FMLP Activates Ras and Raf in Human Neutrophils

Potential Role in Activation of MAP Kinase

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Abstract

Chemoattractants bind to seven transmembrane–spanning, G-protein–linked receptors on polymorphonuclear leukocytes (neutrophils) and induce a variety of functional responses, including activation of microtubule-associated protein (MAP) kinase. Although the pathways by which MAP kinases are activated in neutrophils are unknown, we hypothesized that activation of the Ras/Raf pathway leading to activation of MAP/ERK kinase (MEK) would be induced by the chemoattractant f-met-leu-phe. Human neutrophils exposed to 10 nM FMLP for 30 s exhibited an MAP kinase kinase activity coeluting with MEK-1. Immunoprecipitation of Raf-1 kinase after stimulation with FMLP revealed an activity that phosphorylated MEK, was detectable at 30 s, and peaked at 2–3 min. Immunoprecipitation of Ras from both intact neutrophils labeled with [^{32}P]orthophosphate and electropermeabilized neutrophils incubated with [^{32}P]-GTP was used to determine that FMLP treatment was associated with activation of Ras. Activation of both Ras and Raf was inhibited by treatment of neutrophils with pertussis toxin, indicating predominant linkage to the G_{i2} protein. Although phorbol esters activated Raf, activation induced by FMLP appeared independent of protein kinase C, further suggesting that G_{i2} was linked to Ras and Raf independent of phospholipase C and protein kinase C. Dibutyryl cAMP, which inhibits many neutrophil functional responses, blocked the activation of Raf by FMLP, suggesting that interruption of the Raf/MAP kinase pathway influences neutrophil responses to chemoattractants. These data suggest that G_{i2}-mediated receptor regulation of the Ras/Raf/MAP kinase pathway is a primary response to chemoattractants. (*J. Clin. Invest.* 1994. 94:815–823.) Key words: G-proteins • seven transmembrane–spanning receptors • pertussis toxin • protein kinase C • MAP kinase kinase

Introduction

The neutrophil plays a critical role in nonspecific host defense in part by responding to chemoattractants, leading to accumulation of neutrophils at an inflammatory site (1). The mechanisms by which neutrophils respond to chemotactic stimuli such as f-met-leu-phe (FMLP), although not elucidated as yet, appear to reflect interaction with seven transmembrane–spanning, G-protein–linked receptors, of which the FMLP receptor is an example (2). A variety of studies link ligand interaction with the FMLP receptor to activation of phospholipases (3), by mechanisms that are sensitive to pertussis toxin, and hence ascribed to involvement of G_{i2} (4). Although the best described Gi-linked response is inhibition of adenylate cyclase (5), the α subunit of Gi may activate ion channels (6) and induce mitogenesis (7). Additionally, $\beta\gamma$ subunits may also regulate specific isoforms of phospholipase C β (PLCβ)[1] (8). Activation of PLCβ, which leads to generation of diacylglycerol, has been shown to activate protein kinase C (PKC), and thus a variety of downstream events have been modeled by stimulation of the cell with phorbol esters such as PMA, which directly activate PKC. Despite the undoubted relevance of these pathways to neutrophil activation, the events downstream of G_{i2} regulation of these effectors remain obscure.

The recent description of microtubule-associated protein (MAP) kinase activation after stimulation by FMLP (9, 10) suggests clues to the pathways leading from FMLP to MAP kinase. Kinase pathways which include MAP kinases have been demonstrated in a variety of cells of mammalian, amphibian, and yeast ancestries, speaking to the remarkable evolutionary retention of this pathway (11). MAP kinases are serine/threonine kinases phosphorylating and activating proteins such as Rsk90 (12), cPLA2 (13), and cMyc (14). MAP kinase activation requires phosphorylation on tyrosine and threonine residues (15), induced by a multifunctional MAP kinase kinase, or MEK (MAP ERK kinase), which itself is activated by phosphorylation (16). Recent evidence suggests the existence of several pathways to activation of MEK, including a recently cloned MEKK (17), but the best delineated pathway indicates the involvement of Ras and the kinase Raf which phosphorylates MEK in response to tyrosine kinase–linked receptors (12). Use of this pathway by heterotrimeric G-protein–linked receptors has not been described previously in naturally occurring mammalian systems, but recent evidence suggests that the m2 muscarinic receptor (a seven transmembrane–spanning, G-protein–linked receptor) expressed in Rat 1a cells activates Ras and Raf (18). We questioned whether stimulation through the FMLP receptor could result in activation of Ras, Raf, and MEK, and, if so, whether these events were linked to activation of G_{i2} and if pkc was involved. Recent evidence suggests that this pathway

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1. *Abbreviations used in this paper:* CPTcAMP, 8-(4-chlorophenylthio)-cAMP; MAP, microtubule-associated protein; MEK, MAP ERK kinase; PKC, protein kinase C; PLCβ, phospholipase Cβ.

might be a target for inhibition by cAMP-dependent kinase (19–21). We have also questioned whether FMLP-stimulated Raf activation in neutrophils is negatively regulated by increased intracellular cAMP.

Methods

Reagents. All reagents and plastic ware used in this assay were tested before use for the presence of LPS using the Limulus Amebocyte Lysate kit (Associates of Cape Cod, Inc., Woods Hole, MA). This procedure detects as little as 0.01 ng LPS/ml. Sterile plastics and all reagents tested at the concentration used in the adherence assay were free of detectable LPS contamination. Unless otherwise stated, neutrophils were suspended in Krebs-Ringer phosphate buffer, pH 7.2, with 0.2% dextrose (KRPD) (5% dextrose in 0.2% sodium chloride, injectable; Abbott Laboratories, North Chicago, IL). The salts comprising the buffer were purchased from Mallinckrodt Specialty Chemicals (Paris, KY) and had undetectable LPS levels. All components were freshly diluted with LPS-free saline (0.9% saline for irrigation; Abbott Laboratories) on each experimental day. The PKC inhibitor GF 109203x was purchased from Calbiochem-Novabiochem (La Jolla, CA). Pertussis toxin was purchased from List Biologicals (Campbell, CA). Analogues of cyclic AMP, dibutyryl cAMP, and 8-(4-chlorophenylthio)-cAMP were purchased from Sigma Immunochemicals (St. Louis, MO). Antibody to MAP kinase (anti-rat MAP-kinase R2 (erk1-CT), rabbit polyclonal IgG) was purchased from Upstate Biotechnology Inc. (Lake Placid, NY), and antibody to Raf-1 was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Monoclonal antibody to Ras, clone Y13-259, was a kind gift from Anne Harwood (National Jewish Center, Denver, CO). IP-20 (TTYADFIASGRTGRRNAIHD) and $EGFR_{662-681}$ peptide (RRELVEPLTPSGEAPNQALLR) used for MAP kinase activity assays were synthesized by Macromolecular Resources (Colorado State University, Ft. Collins, CO).

Human neutrophils. Human blood neutrophils were prepared by a method which minimizes LPS exposure, using plasma-Percoll gradients as described (22). After isolation, cells were washed with 50 vol of KRPD and resuspended at a final concentration of 3×10^6/ml.

Permeabilization of neutrophils. Neutrophils were permeabilized by a modification of the method of Grinstein and Furuya (23). Neutrophils were sedimented and resuspended at 10^7/ml in ice-cold permeabilization medium containing 140 mM KCl, 1 mM $MgCl_2$, 10 mM glucose, 1 mM ATP, 10 mM Hepes, pH 7.0, 1 mM EGTA, and 0.193 mM $CaCl_2$ to yield a final Ca^{2+} concentration of 100 nM, calculated using an algorithm described by Fabiato (24). 1 ml of this suspension was transferred to a cuvette (Gene Pulser; Bio-Rad, Cambridge, MA) (0.4 cm interelectrode distance) and subjected to two discharges of 1.9 kV from a 25-μF capacitor using the Bio-Rad Gene Pulser. The cells were quickly centrifuged and washed in fresh ice-cold permeabilization medium after each pulse.

MAP kinase assay. MAP kinase activity was assayed using methods modified from those described previously (25).[2] Neutrophils were incubated with 1 mM PMSF, 10 μg/ml leupeptin, and 10 μg/μl aprotinin for 30 min in KRPD + 0.25% HSA. After stimulation, cells were lysed in 0.5% Triton X-100, 2 mM $MgCl_2$, 1 mM EGTA, 1 mM DTT, 100 μM Na_3VO_4, 50 mM β-glycerophosphate, pH 7.2, containing 2 μg/ml aprotinin and leupeptin, and centrifuged in a microfuge at 15,000 rpm for 10 min to pellet the nuclei. Cell lysates were loaded on an FPLC Mono Q column preequilibrated in 50 mM β-glycerophosphate (pH 7.2), 100 μM Na_3VO_4, 1 mM EGTA, 1 mM DTT using a linear 30-ml gradient of 0–0.35 M NaCl. 20-μl aliquots of each column fraction were assayed in the presence of the $EGF\ receptor_{662-681}$ peptide for 15 min. Immunoblotting of the fractions with anti-MAP kinase antibody showed that the peak of activity corresponded with immunoreactive p44 MAP kinase.

MEK assays. MEK activity was assayed from cell lysates fractionated on a Mono Q FPLC column (26). Cells were lysed in the MAP kinase lysis buffer (described above) at pH 7.2. Nuclei were pelleted by microfuging the lysates for 10 min at 15,000 rpm, and then the supernatants were fractionated on a Mono Q column using a 30-ml gradient from 0 to 0.35 M NaCl. 30-μl aliquots from 1-ml fractions were assayed in the presence of recombinant wild-type MAP kinase (300 ng/reaction) and the $EGF\ receptor_{662-681}$ peptide. The recombinant wild-type MAP kinase was preincubated with protein phosphatase 2A in Hepes (pH 7.2) for 1 h at 37°C to reverse any autophosphorylation of the recombinant protein used in the assay. Aliquots (900 μl) from the Mono Q column fractions were also analyzed by immunoblotting using antisera recognizing the MEK-1 carboxy terminus.

Ras activation assays. Activation of Ras in intact cells was determined by analyzing the ratio of GTP to GDP bound to immunoprecipitated Ras from control and stimulated neutrophils (27). Cells were washed twice in phosphate-free media and then labeled for 3 h with 1 mCi/ml $^{32}P_i$ at 37°C. After stimulation, cells were lysed in Tris/HCl-buffered saline (pH 7.5), 10 mM $MgCl_2$, 1% Nonidet P-40, 10 μg/ml leupeptin, 0.2 U/ml aprotinin, and 200 ng/ml Y13-259 anti-Ras antibody. The nuclei were pelleted by centrifugation of the lysates at 15,000 rpm for 5 min, and the supernatants were incubated on ice for 1 h. Agarose beads coupled to goat anti-rat IgG were then added to the lysates and mixed by rotation for 1 h. Then, the beads were washed six times in lysis buffer minus protease inhibitors and anti-Ras antibody. Ras was eluted from the beads by boiling for 3 min in 0.1% SDS, 2 mM EDTA, 2 mM DTT. Guanine nucleotides were resolved by polyethylinimine cellulose thin layer chromatography using 0.75 M K_2HPO_4, pH 3.4. GDP and GTP were visualized using unlabeled standards. Quantitation of radiolabeled GDP and GTP was accomplished using a Molecular Dynamics PhosphorImager (Sunnyvale, CA).

Ras activation in permeabilized cells. Before the study, neutrophils were incubated in KRPD containing PMSF, leupeptin, and aprotinin (vide supra) for 30 min. After permeabilization, [α-^{32}P]GTP (5 μCi/sample) was added, and the cells were incubated on ice for 1 min, before addition of FMLP or vehicle, whereupon the cells were warmed rapidly to 37°C and incubated for the indicated times before lysis and immunprecipitation of Ras as described.

Raf assays. To assay Raf activity, recombinant kinase inactive MEK was used as a substrate (16, 28). After 30 min of incubation in KRPD containing PMSF, aprotinin, and leupeptin (vide supra), stimulated or control cells were lysed in RIPA (1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 150 mM NaCl, 50 mM Tris/HCl, pH 7.2, 2 μg/ml aprotinin, 10 mM sodium pyrophosphate, 25 mM sodium glycerophosphate). Nuclei were removed by centrifugation in a microfuge, and, after normalization for protein content (0.5–1.0 mg/sample) the supernatants were precleared by incubation with protein A–Sepharose (10 μl) at 4°C for 15 min and pelleted in a microfuge. Samples were then incubated with a 1:100 dilution of rabbit antiserum recognizing the carboxy terminus of c-Raf (Santa Cruz Biotechnology) and protein A–Sepharose for 3 h at 4°C. The pellet was successively washed twice in RIPA, and twice in PAN (10 mM Pipes, pH 7.0, 100 mM NaCl, 2.0 U/ml aprotinin). The final pellet was resuspended in 50 μl PAN, and 40 μl was assayed for Raf activity by the addition of 10× kinase buffer (10 mM $MnCl_2$, 0.9 U/ml aprotinin, 200 mM Pipes, pH 7.0), recombinant kinase inactive MEK (25–50 ng), and [γ-^{32}P]ATP (20 μCi) in a volume of 80 μl and incubated at 30°C for 30 min. Wild-type recombinant MEK was autophosphorylated in parallel as a marker by adding 40 ng of wild-type MEK to the assay mix. The kinase assays were quenched by the addition of 12.5 μl of 5× Laemmli SDS buffer, boiled for 5 min, and centrifuged for 2 min in a microfuge. The supernatant was then electrophoresed on a 10% SDS polyacrylamide gel and analyzed by autoradiography for phosphorylation of MEK.

Statistics. Numerical data were analyzed using one- or two-way

2. Suzuki, N., N. Avdi, S. K. Young, E. L. Elson, and G. S. Worthen, manuscript submitted for publication.

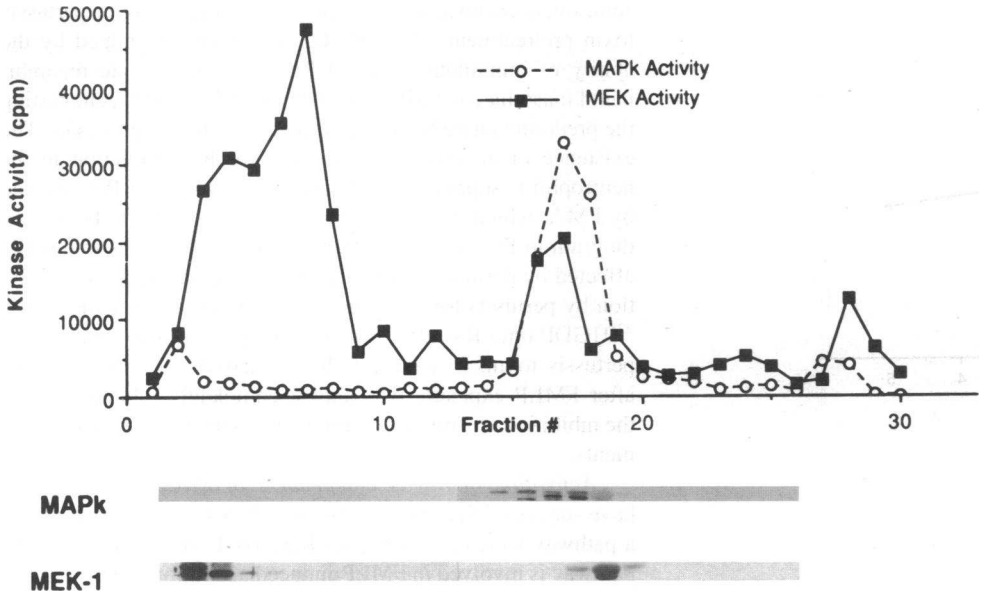

Figure 1. Activation of MAP kinase (*MAPk*) and an MAP kinase kinase (MEK) in human neutrophils after stimulation with FMLP. Cell lysates at 30 s after stimulation with 10^{-8} M FMLP were applied to a MonoQ column and eluted with an NaCl gradient, and fractions were assayed for either MAP kinase activity or MEK activity (by including wild-type recombinant MAP kinase in the kinase reaction). Immunoreactive MAP kinase elutes in fractions 15–17, while immunoreactive MEK elutes in fractions 3–6, corresponding to the activity measured here. Some immunoreactive MEK-1 is detected in fractions 17–19, perhaps linked to MAP kinase.

ANOVA on JMP (SAS, Inc., Cary, NC) running on a Macintosh II. Significance was accepted at $P < 0.5$.

Results

Activation of MEK-1 in human neutrophils by FMLP. We, as well as others (9, 10, 29), have demonstrated the rapid (30 s) and transient activation of MAP kinase in FMLP-stimulated neutrophils.[2] To determine whether activation of MEK-1 was associated with activation of MAP kinase, we examined the elution of MEK activity by Mono-Q chromatography of lysates from neutrophils stimulated with 10^{-8} M FMLP for 30 s. As seen in Fig. 1, when the EGF receptor peptide is the sole phosphate acceptor, the elution profile reveals the typical pattern of MAP kinase (fractions 15–17). However, when recombinant human MAP kinase is provided as a substrate for MEK, then phosphorylation of EGFR is also seen in fractions 3–9. Western blots of the TCA-precipitated material from each fraction reveal the presence of MAP kinase immunoreactivity in fraction 16 and MEK-1 immunoreactivity in fractions 3–6. Interestingly, MEK-1 immunoreactivity is also seen in fractions 17–19, overlapping the distribution of MAP kinase. Thus an MAP kinase kinase activity that coelutes with MEK-1 is stimulated within 30 s after exposure of the neutrophil to FMLP.

Activation of Raf by FMLP. The serine/threonine protein kinase Raf-1 phosphorylates and activates MEK (12, 28, 30). To determine whether Raf-1 is regulated in neutrophils, we examined the time course of Raf activation in response to FMLP. Immunoprecipitated Raf from lysates of control and stimulated cells was used in an in vitro kinase reaction with recombinant kinase–inactive human MEK-1. As seen in Fig. 2, detectable phosphorylation occurred as early as 30 s and peaked at 2 min.

Activation of Ras by FMLP. The mechanisms by which Raf is activated may involve both direct phosphorylation by PKC and interaction with Ras·GTP. Since the latter has been but recently shown to occur in response to activation of serpentine receptors, we explored whether Ras could be shown to be activated in FMLP-stimulated neutrophils. We took two approaches to this question. In the first, Ras was immunoprecipitated from neutrophil lysates after incorporation of $^{32}PO_4$, and the ratio of GTP to GDP was determined as an index of activation. In Fig. 3 *A* the time course of activation of Ras is shown after stimulation with 10^{-8} M FMLP. Ras activation is rapid, with the maximum GTP/GDP ratio found at 30 s. To confirm these data, we used a second approach recently described by Buday and Downward (31). Neutrophils were permeabilized, and [α^{32}P]-GTP was introduced directly into the cytoplasm before stimulation. After stimulation, cells were lysed, Ras was immunoprecipitated, and bound guanine nucleotides were quantified. As described previously, most of the labeled guanine nucleotides

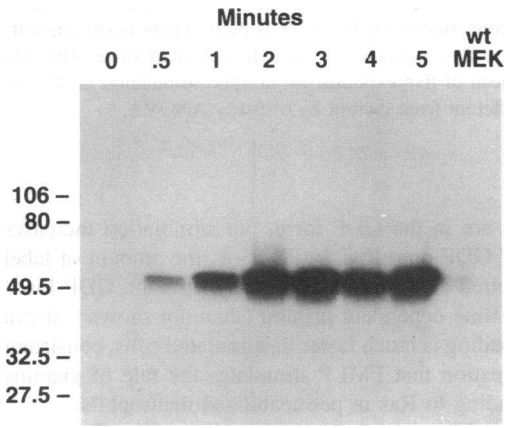

Figure 2. Activation of Raf kinase activity in FMLP-stimulated neutrophils. Raf was immunoprecipitated from neutrophils stimulated with 10^{-8} M FMLP and assayed for its ability to phosphorylate kinase inactive MEK-1 in vitro. Autoradiograms of PAGE of the products of the kinase reaction are shown for neutrophils stimulated for 0.5, 1, 2, 3, and 5 min. The phosphorylation of the 50-kD MEK-1 is shown by reference to the molecular mass markers. A faint band corresponding to autophosphorylated wild-type MEK is shown in the far right lane.

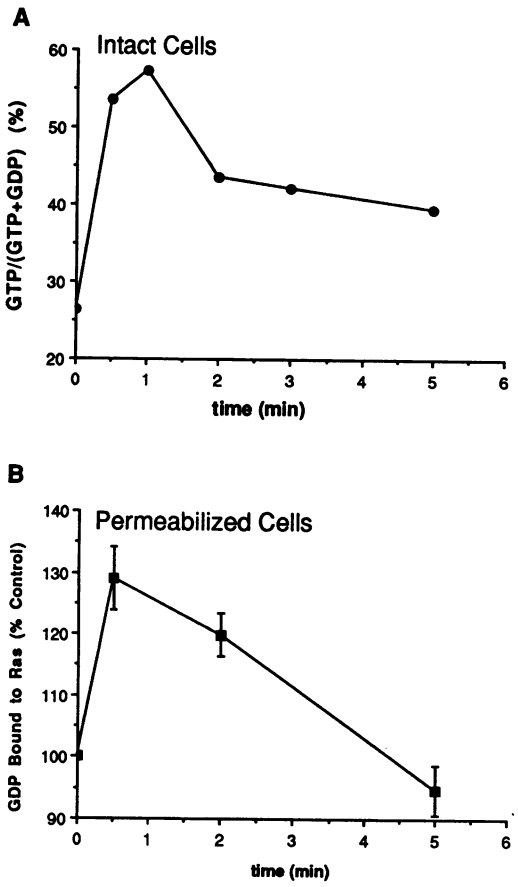

Figure 3. Activation of Ras in FMLP-stimulated neutrophils. (*A*) Ras was immunoprecipitated from intact neutrophils labeled with [^{32}P]-orthophosphate at various times after FMLP, and the ratio of GTP/GDP bound to Ras was determined as an index of activation. A single experiment is shown, representative of four experiments. Stimulation at 30 s was significantly different from control by one-way ANOVA. (*B*) Neutrophils were permeabilized as described, loaded with [^{32}P]GTP and exposed to FMLP, and the amount of labeled guanine nucleotide bound to Ras determined after immunoprecipitation. More than 90% of the added GTP is converted to GDP, and thus plotted here is the amount of GDP bound to Ras, normalized to the unstimulated value. The data represent the mean of five experiments, and the stimulation at 30 s is significantly different from control by one-way ANOVA.

bound to Ras are in the GDP form, but stimulation increases the loading of GDP onto Ras. In Fig. 3 *B*, the amount of label in GDP is plotted as a function of time. Although GDP loads onto Ras in a time-dependent fashion (data not shown), it can be seen that loading is much faster in stimulated cells, consistent with the suggestion that FMLP stimulates the rate of guanine nucleotide binding to Ras in permeabilized neutrophils.

Activation of Ras and Raf is pertussis sensitive. The major pertussis toxin substrate in neutrophils, αi2, is the G-protein α subunit most closely linked to activation via the FMLP receptor (4). To determine whether activation of G_{i2} was involved in FMLP-induced activation of Ras and Raf, neutrophils were exposed to pertussis toxin (500 ng/ml for 2 h) before stimulation. In experiments not shown, this protocol resulted in ADP ribosylation of > 70% of the detectable αi2 (Buhl, A. M., and G. L. Johnson, unpublished observations). As seen in Fig. 4, pertussis toxin pretreatment inhibited Raf activation (as judged by the ability of immunoprecipitated Raf to phosphorylate recombinant kinase-inactive MEK) by between 65 and 80%, suggesting the predominant pathway for activation of Raf requires G_{i2}. The existence of at least two pathways for Raf activation in the neutrophil is supported by the strong activation of Raf induced by PMA, which directly activates PKC. As seen in the autoradiogram in Fig. 4 *B*, activation of Raf by PMA was minimally affected by pertussis toxin, arguing for the specificity of inhibition by pertussis toxin in this system. Increased loading of [α-^{32}P]GDP onto Ras after FMLP exposure was also inhibited by pertussis toxin. In data not shown, activation of Ras at 30 s after FMLP exposure was almost completely inhibited, while the inhibition at 2 min was partial, averaging 65% in four experiments.

Activation of Raf is independent of PKC. Recent studies have suggested that Raf may be directly activated by PKC (32), a pathway which might bypass Ras. To determine whether this pathway is involved in FMLP-induced activation of Raf in intact cells, neutrophils were incubated with the PKC antagonist GF109203x. As seen in Fig. 5 *A*, Raf activation induced by PMA was inhibited in a concentration-dependent fashion by GF109203x over the range of 0.5–5 μM. The response to FMLP (measured 2 min after FMLP) was unaffected by inhibition of PKC by GF109203x. To determine whether the response to FMLP might represent contributions from PKC at different times after stimulation, the effect of 2 μM GF109203x was assessed at 0.5, 2, and 5 min after stimulation with 10 nM FMLP. As seen in Fig. 5 *B*, at none of these time points was GF109203x inhibitory, although it again inhibited PMA activation of Raf. These data suggest that activation of Raf occurs largely through activation of G_{i2} and Ras, with little contribution from PKC that would be activated by $\beta\gamma$ subunit complexes regulating PLCβ.

Increased cAMP inhibits Raf activation. A variety of studies have suggested that increased cAMP in the neutrophil inhibits chemotaxis (33), actin assembly (34), and synthesis of O_2^- (35), but the site of inhibition (presumably reflecting the action of PKA) remains unknown. Since recent studies suggest that a site of action of increased cAMP is to inhibit Raf-1 kinase (19–21), we questioned whether increases in neutrophil cAMP might act to inhibit activation of Raf kinase. In studies not shown, incubation of neutrophils with 1 mM dibutyryl cAMP inhibited not only FMLP-induced Raf but also that due to the phorbol ester PMA. Lower, more physiologically relevant concentrations of cAMP analogues, however, could also be shown to inhibit FMLP-induced Raf activation. As seen in Fig. 6, 10 μM dibutyryl cAMP inhibited Raf activation while exerting little effect on PMA-induced activation of Raf. Furthermore, the effect of increased cAMP is transient. In neutrophils incubated with dibutyryl cAMP for 2 min, there is considerable inhibition of activation. Longer incubations for 5 and 15 min were less effective, suggesting the action of dibutyryl cAMP and PKA activation was transient. To confirm that the effect of dibutyryl cAMP was related to its ability to increase intracellular cAMP levels, we used another cAMP derivative, 8-(4-chlorophenylthio)-cAMP (CPTcAMP). Fig. 7 shows the time course of Raf activation in response to FMLP with or without preincubation (for 3 min) with 60 μM CPTcAMP. Raf activity

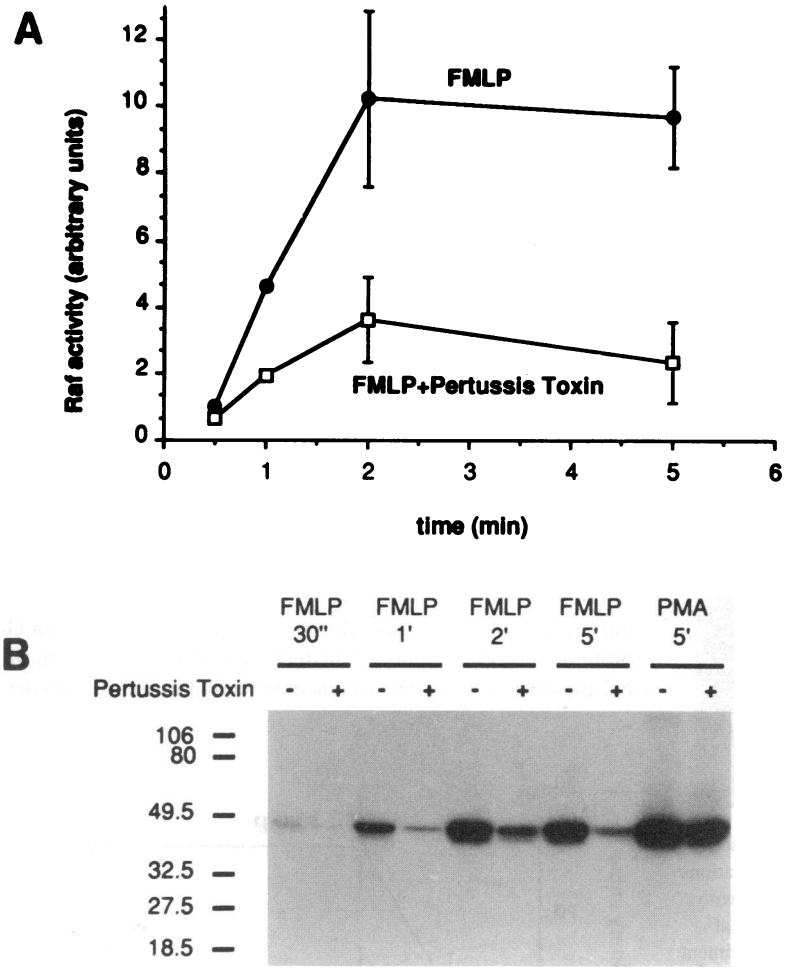

Figure 4. Effect of pertussis toxin on FMLP- and PMA-induced Raf activation. Neutrophils were incubated in the presence or absence of 500 ng/ml pertussis toxin for 2 h before stimulation with 10^{-8} M FMLP. At various times after stimulation, the neutrophils were lysed, and Raf was immunoprecipitated and assayed for activity using kinase-inactive MEK as substrate. (*A*) Quantification of phosphorylation of kinase-inactive MEK in three experiments using the PhosphorImager. Inhibition by pertussis toxin of activation is significant by two-way ANOVA. (*B*) One of the three autoradiograms used to construct the graph in *A* is shown, representative of three with similar results. Intensity of ^{32}P incorporation into kinase-inactive MEK is shown at 0.5, 2, and 5 min after FMLP in the presence or absence of pertussis toxin. Also shown is the effect of PMA to activate Raf-1.

was inhibited significantly at 2 and 5 min after FMLP. The effectiveness of CPTcAMP was also transient, as preincubation with CPTcAMP for 15 min was less effective (data not shown).

A tyrosine kinase inhibitor prevents Raf activation. The cellular mechanisms invoked most commonly for activation of Raf have involved the action of growth factor receptors with intrinsic tyrosine kinase activity. To begin to determine the mechanisms by which G protein–linked receptors might activate Raf, we have questioned whether inhibitors of tyrosine kinases might block FMLP-induced Raf activation. We have used the inhibitor Herbimycin A, suggested to be most specific for Src-family tyrosine kinases, as we have reported that this agent inhibits FMLP-induced MAP kinase activation.[2] As seen in Fig. 8, incubation with 5 μM Herbimycin A prevented Raf activation, raising the possibility that activation of a cellular tyrosine kinase may contribute to activation of Raf and Ras.

Discussion

Stimulation of neutrophils by chemoattractants is critical to establishing an acute inflammatory response and hence to mechanisms of host defense. These same defenses may also induce tissue injury as seen in a variety of inflammatory disorders of the lung and elsewhere. Accordingly, the pathways by which signals from chemoattractants induce neutrophil responses are of considerable importance to mechanisms of disease. Here we suggest that a pathway heretofore implicated in signal transduction through receptors with tyrosine kinase activity may occur upon interaction of FMLP with its receptor in human neutrophils. This receptor, a seven transmembrane–spanning G-protein–linked receptor, is a prototype for chemoattractant receptors in the neutrophil and other leukocytes, but has not previously been suggested to mobilize the recently described Ras/Raf pathway. The pathways suggested to be used in FMLP receptor–mediated activation of Ras, Raf-1, MEK-1, and MAP kinase are indicated schematically in Fig. 9 and will be the subject of this discussion.

It has been considered previously that serpentine receptors such as that represented by the FMLP receptor acted through effectors such as adenylate cyclase, phospholipase C, and K^+ channels. In this study, we have demonstrated that FMLP activates Ras and Raf in a pertussis toxin–sensitive fashion, suggesting that one important activation mechanism involves linkage of G_{i2} to activation of Ras and Raf. Although phospholipase C stimulation can lead to activation of PKC, Raf activation by FMLP was unaffected by a potent and selective PKC antagonist (36) that blocked PMA-induced activation of Raf. These data suggest that, in the neutrophil, direct G-protein–coupled activation of PLC is not the predominant pathway leading to activation of Raf-1. Rather, the inhibition of both Ras and Raf by pertussis

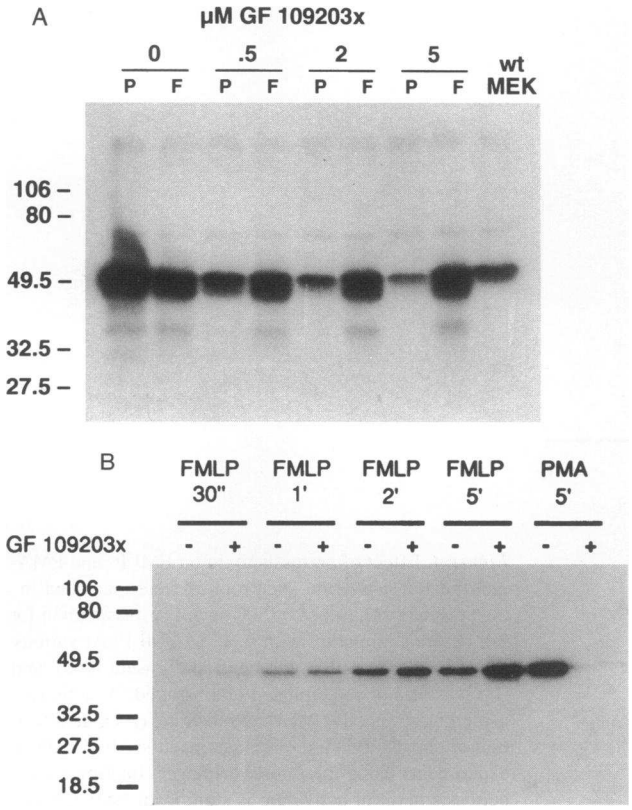

Figure 5. Effect of inhibition of protein kinase C on FMLP-induced Raf activation. Neutrophils were incubated with indicated concentrations of the PKC inhibitor GF109203x for 30 min before stimulation with 10^{-8} M FMLP for various times or 10 ng/ml PMA for 5 min. Raf activation was assessed as described. (*A*) Shown is a single experiment representative of three similar experiments indicating the concentration dependence of inhibition of Raf by GF109203x. The effect of GF109203x to inhibit PMA-induced Raf activation was significant by two-way ANOVA. (*B*) Shown is a single experiment representative of three similar experiments using 2 μM GF examining the time course of Raf activation by FMLP. No significant inhibition of Raf was detected.

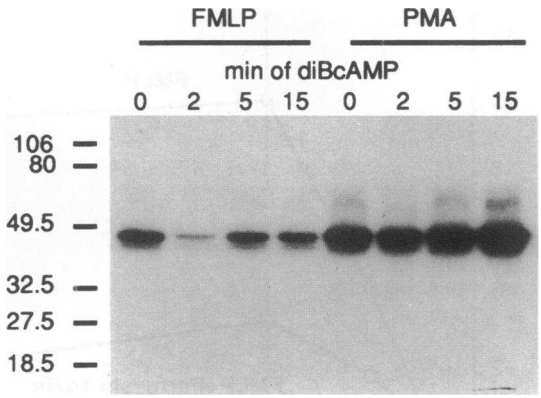

Figure 6. Effect of increased cAMP on Raf activation. Neutrophils were incubated with 10^{-5} M dibutyryl cAMP for the indicated times before stimulation by 10^{-8} M FMLP (2 min) or 10 ng/ml PMA (5 min). Raf activation was assessed as described. Shown is a single experiment representative of three similar studies.

toxin suggests that activation of Ras (12) by αi2·GTP is the predominant pathway for Raf activation.

The products of the Ras family of protooncogenes are small (20–25 kD) guanine nucleotide–binding proteins that are biologically active when bound to GTP and inactive when bound to GDP. They hydrolyze GTP to GDP, and the rate of hydrolysis is regulated by GTPase activating proteins or GAPs (37). Stimulation of guanine nucleotide exchange, induced by Ras exchange factors (38), will lead to GTP loading and activation of Ras. Activation of Ras can thus be accomplished either by inhibiting GAPs or stimulating exchange factors. The former has been described in response to T cell activation (39), while the latter has been demonstrated in Rat 1 fibroblasts (31). Ras activation has been demonstrated in response to a wide variety of stimuli in T cells, mast cells, epithelial cells, and fibroblasts, but has not been described previously in neutrophils. Furthermore, although many stimuli induce Ras activation, including phorbol esters and IL-2 (39), IL-3 and GM-CSF (40, 41), TGF-B (42), EGF (27), and PDGF (43), Ras activation has only recently been suggested to occur after stimulation with G-protein–linked receptors. Transfection of Rat 1a cells with the m2 muscarinic receptor (18) or transfection of the α2 adrener-

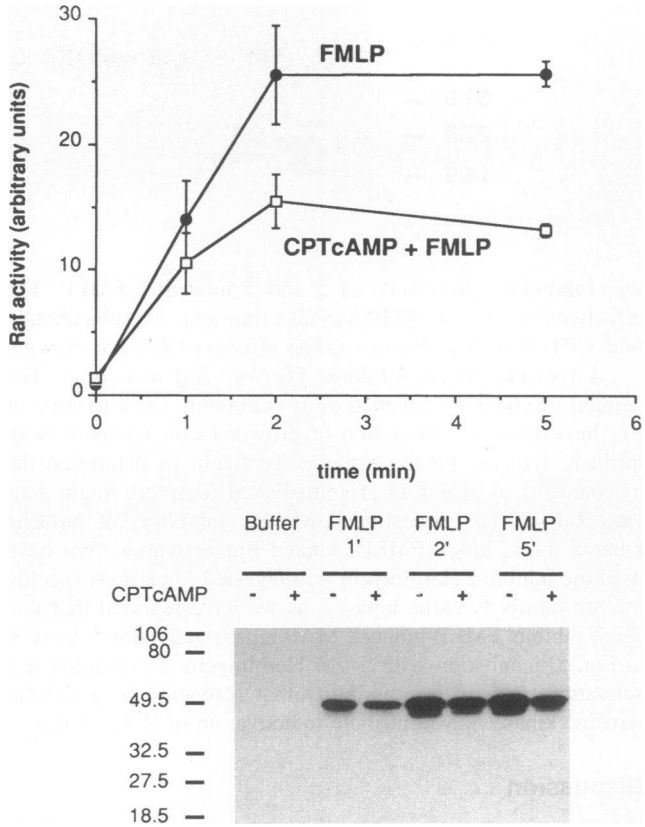

Figure 7. Effect of increased cAMP on Raf activation. Neutrophils were incubated without or with 60 μM CPTcAMP for 3 min before stimulation by 10^{-8} M FMLP. Cells were harvested at the indicated times, and Raf activation was determined. Three experiments were performed and quantified using the PhosphorImager, and means and standard error are shown. The effect of CPTcAMP is significant at 2 and 5 min after FMLP.

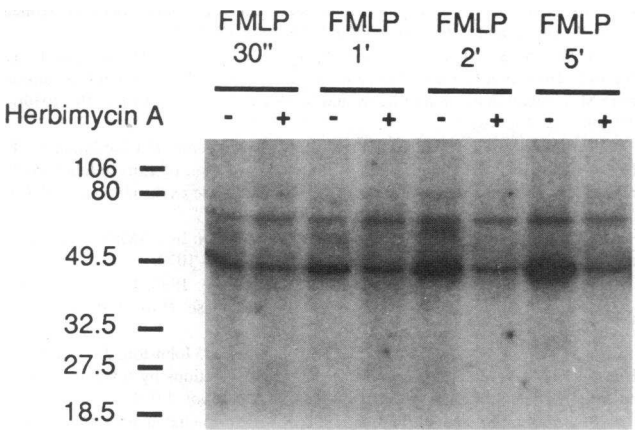

Figure 8. Effect of a tyrosine kinase inhibitor on FMLP-induced Raf activation. Neutrophils were incubated with 5 μM Herbimycin A for 4 h in media before stimulation with 10^{-8} M FMLP. Shown is a single experiment representative of two experiments revealing similar results.

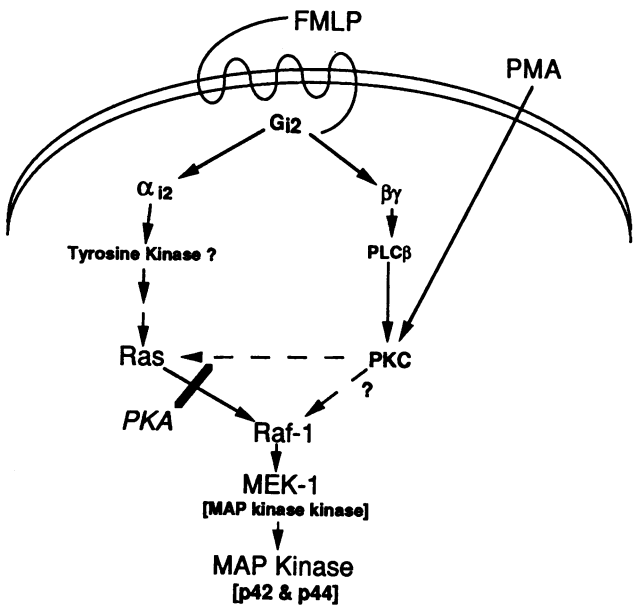

Figure 9. Speculative but simplified scheme to elucidate Gi2-linked signaling pathways activated by FMLP in the human neutrophil. While not comprehensive, the scheme indicates a preferred path for Ras and Raf activation, based on data presented here, that is independent of PKC and might involve a tyrosine kinase(s). It should be recognized, however, that other routes to MEK activation exist, and that several different MEKs, in addition to MEK-1, have already been identified (see text).

gic receptor into Rat 1 cells (44) confers ligand-induced activation of Ras and Raf in a pertussis toxin–sensitive manner, consistent with the notion that these receptors couple largely to G_{i2}. Activation of the lysophosphatidic acid receptor in fibroblasts (also linked, in part, through G_{i2}) has similarly been shown to induce activation of Ras and Raf (45). The data presented here, in the context of other literature, suggest the general notion that serpentine receptors linked to G_{i2} are capable of signaling through Ras and Raf.

The role of Ras, Raf, and MAP kinase in mediating functional responses of neutrophils to chemoattractants is poorly understood. The recent flurry of reports implicating Raf as an important inhibitory target for cAMP-dependent kinase (19, 20, 46) suggested that cAMP might have such effects in neutrophils. Indeed, as shown here, cell-permeant analogues of cAMP potently but transiently inhibited Raf activation by FMLP (Figs. 6 and 7). These results, considered in conjunction with the many studies linking cAMP to inhibition of neutrophil function, might suggest that activation of Raf may be involved in those functional responses. We and others have shown previously that maneuvers that elevate intracellular cAMP attenuate actin assembly (34), chemotaxis (33), adherence (47), O_2^- secretion (35), and liberation of arachidonic acid (48). This latter response is particularly noteworthy, as several groups have demonstrated recently that the cytosolic phospholipase A2 is phosphorylated and activated by MAP kinase (13). While not discounting the potential for other sites of action of cAMP and cAMP-dependent protein kinase, these results suggest the possibility that inhibition of Raf activation interferes with neutrophil function and thus that it may play a critical role in the transduction of such functions. Hence, the regulation of MAP kinase pathways in cells such as the neutrophil is predicted to play at least a modulatory role in specific differentiated functions including chemotaxis and secretion.

More than one effector pathway may lead to Ras, Raf, and MEK activation in the neutrophil. Phorbol ester activation of PKC leads to Ras activation in the neutrophil (data not shown). PKC may also directly phosphorylate and activate Raf (32). In our studies, however, effective inhibition of PMA-induced Raf activation with a PKC antagonist had no effect on FMLP-stimulated Raf activation, suggesting its independence from PKC. Given recent information relating Ras activation to receptors with tyrosine kinase activity, it is tempting to ascribe some component of the activation of Ras and Raf by FMLP to activation of a tyrosine kinase. In this context, we have reported that tyrosine kinase inhibitors attenuate activation of MAP kinase induced by FMLP,[2] and have shown here that the most potent of these inhibitors, Herbimycin A, also blocks Raf activation (Fig. 8). This inhibitor does not interfere with the Ca^{2+} transient induced by FMLP (data not shown), since this event probably is secondary to activation of PLCβ. Despite this suggestive evidence, evidence for direct involvement of a tyrosine kinase in Ras or Raf activation by seven transmembrane–spanning receptors is currently lacking. Similarly, other pathways to MEK activation have been shown to exist. The recent cloning of MEKK, a kinase homologous to Ste11 which plays a critical role in the pheromone pathway (mediated through a serpentine receptor) in budding yeast (17), suggests that MEK activity might reflect the actions of several upstream events, each with its own control system.

The pathway shown here involving Raf-1, MEK-1, and MAP kinase activation indicates not only the activity of these kinases, but also the potential for a far more complex array of interconnected kinases than has heretofore been suggested in neutrophils. Each of the kinases implicated here is a member of a family with several other members. Thus at least two other family members of the MAP kinase family (ERK-3 and -4) (49), MEK (MEK-2 and -3) (50), and Raf (A-Raf and B-Raf) (51) have been shown to coexist in some mammalian systems. Evidence suggesting the presence of another MEK can be adduced from the data in Fig. 1, where MEK activity elutes with-

out concomitant MEK-1 immunoreactivity in fractions 6–9. We have recently obtained evidence linking activation by the C5a receptor to activation of B-Raf in neutrophils (Buhl, A. M., and G. L. Johnson, unpublished observations). These complex and interrelated pathways, before now largely invoked in response to growth factor receptors, must now be considered as part of the response of terminally differentiated cells to seven transmembrane–spanning receptors linked to heterotrimeric G proteins.

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