides (Huang et al., 1984). In addition, vimentin is phosphorylated after neutrophils are stimulated with phorbol myristate acetate, suggesting it may be a substrate for protein kinase C (Huang et al., 1988). This is possible, since it has now been demonstrated that both cAMP-dependent protein kinase and protein kinase C phosphorylate vimentin in vitro at different sites (Inagaki et al., 1987).

In this report, we demonstrate that G-kinase translocates from a diffuse state in the cytoplasm to a highly co-localized distribution with vimentin in fMLP-stimulated neutrophils. This co-localization is transient and is augmented in the presence of 8-Br-cGMP. We also demonstrate for the first time that cGMP levels transiently increase in adherent cells stimulated with fMLP and that these increases correlate with the activation of G-kinase that is co-localized with vimentin. Finally, we demonstrate that G-kinase catalyzes the phosphorylation of vimentin in vitro and in intact neutrophils specifically under conditions of G-kinase co-localization with vimentin and activation by increased levels of cGMP.

**MATERIALS AND METHODS**

**Reagents**—Mono-poly resolving media was obtained from Flow-ICN (Costa Mesa, CA). FITC-goat anti-rabbit IgG and TRITC-sheep antirabbit IgG were obtained from Organon Technika Corp. (Ossining, NY). Mouse anti-porcine lens vimentin was obtained from Dako (Santa Barbara, CA). Rabbit anti-bovine aorta G-kinase was raised and characterized for specificity in smooth muscle cells (Cornwell and Lincoln, 1989) and is cross-reactive with human neutrophil G-kinase, as determined by the immunoprecipitation of a single protein band (Mr, ~77k) shown by Western Blot analysis of neutrophil extracts (Przywansky et al., 1990). Rabbit anti-G-kinase was raised and characterized as reported (Cornwell and Lincoln, 1989). Rabbit anti-human fibroblast vimentin was obtained from Chemicon (Temecula, CA). Purified bovine lens vimentin and staurosporine were purchased from Boehringer Mannheim. Bovine lung G-kinase was purified as previously described using CAMP-agarose affinity chromatography (Lincoln et al., 1977). ATP [γ-32P] and 32P-cGMP were obtained from ICN Radiochemicals (Irvine, CA). H-7 (1-(5-isouquinolinesulfonyl)-2-methylpipеразине, diHCl) and N6-monomethyl-L-arginine were purchased from Calbiochem (San Diego, CA). β-Mercaptoethanol was obtained from Bio-Rad. Nonidet P-40 was obtained from U. S. Biochemicals (Cleveland, OH). fMLP was purchased from Peninsula Laboratories (Belmont, CA). Zapoglobin was purchased from Coulter (Hialeah, FL). The biinchoninic acid protein assay kit was purchased from Pierce Chemical Co. Okadaic acid was a gift from Dr. Robert W. Dickey, Dauphin Island Sea Laboratory, Dauphin Island, AL. All other reagents were purchased from Sigma.

**Cell Preparation**—Neutrophils were isolated from human peripheral blood with Ficoll-hypaque Mono-Polyresolving media. The cells were resuspended at 3 × 10^6 cells/ml in Gey’s balanced salts buffered with 10 mM HEPES, pH 7.3, and supplemented with 1.5 mM CaCl2, 1 mM MgCl2, 0.3 mM MgSO4 (GBS), and 10% human type AB serum. Neutrophils were allowed to adhere to glass coverslips for 15 min or to 60-mm Petri culture dishes for 30 min at 37 °C. Unattached cells were removed by washing with PBS. Cells adherent in neutrophils were >98% viable by trypan blue exclusion. All reagents were checked for endotoxin by the limulus test and contained less than 0.07 ng/ml of endotoxin.

**Immunofluorescence Localization**—Neutrophil monolayers were stimulated with 0.1 μM fMLP in GBS plus 10 μg/ml human type AB serum. Neutrophils were allowed to adhere to glass coverslips for 30 to 5 to 5 min at 37 °C. The culture media was removed and the cells were quickly fixed at room temperature in 1% paraformaldehyde in 0.07% cacodylate buffer containing 0.72% sucrose, pH 7.5, for 10 min, followed by 3.7% formaldehyde in phosphate-buffered saline, pH 7.4 (PBS) for 10 min, and 20 °C methanol for 4 min, and 20 °C acetone for 1 min. Cells were washed in PBS after formaldehyde and acetone. In some instances cells were preincubated with 1 μM 8-Br-cGMP for 15 min, followed by incubation with 0.1 μM fMLP in the presence of 8-Br-cGMP. Fixed monolayers were incubated at 4 °C overnight with rabbit anti-bovine aorta G-kinase, washed in PBS, and stained for 30 min with FITC goat anti-rabbit IgG. The monolayers were then incubated at 4 °C overnight with mouse anti-porcine lens vimentin, washed in PBS, and stained for 30 min with TRITC sheep anti-mouse IgG. Cells were mounted in polyvinyl alcohol and viewed on a Leitz fluorescence microscope. Immunofluorescence photomicrographs were taken on a Leica Orthomath Camera with Kodak T-X 3 film. Immunofluorescence photomicrographs were taken on a Leitz Orthomath Camera with Kodak T-X 3 film. Immunofluorescence photomicrographs were taken on a Leitz Orthomath Camera with Kodak T-X 3 film. Immunofluorescence photomicrographs were taken on a Leitz Orthomath Camera with Kodak T-X 3 film.
of cGMP was determined by radioimmunoassay using [3H]-cGMP and rabbit anti-cGMP as described (Harper and Brooker, 1975).

For cell suspensions, 2 x 10⁶ cells were treated with fMLP in the absence of serum for various times and the reaction stopped by immersing the sample tubes in an ice bath. Cells were microfuged for 5 s, supernatants discarded, and cGMP extracted from the cell pellets with 1 ml of 5% trichloroacetic acid as described above for adherent cells. Protein concentration was determined by dissolving the trichloroacetic acid pellet with 0.5 M NaOH and measuring the amount of protein of each sample using the bicinchoninic acid protein assay kit.

RESULTS

Co-localization of G-kinase and Vimentin in fMLP-stimulated Cells—G-kinase was recently found to transiently localize to filaments in the uropod of polarized, fMLP-treated neutrophils (Pryzwansky et al., 1990). To determine if the IF protein, vimentin, co-localizes with G-kinase, neutrophils were stimulated from 30 s to 5 min with 0.1 μM fMLP, and stained by double-label immunofluorescence microscopy for G-kinase and vimentin. In unstimulated neutrophils, co-localization of G-kinase and vimentin was not observed (Fig. 1, a and b), as G-kinase was diffusely localized in the cytoplasm without any apparent association with vimentin. After 30 s of stimulation with fMLP, some cells began to polarize, and there was diffuse staining of G-kinase in the uropod as well as the rest of the cytoplasm. At this time, some polarized cells displayed co-localization of G-kinase and vimentin primarily in the uropod (Fig. 1, c and d). The most remarkable co-localization was observed within 1–2.5 min, when G-kinase and vimentin localization was predominantly confined to regions which extended from the nuclear cleft, or Hof, into the uropod of polarized cells (Fig. 1, e and f). After 2.5 min of treatment with fMLP, neutrophils became less polarized, as vimentin and G-kinase were compartmentalized to one region of the cell (Fig. 1, g and h). After 5 min of treatment with fMLP, co-localization of G-kinase and vimentin was not evident. At this time, staining for G-kinase at the microtubule organizing center and the nucleus was apparent, while staining for vimentin was further condensed around the nucleus (Fig. 1, i and j). Control cells did not stain when antibody was replaced with preimmune rabbit sera or normal mouse IgG (data not shown). These studies demonstrate that a transient co-localization of G-kinase exists with vimentin and that G-kinase may be targeted to IF, particularly in the uropod during fMLP stimulation.

Effects of 8-Br-cGMP on G-kinase and Vimentin Co-localization—G-kinase is targeted to vimentin only in the presence of fMLP. An elevation in cGMP levels may also enhance G-kinase co-localization with vimentin. To test this possibility, cells were pre-incubated with low concentrations (1 μM) of 8-Br-cGMP (a specific activator of G-kinase) for 15 min and then stimulated with fMLP. 8-Br-cGMP alone did not promote the co-localization of G-kinase and vimentin. However, the co-localization time of G-kinase and vimentin was augmented by 8-Br-cGMP in the presence of fMLP (Fig. 2). At 1 min of stimulation with fMLP, both the filamentous staining of G-kinase and changes in cell shape were enhanced with 8-Br-cGMP (Fig. 2, a and c). Vivid co-localization of G-kinase and vimentin was observed on filaments within the uropod of polarized cells and in the mid-region of bi-directionally locomoting cells. The enhanced co-localization of G-kinase and vimentin was also transient since no co-localization was observed after 5 min in cells which were pre-treated with 8-Br-cGMP and incubated with fMLP (data not shown).

Because co-localization of G-kinase and vimentin was enhanced by cGMP in fMLP-treated cells, it was important to determine whether or not cGMP levels were elevated in response to fMLP. As shown in Fig. 3, a significant increase in cGMP levels was observed when adherent cells were treated with either fMLP, or with fMLP plus L-Arg. A greater and more sustained increase in cGMP levels was observed in the presence of L-Arg. However, even in the absence of L-Arg, fMLP produced significant and transient increases in cGMP. When cells were treated with NMA, an inhibitor of nitric oxide synthase, the levels of cGMP, although elevated, were lower than in L-Arg-treated cells with fMLP (Fig. 4). This suggested that NO production by fMLP may be the physiological mechanism by which fMLP increases cGMP in the neutrophil. Incubations that were performed in the presence of 1 μM 1-isobutyl-3-methyl-xanthine demonstrated a similar trend in cGMP elevation, although the cGMP levels were slightly higher than in the absence of 1-isobutyl-3-methylxanthine shown here (data not shown). Thus, decreased phosphodiesterase activity was not the likely reason for cGMP formation by fMLP. No changes in cGMP levels were detected at any time point in unstimulated cells. Similar to other reports (Simchowitz et al., 1980; Smolen and Weissmann, 1981), we did not detect significant increases in cGMP levels of cells stimulated with fMLP in suspension (data not shown).

Phosphorylation of Vimentin by G-kinase—Because G-kinase co-localizes with vimentin at time points which correlate with increased levels of cGMP, it was of further importance by

Fig. 1. Double label immunofluorescence microscopy of FITC-anti-G-kinase (a, c, e, g, i) and TRITC-anti-vimentin (b, d, f, h, j) of neutrophils incubated with 0.1 μM fMLP from 30 s to 5 min (a and b, unstained; c and d, 0.5 min; e and f, 1 min; g and h, 2.5 min; i and j, 5 min). Co-localization of G-kinase and vimentin is prominent within the uropod of polarized fMLP-stimulated cells (c–f). Note that in randomly migrating cells, only vimentin is found within the uropod (arrows, a and b). Control cells which were incubated with pre-immune serum or normal mouse serum did not stain cells (not shown). Bar in panel j is 10 μm.
to determine if G-kinase is an intracellular regulator of a specific neutrophil function. The co-localization of G-kinase and vimentin suggests that the intermediate filaments could be a substrate for the enzyme. To demonstrate for the first time that vimentin is a substrate for G-kinase, purified vimentin from bovine lens was incubated with purified G-kinase and \([\text{32P}]\text{ATP}\), separated by SDS-polyacrylamide gel electrophoresis, and subjected to autoradiography. The results shown in Fig. 5 demonstrated that vimentin (Mr = 57,000) was phosphorylated by G-kinase in vitro. Some autophosphorylation of G-kinase (77 kDa) is also observed (lanes 1, 3, and 4).

Fig. 2. Double label immunofluorescence microscopy of FITC-anti-G-kinase (a and c) and TRITC-anti-vimentin (b and d) of neutrophils preincubated with 1 mM 8-Br-cGMP for 15 min followed by stimulation with 0.1 mM fMLP for 1 min. Enhanced co-localization is observed in the uropod of polarized cells (arrows, a and b), mid region of highly motile cells (arrowheads, a and b), and as bundles within one region of the cell body (arrow, c and d). Bar, 10 μm.

**FIG. 2.** Double label immunofluorescence microscopy of FITC-anti-G-kinase (a and c) and TRITC-anti-vimentin (b and d) of neutrophils preincubated with 1 mM 8-Br-cGMP for 15 min followed by stimulation with 0.1 mM fMLP for 1 min. Enhanced co-localization is observed in the uropod of polarized cells (arrows, a and b), mid region of highly motile cells (arrowheads, a and b), and as bundles within one region of the cell body (arrow, c and d). Bar, 10 μm.

**FIG. 3.** Time course of cGMP levels in adherent neutrophils treated with 0.1 mM fMLP in the presence (•) or the absence (○) of 0.5 mM L-Arg in the incubation media. L-Arg-treated cells were incubated for 30 min with L-Arg prior to stimulation with fMLP. A transient increase in cGMP levels was observed, with maximal levels achieved between 30 s and 2.5 min. Cyclic GMP levels were higher in the presence of L-Arg. Results represent the mean ± S.E. of three experiments (+L-Arg) and five experiments (−L-Arg) each in duplicate. Student’s paired t test, accounting for different experimental preparations where each time point was compared with the control, showed significance for 30 s to 2.5 min at a range of \(p \leq 0.005-0.03\) without L-Arg and \(p \leq 0.007-0.05\) with L-Arg.

**FIG. 4.** Time course of cGMP levels in adherent neutrophils treated with 0.1 mM fMLP in the presence of 0.5 mM L-Arg (●) or 1 mM NMA (○) in the incubation media. Cells were preincubated for 30 min with L-Arg or NMA prior to stimulation with fMLP. At all time points, NMA-treated neutrophils have a lower cGMP level than L-Arg-treated neutrophils. Results represent the mean ± S.E. of triplicate samples from neutrophils obtained from one blood donor. While cGMP levels varied depending upon the donor, the trend of the neutrophil response was similar.

**FIG. 5.** Phosphorylation of purified vimentin by G-kinase *in vitro*. Lane 1, G-kinase plus 1 mM cGMP; lane 2, vimentin; lanes 3 and 4, vimentin and G-kinase plus 1 mM cGMP. Phosphorylation of vimentin (57 kDa) by G-kinase is shown in lanes 3 and 4. Some autophosphorylation of G-kinase (77 kDa) is also observed (lanes 1, 3, and 4).
Co-localization of G-kinase and Vimentin in Neutrophils

**FIG. 6.** Phosphorylation of vimentin in intact neutrophils stimulated with 0.1 μM fMLP in the presence or absence of 8-Br-cGMP for various times. Stimulation of neutrophils with fMLP for 1, 2.5, and 5 min (lanes 1–3, respectively) reveals maximal phosphorylation of vimentin at 2.5 min (lane 2). Preincubation of neutrophils with 1 μM 8-Br-cGMP and subsequent stimulation with fMLP for 1, 2.5, and 5 min (lanes 4–6, respectively) reveals maximal phosphorylation of vimentin at 1 min (lane 4).

**FIG. 7.** Phosphorylation of vimentin by G-kinase in intact adhered neutrophils. Cells were stimulated with fMLP for 2.5 min or fMLP plus 8-Br-cGMP for 1 min in the presence or absence of H-7 or staurosporine as follows: lane 1, control cells; lane 2, H-7; lane 3, staurosporine; lane 4, cGMP; lane 5, H-7, cGMP; lane 6, staurosporine, cGMP; lane 7, fMLP; lane 8, fMLP, cGMP; lane 9, fMLP, H-7, cGMP; lane 10, fMLP, staurosporine, cGMP.

A significant finding was observed using 8-Br-cGMP. When phosphorylation of vimentin was examined in adherent neutrophils with 8-Br-cGMP alone (i.e., no fMLP treatment), no increase in vimentin phosphorylation was observed over unstimulated control cells (Fig. 7, lanes 1 and 4). However, greater phosphorylation at an earlier time was detected in those neutrophils preincubated with 8-Br-cGMP and then stimulated with fMLP for 1 min (Fig. 7, lane 8). Thus, 8-Br-cGMP by itself was not effective in promoting either vimentin phosphorylation or co-localization of G-kinase and vimentin unless combined with fMLP. These results suggest that maximal phosphorylation of vimentin by 8-Br-cGMP required the co-localization of kinase and substrate by fMLP and suggest that the “targeting” of G-kinase to vimentin in the activated neutrophil is an important process for phosphorylation in the intact cell.

Inhibitors of protein kinase C were added to demonstrate that protein kinase C was not responsible for vimentin phosphorylation. As shown in Fig. 7, the protein kinase C inhibitors, H-7 (lane 9) and staurosporine (lane 10), did not inhibit vimentin phosphorylation in the presence of fMLP. In fact, maximal phosphorylation was observed in those cells stimulated with fMLP and treated with 8-Br-cGMP in the presence of the protein kinase C inhibitors. No phosphorylation was detected in those cells treated with H-7 alone or staurosporine alone (Fig. 7, lanes 2 and 3). Each phosphorylation experiment was performed three times, and these data were consistent. These results suggest that protein kinase C is not responsible for vimentin phosphorylation in fMLP-treated neutrophils.

**DISCUSSION**

In this report, we demonstrate by immunofluorescence microscopy that G-kinase transiently co-localizes with vimentin, the major protein found in neutrophil IF, in fMLP-stimulated neutrophils. Others have estimated that in neutrophils about 2% of the total cellular protein is vimentin (Schmitt-Graff et al., 1987). We estimate that in human neutrophils there is at least a thousand-fold less G-kinase/neuropilin than vimentin, suggesting that cGMP regulates vimentin by phosphorylation in specific cellular compartments (i.e., the uropod), or that G-kinase is simply bound to some of the vimentin. The latter explanation is unlikely since vimentin and G-kinase do not co-localize unless neutrophils are activated. Once activated, however, cGMP levels increase, probably due to the increased Ca++ flux and subsequent NO production (see below). The binding of cGMP to G-kinase might then uncover a vimentin-binding “site” on G-kinase, thus accounting for the augmented co-localization of G-kinase and vimentin observed by 8-Br-cGMP (Fig. 2). This binding site could conceivably be the catalytic site on G-kinase where substrates bind. Regardless, we believe that G-kinase plays a regulatory role in neutrophil activation since G-kinase is not confined to filaments in the uropod of non-activated, randomly locomoting cells (Fig. 1a). We have observed a similar augmentation of vimentin phosphorylation by 8-Br-cGMP in cells treated with A23187 instead of fMLP. Therefore, elevations in intracellular Ca++ are apparently required for this filamentous localization.

Vimentin has been shown to be phosphorylated in vitro by both cAMP-dependent protein kinase (Schmitt-Graff et al., 1987) and protein kinase C (Huang et al., 1988). Vimentin was previously shown to be phosphorylated in fMLP-stimulated neutrophils. However, the kinase was described as not being a calcium or a cAMP-dependent protein kinase (Huang et al., 1984). We demonstrate in this study that the kinase phosphorylating vimentin in the fMLP-stimulated neutrophil is G-kinase, since (i) vimentin is an excellent substrate for purified G-kinase in vitro; (ii) phosphorylation in the presence of fMLP is stimulated by low concentrations of 8-Br-cGMP; and (iii) maximal phosphorylation occurs in the presence of protein kinase C inhibitors. Due to the low levels of G-kinase in neutrophils and its transient compartmentalization during fMLP stimulation, a transient phosphorylation of vimentin upon fMLP stimulation would be predicted, and was, in fact, observed (Fig. 6). As summarized in Table I, and shown in Figs. 6 and 7, phosphorylation was transient and maximal after 2.5 min of fMLP stimulation, the same time in which co-localization of vimentin and G-kinase was maximal. 8-Br-cGMP both enhanced co-localization of G-kinase and vimentin and increased phosphorylation of vimentin at an earlier time point (1 min). The presence of 8-Br-cGMP alone produces little phosphorylation. Similar results have been observed using A23187 instead of fMLP. It is important to emphasize that an activator of neutrophils (i.e. fMLP, A23187) is required to achieve maximal phosphorylation of vimentin. The simplest interpretation of these results is that G-kinase only catalyzes the phosphorylation of vimentin when the two are co-localized. Apparently, the levels of G-kinase are not sufficiently high to trigger phosphorylation in the absence of co-localization.

**TABLE I**

**Summary of phosphorylation of vimentin by G-kinase**

| Experimental treatment | Phosphorylation |
|------------------------|-----------------|
| Unstimulated           |                 |
| H-7                    |                 |
| Staurosporine          |                 |
| 8-Br-cGMP              |                 |
| 8-Br-cGMP + H-7        |                 |
| 8-Br-cGMP + staurosporine |             |
| fMLP                   |                 |
| 8-Br-cGMP + fMLP       | ++++            |
| 8-Br-cGMP + fMLP + staurosporine | ++++ |
| 8-Br-cGMP + fMLP + H-7  | ++++            |

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kinase are too low and possibly contained in compartments other than vimentin in unstimulated cells. Only when cells are first stimulated to co-localize G-kinase and vimentin can phosphorylation occur in the intact cell. The simple activation of G-kinase with 8-Br-cGMP is insufficient.

It would be advantageous to utilize a selective inhibitor of G-kinase in neutrophils. Inhibition of G-kinase activity in living cells would provide the mechanism for assaying the direct effect of G-kinase control of cell function. Additionally, any data concerning the effects of G-kinase on cell function based upon the augmentation of G-kinase stimulation can be supported by converse effects due to the inhibition of G-kinase activity. Unfortunately, the putative inhibitors of G-kinase that are commercially available have only been shown to inhibit the phosphorylation of histones by G-kinase in vitro, and few G-kinase inhibition studies in living cells have been reported to date. Our recent observations suggest that the putative G-kinase selective inhibitor, KT5823, failed to inhibit the phosphorylation of vimentin in vitro at levels as high as 2 mM. In addition, KT5823 failed to inhibit histone phosphorylation by G-kinase as originally reported (Kase et al., 1987). Because a potent and selective inhibitor for G-kinase is not available, we designed our experiments to eliminate the possibility that protein kinase C might be the phosphorylating enzyme by using the selective protein kinase C inhibitors staurosporine and H-7, while optimizing conditions for the activation of G-kinase by adding exogenous 8-Br-cGMP.

G-kinase is an inactive enzyme unless cGMP levels are elevated, and there has been controversy over whether or not cGMP is elevated during neutrophil activation, in large part because the pathway for cGMP formation is unknown. Recently Schmidt and Bohme (1989) have suggested that fMLP produces a transient increase in neutrophil NO, a compound long known to activate guanylate cyclase (Arnold et al., 1977). NO formation is also known to be dependent on the Ca\(^{2+}\)-calmodulin-induced activation of nitric oxide synthase, the enzyme that converts L-Arg to NO (Bredt and Snyder, 1990). Because the rise in Ca\(^{2+}\) by fMLP stimulation is transient (Andersson et al., 1986), it is likely that cGMP increases are also transient and possibly localized. L-Arg augments the increase in cGMP but is not required probably due to the presence of internal stores of the precursor, as has been suggested for endothelial cells (Palmer et al., 1988). We report for the first time that fMLP induces a transient rise in cGMP levels in adhered neutrophils, and these elevations are augmented by L-Arg. Likewise, NMA, an inhibitor of nitric oxide synthase, attenuates the rise in cGMP in response to L-Arg. Consistent with other reports, no significant increases in cGMP levels were detected in neutrophils activated in suspension (Simchowitz et al., 1988; Smolen et al., 1980; Smolen and Weissmann, 1981). To control for variability in the number of cells which attached/died, the amount of cGMP was expressed/milligram of protein. Although the overall trend for each subject was identical, the individual responses between subjects varied as previously reported (Ignarro, 1975). This may be due to the variation in the numbers of fMLP receptors/neutrophil/individual blood donor (Seligmann et al., 1984). Nevertheless, it appears that adherence plays an important role in "priming" cells for demonstrating alterations of cGMP levels, as increasing cGMP levels by fMLP was significant from 30 s to 2.5 min (\(p = 0.003-0.03\)).

A model of G-kinase involvement in neutrophil activation is proposed in Fig. 8. Previous studies have shown that fMLP activation as well as many other neutrophil activation events are a Ca\(^{2+}\)-dependent process (Andersson et al., 1996; Lew, 1989; Naccache and Sha'afi, 1984). As discussed above, our data suggest that intracellular Ca\(^{2+}\) is important in the co-localization of G-kinase and vimentin, and that this co-localization is critical for the phosphorylation of vimentin in the intact cell. In addition, Ca\(^{2+}\) may regulate NO synthesis which stimulates guanylate cyclase, resulting in increased neutrophil cGMP levels. In support of L-Arg as a possible pathway of neutrophil activation, we have found that cGMP levels are elevated by L-Arg in fMLP-treated neutrophils, while this rise is inhibited by NMA. Furthermore, chemotaxis is inhibited by blocking NO synthesis and is restored with either dibutyryl cGMP or with L-Arg, the precursor for NO production (Kaplan et al., 1989). Although we have shown here that cGMP effects neutrophil activation through its specific receptor protein, G-kinase, it remains unclear how the phosphorylation of vimentin functions, if at all, in signaling specific cellular events. Cyclic GMP may play a role in establishing cell polarity and coordinating the various parts of the cytoskeleton responsible for cell movements since it was previously reported that agents which elevate cGMP levels promote an increase in microtubule numbers (Hoffstein, 1980). It is important to point out that 0.1 \(\mu\)M fMLP induces remarkable shape changes which are also coincident with adhesion, aggregation, and granule secretion (Hoffstein et al., 1982). Therefore, in addition to establishing polarity, which is important in cell motility, cGMP may also participate in events controlling adhesion and granule secretion.

Little is known about the biological role of cGMP or G-kinase in neutrophils. This is because neutrophils have only trace amounts of measurable G-kinase activity (Helman et al., 1983) and protein substrates have not been found. Previously, we demonstrated that G-kinase is present in human neutrophils by Western blot analysis of neutrophil extracts using polyclonal rabbit antibody against bovine aorta G-kinase (Pryzwansky et al., 1990). The cross-reactivity between bovine and human G-kinase is not surprising since there is a 99.5% identity between the bovine enzyme and human placental G-kinase (Sandberg et al., 1989), the enzymes function similarly, and G-kinase antibodies raised against the bovine G-kinase cross-react with G-kinases isolated from various tissues from several species including human (Pryzwansky et al., 1990; Walter, 1981, 1984). Analysis of the neutrophil extracts suggests that there are low levels of G-kinase in neutrophils (approximately 100-fold lower than in smooth muscle cells) and that most of the G-kinase is associated with the cytoskeleton (Pryzwansky et al., 1990). Of particular importance is that the localization of G-kinase, as demonstrated by confocal laser-scanning microscopy, is transiently
restricted to specific subcellular compartments in fMLP-stimulated neutrophils (Pryzwansky et al., 1990), implying that the physiologic substrates and function of G-kinase are also restricted to subcellular compartments. Because the levels of G-kinase are focally localized, the overall levels of cGMP and G-kinase may be of secondary importance in neutrophil function, thus supporting the concept of compartmentalization. The concept of functional compartments regulating cyclic nucleotide effectors has been described in neutrophils (Pryzwansky et al., 1981) and other cellular systems (Earp and Stein, 1978; Hayes and Brunton, 1982; Naef, 1983).

These studies focus on the emerging concept of targeting of regulatory enzymes such as protein kinases and phosphatases to subcellular structures and organelles containing substrates for these enzymes. Conventional wisdom is that kinases phosphorylate substrates by recognizing specific primary sequence motifs within the substrate. However, vimentin is a substrate for several kinases. That G-kinase is targeted to vimentin, since neither cAMP-dependent protein kinase, protein kinase C, nor Ca2+-dependent kinases catalyze its phosphorylation (Huang et al., 1984). These findings suggest the presence of G-kinase targeting or "binding" proteins on the IF, whose nature is yet unknown. In smooth muscle, we have similarly concluded that G-kinase is targeted to the sarcoplasmic reticulum with its substrates, including phospholamban (Cornwell et al., 1991). Conceivably, other in vitro substrates for different kinases will be shown to be targeted with G-kinase in specific cell types, thus accounting for cGMP-dependent protein phosphorylation in these cases. As for neutrophils, the effects of vimentin phosphorylation on neutrophil regulation and the nature of G-kinase control of traditional neutrophil functions such as chemotaxis and degranulation remain unknown, as further studies are most certainly in order.

Acknowledgments—We thank Harriette Nichols for her technical help in quantitating GMP levels, Elizabeth Parker for photographic assistance, Dr. Maureane Hoffmann and Dr. Nadia Malouf for technical advice, Dr. Sheldon Earp for help with the in vitro phosphorylation studies, and Dr. Michael Simons for biostatistical consultation.

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