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Differential fMet-Leu-Phe- and Platelet-activating Factor-induced Signaling Toward Ral Activation in Primary Human Neutrophils*

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We have measured the activation of the small GTPase Ral in human neutrophils after stimulation with fMet-Leu-Phe (fMLP), platelet activating factor (PAF), and granulocyte macrophage-colony stimulating factor and compared it with the activation of two other small GTPases, Ras and Rap1. We found that fMLP and PAF, but not granulocyte macrophage-colony stimulating factor, induce Ral activation. All three stimuli induce the activation of both Ras and Rap1. Utilizing specific inhibitors we demonstrate that fMLP-induced Ral activation is mediated by pertussis toxin-sensitive G-proteins and partially by Src-like kinases, whereas fMLP-induced Ras activation is independent of Src-like kinases. PAF-induced Ral activation is mediated by pertussis toxin-insensitive proteins, Src-like kinases and phosphatidylinositol 3-kinase. Phosphatidylinositol 3-kinase is not involved in PAF-induced Ras activation. The calcium ionophore ionomycin activates Ral, but calcium depletion partially inhibits fMLP- and PAF-induced Ral activation, whereas Ras activation was not affected. In addition, 12-*O*-tetradecanoylphorbol-13-acetate-induced activation of Ral is completely abolished by inhibitors of protein kinase C, whereas 12-*O*-tetradecanoylphorbol-13-acetate-induced Ras activation is largely insensitive. We conclude that in neutrophils Ral activation is mediated by multiple pathways, and that fMLP and PAF induce Ral activation differently.

Neutrophils play an important role in the host defense to invading microbial pathogens. Upon infection neutrophils become activated through interaction with chemoattractants and cytokines. These ligands bind to a variety of cell surface receptors, including heterotrimeric G-protein-coupled receptors for fMet-Leu-Phe (fMLP)[1] and platelet activating factor (PAF), and tyrosine kinase-associated receptors for granulocyte-macrophage colony stimulating factor (GM-CSF). Receptor activation triggers intracellular signal transduction pathways, resulting in the correct biological response, for instance, migration, phagocytosis, antibody-dependent cell-mediated cytotoxicity, degranulation, and superoxide production. Improper functioning of neutrophils is implicated in the pathogenesis of a variety of inflammatory diseases resulting in tissue damage (1–5).

Activation of small GTPases of the Ras superfamily is thought to play a critical role in the regulation of neutrophil function (6). For instance, Rac1 is implicated in the assembly of the NADPH oxidase complex which generates the respiratory burst and has been demonstrated to be activated (7). Also Ras and its close relative Rap1 are rapidly activated after stimulation of primary human neutrophils with fMLP, PAF, and GM-CSF (8, 9), suggesting a role in initial events of neutrophil activation. Ras controls the Raf-MEK-ERK kinase cascade and, in addition, the regulation of the actin cytoskeleton (10, 11). The function of Rap1 is still largely unclear, although for neutrophils it has been suggested that Rap1 plays a role in the regulation of the respiratory burst (12, 13).

Recent attention has focused on the small GTPase Ral. Ral comprises of two very closely related proteins RalA and RalB and is abundantly expressed in human neutrophils. By yeast two-hybrid analysis several Ral-specific guanine nucleotide exchange factors (Ral-GEFs) were found to bind directly to the active, GTP-bound form of the small GTPases Ras, Rap1, and two other close relatives, R-Ras and TC21 (14–18). This suggested that Ral may be a downstream target for these Ras-like GTPases. Indeed, in NIH3T3-A14 fibroblasts it was shown that Ras mediates insulin and epidermal growth factor-induced activation of Ral (19–22). In platelets, where Ral is also abundantly expressed, Ral activation is mediated by calcium and correlated with the activation of Rap1 rather than Ras (23). Also in Rat2 fibroblasts Ral activation induced by epidermal growth factor and endothelin is mediated by intracellular calcium levels ($[Ca^{2+}]_i$) independently of Ras (24, 25).

We have investigated fMLP- and PAF-induced Ral activation in in human neutrophils and the possible involvement of Ras and Rap1. In addition, utilizing specific inhibitors and $[Ca^{2+}]_i$ depletion we analyzed the mechanism of Ral activation. From the results obtained we conclude that in human neutrophils Ral is a downstream target of both fMLP and PAF, each with different signaling pathways toward Ral. Furthermore, we conclude that besides Ras and/or Rap1, $[Ca^{2+}]_i$ elevation, diacylglycerol/PKC, Src-like kinases, and PI 3-kinase are involved in the regulation of Ral activation.

EXPERIMENTAL PROCEDURES

Isolation of Human Neutrophils—Blood was obtained from healthy volunteers from the Red Cross Blood Bank, Utrecht, The Netherlands. Mixed granulocytes were isolated from the buffy-coat of 500 ml of 0.4%

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[1] The abbreviations used are: fMLP, formyl methionyl leucyl phenylalanine; PAF, platelet activating factor; GM-CSF, granulocyte macrophage-colony stimulating factor; PI 3-kinase, phosphatidylinositol 3-Kinase; PKC, protein kinase C; GEF, guanine nucleotide exchange factor; RalGDS-RBD, Ral guanine nucleotide dissociating stimulator-Rap1-binding domain; Raf1-RBD, Raf1-Ras-binding domain; RLIP76-RBD, Ral interacting protein of 76-kDa Ral-binding domain; TPA, 12-*O*-tetradecanoylphorbol-13-acetate; $[Ca^{2+}]_i$, concentration of intracellular calcium; PP1, 4-amino-5-(4-methylphenyl)-7-(t-butyl)pyrazolo[3,4-*d*]pyrimidine; GST, glutathione *S*-transferase.

(w/v) trisodium citrate (pH 7.4)-treated blood as described previously (26). Mononuclear cells were removed by centrifugation over isotonic Percoll (1.078 g/ml) (Amersham Pharmacia Biotech). After lysis of the erythrocytes in isotonic NH_4Cl solution, neutrophils were washed and resuspended in incubation buffer (20 mM HEPES, 132 mM NaCl, 6 mM KCl, 1 mM $MgSO_4$, 1.2 mM KH_2PO_4, 5 mM glucose, 1 mM $CaCl_2$) containing 0.5% human serum albumin (Central Laboratory of The Netherlands Red Cross Blood Transfusion Service, Amsterdam, The Netherlands). Neutrophils were incubated for 30 min at 37 °C before stimulation. Neutrophils isolated in this manner were unprimed. In all experiments a concentration of 10^7 cells/ml was used for stimulation.

Neutrophil Stimulation—1 ml of neutrophil suspension was stimulated with one of the following stimuli: fMLP (1 μM), PAF (1 μM), TPA (100 ng/ml), thapsigargin (100 nM) (all from Sigma), GM-CSF (0.1 nM) (Genzyme), and ionomycin (100 nM) (Calbiochem). In some experiments cells were preincubated with GF109203X (5 μM), Ly294002 (10 μM), PP1 (50 μM) (Biomol), Ro 31-8220 (5 μM) (Calbiochem), and PD98059 (10 μM) (Sigma). After stimulation 0.5 ml 3 × Lysis buffer (1 × Lysis buffer: NaCl (100 mM), Tris-HCl (10 mM), pH 7.4, 1% Nonidet P-40, 10% glycerol, $MgCl_2$ (2 mM), phenylmethylsulfonyl fluoride (2 mM), benzamidine (2 μM), aprotinin (2 μM), leupeptin (2 μM), trypsin inhibitor (2 μg/ml)) was added to lyse the cells.

Ral, Ras, and Rap1 Activation Assay—After stimulation and lysis, samples were put on ice for 5 min and clarified by centrifugation at 14,000 rpm in an Eppendorf centrifuge for 8 min at 4 °C. Per cell lysate 100 μl of bacterial lysate containing GST-Ral-binding domain of RLIP76 (GST-RLIP-RBD) and 100 μl of bacterial lysate containing GST-Rap-binding domain of Ral GDS (GST-RalGDS-RBD) or 100 μl of bacterial lysate containing GST-Ras-binding domain of Raf (GST-Raf-RBD) isolated as described in Refs. 23, 27, and 28, was precoupled for 1 h to 50 μl of 10% glutathione beads. After coupling, beads were washed 4 times with Lysis buffer, added to the cell lysate and incubated for 30 min at 4 °C. Samples were washed 3 times in Lysis buffer and bound proteins were eluted in 15 μl of Laemmli sample buffer. The samples were separated by SDS-polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride membranes (Immobilon-P, Millipore). RalA and Rap1 were detected with monoclonal antibodies (Transduction Laboratories) and horseradish peroxidase-coupled goat anti-mouse (Bio-Rad) using enhanced chemiluminescence (NEN Life Science Products Inc.). To detect Ras Y13–259 and horseradish peroxidase-coupled rabbit anti-rat (Santa Cruz) were used. RalB was detected with a goat polyclonal (Santa Cruz) and horseradish peroxidase-coupled donkey anti-goat (Santa Cruz). Band intensities were measured after scanning the blots with the use of NIH image 1.62 (Macintosh). All experiments were done at least three times and representative experiments are shown.

ERK2 Phosphorylation—10^6 Neutrophils were stimulated and lysed in 1% Triton, 150 mM NaCl, 50 mM Tris-NaCl (pH 8.0), 400 μM $NaVO_4$, benzamidine (2 μM), aprotinin (2 μM), and leupeptin (2 μM). 5 × Laemmli sample buffer was added and samples were boiled for 5 min at 95 °C. Phosphorylated ERK was detected as described previously (29). Samples were put on 10% SDS-polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride. ERK2 was detected with polyclonal antibodies and horseradish peroxidase-coupled goat anti-rabbit using enhanced chemiluminescence.

Depletion of Intracellular Free Ca^{2+}—Neutrophils were Ca^{2+} depleted as described before (30). Neutrophils were suspended in Ca^{2+}-free incubation buffer supplemented with 1 mM EGTA. Indo-1/AM (Molecular Probes) was added to 1-ml aliquots of suspended cells (10^7 cells/ml) at a final concentration of 1.5 μM and incubated for 40 min at 37 °C. To deplete the internal Ca^{2+} stores, 100 nM thapsigargin was added 10 min prior to washing. Cells were washed once with Ca^{2+}-free incubation buffer containing 1 mM EGTA. Under these conditions we did not observe any increase in intracellular calcium when the cells were treated with fMLP (not shown, but see Ref. 9).

RESULTS

fMLP and PAF Induce Ral Activation—Utilizing activation-specific probes which recognize the active GTP-bound form, but not the inactive GDP-bound form of small GTPases (23, 27, 28), we have analyzed activation of Ral, Ras, and Rap1 after stimulation with fMLP. As shown in Fig. 1*A* treatment of resting primary human neutrophils with fMLP (1 μM) resulted in a very rapid but transient activation of Ral. Activation was maximal after 30 s and returned to basal levels after 10 min. The pattern of activation was the same in every experiment but the maximal fold activation was donor-specific and varied between 5- and 25-fold.

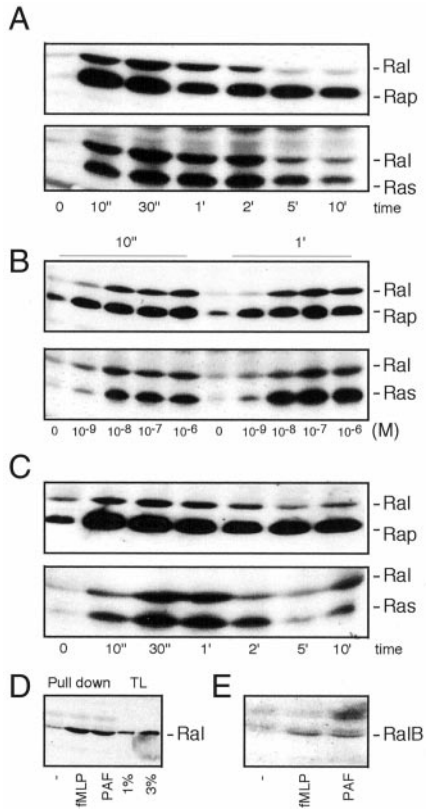

FIG. 1. **fMLP- and PAF-induced activation of the small GTPases Ral, Rap1, and Ras.** *A,* neutrophils were stimulated with 1 μM fMLP for the time indicated. Ral and Rap1 or Ras activity were determined in the same cell lysate by precipitating the GTP-bound GTPases with activation specific probes: for Rap1 (RalGDS-RBD), Ras (Raf-RBD), and Ral (RLIP76-RBD). After gel electrophoresis and Western blotting, Ral, Ras, and Rap1 were detected with mouse monoclonals against Rap1, Ras, and Ral. *B,* neutrophils were stimulated with various concentrations of fMLP for 10 s or 1 min for Ral and Rap1 activity, or Ras and Ral activity were determined as described in *A. C,* PAF-induced activation of the small GTPases Ral, Rap1, and Ras. Neutrophils were stimulated with 1 μM PAF. Stimulations and activation assays were done as described in *A*. Elevated levels of Ral and Ras GTP in the *lower panel* at 10 min was observed in two out of nine experiments and is a clear example of donor variation. The *top panel* is from a different donor and Ral-GTP is low. *D,* neutrophils were stimulated and lysed as described in *A*. Next to the pull downs, 1 and 3% of the original amount of neutrophil lysates were put on gel. Quantification of the amount of Ral-GTP detected after fMLP and PAF stimulation indicates that about 5% of Ral is recovered with the use of RLIP76-RBD. *E,* neutrophils were stimulated and lysed as described in *A*. RalB was detected with a αRalB.

A similar activation profile was observed for Ras, whereas Rap1 showed the biphasic activation with a major peak at 30 s and a minor peak at 5 min as reported previously (9). Activation of Ral was already observed with fMLP at 1 nM and maximal at 10 nM. Ras activation showed a similar sensitivity for fMLP (Fig. 1*B*). Maximal Rap1 activation was already detected after 1 nM fMLP stimulation. This demonstrated that, similar to Ras and Rap1, Ral is very rapidly activated by fMLP at a concentration known to induce neutrophil effector functions (8, 31, 32).

Ral activation was also observed after stimulation of neutrophils with PAF (Fig. 1*C*). Activation peaked between 30–60 s and decreased afterward. After 10 min PAF stimulation, no significant Ral activation was detected. Ras followed similar kinetics as Ral, whereas Rap1 activation was still detected after 10 min.

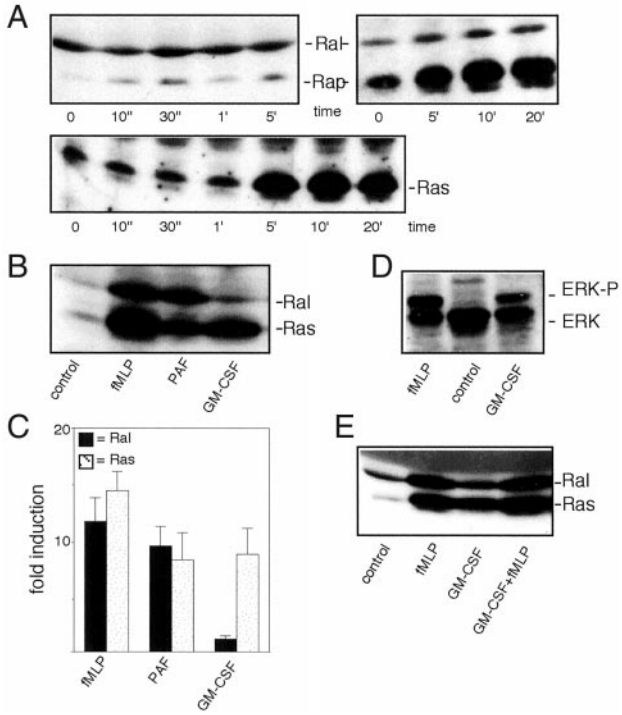

FIG. 2. **Ral activation after GM-CSF stimulation.** *A,* neutrophils were stimulated with 0.1 nM GM-CSF as described in the legend to Fig. 1*A*. *B,* levels of Ras activation after 1 min fMLP (1 μM), 1 min PAF (1 μM), and 10 min GM-CSF (0.1 nM) stimulation were compared. Activation was determined as described in the legend to Fig. 1*A*. *C,* the amount of Ral- and Ras-GTP of Fig. 3*B* was quantified and depicted as fold induction compared with the amount of Ral- and Ras-GTP detected in non-stimulated neutrophils. *D,* activation of ERK after stimulation with 1 min fMLP (1 μM) or 10 min GM-CSF (0.1 nM) was determined by mobility shifts. ERK-P is the active form. *E,* GM-CSF has no inhibitory effect on fMLP-induced Ral activation. Neutrophils were treated for 20 min with 0.1 nM GM-CSF. Afterward neutrophils were stimulated with 1 μM fMLP for the indicated time points. Ral and Ras activation was determined as described.

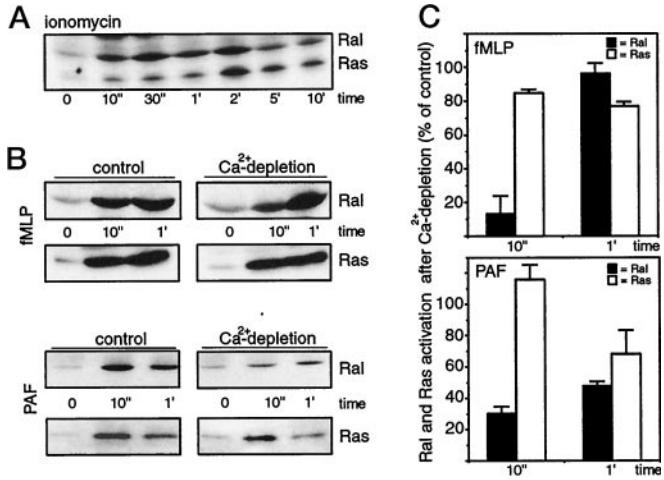

FIG. 3. **Calcium dependence of Ral activation.** *A,* Ral and Ras activation after stimulation with 100 nM ionomycin for the indicated time points. *B,* neutrophils were depleted of intracellular Ca^{2+}. Afterward neutrophils were stimulated with 1 μM fMLP or 1 μM PAF. *C,* the amount of Ral-GTP detected in calium-depleted neutrophils was quantified and depicted as % activation. 100% is the induction of Ral-GTP detected after fMLP or PAF stimulation for the indicated time in control neutrophils.

With the use of RLIP76-RBD, 5–10% of Ral-GTP is recovered (23). Because approximately 4–5% of the total amount of Ral present in neutrophils is recovered by RLIP76-RBD (Fig. 1*D*) after fMLP stimulation, this implied that a significant amount of Ral is in the GTP bound state.

With the use of a RalB specific antibody we could also detect RalB activation after 1 min fMLP and PAF stimulation. Apparently both RalA and RalB are activated in neutrophils. Because the RalA antibody is more sensitive than the RalB antibody we focussed on the activation mechanism of Ral detected with the RalA antibody.

GM-CSF Activates Ras and Rap1, but Not Ral—We have measured Ral activation after GM-CSF stimulation and as shown in Fig. 2*A*, Ral was not significantly activated in the first 20 min after stimulation. Interestingly GM-CSF did induce both Ras and Rap1 activation (Fig. 2*A*). Furthermore, GM-CSF induced Ras activation was comparable to fMLP and PAF induced Ras activation (Fig. 2, *B* and *C*), suggesting that the level of Ras activation is not the limiting factor for Ral activation. A downstream target of Ras, ERK was activated after GM-CSF stimulation (Fig. 2*D*), indicating that the GM-CSF induced Ras activation is functional toward downstream targets. This implies that GM-CSF either induces an inhibitory signal which prevents Ras (and/or Rap1) mediated activation of Ral, or that Ras (and/or Rap1) activation is not sufficient for Ral activation.

To discriminate between these two possibilities, we treated neutrophils for 10 min with GM-CSF and subsequently with fMLP. We did not observe any inhibitory effect of GM-CSF on fMLP-induced Ral activation (Fig. 2*E*). This implies that GM-CSF-induced Ras (and/or Rap1) is not sufficient for Ral activation.

Calcium and Diacylglycerol Signaling Activate Ral—If indeed Ras (and/or Rap1) activation is not sufficient for Ral activation, it may be that fMLP- and PAF-induced Ral activation is not mediated by Ras (and/or Rap1) but via different signaling pathways not induced by GM-CSF.

After stimulation with fMLP or PAF, phospholipase β is activated resulting in diacylglycerol-mediated activation of protein kinase C (PKC) and inositol 1,4,5-triphosphate-mediated Ca^{2+} mobilization (6, 33). Since an increase in $[Ca^{2+}]_i$ has been implicated in the activation of Ral (23, 24) and GM-CSF does not give rise to $[Ca^{2+}]_i$ (30) in neutrophils, we have investigated whether calcium is involved in Ral activation in neutrophils. Indeed, the calcium ionophore ionomycin did induce the activation of Ral, showing that changes in $[Ca^{2+}]_i$ are sufficient to induce Ral activation (Fig. 3*A*). However, only an inhibition of the early fMLP-induced Ral activation, at 10 s, was observed in calcium-depleted neutrophils, indicating that a change in $[Ca^{2+}]_i$ is not the sole mechanism by which fMLP can activate Ral. Compared with the fMLP-induced Ral activation, PAF-induced Ral activation is more sensitive to calcium depletion. Not only the 10-s induced Ral activation is blocked, but also the 1-min Ral activation is inhibited by more than 50% (Fig. 3, *B* and *C*). Calcium depletion did not affect the 10-s fMLP- and PAF-induced Ras activation (Fig. 3, *B* and *C*), indicating that the calcium-dependent Ral activation is unlikely to be mediated via Ras.

Although GM-CSF induces diacylglycerol formation, there is no evidence that classical PKCs are activated (34, 35). Therefore we also investigated the involvement of PKC in Ral activation. As shown in Fig. 4 TPA is a strong activator of both Ral and Ras (Fig. 4) and Rap1 (9). To investigate whether TPA-induced Ral activation is mediated via PKC we used the protein kinase C inhibitors bisindolylmaleimide (GF109203X) and Ro 31-8220. Interestingly, these inhibitors did not or only partially inhibited TPA-induced activation of Ras, whereas TPA-induced Ral activation was completely inhibited. These results indicated that TPA-induced Ral activation is mediated via PKC and not via Ras.

We next investigated whether PKC is an essential compo-

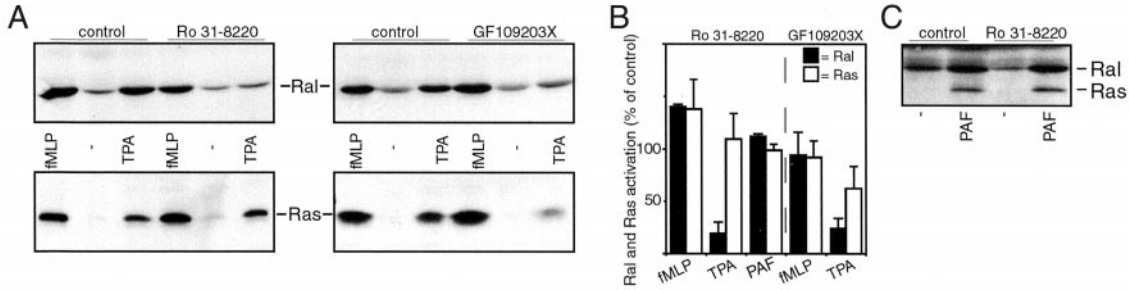

FIG. 4. **PKC is not involved in fMLP- and PAF-induced Ral and Ras activation, whereas TPA-induced Ral activation is inhibited by PKC inhibitors.** *A,* neutrophils were pretreated with 3 μM GF109203X, 5 μM Ro 31–8220, or solvent for 10 min. Afterward neutrophils were stimulated with 1 μM fMLP for 1 min or 100 ng/ml TPA for 5 min. Neutrophils were only pretreated with 5 μM Ro 31–8220 in the case of 1 μM PAF stimulation. Ras and Ral activations were determined as described in legend to Fig. 1*A*. *B,* detected Ral- and Ras-GTP were quantified and depicted as described in the legend to Fig. 3*C*. 100% is the induction of Ral-GTP detected from stimulated neutrophils treated with solvent. *C,* neutrophils were pretreated with 5 μM Ro 31–8220 or solvent for 10 min. Afterward neutrophils were stimulated with 1 μM PAF for 1 min. Ras and Ral activation were determined as described in legend to Fig. 1*A*. 100% is the induction of Ral-GTP detected from stimulated neutrophils treated with solvent.

nent in fMLP- and PAF-induced activation of Ral. However, the fMLP-induced activation of both Ral and Ras was not affected by both inhibitors (Fig. 4). Also PAF-induced Ral and Ras activation is not affected by Ro 31-8220 (Fig. 4). These results show that fMLP- and PAF-mediated Ral activation can be independent of PKCs. Double inhibition of fMLP stimulation by calcium depletion and GF109203X only partially inhibited, but did not block fMLP-induced Ral activation (Fig. 5), again indicating that fMLP-induced Ral activation can be independent of changes in $[Ca^{2+}]_i$ and PKCs.

PI 3-Kinase and Src-like Kinases Partially Mediate Ral Activation—In search for an additional pathway toward Ral activation we tested whether PI 3-kinase is involved in fMLP- and PAF-induced Ral activation, since PI 3-kinase is implicated in fMLP- and PAF-induced signaling (8, 36). Ly294002, a PI 3-kinase inhibitor did not inhibit the fMLP-induced Ral activation, whereas the PAF-induced Ral activation was partially inhibited (Fig. 6A). Neither fMLP- nor PAF-induced Ras activation are inhibited by LY294002 (Fig. 6*B*). These results indicated that besides calcium and PKC, PI 3-kinase can mediate PAF-induced Ral activation.

In addition, we tested whether fMLP- and PAF-induced Ral activation is mediated by Src-like kinases, which are known to be phosphorylated after fMLP and PAF stimulation (6, 33). After inhibition of Src-like kinases with PP1 (37), both the fMLP- and PAF-induced Ral activation were partially inhibited, under conditions that fMLP-induced respiratory burst was completely blocked (data not shown). The PP1 inhibition on PAF-induced Ral activation is more severe (Fig. 6*A*). fMLP-induced Ras activation is also, but mildly, affected by inhibition with PP1, whereas the PAF induced Ras activation is inhibited by approximately 75% (Fig. 6*B*). These results indicate that Src-like kinases partially mediate fMLP- and, more clearly, PAF-induced Ral and Ras activation. PD98059, a MEK inhibitor did not inhibit fMLP- and PAF-induced Ral and Ras activation, indicating that fMLP- and PAF-induced Ral and Ras activation are independent of MEK (Fig. 6).

Difference in Signal Transduction Toward Ral between fMLP and PAF—Our results suggested that fMLP-induced Ral activation is regulated differently than PAF-induced Ral activation. It is known that PAF and fMLP mediate signaling via different G-proteins. For instance, PAF-induced Ca^{2+} mobilization is not inhibited by pertussis toxin, whereas the fMLP-induced Ca^{2+} mobilization is (38, 39). Therefore we tested whether fMLP- and PAF-induced Ral activation could be blocked by preincubation with pertussis toxin. Indeed fMLP-induced Ral activation is inhibited by approximately 80%, but PAF-induced Ral activation is only inhibited by 20% (Fig. 7).

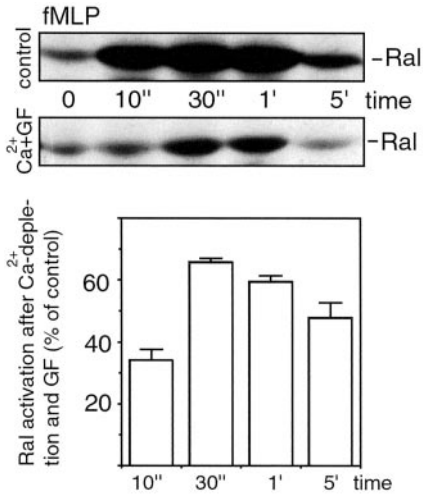

FIG. 5. **Inhibition of both calcium and PKC signaling only partially inhibits fMLP-induced Ral activation.** Neutrophils were calcium depleted and pretreated with 3 μM GF109203X or solvent for 10 min. Afterward neutrophils were stimulated with 1 μM fMLP for the indicated time points. Ral activation was determined as described in the legend Fig. 1*A*. 100% is the induction of Ral-GTP detected after fMLP or PAF stimulation for the indicated time in control neutrophils.

From these results we concluded that the fMLP-induced Ral activation is mostly regulated by pertussis toxin-sensitive G-proteins, whereas the PAF-induced Ral activation is mostly regulated by pertussis toxin-insensitive G-proteins.

DISCUSSION

In this study we show that the small GTPase Ral is rapidly activated after stimulation of primary human neutrophils with fMLP and PAF with similar kinetics as observed for the activation of Ras and Rap1. This correlation in activation between Ras and Ral is compatible with the finding that Ral activation is mediated by direct binding of Ras to exchange factors for Ral (RalGEFs) (19–22). Also Rap1 may mediate the activation of Ral, since Rap1 binds to Ral-GEFs *in vitro* (14, 15, 18) and in co-transfection studies in COS7 cells, active Rap1 induces Ral activation (25). Surprisingly, although GM-CSF did induce the activation of both Ras and Rap1, it fails to induce Ral activation. This implies that in neutrophils Ras and Rap1 activation does not necessarily lead to Ral activation.

It is unlikely that an inhibitory signal by GM-CSF results in the failure of Ras and/or Rap1 to activate Ral, since GM-CSF did not inhibit fMLP-induced Ral activation. More plausible explanations would be either that: (i) GM-CSF fails to provide

an essential auxiliary signal, (ii) GM-CSF activates a pool of Ras and Rap1 which is spatially separated from Ral-GEF and Ral, or (iii) Ral activation is independent of Ras and Rap1.

An alternative signaling pathway implicated in Ral activation is a change in $[Ca^{2+}]_i$ (23–25). Thus, if Ras and/or Rap1 are indeed unable to activate Ral, calcium may be responsible for fMLP- and PAF-induced Ral activation. This would be compatible with the notion that GM-CSF does not induce an increase in $[Ca^{2+}]_i$ (30, 40). Indeed, in neutrophils the calcium ionophore ionomycin induces Ral activation, showing that a rise in $[Ca^{2+}]_i$ is sufficient to activate Ral. However, both calcium-dependent and -independent pathways are used by fMLP and PAF to activate Ral. Because calcium depletion only partially affected Ral activation and did not affect the fMLP-induced Ras and Rap1 activation (9), this again indicated that Ras and Rap1 activation are not sufficient for Ral activation.

After stimulation of neutrophils with the phorbol ester TPA we observed activation of Ral, as well as Ras and Rap1 (9). Since TPA-induced Ral activation is sensitive to PKC inhibitors, this Ral activation is likely to be mediated by PKC. In contrast, Ras and Rap1 activation by TPA are largely independent of PKC (9 and Fig. 4). Most likely TPA-induced Ras and Rap1 activation is mediated by specific GEFs with diacylglycerol-binding domains (41–43). It should be noted that both in platelets and Rat1 NIH3T3-A14 fibroblasts, TPA fails to activate Ral, showing the cell type specificity of the regulation of Ral activation (25, 44).

fMLP-induced Ral activation was insensitive to PKC inhibitors and, in addition, inhibition by both calcium depletion and GF109203X only partially inhibited fMLP-induced Ral activation. This implies that besides diacylglycerol to activate PKC and calcium, other mediators of Ral activation may exist. Also PAF-induced Ral activation is insensitive to PKC inhibition, indicating that PAF may activate Ral by mediators other than calcium and PKC as well. To obtain further insight into a calcium and PKC independent pathway, we investigated fMLP- and PAF-induced Ral activation after pretreatment with various pharmacological inhibitors.

fMLP-induced Ral activation is insensitive to PI 3-kinase inhibition, whereas PAF-induced Ral activation is partially inhibited by PI 3-kinase inhibition. This implies that PI 3-kinase may, in part, be involved in PAF-induced Ral activation.

In accordance with previous reports in which tyrosine kinase-independent fMLP-induced Ras activation was found (48, 49), fMLP-induced Ras activation is independent of Src-like kinases. In contrast fMLP-induced Ral activation can be partially mediated by Src-like kinases. After PAF stimulation not only Ral but also Ras activation is mediated by Src-like kinases, suggesting that PAF and fMLP activate Ras and Ral by a different mechanism.

In addition, calcium depletion inhibited PAF-induced Ral activation more strongly than fMLP-induced Ral activation. Pertussis toxin inhibited fMLP-induced Ral activation, whereas PAF-induced Ral activation was largely insensitive to the same treatment, indicating that fMLP-induced signaling is mediated by pertussis toxin-sensitive Gα-proteins, as G_{i2} (50, 51), whereas PAF-induced signaling is mediated by pertussis toxin-insensitive G-proteins, as G_{i3}, $G\alpha_s$, or $G\alpha_q$ (52). This is in agreement with previous reports, where pertussis toxin inhibited fMLP-induced elevation of $[Ca^{2+}]_i$, but not PAF induced elevation of $[Ca^{2+}]_i$ (38, 39).

The biological function for Ral in neutrophils is still unclear. Since Ral is activated only after fMLP and PAF stimulation,

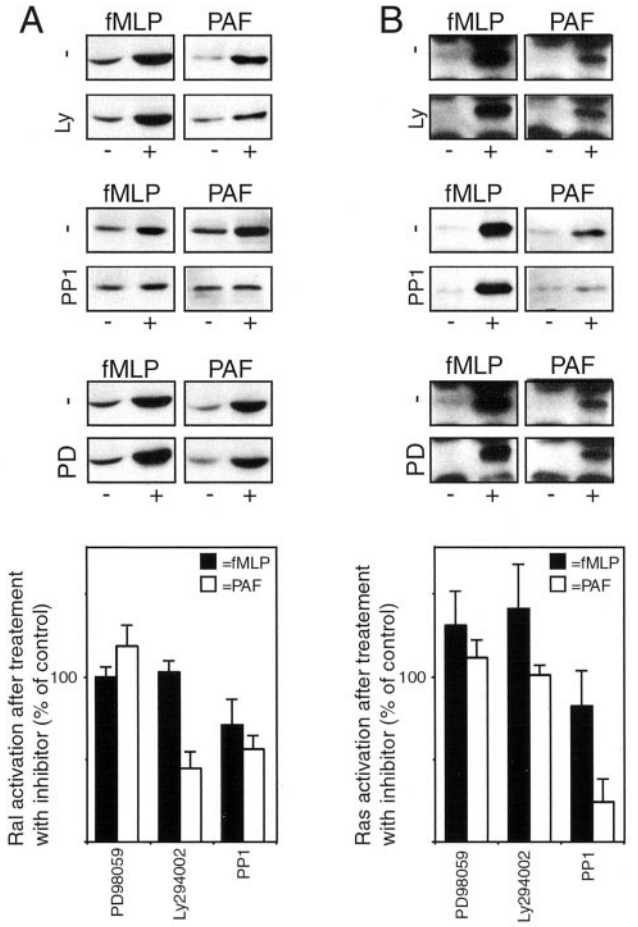

FIG. 6. **PAF-induced Ral activation is partially mediated by PI 3-kinase, whereas the fMLP-induced Ral activation is PI 3-kinase independent.** Neutrophils were pretreated with 10 μM PD98059, 10 μM LY294002, 50 μM PP1, or solvent for 10 min. Afterward neutrophils were stimulated with 1 μM fMLP or 1 μM PAF for 1 min. *A,* Ral activation was determined and quantified as described in legends to Figs. 1*A* and 3*C*. 100% is the induction of Ral-GTP detected from stimulated neutrophils treated with solvent. *B,* Ras activation determined and quantified as described in legends to Figs. 1*A* and 3*C*. 100% is the induction of Ral-GTP detected from stimulated neutrophils treated with solvent.

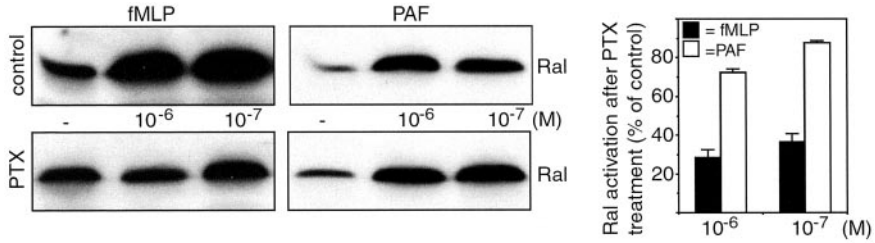

FIG. 7. **Pertussis toxin inhibits fMLP-induced Ral activation but not PAF-induced Ral activation.** Neutrophils were pretreated with 300 ng/ml pertussis toxin or solvent for 1 h. Afterward neutrophils were stimulated with 1 or 0.1 μM fMLP or 1 or 0.1 μM PAF for 1 min. Ral activation was determined and quantified as described in the legends to Figs. 1*A* and 3*C*. 100% is the induction of Ral-GTP detected from stimulated neutrophils treated with solvent.

but not after GM-CSF stimulation, Ral may be involved in a cellular response specific for fMLP and PAF, such as chemotaxis (directional cell movement), endo-/phagocytosis, degranulation, and respiratory burst. In the active GTP-bound form, Ral associates with RLIP76 (53, 54). This protein is a GTPase-activating protein for the small GTPase Cdc42, a GTPase involved in the control of filopodia formation in fibroblasts (55) and chemotaxis in macrophages (56). Recently it was reported that Ral in the GTP bound form also binds to ABP280/filamin 1, a protein involved in cross-linking of actin filaments. This complex may mediate Cdc42-induced filopodia formation (57). Furthermore, it has been suggested that RLIP76 may have a function in endocytosis via recently discovered Reps1 and POB1 (58, 59). Finally, Ral was reported to associate with the small GTPase Arf and phospholipase D_1 in a nucleotide independent manner (60–63). Both phospholipase D_1 and Arf are implicated in neutrophil vesicle transport and degranulation (64). Although the physiological relevance of these interactions remains to be established, they may indicate that Ral plays a role in the control of the actin cytoskeleton and processes related to this, such as establishing cell polarity, migration, and vesicular transport (55, 64).

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