Amantadine inhibits platelet-activating factor induced clathrin-mediated endocytosis in human neutrophils

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Eckels PC, Banerjee A, Moore EE, McLaughlin NJ, Gries LM, Kelher MR, England KM, Gamboni-Robertson F, Khan SY, Silliman CC. Amantadine inhibits platelet-activating factor induced clathrin-mediated endocytosis in human neutrophils. Am J Physiol Cell Physiol 297: C886–C897, 2009.—Receptor signaling is integral for adhesion, emigration, phagocytosis, and reactive oxygen species production in polymorphonuclear neutrophils (PMNs). Priming is an important part of PMN emigration, but it can also lead to PMN-mediated organ injury in the host. Platelet-activating factor (PAF) primes PMNs through activation of a specific G protein-coupled receptor. We hypothesize that PAF priming of PMNs requires clathrin-mediated endocytosis (CME) of the PAF receptor (PAF-R), and, therefore, amantadine, known to inhibit CME, significantly antagonizes PAF signaling. PMNs were isolated by standard techniques to ensure 98% purity and tested for viability. Amantadine (1 mM) significantly inhibited PAF-mediated changes in the cellular distribution of clathrin and the physical colocalization [fluorescence resonance energy transfer positive (FRET⁺)] of early endosome antigen-1 and Rab5a, known components of CME and similar to hypertonic saline, a known inhibitor of CME. Furthermore, amantadine had no effect on the PAF-induced cytotoxic calcium flux; however, phosphorylation of p38 MAPK was significantly decreased. Amantadine inhibited PAF-mediated changes in PMN physiology, including priming of the NADPH oxidase and shape change with lesser inhibition of increases in CD11b surface expression and elastase release. Furthermore, rimantadine, an amantadine analog, was a more potent inhibitor of PAF priming of the N-formyl-methionyl-leucyl-phenylalanine-activated oxidase. PAF priming of PMNs requires clathrin-mediated endocytosis that is inhibited when PMNs are pretreated with either amantadine or rimantadine. Thus, amantadine and rimantadine have the potential to ameliorate PMN-mediated tissue damage in humans.

G protein-coupled receptor; clathrin-coated vesicle; polymorphonuclear neutrophils

POLYMORPHONUCLEAR NEUTROPHILS (PMNs) circulate for 12–18 h before migrating into tissue (36). Emigration of PMNs from the vasculature into tissue is a complex, coordinated process that is vital for the eradication of infectious pathogens (1, 6). This innate process of tissue migration initiates PMN priming and augmentation of the microbicidal response, both oxidative and nonoxidative, to a subsequent stimulus (1, 67). Priming is an integral part of PMN emigration and changes PMNs from a nonadherent to an adherent phenotype (1, 6, 60, 66, 67). In addition, priming causes rapid actin redistribution, allowing for shape change and chemotaxis, and maximizes the microbicidal response to invading microbes for effective elimination (24, 31, 60, 67, 74). Priming begins via vascular endothelial attraction of PMNs to their surface through the release of chemokines followed by E- and P-selectin-mediated tethering through contact with obligate ligands (PSGL-1) on the PMN surface and firm adhesion of the PMNs through β2-integrins and ligands [e.g., intercellular adhesion molecule-1 (ICAM-1), vascular cellular adhesion molecule (VCAM)] on the endothelial cell (EC) surface. PMNs then diapedes through the EC, which involves CD31 on both PMNs and EC, chemotax to the site of infection, and phagocytize and kill the pathogenic invaders (1, 6, 22, 24, 74). Furthermore, priming changes the PMN phenotype to “hyper-responsive,” such that the PMNs microbial arsenal can be activated by normally innocuous stimuli (74), and in two-event models of PMN-mediated lung injury, PMN priming agents are etiologic; therefore, interruption of the priming signal can ameliorate or inhibit tissue damage (49, 61).

Platelet-activating factor (PAF) is an effective physiological priming agent, and its receptor is a member of the G protein-coupled receptor family (GPCR) (37, 49, 58, 67). A characteristic of GPCRs is clathrin-mediated endocytosis (CME), which may be sensitive to weak bases such as amantadine (33). Amantadine has classically been used for the prevention and treatment of influenza A and Parkinson’s disease (46, 54). Amantadine has been further described to inhibit neuronal sodium channel activity (28) and acts as a low-affinity N-methyl-D-aspartate (NMDA) receptor antagonist (39, 48); however, its specificity inhibiting CME of the PAF receptor is not well understood although it is frequently used as a clathrin inhibitor (3, 29, 47, 50, 52, 65). Recent work has characterized the early events in PAF-mediated CME (25) including internalization of the ligand:receptor complex and sequestration of these ligated receptors to clathrin-coated vesicles (CCVs) (55). In addition, the PMN is unique for it does not contain caveolin and thus serves as an excellent cellular model to study CME (56). Therefore, we hypothesize that amantadine inhibits CME, which is required for PAF signaling and for PAF-mediated changes in PMN physiology. We will also compare the effects of amantadine to hypertonic saline, a well-described antagonist of CME, to support this hypothesis (9, 26, 57).

MATERIALS AND METHODS

Unless otherwise indicated, all chemicals were purchased from Sigma Chemical (St. Louis, MO). Acrylamide, N’-methylene-bis-acrylamide, and N,N,N’,N’-tetramethylethylenediamine (TEMED) were obtained from Bio-Rad (Hercules, CA). X-ray and enhanced chemiluminescence reagents were obtained from E. I. DuPont, (Wil-
Amantadine was isolated by standard techniques (59). Heparinized whole blood was drawn from healthy human donors after obtaining informed consent and employing a protocol approved by the Colorado Multiple Institutional Review Board and Human Subjects Committee at the University of Colorado School of Medicine. PMNs were isolated by dextran sedimentation, ficoll-hypaque gradient centrifugation, and hypotonic lysis as previously described (59). Cells were resuspended to a concentration of 2.5 × 10⁶ cells/ml in Krebs-Ringer-phosphate buffer with 2% dextrose (KRPD) (pH 7.35) and used immediately for all subsequent manipulations. In all experiments, phosphate buffer with 2% dextrose (KRPD) (pH 7.35) and used.

Fluorescent Visualization of Clathrin

PMNs (5.0 × 10⁶ cells) were warmed to 37°C, incubated for 5 min with buffer (control) or inhibitors, and were then primed with either buffer or PAF for 3 and 5 min. The reaction was stopped with the addition of an equal volume of cold, fresh 8% paraformaldehyde (PFA) and incubated at 4°C for 5 min. Approximately 1.5 × 10⁶ PMNs were smeared on microscope slides and allowed to dry overnight. Following three washes with phosphate-buffered saline (PBS), cells were permeabilized with acetone:methanol (70:30) at −20°C for 10 min. The slides were air dried and blocked for 1 h at room temperature with 10% donkey serum and incubated overnight at 4°C with goat anti-clathrin primary antibody in PBS with 1% BSA. After washing three times with PBS, an AlexaFluor 488 donkey anti-goat secondary antibody (red) was added along with anti-quenching media, and the nuclear region (blue) was visualized using bis-benzimide (60).

Subcellular Localization and Western Blot Analysis of Clathrin

PMNs (1 × 10⁶/ml) were warmed to 37°C with gentle agitation, pretreated with buffer, amantadine, or HTS for 5 min and then stimulated with buffer or PAF for 1–3 min and placed into ice-cold relaxation buffer [10 mM PIPES (pH 7.4), 3 mM NaCl, 100 mM KCl, 3.5 M MgCl₂, 1.2 mM EGTA, 10 μg/ml leupeptin, 40 mM sodium orthovanadate, 1 M triethanolphosphate, and 50 μg/ml PMSF] and immediately sonicated (2 × 30 s). The lysates were placed onto sucrose gradients and ultracentrifuged as documented previously (37, 38, 60). Purity of fractions was determined using p47phox as a cytosol marker and the PAF receptor as a membrane marker (data not shown). Before protein separation, the fractions were placed in Laemmli digestion buffer containing 40 mM sodium orthovanadate, 1 M triethanolphosphate, 10 μg/ml leupeptin, and 100 mM PMSF (an inhibitor cocktail) (17). Samples were boiled for 15 min and proteins were separated by SDS-PAGE. The proteins were transferred to nitrocellulose, blocked with 5% BSA (fraction V) overnight, and incubated with a primary antibody to clathrin heavy chain. After washing in Trizma-buffered saline plus 0.1% Tween 20 (TBST), the blots were incubated with horseradish peroxidase (HRP)-linked goat anti-rabbit secondary antibody and visualized by enhanced chemiluminescence and exposure of X-ray film (17, 60).

FRET Analysis of Rab5a and EEA-1

Isolated PMNs were incubated at 37°C with buffer, 2 μM PAF (1 min), 1 mM amantadine (5 min), or amantadine followed by PAF, and then fixed in 4% paraformaldehyde at 4°C for 20 min and smeared onto slides. FRET determinations were obtained by direct acceptor photobleaching FRET (adFRET), as previously described (37). Within this context, the ability of the two secondary antibodies to FRET was acquired between rhodamine (Jackson ImmunoResearch; excitation, 550 nm; emission, 570 nm, acquired on the Cy3 channel) and AlexaFluor 488 (Molecular Probes; excitation 495 nm; emission 519 nm, acquired on the FITC channel). In all cases, an initial image was acquired of the donor and acceptor channels, and following capture, a region of interest was defined, a mask applied, and the parameters of the acceptor channel (rhodamine 610-Cy5 or rhodamine 540-Cy3). FRET efficiencies (Ei) were calculated using the following equation:

\[ E_i = \frac{I_{\text{post,i}} - I_{\text{pre,i}}}{I_{\text{pre,i}}} \]

where \( I_{\text{pre,i}} \) is the mean intensity of the donor pre-photo-bleach image and \( I_{\text{post,i}} \) is the mean intensity of the donor post-photo-bleach image (73). Images are displayed in pseudocolor where blue indicates little FRET and red indicates the most FRET.

PAF Receptor Surface Expression Via Flow Cytometry

Isolated PMNs (1 × 10⁶ cells) were preincubated with amantadine, rimantadine, or buffer before incubation with PAF at 37°C. The reaction was stopped with the addition of an equal volume of cold, fresh 8% paraformaldehyde and incubated at 4°C for 5 min. PMN were coincubated with a PAF receptor antibody and Fc-receptor blocker (Accurate Chemical, Westbury, NY), to reduce nonspecific binding, for 30 min at 4°C and washed with cold KRPM. Cells were resuspended in a 4% PFA-PBS solution and analyzed on a Beckman FC500 flow cytometer within 24 h.

Measurement of Cytosolic Ca²⁺

In selected experiments, PMN cytosolic Ca²⁺ levels were determined by indo-1 AM loading of PMNs and analyzed in a Perkin-Elmer LS50B spectrofluorimeter over real time (Perkin-Elmer, Norwalk, CT), as previously described, employing the Grynkiewicz equation (60). Briefly, following loading of indo-1 AM, PMNs were either incubated in amantadine (1 mM), HTS (180 mmol/l), or buffer, stimulated with PAF, and in some cases stimulated a second time again with PAF following a return to basal calcium levels.

Western Blot Analysis of Phosphorylated p38 MAPK

PMNs were warmed to 37°C with gentle agitation. PMNs were pretreated with buffer, HTS, or amantadine for 5 min and stimulated with buffer, PAF, or N-formyl-methionyl-leucyl-phenylalanine (fMLP) for 1 min and immediately lysed in Laemmli digestion buffer containing 40 mM sodium orthovanadate, 1 M triethanolphosphate, 10 μg/ml leupeptin, and 100 mM PMSF (an inhibitor cocktail) (17). Samples were boiled for 60 min, and proteins were separated by...
The proteins were transferred to nitrocellulose, cross-linked, blocked with 5% BSA (fraction V) overnight, and incubated with a primary antibody to diposphorylated (Thr180/Tyr182) p38 MAPK (Cell Signaling). Washed in TBST, the blots were incubated with HRP-linked goat anti-rabbit secondary antibody and visualized by enhanced chemiluminescence and exposure of X-ray film (17, 60). For evaluation of p38 MAPK, the blots were stripped, incubated with a primary antibody to total p38 MAPK, washed, and visualized as detailed above (17, 60).

**Immunoprecipitation of EEA-1 and Rab5a and Western Blot Analysis of Proteins From PMNs**

PMNs (5 × 10⁶) were incubated with buffer, 2 μM PAF for 1–5 min, 1 mM amantadine, 180 mM HTS, or amantadine + PAF or HTS + PAF as indicated. The reactions were stopped with the addition of ice-cold relaxation buffer (10 mM PIPES (pH 7.4), 3 mM NaCl, 100 mM KCl, 3.5 mM MgCl₂, 1.2 mM EGTA, 10 μg/ml leupeptin, 40 μM sodium orthovanadate, 1 mM nitrophenylphosphate, and 50 μg/ml PMSF) and immediately sonicated (2 × 30 s). Lysates were cleared and immunoprecipitated with beads linked to specific antibodies against EEA-1 or Rab5a overnight at 4°C as previously described (51). For whole cell lysates, the PMNs were lysed employing Laemmli digestion buffer and boiled for 60 min. In either case, proteins were separated by 7.5% or 10% SDS-PAGE, transferred to nitrocellulose membranes, which were cross-linked and then probed with specific antibodies to EEA-1 or Rab5a.

**PAF-Mediated Changes in PMN Morphology**

Aliquots of freshly isolated PMNs (5.0 × 10⁶ cells) were added to microcentrifuge tubes, warmed to 37°C using adjacent plate warmer, and incubated with buffer, PAF (5 min), 1 mM amantadine (5 min), 100 μM rimantadine (5 min), or either amantadine or rimantadine (5 min) followed by PAF (5 min). Cells were fixed in freshly prepared 4% PFA/PBS (pH 7.4) for 20 min. PMNs were smeared on Superfrost/Plus microscopy slides (Fisher Scientific), allowed to air dry overnight, washed three times with PBS, and mounted with Biomena Gel/Mount (Foster City, CA) and a coverslip. Cell response to PAF was observed under bright field illumination using a Zeiss LSM microscope (60).

**CD11b Surface Expression**

PMNs (1.0 × 10⁶) were incubated for 5 min with buffer or amantadine. Cells were stimulated with buffer or PAF for 5 min, pelleted, and the reaction was stopped by the addition of ice-cold buffer. The PMNs were then incubated for 30 min at 4°C with 1 μg/ml of a monoclonal antibody to CD11b (Clone Bear1, Beckman Coulter) or isotype control. Cells were fixed in 4% PFA for 10 min, diluted to 1% PFA with KRPD, and analyzed within 24 h via flow cytometry with the data expressed as mean fluorescence intensity (60).

**Elastase Release**

PMNs (1.25 × 10⁶) were warmed to 37°C and incubated for 5 min with buffer (control) or amantadine. The PMNs were primed for 5 min with buffer or PAF, and subsequently stimulated with buffer or fMLP for 5 min. Cells were pelleted and the amount of elastase released in the supernatant was quantified spectrophotometrically by the reduction of the specific substrate methoxy-succinyl-alanly-alanly-prolyl-valyl p-nitroanilide (AAPVNA) at 405 nM (60). Elastase reactions were run in duplicate, and wells containing 5 mM of the specific elastase inhibitor methoxy-succinyl-alanly-alanly-prolyl-valyl chloromethyl ketone (AAPVCK) were run in conjunction. Elastase is reported as percentage of total cellular elastase from an identical number of cells treated with 0.1% Triton X-100.

**PAF Priming of the NADPH Oxidase**

The maximal rate (V_max) of superoxide anion generation was measured by monitoring the superoxide dismutase-inhibitable reduction of cytochrome c at 550 nm in a Molecular Devices microplate reader (Menlo Park, CA) as previously described (59). PMNs were preincubated with buffer, amantadine, or rimantadine for 5 min at 37°C with gentle agitation. Following preincubation, PMNs (3.75 × 10⁵ cells) were primed for 3 min with PAF or buffer, activated with fMLP or buffer, and the maximal rate of O₂ production was measured.

**Statistical Analysis**

Statistical differences among groups were determined by a paired or an independent ANOVA followed by a Tukey or Bonferroni post hoc analysis for multiple comparisons based on the equality of variance. All data are presented as means ± SE; statistical significance was determined at the P < 0.05 level.

**RESULTS**

**Clathrin Reorganization**

Because PAF ligation of the platelet-activating factor receptor (PAFr) results in CME in PMNs, we examined the effects of amantadine on PAF-mediated clathrin reorganization in isolated PMNs (33, 37) (Fig. 1). Quiescent buffer-treated PMNs exhibited an almost uniform distribution of clathrin (Fig. 1a). In contrast, PAF (2 μM) priming of PMNs caused a redistribution of clathrin at 3 min (Fig. 1a), with further progression at 5 min as demonstrated by the appearance of a number of punctate fluorescent structures at the membrane and within the cytoplasm (Fig. 1ac). Amantadine (1 mM) alone did not affect clathrin distribution in buffer-treated PMNs (Fig. 1a), similar to buffer-treated controls (Fig. 1aa). However, amantadine pretreatment abolished PAF-mediated clathrin reorganization at both 3 and 5 min such that clathrin remained uniformly distributed throughout the cytoplasm without the formation of the fluorescent punctate structures in either the cytoplasm or the membrane (Fig. 1ac, ad, and f). This distribution in amantadine-pretreated PMNs primed with PAF was similar to buffer- or amantadine-pretreated PMNs (Fig. 1aa, a and d). To confirm these data, PMNs were treated with buffer, amantadine (5 min), PAF (5 min), or amantadine (5 min) then PAF (5 min) and subcellular fractions were made, and clathrin immunoreactivity was measured in the PMN membrane. As compared with buffer-treated PMNs, PAF (5 min) elicited increased amounts of clathrin immunoreactivity in the PMN membrane, which was inhibited by pretreatment of these PMNs by 1 mM amantadine (Fig. 1b). HTS caused similar inhibition of PAF-mediated changes in clathrin spatial organization similar to that of amantadine (Fig. 2). Compared with controls (Fig. 2aa) PAF induced clathrin reorganization at 3 and 5 min (Fig. 2a, b, and c), which was inhibited by pretreatment with HTS (Fig. 2a, e, and f). Importantly, HTS did not affect clathrin distribution of control PMNs (Fig. 2ad). In addition, the subcellular fractions of PMNs, treated with buffer, HTS (5 min), PAF (3 min) and HTS (5 min) then PAF (5 min), demonstrated that the PAF-elicited increases of clathrin in the PMN membrane were inhibited by HTS (Fig. 2b).
FRET Analysis of EEA-1 and Rab5a in Response to Amantadine

Because CME initiates the assembly of the endosomal components EEA-1 and Rab5a-GTPase in the formation of CCVs, we investigated if amantadine and HTS alter this physical association (Fig. 3) (2, 5). In control PMNs, there was a negligible amount of FRET between the two proteins (Fig. 3m). In contrast, PAF elicited a physical (FRET+) interaction beginning at 1 min between Rab5a and the EEA-1 sorter protein (Fig. 3n), similar to previous data (38). Amantadine (1 mM) alone did not cause any significant FRET+ interactions between EEA-1 and Rab5a (Fig. 3o); however, amantadine pretreatment did inhibit the PAF-mediated FRET+ interaction between EEA-1 and Rab5a (Fig. 3p).

Amantadine Inhibits EEA-1 and Rab5a Coprecipitation

Recruitment of Rab5a to CCVs has been described as a preliminary to receptor endocytosis (2), and since both EEA-1 and Rab5a can be phosphorylated by p38 MAPK (34), and amantadine inhibits phosphorylation of p38 MAPK, we hypothesized that amantadine would inhibit PAF-mediated colocalization of EEA-1 and Rab5a. PMNs were treated with buffer, HTS (5 min), amantadine (5 min), PAF (1 or 3 min), amantadine (5 min) then PAF (1 or 3 min), or HTS (5 min) followed by PAF (1 or 3 min), the PMNs were lysed immediately, and Rab5a or EEA-1 was immunoprecipitated. The proteins in the resultant immunoprecipitates were separated by SDS-PAGE and immunoblotted for EEA-1 or Rab5a, respectively (Fig. 4). Compared with buffer-treated controls, PAF induced colocalization of EEA-1 with Rab5a at 1 min, which persisted to 3 min (Fig. 4). Amantadine alone did not cause colocalization of EEA-1 with Rab5a, but amantadine pretreatment did inhibit the PAF-induced colocalization of EEA-1 with Rab5a (Fig. 4, A and B). In addition, HTS alone did not affect the EEA-1:Rab5a colocalization, but pretreatment did inhibit the PAF-mediated colocalization of EEA-1 with Rab5a (Fig. 4C).

Amantadine and HTS Inhibit PAF-Mediated Internalization of the PAFr on PMNs

CME causes internalization of the activated GPCR, and in the case of PMNs, PAF causes internalization of the PAFr...
The surface expression of the PAF receptor (PAFr) was determined in quiescent PMNs by flow cytometry, which was unaffected by amantadine alone (Fig. 5). PAF caused a decrease in PAFr surface expression by 35% as compared with controls or amantadine or rimantadine alone (P < 0.05; n = 4). Both amantadine and rimantadine (1 μM) (an effective derivative of amantadine) significantly inhibited the loss of PAFr surface expression on PMNs (P < 0.05; n = 4) (16, 51, 53, 71).

Amantadine Does Not Affect PAF-Mediated Changes in Cytosolic Ca²⁺

PAF elicits rapid increases in cytosolic Ca²⁺ concentration in human PMNs, and this Ca²⁺ flux was used to test the integrity of receptor ligation and subsequent heterotrimeric G protein signaling (60). Stimulation of PMNs with 2 μM PAF caused a rapid increase in cytosolic Ca²⁺ concentration ([Ca²⁺]), which returned to basal levels after ~160 s (Fig. 6). Pretreatment of PMNs with 1 mM amantadine or 180 mM HTS had little effect on the PAF-induced cytosolic Ca²⁺ flux (Fig. 6), and to quantify the lack of effect on the rise in cytosolic Ca²⁺, the baseline Ca²⁺, the maximal Ca²⁺ concentration reached, and the time to reach maximal Ca²⁺ concentration were calculated and confirmed that neither amantadine nor HTS affected PAF-mediated changes in cytosolic Ca²⁺ (Table 1).

Amantadine and HTS Inhibit PAF-Mediated Activation of p38 MAPK

PAF priming of PMNs caused activation, dual phosphorylation (Thr180/Tyr182), of p38 MAPK at 1 min (17). Consistent with these data, PMNs treated with PAF induced dual phosphorylation of p38 MAPK, when compared with treatment with buffer alone. However, amantadine pretreatment of PMNs caused a concentration-dependant inhibition of PAF-induced phosphorylation of p38 MAPK, and amantadine alone did not cause activation of p38 MAPK (Fig. 7A). Furthermore, HTS also inhibited PAF-induced activation of p38 MAPK and alone did not elicit activation of p38 MAPK (Fig. 7B).
Amantadine Inhibits PAF-Induced Changes in PMN Physiology

Changes in PMN morphology. Because amantadine appeared to inhibit PAF-mediated CME, we examined its effect on PAF-induced PMN physiology. Membrane ruffling and pseudopodia formation are characteristics of primed PMNs, which is analogous to a higher degree of actin rearrangement (14, 27). In contrast, quiescent PMNs are spherical in shape, displaying a smooth outer edge and minimal cytoskeletal rearrangement (Fig. 8A). As expected, PMNs challenged with PAF for 5 min demonstrated classic membrane ruffling and pseudopodia-like protrusions (Fig. 8B). However, PMNs incubated with 1 mM amantadine or 100 μM rimantadine and then stimulated with PAF for 5 min failed to exhibit these changes (Fig. 8C and D).

Inhibition of PAF priming of NADPH oxidase with amantadine and rimantadine. To determine whether PAF priming of the respiratory burst depends on clathrin-mediated endocytosis, PMNs were pretreated with amantadine and subsequently primed with PAF, activated with fMLP, and the maximal rate of the superoxide anion \( \left( \text{O}_2^- \right) \) was measured \( (V_{\text{max}}) \). Amantadine did not inhibit fMLP-activation of the oxidase. In contrast, amantadine significantly inhibited PAF priming of the fMLP-activated oxidase burst as compared with buffer-treated con-
Fig. 4. Amantadine inhibits EEA-1 and Rab5a coprecipitation. PMNs (5 x 10^6 cells) were incubated with buffer, 1 mM amantadine (5 min), 180 mM HTS (5 min), 2 μM PAF (1 min or 3 min), amantadine + PAF, or HTS + PAF. Reactions were stopped by lysing the PMNs, and the lysates were cleared before immunoprecipitation (IP) of EEA-1 or Rab5a. The beads were lysed, and the proteins were separated by SDS-PAGE and transferred to nitrocellulose membranes, which were cross-linked. The IPs of EEA-1 were probed for Rab5a and the IPs of Rab5a were probed for EEA-1. Amantadine alone did not cause coprecipitation of EEA-1 with Rab5a (A) and as compared with controls or amantadine-treated PMNs, PAF caused coprecipitation of EEA-1 with Rab5a at 3 and 5 min regardless of which protein was immunoprecipitated (A and B). Both amantadine (A and B) and HTS (C) pretreatment inhibited coprecipitation of these two proteins. These images are representative of three individual experiments, and although the presented images are from the same gel, the order has been changed for greater clarity to keep the identical time points as denoted by the white breaks in the gel.

CD11b surface expression. Under chemoattractant challenge, PMNs increase β2-integrin surface expression via fusion of the secretory vesicles and secondary granules with the plasma membrane (75). Incubation of PMNs with PAF caused increased surface expression of CD11b as compared with buffer-treated controls (P < 0.05, n = 6). Amantadine attenuated the PAF-elicited increase in cell surface-labeled CD11b at 1 mM (P < 0.05, n = 6) but did not affect the CD11b surface expression of buffer-treated controls (P = 0.11, n = 6) (Fig. 9A).

Amantadine inhibits PAF priming of elastase release. Elastase release was employed as a direct measurement of azurophilic degranulation. PMNs treated with buffer and incubated with PAF or fMLP released very little elastase, however, elastase release was greatly increased when PMNs were primed with PAF and activated with fMLP (P < 0.05, n = 6). Amantadine pretreatment had little effect on elastase release when PMNs were incubated with PAF or fMLP alone. In contrast, pretreatment of PMNs with amantadine significantly diminished the PAF/fMLP-induced degranulation in PMNs (P < 0.05, n = 6) (Fig. 9B).

DISCUSSION

The results of the present study demonstrate that PAF priming of PMNs elicited clathrin recruitment to the plasma membrane, a corrected FRET + interaction (FRET+) -positive colocalization of EEA-1 and Rab5a, and activation of p38 MAPK. Amantadine 1) abolished clathrin recruitment to the membrane and it remained uniformly distributed throughout the cytoplasm, 2) disrupted endosome formation by inhibiting the FRET+, colocalization of EEA-1 and Rab5a, and 3) abrogated the activation of the p38 MAPK. Furthermore, amantadine abrogated PAF-mediated changes in PMN physiology including cytoskeletal rearrangement of actin and priming of the respiratory burst. In addition, amantadine attenuated PAF-mediated increases in β2-integrin surface expression, and priming azurophilic granule release. Amantadine and rimantadine antagonism of the described PAF-mediated changes in PMN physiology is identical to the inhibitory effects of HTS on PAF priming as we have published previously (9, 26, 57). Importantly, amantadine, similar to HTS, did not affect the PAF-induced cytosolic Ca2+ flux, which is presumably the direct result of activation of the heterotrimeric G proteins linked to the PAFr (34). Therefore, the hypothesis that amantadine inhibition is due to the possible disruption of ligand binding or interruption in PAF-mediated signal transduction, as has...
been shown for the NMDA-type glutamate receptor, appears moot (20). Taken together, these data provide evidence that amantadine does not affect the PAF:PAF receptor interaction because of its inability to diminish the PAF-induced increases in cytosolic Ca\(^{2+}\). Instead, we observed that amantadine inhibited clathrin reorganization into clathrin-coated pits with subsequent decrease in dual phosphorylation (activation) of p38 MAPK. Many of the cellular responses elicited by PAF are directly influenced by p38 MAPK activation, priming of the respiratory burst, and actin reorganization; however, the effects on intracellular granules, whether due to the specific granules binding to the membrane and increasing the surface expression of CD11b or to the release of proteases from azurophilic granules, is not as well understood and have been proposed to involve multiple mechanisms including MAPKs and changes in cytosolic Ca\(^{2+}\) (18, 44, 45). Importantly, amantadine does inhibit the PAF-induced activation of p38 MAP kinase but did not affect the PAF-mediated increases in cytosolic Ca\(^{2+}\), which may be the reason that amantadine only attenuates the PAF-elicted changes in PMN physiology related to the movement or release of granule constituents including both the release of elastase and the increased surface expression of CD11b (7, 42, 69). Previous work has determined that chelation of cytosolic Ca\(^{2+}\) does significantly attenuate PAF-induced increases in the surface expression of CD11b (18). Lastly, physiologic concentration of HTS (180 mM) inhibits fMLP-elicited activation of PMNs; however, amantadine did not as documented by its inability to decrease the amount of fMLP-activation of the oxidase in the priming experiments (Table 2) (9–12). Although PAF and fMLP are both effective chemoattractants, they mediate their actions through very diverse signaling pathways, and previous work has documented that PAF-mediated CME in PMNs directly causes actin reorganization and priming of the oxidase through phosphorylation and translocation of the cytosolic components of the NADPH oxidase (8, 35, 37, 38, 44). Little data are available with respect to fMLP inducing CME via ligation of its receptor, and further work is needed.

The sorter protein EEA-1 is a component of the endosomal fusion machinery that binds to the GTP-loaded form of Rab5a, a small GTPase that controls endocytosis and early endosome dynamics (13, 21). A 30-amino acid region upstream of the FYVE domain of EEA-1 was shown to be essential for Rab5a binding in vitro, although the functional FYVE domain was also required for efficient interaction (32). Because PMN migration demands reorganization of actin and changes in β2-integrin surface expression, the effects of amantadine preincubation were tested on PAF-induced changes in cell morphology and CD11b surface expression (6, 14). Changes in the actin cytoskeleton are required for the observed changes in cellular morphology as well as chemotaxis, which is directly related to increases in the surface expression of the β2-integrins (14). Rab5a is vital for these changes in PMN morphology, and amantadine blocked recruitment of Rab5a to the endosome (30, 72).

The role of CME in GPCR cell signaling has become an area of intense research (15, 19). Several different processes appear to be conserved among many GPCR and CME events including receptor ligation, which activates the associated heterotrimeric G protein, resulting in an increase in second messengers (19). Arrestin binding uncouples the receptor from the Gα, and Gβγ subunits, and this desensitization is accompanied by the concomitant sequestering of receptors

### Table 1. Effects of amantadine and hypertonic saline on PAF-mediated changes in cytosolic Ca\(^{2+}\) concentration

<table>
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<tr>
<th>Treatment</th>
<th>Resting ([\text{Ca}^{2+}])(_{\text{CYTO}}), nM</th>
<th>Peak ([\text{Ca}^{2+}])(_{\text{CYTO}}), nM</th>
<th>Time to Peak, s</th>
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<tr>
<td>PAF</td>
<td>106 ± 15</td>
<td>941 ± 64</td>
<td>22 ± 0.8</td>
</tr>
<tr>
<td>Amantadine + PAF</td>
<td>104 ± 8</td>
<td>940 ± 43</td>
<td>22 ± 0.5</td>
</tr>
<tr>
<td>HTS + PAF</td>
<td>102 ± 7</td>
<td>984 ± 73</td>
<td>21 ± 0.6</td>
</tr>
</tbody>
</table>

Values are means ± SE and represent 4 identical experiments performed on polymorphonuclear neutrophils (PMNs) from disparate donors. PAF, platelet-activating factor; HTS, hypertonic saline; \([\text{Ca}^{2+}]\)\(_{\text{CYTO}}\), cytosolic Ca\(^{2+}\) concentration.
to clathrin-coated pits (19). This ends the initial stage of receptor signaling; however, sequestering receptors to clathrin-coated pits induces recruitment of endocytotic machinery and signaling complexes to the membrane (4, 15, 19, 34, 56, 64). Such signaling cascades post receptor-ligand binding have been implicated as potential platforms for staging the assembly of signaling complexes that can be trafficked to various intracellular destinations (23, 62). Indeed, PAF and its obligate receptor have the ability to induce the formation of such scaffolding complexes as the ASK1/MKK3/p38 MAPK signalosome that has been shown to be required for CME in which clathrin plays a key role (37). The diversity of clathrin-binding proteins suggests that clathrin-coated pits can act as signaling microdomains that regulate signaling in a temporal and spatial manner (15, 64). The majority of available data have come from studies of transfected cell lines, such as the Chinese hamster ovary (CHO) or COS7 cell lines, used to represent hematopoietic cells, many of which are transformed to become virtually immortal (2, 5, 40, 41, 63, 68). Although important data may be gleaned from experimentation within these models, they may not demonstrate physiologic relevance because they are terminally differentiated cells versus primary cells, e.g., PMNs.

The goal of the present study was to investigate inhibition of CME using PAF priming of PMNs as a model. However, the concentrations of amantadine employed herein are not physiological. The average maximal concentration ($C_{\text{max}}$) of amantadine and HTS inhibit PAF activation of p38 MAPK. PMNs were treated with buffer, amantadine (100 µM or 1 mM) for 5 min (A) or HTS (180 mM; B) and stimulated with buffer or 2 µM PAF for 1 min. Activation of p38 MAPK was determined by SDS-PAGE electrophoresis and immunoblotting with an antibody against dual phosphorylated (activated) p38 MAPK (Thr180/Tyr182). Following these experiments, the blots were stripped and incubated with an antibody against p38 MAPK to ensure that the concentrations of this enzyme were constant per well, and the observed differences in p38 MAPK activation were not due to loading unequal amounts of this enzyme. Compared with buffer-treated and amantadine-treated PMNs, PAF elicited activation of p38 MAPK as demonstrated by a band immunoreactivity to the activated p38 MAPK (A and B). Amantadine (A) and HTS (B) pretreatment caused inhibition of PAF-induced p38 MAPK activation that was concentration dependent for amantadine (A). The total amounts of p38 MAPK did not differ across the wells as demonstrated by the immunoblots of the stripped gels (A and B). These images are representative of three individual experiments, and the white breaks are to demonstrate that data from different time points that are not discussed have been removed and that these bands are from the identical gel.

Fig. 7. Amantadine and HTS inhibit PAF activation of p38 MAPK. PMNs were treated with buffer, amantadine (100 µM or 1 mM) for 5 min (A) or HTS (180 mM; B) and stimulated with buffer or 2 µM PAF for 1 min. Activation of p38 MAPK was determined by SDS-PAGE electrophoresis and immunoblotting with an antibody against dual phosphorylated (activated) p38 MAPK (Thr180/Tyr182). Following these experiments, the blots were stripped and incubated with an antibody against p38 MAPK to ensure that the concentrations of this enzyme were constant per well, and the observed differences in p38 MAPK activation were not due to loading unequal amounts of this enzyme. Compared with buffer-treated and amantadine-treated PMNs, PAF elicited activation of p38 MAPK as demonstrated by a band immunoreactivity to the activated p38 MAPK (A and B). Amantadine (A) and HTS (B) pretreatment caused inhibition of PAF-induced p38 MAPK activation that was concentration dependent for amantadine (A). The total amounts of p38 MAPK did not differ across the wells as demonstrated by the immunoblots of the stripped gels (A and B). These images are representative of three individual experiments, and the white breaks are to demonstrate that data from different time points that are not discussed have been removed and that these bands are from the identical gel.

Fig. 8. Inhibition of PAF-mediated cytoskeletal changes by amantadine. PMNs were preincubated with buffer (A), 2 µM PAF (5 min) (B), or preincubated with amantadine (1 mM) (C) or rimantadine (1 µM) (D) for 5 min at 37°C, and subsequently stimulated with PAF for 5 min. PMNs were visualized using phase-contrast microscopy (Nomarski). Membrane ruffling and pseudopodia formation were observed in the PAF-primed cells (B) that is characteristic of chemotactically challenged PMNs. In contrast, amantadine- and rimantadine-pretreated PMNs appeared spherical in shape, displaying a smooth outer edge (C and D). These images represent three individual experiments.
Tadine is an analog of amantadine, differing only in the replacement of the amino group at position one with a methyl amine group; rimantadine pretreatment significantly inhibited PAF priming of the fMLP-activated oxidase at clinically achievable concentrations. Rimantadine to significantly inhibit PAF priming of the fMLP-activated respiratory burst at concentrations 10-fold lower (70). The Cmax of rimantadine is 74 ng/ml (0.3 μM) 6 h after an initial dose of 100 mg, indicating that physiologic concentrations are close to the in vitro concentrations required to inhibit PAF-mediated changes in PMN physiology (25, 43). Thus, rimantadine may provide a better means to curtail the effect of PMN-mediated organ injury, e.g., the acute respiratory distress syndrome and postinjury organ failure, yet its effects on other clathrin-dependent and -independent endocytotic mechanisms in different tissues need to be more clearly defined. Importantly, both amantadine and rimantadine were effective in inhibiting PAF signaling and PAF-induced changes in PMN physiology similar to HTS. It is important to remember that HTS represents a clinically relevant strategy for attenuating the cytotoxic responses of PMNs and is known to inhibit CME (9, 26, 57). Therefore, HTS has served as a competent foil for amantadine and rimantadine, thus implicating their ability to inhibit CME in a similar fashion.

In conclusion, amantadine inhibited multiple PAF-mediated changes in PMN physiology at the level of clathrin recruitment to the membrane such that downstream events including early endosomal formation are dependent on EEA-1 and Rab5a.

Table 2. Amantadine and rimantadine inhibition of PAF priming of the fMLP-activated respiratory burst

<table>
<thead>
<tr>
<th></th>
<th>Buffer</th>
<th>10 μM</th>
<th>100 μM</th>
<th>1 mM</th>
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<tbody>
<tr>
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<td>0.2±0.1</td>
<td>0.2±0.1</td>
<td>0.2±0.1</td>
</tr>
<tr>
<td>fMLP</td>
<td>0.8±0.3</td>
<td>0.9±0.2</td>
<td>0.6±0.2</td>
<td>0.6±0.1</td>
</tr>
<tr>
<td>PAF/fMLP</td>
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<td>2.5±0.2*</td>
<td>1.8±0.3†</td>
<td>1.6±0.2†</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>Buffer</th>
<th>1 μM</th>
<th>10 μM</th>
<th>100 μM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Buffer</td>
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<td>0.2±0.1</td>
<td>0.3±0.1</td>
<td>0.1±0.1</td>
</tr>
<tr>
<td>fMLP</td>
<td>1.0±0.3</td>
<td>0.9±0.1</td>
<td>0.8±0.1</td>
<td>0.7±0.2</td>
</tr>
<tr>
<td>PAF/fMLP</td>
<td>4.9±0.3*</td>
<td>4.2±0.6*</td>
<td>3.7±0.4†</td>
<td>2.8±0.4†</td>
</tr>
</tbody>
</table>

Values (in nmol O2/min) are means ± SE. *P < 0.05 compared with buffer-primed PMNs activated with N-formyl-methionyl-leucyl-phenylalanine (fMLP). † P < 0.05 compared with buffer-pretreated PAF/fMLP.

Fig. 9, A: amantadine inhibition of PAF-mediated changes in CD11b surface expression. PMNs were preincubated with buffer (black bar) or amantadine [100 μM (shaded bar) or 1 mM (open bar)] for 5 min and subsequently stimulated with 2 μM PAF. The PMNs were then incubated with phycoerythrin-labeled antibodies to CD11b and fixed in paraformaldehyde, and CD11b cell surface labeling was measured by flow cytometry. Amantadine alone did not affect the amount of cell surface accessible CD11b as compared with buffer controls. In contrast, PAF increased extracellularly available CD11b as compared with buffer-treated control PMNs. Amantadine pretreatment inhibited the PAF-induced increase in extracellularly accessible CD11b in a concentration-dependent fashion. Data are expressed as mean fluorescence intensity ± SE. *Statistical differences between buffer-treated and PAF-primed PMNs (P < 0.05, n = 6); †statistical differences from PAF and PAF/N-formyl-methionyl-leucyl-phenylalanine (fMLP) buffer-treated groups (P < 0.05, n = 6). B: amantadine inhibition of PAF-mediated elastase release. PMNs were incubated with buffer or amantadine (100 μM–1 mM) for 5 min and primed with 2 μM PAF or buffer and activated with fMLP (1 μM) or buffer. Amantadine at either concentration had a significant decrease on the PAF/fMLP elastase release (P < 0.05, n = 6). *Statistical significance (P < 0.05) compared with PAF/fMLP and control PMNs. This figure is representative of six individual experiments.
colocalization. Furthermore, rimantadine appears to be a more physiological inhibitor than amantadine because of its ability to inhibit PAF priming at clinically relevant concentrations (25, 43), and may represent an effective pharmacological approach to reduce PMN-mediated tissue damage in the injured or critically ill patient.

NOTE ADDED IN PROOF

Figures 4 and 7 and the legends differ from the early posted online version of this article because of questions raised by the editors with regard to the presentation of these figures. Specifically, for Fig. 4 (A and C), spaces have been added to show that the image is not one contiguous gel. As the legend indicates, these images are representative of three individual experiments and although the presented images are from the same gel, the order has been changed for greater clarity to keep the identical time points in order. Figure 7 has been replaced with that from the identical gel, and it shows results from three individual experiments, with white breaks to demonstrate that data from different time points that are not discussed in the article have been removed.

GRANTS

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