ADP-ribosylation Factor and Rho Proteins Mediate fMLP-dependent Activation of Phospholipase D in Human Neutrophils*

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Phospholipase D (PLD)1 is an important signal-transducing enzyme in a wide variety of cells and catalyzes the hydrolysis of phosphatidylcholine (PC) to produce the potential second messenger phosphatic acid (PA) (1–4). Studies in neutrophils and HL60 cells have identified a requirement for both cytosolic and membrane components for PLD activation when stimulated by the nonhydrolyzable analog of GTP, GTP

In this study we have examined the recruitment of ARF and Rho proteins to membranes by fMLP and the ability of these proteins to regulate receptor-controlled PLD in human neutrophils. We had reported previously that fMLP-dependent activation was compromised in differentiated HL60 cells depleted of their cytosolic contents (26). We report that both ARF and Rho proteins are regulated by the fMLP receptor via a pertussis toxin-sensitive heterotrimeric G protein. Although activation of PLD in intact neutrophils is inhibited by wortmannin, a relatively selective inhibitor of PI 3-kinase, wortmannin treatment is not inhibitory to the fMLP-stimulated recruitment of ARF and Rho to membranes. We conclude that the activation of PLD by the fMLP receptor is dependent on receptor-activated ARF and Rho proteins in human neutrophils coupled via G proteins and is not obligatorily dependent on PI 3-kinase activation.

EXPERIMENTAL PROCEDURES

Materials—Neutrophils were purified from blood from healthy volunteers or isolated from buffy coats that were obtained from the North London Blood Transfusion Center. Recombinant ARF1 and myr-ARF1 proteins were purified from Escherichia coli as described previously (6). Anti-RhoA antibodies were obtained from Santa Cruz Biotechnology Ltd. The anti-ARF antibodies used have been described previously (27). All other reagents were obtained as described previously (26).

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Preparation of Neutrophils—Neutrophils were prepared according to established procedures (28). 50 ml of anti-coagulated blood or a buffy coat pack was mixed with an equal volume of 2% dextran solution in phosphate-buffered saline, pH 7.2, to aggregate erythrocytes. After 20 min at room temperature, the leukocyte-rich upper layer was removed and layered onto 10 ml of Lymphoprep and centrifuged at 2,000 rpm for 20 min to separate neutrophils from other white cells. Contaminating erythrocytes were removed by hypotonic lysis.

Analysis of ARF and Rho Leakage from Permeabilized Neutrophils—Neutrophils were permeabilized for varying lengths of time with 0.4 IU/ml streptolysin O. At the required time points, 1-ml aliquots were removed and centrifuged. The proteins from the supernatants (after precipitation with trichloroacetic acid) and cell pellets were resuspended in sample buffer and analyzed for ARF and RhoA proteins by Western blot analysis using appropriate antibodies.

Labeling of Neutrophils with [3H]Alkyllyso-PC—Neutrophils were washed twice in HEPES buffer (20 mM HEPES, 137 mM NaCl, 3 mM KCl, 1 mM MgCl₂, 1 mM CaCl₂, 1 mg/ml glucose, and 0.1 mg/ml bovine serum albumin, pH 7.2) and finally resuspended in 1.5 ml. The cells were incubated for 30 min at 37 °C with [3H]alkyllyso-PC (100 nM). The cells were harvested by centrifugation to remove unincorporated label, and the cells were washed with either HEPES buffer (for intact cell experiments) or PIPES buffer (20 mM PIPES, 137 mM NaCl, 3 mM KCl, 1 mM MgCl₂, and 0.1 mg/ml bovine serum albumin, pH 6.8) for permeabilized cell experiments.

Assay for PLD Activity in Intact Neutrophils—Neutrophils were suspended in HEPES buffer and pretreated with 5 μM cytochalasin B for 5 min. 50-μl aliquots were transferred to tubes containing 2% EtOH (1% final in the assay) in the presence or absence of FMLP (1 μM final). After a 10-min incubation at 37 °C, assays were quenched with 700 μl of CHCl₃:MeOH (1:1). After phase separation with 250 μl of water, the chloroform phase was recovered. The chloroform phase was dried under vacuum and redissolved in 50 μl of chloroform. Samples were spotted onto Whatman LK6TLC silica plates. The plates were developed in chloroform:methanol:acetic acid:water (75:45:3:1), dried at room temperature, and the lipid spots localized with iodine vapors. The spots corresponding to PEt and PC were excised after iodine sublimation and analyzed by TLC.

RESULTS

ARF Restores FMLP-dependent PLD Activity in Cytosol-depleted Human Neutrophils—To examine a requirement for ARF proteins in FMLP-stimulated PLD activity, human neutrophils were permeabilized with streptolysin O for 10 min to deplete the cells of their freely diffusible cytosolic proteins. Fig. IA illustrates that this protocol depletes the majority of the ARF proteins from the permeabilized cells, and they are recovered in the external medium. This loss is coincident with the inability of FMLP to stimulate PLD activity in cytosol-depleted cells (Ref. 26 and Fig. IB). FMLP regains the ability to stimulate PLD activity provided that myr-ARF1 is also added to the permeabilized cells (Fig. IB). It was noted that adding myr-ARF1 alone raised the basal activity of PLD, and this was dependent on the presence of GTP. In the reconstituted assay, the time course of PLD activation by FMLP reached a maximum at 30 min. Fig. 1, C and D, illustrates that reconstitution of PLD activity with myr-ARF1 and fMLP is concentration-dependent. For the remainder of the experiments, FMLP was used at 1 μM, and myr-ARF1 was used at 50 μg/ml.

Myristoylation of ARF Is Essential for FMLP- but Not GTPγS-dependent PLD Activity—ARF proteins are myristoylated at their NH₂ terminus, and this lipid modification is thought to be important for efficient guanine nucleotide exchange catalyzed by the ARF exchange factors (32–34). Both myristoylated (myr-ARF1) and nonmyristoylated (ARF1) ARF proteins were examined for their ability to restore FMLP-dependent PLD activity in cytosol-depleted neutrophils. Fig. 2 illustrates that myristoylation is essential for the restoration of FMLP-dependent PLD activity and also for the response observed with GTP alone. Consistent with our own observation (6) and those of others (5, 35), myristoylation is not required for GTPγS-dependent stimulation of PLD (Fig. 2). However, a 100-fold higher concentration of nonmyristoylated ARF1 is required for maximal stimulation compared with fully myristoylated ARF1 for GTPγS-stimulated PLD activity (6). Therefore, concentrations of recombinant ARF proteins used to examine the requirement for myristoylation take this into account; for nonmyristoylated ARF, a concentration of 750 μg/ml is used compared with 50 μg/ml for myr-ARF1. (Effective myr-ARF1 used is approximately 5 μg/ml (500 nM) because of the 10% efficiency of myristoylation in E. coli determined by mass spectroscopy analysis.) These concentrations of recombinant myr-ARF1 and non-myr-ARF1 reflect the equivalent loading of GTPγS when measured in vitro. Fig. 2 also illustrates that the level of PLD stimulated by these proteins is similar in magnitude when GTPγS is the activator.

Pertussis Toxin Inhibits Myr-ARF1-restored FMLP-dependent PLD Activity—The FMLP receptor is coupled to the pertussis toxin-sensitive heterotrimeric G proteins, G₁₂ and G₁₃ (36), and βγ subunits are the direct regulators of phospholipase Cβ₂ and PI 3-kinase (γ isoform) (37, 38). To address the question of whether activation of the PLD activity by the FMLP receptor in the reconstituted cells also requires a prior activation of G proteins, we examined the influence of pertussis toxin pretreatment. Initially we confirmed that, as reported previously (39, 40), pertussis toxin pretreatment led to inhibition of the FMLP-stimulated PLD activity in intact cells (Fig. 3A). Pertussis toxin pretreatment also inhibits fMLP-stimulated PLD activity when the agonist and the permeabilizing agent streptolysin O are added simultaneously, conditions in which the cytosolic proteins are still present (Fig. 3B). To establish a requirement for G₁₂ proteins in the regulation of PLD activity by myr-ARF1,
we examined the effect of pertussis toxin pretreatment on the myr-ARF1-restored fMLP-dependent PLD activity in cytosol-depleted neutrophils (Fig. 3C). Although the stimulation of PLD activity observed in the combined presence of GTP and myr-ARF1 was not inhibited significantly by pertussis toxin treatment, the fMLP-stimulated activity was inhibited (Fig. 3C). These results confirm that regulation of myr-ARF1 by the fMLP receptor is indirect and that one intervening component in the pathway leading to myr-ARF1 activation has to include the heterotrimeric Gi proteins.

**Rho Proteins Participate in PLD Activation by the fMLP Receptor**—Previous studies in a variety of cells have indicated that not only ARF but also Rho can activate PLD activity (1–4). It has been reported that in HL60 cells, endogenous Rho is unlikely to play a physiological role in PLD activation (16). However, these experiments were performed using GTPγS as an activator. To examine the contribution of Rho proteins in the system analyzed here, we first established whether Rho proteins leaked out of permeabilized cells. Neutrophils were permeabilized with streptolysin O for various lengths of time, and the supernatants and the cell pellets were analyzed for Rho proteins. The majority of RhoA remains cell-associated in the permeabilized cells even after 30 min. The amount of Rho released in the supernatant was low compared with that retained in the cells (Fig. 4). Under these conditions, the majority of the ARF proteins was found to leak out of the permeabilized cells (Fig. 1A). Because Rho proteins are found mainly to be cytosolic when cells are disrupted by homogenization (27), this would suggest that RhoA proteins do not behave as freely diffusible proteins under conditions in which the cellular architecture is maintained, as is the case in permeabilized cells. In addition to RhoA, Rac proteins were also retained in the permeabilized cells (data not shown).

Despite the retention of Rho proteins, the stimulation of PLD activity by fMLP is impaired in the cytosol-depleted cells (Fig. 1B), which would suggest that RhoA proteins do not play a major role in fMLP-stimulated PLD activation. However, RhoA has been shown to be a poor activator of PLD activity by itself; but when it is present together with ARF, a synergistic activation is observed (10). Thus, it was still possible that RhoA could be a contributory factor in the myr-ARF1-reconstituted response stimulated by fMLP in the permeabilized cells.

C3 transferase ADP-ribosylates Rho proteins, thereby inactivating them. To investigate a role for Rho proteins, permeabilized cells were treated with C3 transferase. The myr-ARF1-restored fMLP-dependent PLD activity was partially reduced when Rho proteins were inactivated (Fig. 5). This experiment uncovers the Rho component to the PLD response observed in the presence of myr-ARF. Thus ARF and RhoA proteins act synergistically to regulate fMLP-stimulated PLD activity. ARF
ARF and Rho Mediate fMLP-stimulated PLD activity.

ARF and Rho Mediate fMLP-stimulated PLD activity. Cytosol-depleted neutrophils were incubated as described in Fig. 1B in the presence or absence of 100 μM GTP, 1 μM fMLP, and 10 μM GTP/PPi as indicated. Myr-rARF1 was used at 50 μg/ml and non-myr-rARF1 at 750 μg/ml. The radioactivity incorporated into PEt was measured as described under “Experimental Procedures.” Results are means ± S.E. from three independent experiments done in duplicate.

Can stimulate PLD activity in the absence of Rho (C3-treated cells), whereas Rho requires the presence of ARF.

ARF and Rho Are Translocated to the Membrane Fraction on fMLP Stimulation—ARF and RhoA are found predominantly in the postnuclear supernatant where they are present in a GDP-bound state. Nucleotide exchange and therefore activation results in the stable interaction of ARF and Rho with membranes (41, 42). The data presented indicate that ARF and Rho are required for fMLP stimulation of PLD in a reconstituted system. To verify that ARF and Rho proteins are activated in intact cells, the translocation of ARF and Rho proteins to the membranes was examined upon stimulation with fMLP. Intact neutrophils were pretreated with cytochalasin B and incubated in the presence or absence of fMLP for 1 and 10 min. At the end of the incubation, the cells were recovered and the membrane fractions prepared. The samples were run on SDS-PAGE, blotted to polyvinylidene difluoride, and probed with the appropriate antibodies. Both ARF and Rho translocated to the membrane fraction within a minute of stimulation. (Maximal activation of PLD activity by fMLP in intact cells occurs at 1 min (28, 43).) ARF proteins remained membrane-associated even after 10 min, but the association of Rho was diminished at 10 min (see Fig. 6A).

PLD activation by fMLP in intact neutrophils is enhanced greatly by pretreatment with cytochalasin B (43). Several other neutrophil responses, including degranulation, respiratory burst, phospholipase A2 activation, and protein kinase C translocation, are also potentiated greatly by cytochalasin B (44–47). The priming of human neutrophils by cytochalasin B can also be mimicked by other physiological agonists, e.g. low concentrations of C5a, fMLP, or tumor necrosis factor, and is therefore of physiological relevance. To examine whether translocation of ARF and Rho was dependent on priming, the cells were stimulated with fMLP for 1 min with or without cytochalasin B pretreatment. The translocation of ARF was entirely dependent on cytochalasin B pretreatment, whereas some RhoA translocation could be observed in its absence. Cytochalasin B enhanced RhoA translocation (Fig. 6B). These data indicate that similar to the activation of PLD by fMLP, the translocation of the two regulators, ARF and RhoA, is more efficient in primed cells.

Fig. 6C illustrates that in permeabilized cells, translocation
ARF and Rho Mediate fMLP-stimulated PLD

Potential Role of PI 3-Kinase in PLD Activation—Although we have established that ARF and Rho proteins are involved in the stimulation of PLD via the fMLP receptor, it remains to be ascertained how the monomeric GTPases are activated by the fMLP receptor. Activation of Gi proteins is clearly an intermediary step as observed by the inhibition with pertussis toxin treatment. A potential candidate that could couple Gi proteins to ARF and Rho activation is PI(3)P, the product of PI 3-kinase activation. Wortmannin, a direct inhibitor of PI 3-kinase (48, 49), has been reported to block the activation of fMLP-stimulated PLD activity in intact human neutrophils (48, 50). PI(3)P could potentially recruit the ARF and Rho exchange factors to the plasma membrane via their PH (pleckstrin homology) domains (34, 52–55). The ARF exchange factors, ARNO, cytohesin, and GRP1, all contain a PH domain that can bind phosphoinositides and a SEC7 domain that is responsible for the exchange of GDP for GTP (34, 52–54). The guanine nucleotide exchange factors for Rho proteins all contain a DbI homology domain responsible for GDP-GTP exchange and also a PH domain (55). To test for this possibility, we examined whether wortmannin could inhibit the translocation of ARF and Rho proteins in fMLP-stimulated intact cells. Contrary to our expectations, wortmannin did not block the translocation of ARF proteins to membranes and marginally inhibited RhoA translocation (Fig. 6A). We observed consistently that wortmannin alone increased the amount of Rho proteins associated in the membranes slightly.

To check that wortmannin was effective in the inhibition of PLD activity in intact cells in our hands, we confirmed that wortmannin inhibited the fMLP-stimulated PLD activation in intact cells as reported previously (48). However, only a partial inhibition by wortmannin was observed in acutely permeabilized cells examined under conditions in which cytosolic proteins are still present during the time of stimulation (data not shown). We next examined the reconstitution of PLD activity by myr-ARF1 in the cytosol-depleted neutrophils, and this was not inhibited by wortmannin (data not shown). The lack of involvement of PI 3-kinase in the activation of fMLP-stimulated PLD in the cytosol-depleted cells is also in line with our recent observations (56). We have shown that fMLP is unable to stimulate PI 3-kinase activation in cytosol-depleted cells because of the loss of PI 3-kinase (γ isoform) and PTP (phosphatidylinositol transfer protein) from the permeabilized cells. Collectively, these results exclude the possibility that PI(3)P is an absolute requirement in the regulation of ARF and Rho proteins in human neutrophils. However, it is still possible that in intact cells PI 3-kinase has a modulatory function because wortmannin is an inhibitor in intact cells. In intact cells the activation of PLD activity is rapid and occurs within 1 min, whereas the reconstituted activity in cytosol-depleted cells is slow, and PI(3)P could potentially have a bearing on the kinetics of activation.

FIG. 5. C3 partially inhibits the fMLP/GTP response restored by myr-ARF. Neutrophils were labeled and depleted of cytosol by permeabilization for 10 min and then divided into two sets. Half of the cells were treated with C3 for 10 min before use in a reconstitution assay. Cells were incubated in the presence of 1 mM MgATP, 100 μM GTP, 2 mM MgCl₂, 1 mM CaCl₂, and 1% EtOH. Incubations were carried out at 50 μg/ml myr-ARF1 and 1 μM fMLP as indicated. The radioactivity incorporated into PI(3)P was measured as described under “Experimental Procedures” and is expressed as a percentage of dpm present in PC. The results are from a single experiment representative of two others.

FIG. 6. Panel A, wortmannin does not inhibit the fMLP-stimulated translocation of ARF or Rho to membranes. Intact cells were pretreated with 100 nM wortmannin as indicated. After treatment with 5 μM cytochalasin B, cells were stimulated with 1 μM fMLP as indicated. After a 1- or 10-min incubation, the cells were harvested and the membrane fractions obtained as detailed under “Experimental Procedures.” Equivalents of 10⁶ cells/lane were run on SDS-PAGE, transferred onto polyvinylidene difluoride, and the blots were probed with anti-ARF antibodies or anti-Rho antibodies. A representative blot repeated on four independent occasions is shown. Panel B, effect of cytochalasin B treatment on the translocation of ARF and Rho to the membrane fraction. Intact cells were incubated in the presence or absence of 1 μM fMLP and 5 μM cytochalasin B. After a 1-min incubation, the cells were harvested, and the membrane fractions were obtained and analyzed as in panel A. A representative blot repeated on two independent occasions is shown. Panel C, translocation of ARF to membranes by fMLP and GTP·S in permeabilized cells. Neutrophils were permeabilized with streptolysin in the presence of 1 mM MgATP, 2 mM MgCl₂, and 1 mM CaCl₂. GTP (100 μM), GTP·S (10 μM), and fMLP (1 μM) were added as indicated. After a 10-min incubation, the cells were harvested, and the membrane fractions were obtained and analyzed as for panel A. A representative blot repeated on two independent occasions is shown.
to stimulate PLD activity could be caused by the activation of protein kinase C isoforms. Alternatively, PMA could cause translocation of ARF proteins to membranes as reported previously for rat basophilic leukemia mast cells (58). In human neutrophils, we observe that PMA recruits both ARF and Rho to membranes (Fig. 7A) under conditions in which no PI 3-kinase activation occurs.

We examined the consequences of this PMA-stimulated recruitment of ARF and RhoA to membranes. Intact cells were pretreated with PMA to translocate ARF and RhoA to membranes. The cells were then permeabilized with streptolysin O for 10 min and washed. The resulting cytosol-depleted cells were incubated with or without GTPγS. In the control cells, GTPγS stimulated a small level of PLD activity. In the PMA-treated cells, the basal activity was substantially higher compared with control cells, which most likely reflects the recruitment of protein kinase C to the membranes. The addition of GTPγS to these cells showed an enhanced response compared with control cells (Fig. 7B).

**DISCUSSION**

Activation of many G protein-coupled receptors and receptor tyrosine kinases in intact cells leads to stimulation of PLD activity, but the intervening components that lead to PLD activation remain to be delineated. Mammalian PLD has three known members, hPLD1a, hPLD1b, and PLD2; hPLD1a and hPLD1b differ by lacking a 38-amino acid insert, and both are regulated by ARF and Rho proteins. PLD2 is constitutively active. In HL60 cells, a cell line that is related to neutrophils, the predominant isof orm that is expressed is hPLD1 (59).

**ARF and Rho Proteins Are Involved in the Coupling of the fMLP Receptor to PLD via a Heterotrimeric G Protein in Human Neutrophils**—In this study, we present results demonstrating that ARF and Rho proteins are involved in PLD regulation in fMLP-stimulated human neutrophils. Extended permeabilization depleted the cells of ARF proteins but not RhoA, and this compromised the ability of fMLP to stimulate PLD activity (Fig. 3). Myr-ARF1 (but not non-myr-ARF1) restores fMLP-dependent PLD activity in these cytosol-depleted cells (Fig. 1A). This result was surprising initially because we had previously shown that nonmyristoylated ARF proteins were equally competent at activating PLD when GTPγS was used as a stimulus. Higher concentrations of nonmyristoylated ARF are required to elicit a response similar to that of myristoylated protein in GTPγS-dependent PLD activation (5, 6). Myristoylation is clearly required for efficient nucleotide exchange at physiological concentrations of Mg2+ when the receptor-driven system is used (32, 34, 60). When GTPγS is used, the exchange factors must be bypassed, and the limited exchange that occurs on nonmyristoylated ARF could be the result of basal turnover. The recently described guanine nucleotide exchange factor for ARF (ARNO) has been shown to promote exchange rapidly on myristoylated proteins but not nonmyristoylated ARF (34, 60).

Unlike ARF (Fig. 1A), RhoA does not leak significantly from the cells upon permeabilization (Fig. 5). Although the presence of RhoA was insufficient to elicit a response to fMLP in ARF-depleted cells, in the presence of ARF a role for RhoA could be identified clearly. Treatment of cells with C3 transferase partially inhibited the reconstitution of ARF-regulated PLD activity, revealing the Rho component of fMLP-stimulated PLD activation. Synergistic activation by ARF and Rho has been noted for the expressed hPLD1a, and the data provided here reinforce the concept that multiple inputs are involved in the activation of PLD in intact cells triggered with a physiological stimulus. From *in vitro* studies, ARF is more active than RhoA in activating hPLD1a, and the data presented in this paper show that although ARF can activate PLD activity in the absence of Rho, Rho activation becomes apparent only in the presence of ARF. Further evidence that fMLP receptors can activate ARF and Rho was obtained from the observation that fMLP stimulates their translocation to the membrane fraction (Fig. 6). The conclusions drawn from our studies are at variance with those of Wakelam and co-workers (16), who reported that Rho was not a physiological activator of PLD in HL60 cells (16). However, their studies did not include an investigation with fMLP as a stimulus but instead used GTPγS, and this could possibly account for the different conclusions.

To elucidate the pathway leading to ARF and RhoA activation by the fMLP receptor, we have considered the following...
sequence that could lead to ARF and RhoA activation (Fig. 8). It is established that the fMLP receptor is coupled to the pertussis toxin-sensitive G proteins G\(_{i2}\) and G\(_{i3}\) and that the pretreatment of intact cells with pertussis toxin results in the inhibition of fMLP-stimulated PLD activity (39, 40). We established that the myr-ARF-restored fMLP-dependent activity was also sensitive to pertussis toxin treatment, supporting the idea that the heterotrimeric G proteins of the G\(_{i}\) family can lead to ARF and RhoA activation.

How do activated G\(_{i}\) or \(\beta\gamma\) subunits activate ARF and Rho? We tested the possibility that PI 3-kinase activation could be important in this process because it is known that wortmannin, an inhibitor of PI 3-kinase, can inhibit PLD activity in intact cells. Protein G\(\beta\gamma\) subunits regulate the PI 3-kinase \(\gamma\) isoform directly in these cells. This model is attractive because several exchange factors for ARF proteins have been identified recently. Proteins with SEC7 domains function as guanine nucleotide exchange factors for ARF; in mammalian cells, ARNO, GRP1, and cytohesin have been cloned recently (34, 52–54). ARNO, GRP1, and cytohesin have PH domains and have been shown to bind phosphoinositides. Rho exchange factors are proteins with PH domains and with a DBL domain that functions as the guanine nucleotide exchange factor. Taking into consideration the observation that wortmannin inhibits activation of PLD activity in intact human neutrophils, the activation of the PI 3-kinase pathway by protein G\(\beta\gamma\) subunits could potentially provide the link to ARF activation. However, an alternative possibility is that these PH domains are direct targets for the \(\beta\gamma\) subunits because several proteins with PH domains do bind protein G\(\beta\gamma\) (61).

We tested the requirement of PI 3-kinase as an intermediary step to ARF and RhoA activation in two ways. In the reconstituted assay, we found that wortmannin had no effect on the myr-ARF-reconstituted PLD activity stimulated by fMLP. This lack of inhibition is in keeping with our previous study in which we showed that in the cytosol-depleted cells, fMLP stimulation of PI\(_{3}\) production is greatly attenuated because of leakage of the PI 3-kinase \(\gamma\) isoform (56). The second approach was to examine whether the translocation of ARF and RhoA to membranes was inhibited by wortmannin in intact cells. ARF translocation was unaffected, and that of Rho was inhibited slightly. We conclude that PI 3-kinase is not involved obligatorily in the activation of ARF and Rho, and the most likely possibility is that protein G\(\beta\gamma\) interacts directly with the PH domains of the appropriate exchange factors. We are currently identifying the specific nature of the exchange factors that are present in neutrophils. Brefeldin A does not influence fMLP-stimulated PLD activation, indicating that the exchange factor is brefeldin A-insensitive (data not shown).

One interesting facet of neutrophil physiology is the need to prime the cells before they acquire the potential to activate many of their downstream functional responses, such as degranulation and the oxidative burst. However, PI 3-kinase and phospholipase C\(\beta\) activation by fMLP is not dependent on priming, unlike PLD and phospholipase A\(_2\). We have observed that recruitment of ARF to the membranes is absolutely dependent on priming and that of Rho less so. In human neutrophils, we have shown recently that the ARF-regulated PLD is localized at the secretory vesicles and that upon priming, these vesicles fuse with the plasma membrane translocating the PLD in the process (51). The requirement for neutrophils to be primed would suggest that a component, possibly PLD, has to be present at the appropriate membrane for ARF to be recruited.

The model illustrating the molecular components that participate in the activation of PLD activity is summarized in Fig. 8. fMLP activates G\(_i\) proteins, and G\(\beta\gamma\) targets the ARF and Rho exchange factors directly for recruitment of ARF and Rho to the plasma membrane where the PLD is present. This model should be contrasted with insulin-mediated activation of PLD activity. Insulin also causes the translocation of ARF and Rho proteins, but in this case PI 3-kinase does appear to be the intermediary for the activation of the appropriate exchange factors. Because these exchange factors belong to a large family of proteins, it is likely that different cells utilize a different subset of exchange factors, and hence different intermediate components may be required. Insulin, which activates a receptor tyrosine kinase, uses PI 3-kinase to activate ARF and Rho exchange factors; and fMLP, which activates G protein-coupled receptors, uses G\(\beta\gamma\) to do the same. The mechanism whereby PMA causes the translocation of ARF and Rho is not known.

**Acknowledgment—**We thank Dr. Anne Ridley for the gift of C3 transerase.

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