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# Selective Inhibition of Protein Kinase C, Mitogen-Activated Protein Kinase, and Neutrophil Activation in Response to Calcium Pyrophosphate Dihydrate Crystals, Formyl-Methionyl-Leucyl-Phenylalanine, and Phorbol Ester by O-(Chloroacetyl-carbamoyl) fumagillol (AGM-1470; TNP-470)

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**ABSTRACT.** The effect of O-(chloroacetyl-carbamoyl) fumagillol (AGM-1470; TNP-470) was investigated on protein kinase C (PKC) and mitogen-activated protein kinase (MAPK) activation in neutrophils stimulated by plasma-opsonized crystals of calcium pyrophosphate dihydrate (triclinic) [CPPD(T)], formyl-Met-Leu-Phe (fMLP), and phorbol 12-myristate 13-acetate (PMA). Neutrophil respiratory burst responses also were determined in AGM-1470-pretreated cells stimulated with the same agonists, using chemiluminescence and superoxide anion generation assays. AGM-1470 (5 μM) effectively inhibited PKC activation in cells treated with CPPD(T) crystals (50 mg/mL, 2 min) and fMLP (1 μM, 1 min), but had no effect on PMA-treated cells (0.5 μM, 5 min). AGM-1470 blocked MAPK activity completely and reduced neutrophil activation induced by fMLP and PMA but not by CPPD(T). The degree of inhibition of the respiratory burst plateaued at approximately 46 ± 9 and 54 ± 3% in fMLP- and PMA-treated cells, respectively. These data indicate that activation of neutrophil respiratory burst activity may be mediated through the MAPK pathway. AGM-1470 pretreatment did not inhibit CPPD(T) crystal- or fMLP-stimulated phosphatidylinositol 3-kinase (PI 3-kinase) activity. These findings, coupled with further observations that the PI 3-kinase inhibitor wortmannin (10 nM) inhibited fMLP- and CPPD(T) crystal-induced but not PMA-induced chemiluminescence, indicate that at least two distinct signaling pathways (mediated by PI 3-kinase or MAPK) lead to neutrophil respiratory burst responses. PKC may also be required in the MAPK-stimulated pathway. We propose that the inhibitory effect of AGM-1470 on the neutrophil respiratory burst may be due to its ability to inhibit PKC and MAPK activation. BIOCHEM PHARMACOL **58**;12:1869–1880, 1999. © 1999 Elsevier Science Inc.

**KEY WORDS.** neutrophils; cellular activation; PKC; MAP kinase; rheumatoid arthritis; signal transduction

The primary function of neutrophils is the identification, migration, and destruction of microbial pathogens, hence protecting the host from infections. At the onset of phagocytosis, antimicrobial enzyme systems including $O_2^-$/$H_2O_2$-generating NADPH oxidase, myeloperoxidase, proteases, and hydrolases are activated and delivered into phagocytotic vesicles in high concentrations [1–3]. The inflammatory disease known as acute pseudogout arises from the deposition of CPPD¶ crystals in the synovial joints of humans [4]. Both monoclinic and triclinic crystalline forms of CPPD are able to activate neutrophils in the joint, a process that is thought to be a key factor in the pathophysiology of the disease [4–6]. CPPD(T) crystals have been shown to adsorb synovial protein components including small amounts of IgG (synovial fluid is an ultrafiltrate of plasma). *In vitro*, both uncoated and plasma- or synovial fluid-coated crystals have been shown to induce neutrophil activation, includ-

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¶ *Abbreviations*: BIM, bisindoylmaleimide; CPPD, calcium pyrophosphate dihydrate; CPPD(T), calcium pyrophosphate dihydrate (triclinic); DAG, diacylglycerol; DO, diolein (DAG homolog); ERK, extracellular signal-related kinase; fMLP, formyl-Met-Leu-Phe; MAPK, mitogen-activated protein kinase; MBP, myelin basic protein; $p70^{S6K}$, 70-kDa ribosomal protein S6 kinase; PI, phosphatidylinositol; PKC, protein kinase C; PMA, phorbol 12-myristate 13-acetate; and PS, phosphatidylserine.

ing superoxide anion production and lysosomal enzyme release [5, 7, 8]. Most studies on signal transduction pathways leading to neutrophil activation have used chemoattractants such as fMLP, C5a, platelet-activating factor, leukotriene $B_4$, and interleukin-8. When activated by the chemoattractant, neutrophil responses include cytoskeletal rearrangement, degranulation, and a respiratory burst catalyzed by NADPH oxidase [1, 3]. Studies in our laboratory have focused on elucidating the signal transduction pathways involved in microcrystal-induced neutrophil oxidase activation and degranulation responses. The activation of neutrophils by chemoattractants proceeds via intracellular signaling pathways that include many of the components identified in particulate-activated and PMA-induced neutrophils.

Neutrophil activation by both fMLP and CPPD(T) crystals has been demonstrated to activate MAPK [9–11], together with neutrophil chemiluminescence, superoxide anion generation, and degranulation [11, 12]. We reported a possible link between CPPD(T) crystal-induced MAPK activation and neutrophil oxidative and degranulation responses. We demonstrated that the anti-cancer and anti-arthritic drug taxol [13, 14] effectively disrupted MAPK and neutrophil activation stimulated with CPPD(T) crystals [11]. *O*-(Chloroacetyl-carbamoyl) fumagillol (AGM-1470; TNP-470) is another compound that has been demonstrated to inhibit rat adjuvant and collagen-induced arthritis, two animal models of rheumatoid arthritis [14–16]. AGM-1470 is being considered as a new treatment option for rheumatoid arthritis, but its mechanism of action in this angiogenesis-dependent disease is not yet established. To gain insight into the mechanism of AGM-1470 and a greater understanding of the signaling events in neutrophils leading to oxidative and degranulation responses, we utilized a neutrophil system activated by CPPD(T) crystals, fMLP, and PMA. We investigated the effects of AGM-1470 on agonist-induced PKC, MAPK, and neutrophil activation based on chemiluminescence and superoxide anion generation. We found a PI 3-kinase-independent correlation between MAPK and neutrophil activation.

## MATERIALS AND METHODS

### *Materials*

Unless otherwise stated, all chemicals were obtained from the Sigma Chemical Co., and all antibodies were obtained from Kinetek Pharmaceuticals Inc.

### *Preparation and Opsonization of Crystals*

CPPD(T) crystals were prepared and characterized as previously reported [17]. Plasma-opsonized crystals were used in all studies involving CPPD(T) crystal incubation. Opsonization of crystals was carried out using 50% heparinized plasma and HBSS (Hanks' balanced salt solution, pH 7.4) at 37° for 30 min immediately prior to experiments. Twenty-five milligrams of CPPD(T) crystals was weighed into 1.5-mL Eppendorf tubes, followed by 0.5 mL of 50% fresh human plasma. The tubes were capped and tumbled end-over-end at 30 rpm for 30 min at 37°. Then the tubes were centrifuged at 1000 *g*, and crystals were washed in HBSS and centrifuged.

### *Neutrophil Preparation*

Neutrophils were prepared from freshly collected, citrated human whole blood by dextran sedimentation and Ficoll Paque density centrifugation. Briefly, 400 mL of blood was mixed with 80 mL of 4% dextran T500 (Pharmacia LKB Biotechnology) in HBSS and allowed to settle for 1 hr. Plasma was collected continuously, and 5 mL was applied to 5 mL of Ficoll Paque (Pharmacia) in 15-mL polypropylene tubes. Following centrifugation at 500 *g* for 30 min, the neutrophil pellets were washed free of erythrocytes by 20 sec of hypotonic shock in $dH_20$. Neutrophils were resuspended in HBSS, kept on ice, and used for experiments within 3 hr. Neutrophils prepared under these conditions yielded cell suspensions that contained over 95% neutrophils with greater than 90% cell viability (assessed by trypan blue exclusion).

### *Incubation of Neutrophils with Agonists and Inhibitors*

Stock solutions of 28 mM taxol (Hauser Chemical Co.), 20 mM AGM-1470 (synthesized at BioChem Pharma), 0.25 mM PMA, 0.25 mM cytochalasin B, 0.5 mM fMLP, 100 mM compound 3 (Ro-31–8221), and 10 mM wortmannin in DMSO were prepared freshly before each experiment. Equal volumes of solutions were added to neutrophils at $5 \times 10^6$ cells/mL under mild vortexing conditions to achieve concentrations of 28 μM taxol, 0.5 μM PMA, 0.5 μM cytochalasin B, 1 μM fMLP, 10 nM compound 3, 10 nM wortmannin, and 300 nM to 50 μM AGM-1470. Except during chemiluminescence analysis, all experiments with AGM-1470 were performed using a concentration of 5 μM. The final DMSO concentration never exceeded 0.5%, which has been shown to have no effect on control cell responses [11]. In all experiments involving fMLP, cells were pretreated with cytochalasin B (0.5 μM) for 1 min prior to the addition of fMLP (1 μM).

### *MonoQ Chromatography*

Neutrophil lysates were fractionated on a MonoQ anion exchange column to partially purify the MAPK and PKC proteins before conducting phosphotransferase assays with the appropriate substrates. Following incubation with the appropriate inhibitors and agonists, the cells were washed in cold lysis buffer [2 mM HEPES (pH 7.5), 0.34 M sucrose, 2 mM β-mercaptoethanol, 100 μM sodium orthovanadate, 0.1 μM sodium fluoride, 60 mM β-glycerophosphate, 1% Triton X-100, 1 μM pepstatin, and 10 μg/mL each of aprotinin, leupeptin, and soybean trypsin inhibitor]. The

homogenates were centrifuged at 4° for 10 min at 8000 *g*, and 2 mg of the supernatant protein in a 2-mL dilution with buffer A [25 mM β-glycerophosphate, 10 mM MOPS (pH 7.2), 5 mM EGTA, 2 mM $MgCl_2$, 1 mM dithiothreitol, and 2 mM sodium orthovanadate] was loaded onto a 1-mL MonoQ column (Pharmacia LKB Biotechnology) equilibrated with buffer A. The column was developed with a 10-mL, linear 0 to 0.8 M NaCl gradient in buffer A at a flow rate of 0.8 mL/min with a fast protein liquid chromatography (FPLC) system (Pharmacia LKB Biotechnology), and 250-μL fractions were collected.

### *Kinase Assays*

MAPK and PKC in the MonoQ fractions (5-μL aliquots) were assayed at 30° using the substrate MBP (1 mg/mL) and histone H1, respectively, in assay buffer consisting of: 20 mM MOPS (pH 7.2), 25 mM β-glycerophosphate, 5 mM EGTA, 2 mM EDTA, 20 mM $MgCl_2$, 2 mM sodium orthovanadate, 1 mM dithiothreitol, 500 nM cAMP-dependent protein kinase inhibitor peptide, and 50 μM [γ-$^{32}$P]ATP (≈ 2000 cpm/pmol) in a final volume of 25 μL. The reactions were allowed to proceed for 10 min, after which 20 μL of reaction mixture was spotted onto 1.5 × 2 cm P-81 phosphocellulose filter papers. The papers were washed extensively with ten changes of 1% phosphoric acid, after which the adsorbed radioactivity was quantitated by liquid scintillation counting in a Packard TriCarb 4530 counter.

Five hundred micrograms of total protein (bicinchoninic acid assay, Pierce) from extract supernatants was diluted into immunoprecipitation buffer [50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 5% glycerol, 10 mM sodium fluoride, 5 mM EGTA, 1 mM EDTA, and 30 mM β-glycerophosphate], and the solutions were incubated with the appropriate antibodies [MAPK: anti-ERK1-CT (UBI); PKC: anti-PKC Mab to the catalytic subunit (Kinetek Pharmaceuticals Inc.)] for 4 hr at 4°. Protein A-agarose beads (30 μL) (Pharmacia), which were preincubated in cold (4°) immunoprecipitation buffer, were added, and the samples were incubated further for 1 hr at 4°. The beads were washed twice with immunoprecipitation buffer and twice with KII buffer [12.5 mM MOPS (pH 7.5), 12 mM β-glycerophosphate, 5 mM EGTA, 7.5 mM $MgCl_2$, 50 mM NaF, and 0.25 mM dithiothreitol]. The beads were resuspended in KII buffer supplemented with 10 mM $MgCl_2$ and 5% glycerol, and the reactions were initiated with 10 μL [γ-$^{32}$P]ATP (≈ 2000 cpm/pmol) in a final volume of 40 μL and incubated for 20 min at 30°. The reactions were terminated with the addition of 5X SDS–sample buffer [125 mM Tris–HCl (pH 6.8), 4% SDS, 0.01% bromophenol blue, 10% β-mercaptoethanol, and 20% glycerol], boiled for 5 min, and loaded onto an SDS-PAGE gel. Following transfer of protein as described above, the membrane was immunoblotted for the appropriate protein, exposed to film, and the bands representing ERK1 and ERK2 proteins were excised and subjected to Cerenkov counting. PI 3-kinase was immunoprecipitated from neutrophil lysates (500 μg of total protein) with 0.5 μg of anti-p85 antibody (Santa Cruz Laboratories) for 3 hr at 4° with rotation and collected with 25 μL of protein A-conjugated Sepharose (Pharmacia). Then the beads were washed twice with lysis buffer and three times with 10 mM Tris-HCl, pH 7.4. PI (10 μg) was sonicated briefly in 30 mM HEPES, pH 7.4, then mixed with the beads, and incubated for 10 min on ice. The reaction was started by adding 10 μCi of [γ-$^{32}$P]ATP in 40 μL of kinase buffer [30 mM HEPES (pH 7.4), 30 mM $MgCl_2$, 200 μM adenosine, and 50 μM ATP] and incubating for 15 min at room temperature. One hundred microliters of 1 N HCl was added to stop the reaction, and PI 3-phosphate was extracted using 200 μL of chloroform: methanol (1:1). PI 3-phosphate was resolved from residual [γ-$^{32}$P]ATP in the organic layer by TLC on oxalate-treated silica gel 60 plates (E. Merck) using chloroform:methanol: water:concentrated $NH_4OH$ (45:35:7.5:2.5). PI 3-kinase activity was determined by scintillation counting of the PI 3-phosphate zone on the chromatogram after autoradiography.

### *Electrophoresis and Immunoblotting*

SDS-PAGE of the neutrophil lysates was performed on 1.5-mL thick gels. An 11% separating gel and a 4% stacking gel were used. Samples were boiled for 5 min in the presence of 5X SDS–sample buffer and electrophoresed for approximately 12 hr at 10 mA. Subsequently, the separating gel was soaked in transfer buffer (25 mM Tris, 192 mM glycine, and 20% methanol) for 10 min and then sandwiched with a nitrocellulose membrane. Proteins were transferred for 3 hr at 300 mA. The membrane was blocked with TBS [Tris-buffered saline, 20 mM Tris-HCl (pH 7.4), 0.25 M NaCl] containing 5% BSA for 3 hr at room temperature, and then was washed three times in TBS containing 0.05% Tween (TTBS) for 15 min. The membranes were incubated overnight at room temperature with primary antibody [MAPK: anti-ERK1-CT; PKC: anti-PKC Mab, anti-PKCδ (Gibco BRL)]. Membranes then were washed with TTBS three times before a 1-hr incubation at room temperature with secondary antibody (goat anti-mouse IgG coupled to horseradish peroxidase in 2.5% skim milk-TTBS for blots with anti-PKC-catalytic subunit Mab; and goat anti-rabbit IgG coupled to alkaline phosphatase in TTBS for blots probed with anti-PKCδ and anti-ERK1-CT antibodies). Next, the membranes were rinsed three times in TTBS and once in TBS before incubation with 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium color development solution (a mixture of 3% nitroblue tetrazolium in 1 mL of 70% dimethylformamide and 1.5% 5-bromo-4-chloro-3-indolyl phosphate in 1 mL of 100% dimethylformamide) before being added to 100 mL of buffer (0.1 M $NaHCO_3$, 10 mM $MgCl_2$, pH 9.8) for detection of alkaline phosphatase-conjugated secondary antibodies. The color was developed in 2–10 min, and the reaction was

stopped by rinsing the membranes in water. For detection of horseradish peroxidase-conjugated secondary antibodies, membranes were washed as described previously and subjected to enhanced chemiluminescence detection (Amersham).

### *Chemiluminescence Assay*

Chemiluminescence studies were performed using a cell concentration of $5 \times 10^6$ cells/mL in HBSS with the appropriate agonist. In all experiments, 0.5 mL of cell suspension was added to 25 mg of CPPD(T), 1 μL of 0.5 mM fMLP, or 1 μL of 0.25 mM PMA in 1.5-mL capped Eppendorf tubes. To each tube was added 5 μL of luminol dissolved in 25% DMSO in HBSS to a final concentration of 1 μM, and the samples were mixed at 37° to initiate neutrophil activation by one of the three agonists indicated. Chemiluminescence was monitored using the LKB Luminometer (model 1250) at 37°, with shaking immediately prior to measurements to resuspend the crystals. Control tubes contained cells, AGM-1470 (5 μM), and luminol (agonists absent).

### *Superoxide Anion Generation*

Superoxide anion concentrations were measured using the superoxide dismutase-inhibitable reduction of cytochrome *c* assay [11]. Twenty-five milligrams of plasma-opsonized CPPD(T) crystals (50 mg/mL), fMLP (1 μM), or PMA (0.5 μM) was added to 0.5 mL of cell suspension at 37° containing ferricytochrome *c* (horse heart, type 3, Sigma) (final concentration 1 mg/mL), and the cells were activated by shaking the capped tubes. After a 5-min incubation, tubes were centrifuged at 10,000 *g* for 10 sec, and the supernatant was collected for spectrophotometric analysis at 550 nm. Readings were taken at 540, 550, and 560 nm, and the peak height was calculated by subtracting the average of the 540 and 560 nm readings from the reading at 550 nm. This procedure corrects for baseline differences between different samples. The change in absorbance resulting from the reduction of cytochrome *c* was calculated by subtracting a blank value from the corresponding incubated sample value. Control tubes were set up under the same conditions with the inclusion of superoxide dismutase at 600 U/mL. The measure of $O_2^-$ generated in the whole reaction was calculated from the following formula:

$$O_2^- \ (nmol) \ = \ ABS_{550nm} \times 0.5 \text{ mL (incubation mixture volume)} \times 47.4$$

## RESULTS

### *Effect of AGM-1470 on CPPD(T) Crystal-, fMLP-, or PMA-Induced PKC Activity*

In these experiments, the total pool of PKC was used to investigate the activation of PKC, as it was not possible to effectively separate membrane and cytosolic fractions of PKC in crystal-activated cells due to partial phagocytosis and clumping effects. Following MonoQ fractionation of neutrophil homogenates from cells incubated with CPPD(T) crystals with and without AGM-1470 (5 μM) pretreatment, and from control cells, the column fractions were assayed for histone H1 phosphotransferase activity (Fig. 1). CPPD(T) crystals induced strong activation of PKC as indicated by the phosphotransferase peak (fractions 25–28) in Fig. 1B. This activation of PKC was not present in control cells (Fig. 1A) and was inhibited by AGM-1470 in CPPD(T) crystal-activated neutrophils (Fig. 1C). To distinguish whether a peak represented PKC activity or the activity of another kinase that co-eluted and phosphorylated histone H1, each fraction was assayed in the presence of the PKC cofactors PS, the DAG homolog DO, and calcium, or magnesium alone. The conventional isoenzymes of PKC (α, β, βII, and γ) have been shown to require PS, DAG, and calcium, whereas the nonconventional PKC isoforms (δ, ϵ, and η) are calcium independent and require only PS and DAG. The other, atypical isoforms are not activated by DAG and do not require calcium as a cofactor [18, 19]. Therefore, the peak observed in control cells (Fig. 1A) was unlikely to arise from PKC, since the peak representing the histone H1 phosphotransferase activity in the presence of the cofactors had approximately the same area as the peak assayed with magnesium alone. The peak (fractions 25–28) in Fig. 1B corresponded to a calcium-independent isoform of PKC, since the two peaks assayed with PS and DO with and without calcium represented approximately the same area and significantly exceeded the activity detected in the fractions assayed with magnesium alone. The presence of PKC in fractions 24–28 was confirmed further following SDS–PAGE and western analysis using a monoclonal antibody to the catalytic subunit of PKC (Fig. 1B, inset), which visualized 90- and 93-kDa bands and a few other less well-defined bands. The identity of the PKC isoform is not known at this time. This PKC histone H1 phosphotransferase peak was abolished completely in cells treated for 2 min with CPPD(T) crystals after being pretreated with AGM-1470 (Fig. 1C).

The PKC phosphotransferase peak that eluted in fractions 25–28 also was induced by fMLP and PMA (Fig. 2, A and C, respectively); this peak probably contained nonconventional, calcium-independent isoforms, since the degrees of calcium-dependent (PS/DO, $Ca^{2+}$) and calcium-independent (PS/DO) histone H1 phosphotransferase activities were inhibited similarly by AGM-1470 pretreatment. PMA also appeared to induce a calcium-independent histone H1 phosphotransferase activity that exceeded the activity of the fractions assayed with the PKC cofactors, and, therefore, may represent the activity of some isoforms that were not induced following CPPD(T) crystal incubation. AGM-1470 pretreatment of neutrophils resulted in the inhibition of fMLP-induced PKC activity (Fig. 2B), whereas none of the activated peaks in the PMA-induced profile were inhibited significantly by AGM-1470 incubation (Fig. 2D).

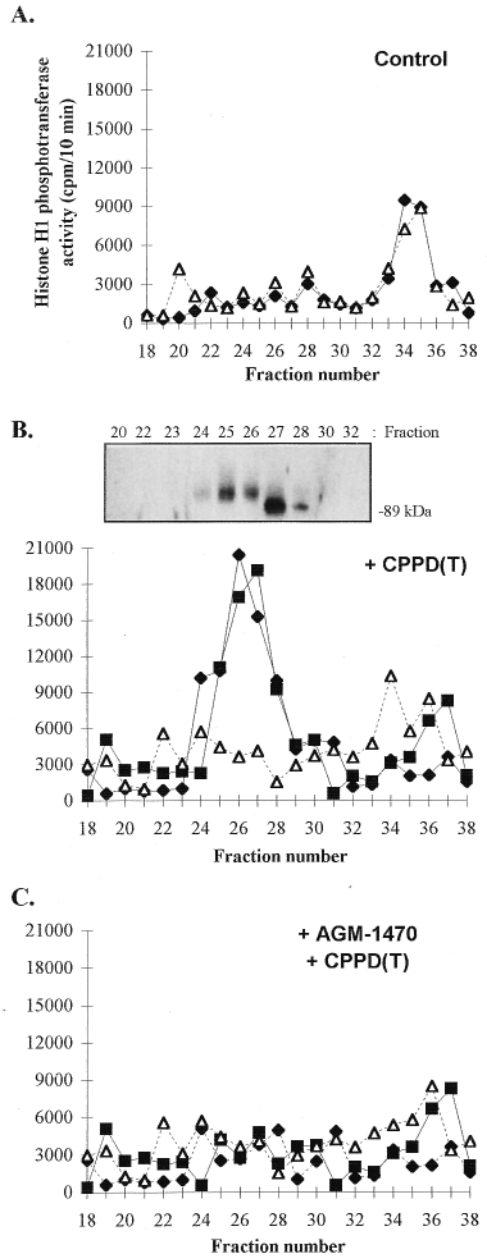


**FIG. 1. Effect of AGM-1470 inhibition on CPPD(T) crystal activation of PKC in neutrophils. MonoQ fractions from neutrophil ($5 \times 10^6$ cells/mL) extracts derived from (A) untreated (control) cells, (B) CPPD(T) crystal (50 mg/mL, 2 min) incubated cells, or (C) cells incubated with 5 μM AGM-1470 for 60 min prior to CPPD(T) incubation were incubated with [$\gamma$-$^{32}$P]ATP and histone H1 in the presence of the PKC cofactors PS and DO with and without calcium (PS/DO, $Ca^{2+}$, ♦; PS/DO, ■), or magnesium (△). Fractions (20–32) from condition (B) were subjected to SDS–PAGE and western analysis using a monoclonal antibody to the catalytic subunit of PKC (B, inset). These results are representative of three individual experiments.**

### *Effect of AGM-1470 on CPPD(T) Crystal-, fMLP-, or PMA-Induced MAPK Activity*

The MAPK ERK1 was immunoprecipitated from homogenates of neutrophils incubated with CPPD(T) crystals or fMLP, and the effect of AGM-1470 or taxol (positive control) on the MBP phosphotransferase activity of this MAPK isoform was assessed (Fig. 3A). CPPD(T) crystals induced a strong activation of MAPK that was inhibited completely by taxol preincubation and partially by AGM-1470 preincubation, as seen in Fig. 3A. Figure 3B shows the same nitrocellulose transfer used in Fig. 3A that was probed with anti-ERK-CT antibody, which cross-reacts with both the ERK1 and ERK2 isoforms of MAPK. The broad band in lane 2 indicates a reduction in band mobility of MAPK associated with activation of this enzyme by CPPD(T). Taxol inhibited this activation (lane 5), whereas AGM-1470 had little effect (lane 4). fMLP also activated MAPK, and this was inhibited strongly by AGM-1470 pretreatment (Fig. 3, A and C). The MBP bands in Fig. 3A were excised and analyzed by Cerenkov counting as shown in Fig. 3A (bottom), and the experiment was repeated two additional times, statistically represented in Fig. 3C. AGM-1470 failed to inhibit CPPD(T)-induced MAPK activation, but was able to inhibit fMLP-induced activation.

Following MonoQ fractionation of lysates of neutrophils incubated with CPPD(T) crystals, fMLP, or PMA, with or without AGM-1470 pretreatment, multiple peaks of MBP phosphotransferase activity were observed (Figs. 4 and 5). The identities of the first and second peaks are unknown, but similar to MAPK activation experiments assayed with this method previously [11, 20, 21], immunoblotting with the anti-ERK1-CT antibody revealed that MAPK was detected in the fractions of the third peak (fractions 24–27) as illustrated in Fig. 4B. Figure 4 illustrates the CPPD(T) crystal-induced activation of the MAPK-associated MBP phosphotransferase peak, which exceeded the activity in the third peak of the control (Fig. 4A). The MAPK activation induced by CPPD(T) crystal incubation was not inhibited by pretreatment with AGM-1470 (Fig. 4A). Figure 5 demonstrates that AGM-1470 inhibited the MAPK-associated MBP phosphotransferase activity induced by fMLP and PMA, which is consistent with the MAPK-associated MBP phosphotransferase activity results obtained from immunoprecipitated protein (Fig. 3). We propose that the results presented in Figs. 3–5 indicate that AGM-1470 selectively inhibited fMLP- and PMA-induced MAPK activity in neutrophils, but not CPPD crystal-induced MAPK activity.

### *Effects of AGM-1470 on CPPD(T)-, fMLP-, and PMA-Induced Respiratory Burst Activity Measured by Chemiluminescence and Superoxide Anion Generation*

Chemiluminescence arises primarily from the light emission associated with the myeloperoxidase-initiated breakdown of hydrogen peroxide, a product of superoxide anion. Chemiluminescence, therefore, only measures oxidase activation indirectly, so we also assayed respiratory burst activity via discontinuous measurements of superoxide anion concentrations as measured by the superoxide dismutase-inhibitable reduction of cytochrome *c*.

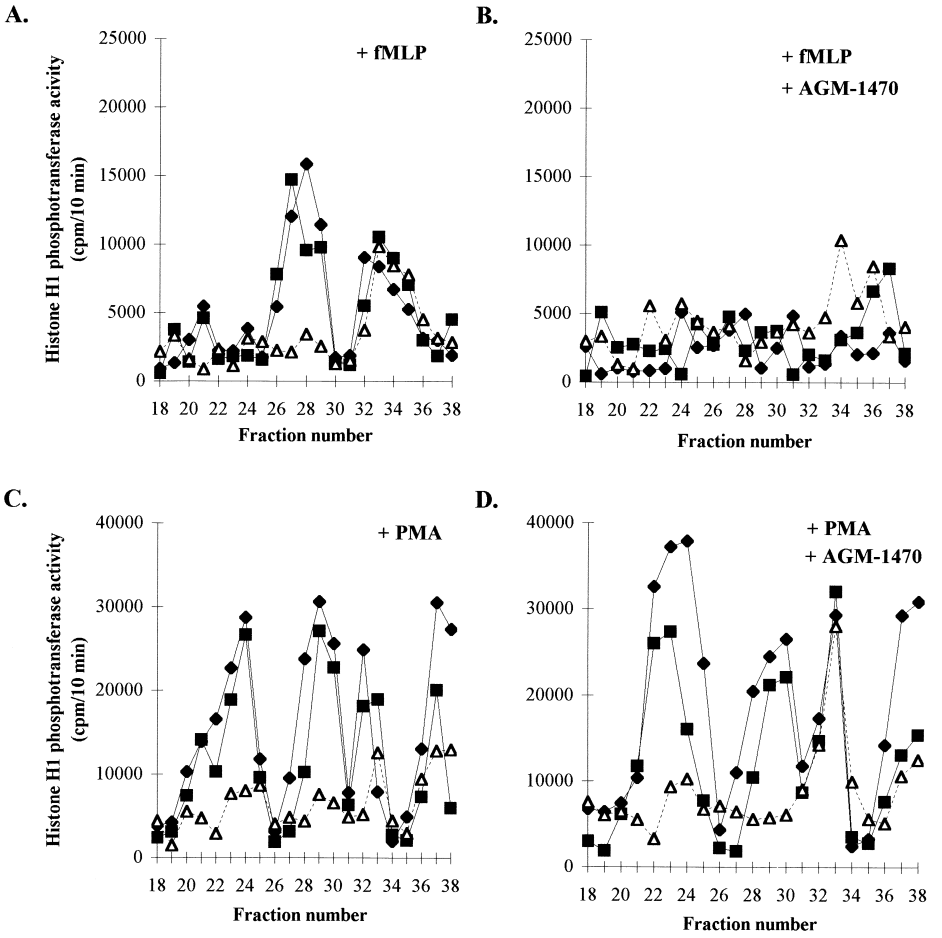


**FIG. 2. Differential effect of AGM-1470 on fMLP- and PMA-induced PKC activity in neutrophils. MonoQ fractionation was conducted on extracts from neutrophils stimulated with (A) fMLP (1 μM, 1 min), (C) PMA (0.5 μM, 5 min), (B) fMLP (1 μM, 1 min) on AGM-1470- (0.5 μM, 1 hr) pretreated neutrophils, and (D) PMA (0.5 μM, 5 min) on AGM-1470- (0.5 μM, 1 hr) pretreated neutrophils. PKC activity was determined by analyzing histone H1 phosphotransferase in the presence of the calcium-dependent PKC cofactors [(PS/DO, $Ca^{2+}$, ◆) and calcium-independent PKC cofactors (PS/DO, ■), or magnesium without cofactors (△)]. These results are representative of three individual experiments.**

CPPD(T) crystal-, fMLP-, and PMA-induced stimulation of neutrophil chemiluminescence reached peak values in approximately 2–4 min (Fig. 6, A–C, respectively). Time courses for CPPD(T) crystal- and PMA-treated cells were completed within 15 min, and for fMLP, within 3.5 min. AGM-1470 was not effective in inhibiting CPPD(T) crystal-induced chemiluminescence (Fig. 6A), whereas activation in response to fMLP and PMA was reduced significantly by the drug (Fig. 6, B and C, respectively). AGM-1470 also effectively inhibited the superoxide anion generation stimulated in response to fMLP and PMA (Fig. 7). The inhibition of superoxide anion generation by AGM-1470 (5 μM) pretreatment was not complete. fMLP induction was inhibited by 46 ± 9%, and PMA was inhibited by 54 ± 3%. These results demonstrate that AGM-1470 inhibited neutrophil respiratory burst activity based on chemiluminescence and superoxide anion production assays in cells treated with fMLP or PMA. AGM-1470 was ineffective in inhibiting respiratory burst activation induced with CPPD(T) crystals. The finding that AGM-1470 selectively inhibited fMLP- and PMA-induced respiratory burst correlated with the observation that AGM-1470 pretreatment selectively inhibited fMLP- and PMA-stimulated MAPK activity, but not CPPD(T) crystal-induced kinase activation.

### *PI 3-Kinase-Independent Inhibition of MAPK and Neutrophil Activation by AGM-1470*

To determine if PI 3-kinase activity was involved in the mechanism of inhibition of neutrophil activation by AGM-1470, we first analyzed the effects of wortmannin treatment on fMLP- and PMA-induced chemiluminescence. Figure 8 shows the effect of wortmannin pretreatment on fMLP-treated (panel A) and PMA-treated (panel B) neutrophils. Wortmannin inhibited fMLP-induced neutrophil respiratory burst as measured by chemiluminescence, but no effect was observed in cells stimulated with PMA. We next examined the effects of AGM-1470 on PI 3-kinase activity in cells stimulated with CPPD(T) crystals or fMLP. Immunoprecipitation studies were conducted on crude neutrophil lysates with the monoclonal anti-p85 PI 3-kinase antibody, and the activities of the immunoprecipitates were assessed using phosphatidylinositol as the substrate. We have previously described the use of this assay to quantitate the PI 3-kinase levels following detergent lysis [12]. The results of the autoradiography of the TLC-separated phosphorylated substrate PI 3-phosphate are shown in Fig. 9A. The spots were excised and counted for [$\gamma$-$^{32}$P]ATP incorporation, and the results are illustrated in Fig. 9B. Activation of PI 3-kinase in response to CPPD(T) crystals and fMLP treatment was observed, as [$\gamma$-$^{32}$P]ATP incorporation in these

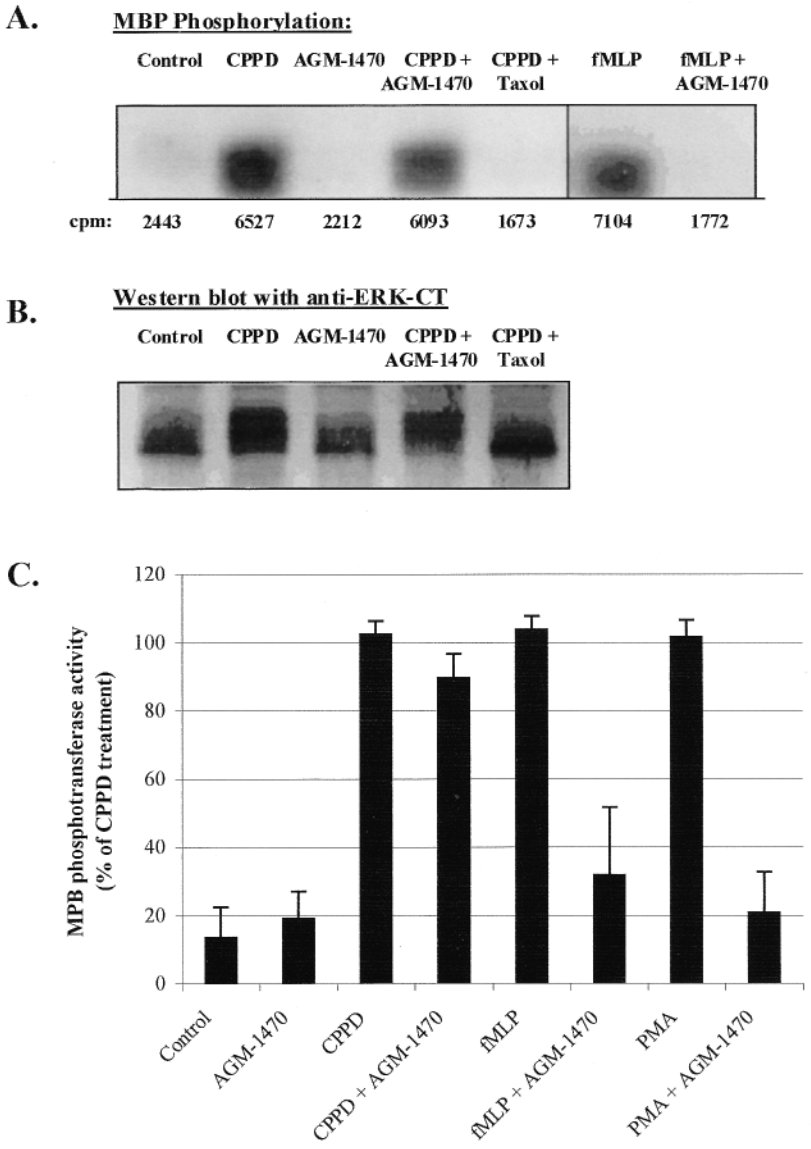


**FIG. 3. Effect of AGM-1470 on fMLP- or CPPD(T)-stimulated MAPK activity in neutrophils. MAPK immunoprecipitates from 500 μg of total protein extracts were incubated with [γ-$^{32}$P]ATP and MBP substrate for 30 min and then subjected to SDS–PAGE. Phosphorylated MAPK was transferred to nitrocellulose and subjected to autoradiography. (A) Lane 1, control; lane 2, CPPD(T); lane 3, AGM-1470 (5 μM), 1-hr incubation; lane 4, AGM-1470 pretreatment followed by CPPD(T) incubation for 2 min; lane 5, taxol (28 μM) pretreatment followed by CPPD(T) incubation for 2 min (positive control); lane 6, fMLP (1 μM); lane 7, AGM-1470 pretreatment followed by fMLP incubation for 1 min. (B)Western analysis with the anti-ERK1-CT antibody (UBI) of the immunoprecipitates described in (A). (C) MBP bands were excised from the membrane, and phosphorylation was quantitated by Cerenkov counting. Values represent the means ± SD, N = 3.**

samples was significantly greater than in control cells, and CPPD(T) crystal-treated cells preincubated with wortmannin demonstrated negligible activity. Pretreatment with AGM-1470 did not inhibit PI 3-kinase activation in response to either CPPD(T) crystals or fMLP.

## DISCUSSION

AGM-1470 has been investigated recently as a new treatment option for rheumatoid arthritis. Suppression of collagen- and complete Freund's adjuvant-induced arthritis in rats has been observed following AGM-1470 treatment [13–16]. In collagen-induced arthritis and complete Freund's adjuvant-induced synovitis models, AGM-1470 effectively prevented pannus formation and reduced vascular endothelial growth factor expression and neovascularization in the area of the joint where arthritis was induced, in both preventative and suppressive protocols [14–16]. Furthermore, histologic sections revealed significant alleviation of destruction of bone/cartilage [13]. Combination therapy with cyclosporin [15] or taxol [14], two compounds that have demonstrated efficacy in reducing arthritis, demonstrated reduction in collagen-induced arthritis symptoms more effectively than single-agent treatments with negligible immunosuppression.

The mechanism(s) of action of AGM-1470 is not yet clear other than its ability to inhibit proliferation by inducing a $G_1$ phase block due to suppression of cyclin D expression, as was observed in human umbilical vascular endothelial cells and WiDr human tumor cells following incubation with the drug [22]. We have demonstrated recently that AGM-1470 effectively inhibited PKC activity in human umbilical vascular endothelial cells stimulated with vascular endothelial growth factor and basic fibroblast growth factor (manuscript in progress). The results presented in this report illustrate that neutrophil activation by CPPD(T) crystals, fMLP, and PMA activated PKC as observed in MonoQ-fractionated histone H1 phosphotrans-

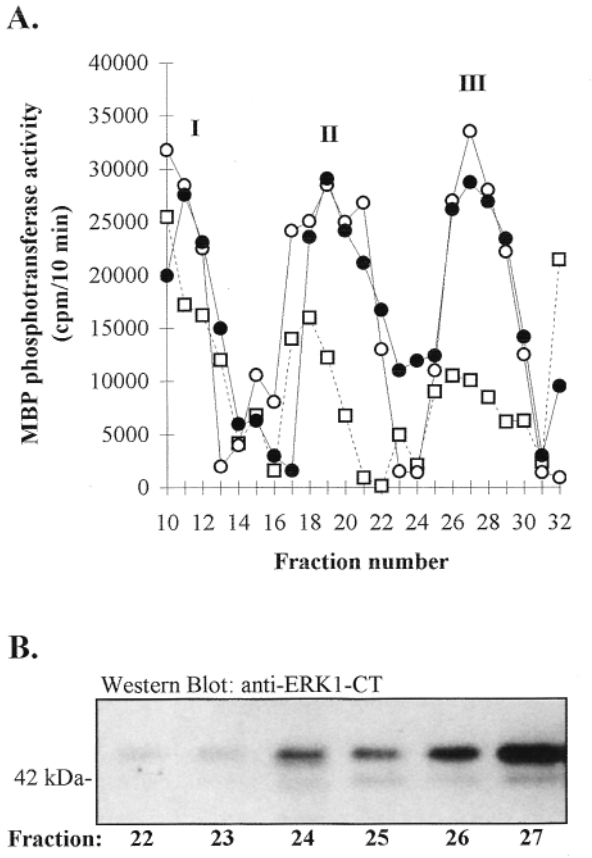


**FIG. 4. MonoQ chromatography of CPPD(T) crystal-stimulated MBP kinases: effect of AGM-1470 preincubation. (A) Neutrophil (5 × $10^6$ cells/mL) extracts from control (□), CPPD(T)-(50 mg/mL) treated (○), and CPPD(T) crystal-treated cells following a 60-min AGM-1470 (5 μM) pretreatment (●) were fractionated over a MonoQ anion-exchange column (with 0–800 mM linear NaCl gradient) and assayed for MBP phosphotransferase activity as described in Materials and Methods. (B) Eluent fractions 22–27 from neutrophils were subjected to SDS–PAGE, transferred to nitrocellulose, and immunoblotted with anti-ERK1-CT antibody. These results are representative of four individual experiments.**

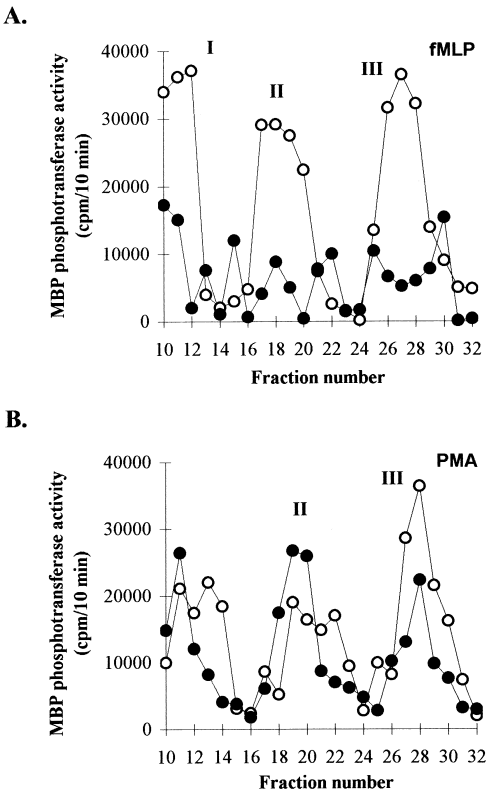


**FIG. 5. MonoQ chromatography of fMLP- and PMA-stimulated MBP kinases: effect of AGM-1470 preincubation. (A) Neutrophil (5 × $10^6$ cells/mL) extracts from fMLP-treated cells (1 μM, 1 min) (○), and fMLP- and AGM-1470-pretreated cells (●), and (B) PMA (0.5 μM, 5 min) (○) and PMA with AGM-1470-pretreated cells (●) were fractionated over a MonoQ anion-exchange column and assayed for MBP phosphotransferase activity as described in Materials and Methods. These results are representative of three individual experiments.**

ferase assays in the presence of PKC cofactors (Figs. 1 and 2). AGM-1470 inhibited PKC activation induced by CPPD(T) crystals and fMLP but not by PMA.

Although AGM-1470 pretreatment inhibited PKC activity in neutrophils stimulated with CPPD(T) crystals or fMLP, neutrophil oxidative responses were not affected in cells treated with CPPD(T) crystals. Furthermore, PMA-treated neutrophils preincubated with AGM-1470 were observed to display significantly reduced chemiluminescence and superoxide anion generation. These observations indicate that AGM-1470 may inhibit a mediator of neutrophil activation other than PKC. Since AGM-1470 did not affect CPPD(T) crystal-induced neutrophil respiratory burst but inhibited CPPD(T)-induced PKC activation, the inhibitory effect likely was centered on an enzyme on a signal transduction pathway that is distinct from, but parallel to, the PKC signaling pathway. To investigate the role of PKC in the neutrophil respiratory burst induced by CPPD(T) crystals, fMLP, and PMA, we preincubated neutrophils with specific inhibitors of PKC: Roche compound 3 and BIM. Using relatively high concentrations of these agents (1 μM compound 3 and 2–5 μM BIM) caused almost complete inhibition of both PMA- and fMLP-induced respiratory burst, but only 87% (compound 3) and 71% (BIM) inhibition of CPPD(T) crystal-induced respiratory burst (unpublished data). These PKC inhibition data indicate that PKC does have an important role in the signal transduction pathway leading to activation by all three agents. However, the lack of complete inhibition of crystal-induced activation indicates that signaling pathways for this agonist may not depend completely on the involvement of PKC, mirroring the AGM-1470 data for this agonist.

The possible association of the MAPK pathway with neutrophil activation in response to fMLP stimulation has been suggested previously [9, 23, 24]. Wortmannin, a PI 3-kinase inhibitor that represses CPPD(T) crystal-induced neutrophil chemiluminescence and superoxide anion generation [12], has been demonstrated to inhibit interleukin-8-induced neutrophil MAPK activation and degranulation responses [25]. Both uncoated and plasma-opsonized CP-

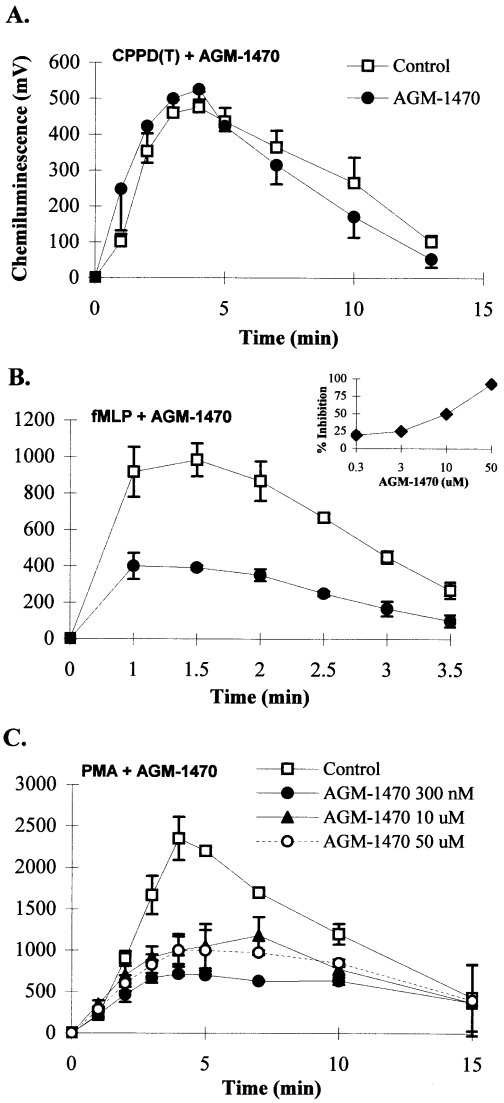


**FIG. 6. Effect of AGM-1470 on CPPD(T)-, fMLP-, and PMA-induced luminol-enhanced chemiluminescent responses in neutrophils. Shown is the time course of luminol-enhanced chemiluminescence due to (A) CPPD(T) crystals (50 mg/mL), (B) fMLP (1 μM) in the presence (●) or absence (□) of AGM-1470 (5 μM), and (C) PMA (0.5 μM) in the presence or absence of various concentrations of AGM-1470. The inset in panel B shows the concentration dependence of AGM-1470 inhibition of fMLP-induced peak (1.5 min) chemiluminescence. Values represent the means ± SD of three experiments.**

PD(T) crystals have been shown recently to activate MAPK, with peak activation occurring within 5 min of CPPD(T) crystal incubation of neutrophils [11]. Neutrophil oxidative and degranulation responses have been found to peak at approximately 5 min following crystal incubation, and it has been observed that CPPD(T) crystal-induced MAPK activity and neutrophil activation are inhibited by taxol pretreatment [11]. This inhibition of MAPK and neutrophil activation by taxol in response to CPPD(T) crystal incubation, and reports implicating the involvement of MAPK in chemoattractant-stimulated neutrophil responses [10, 12, 26], further support a role for

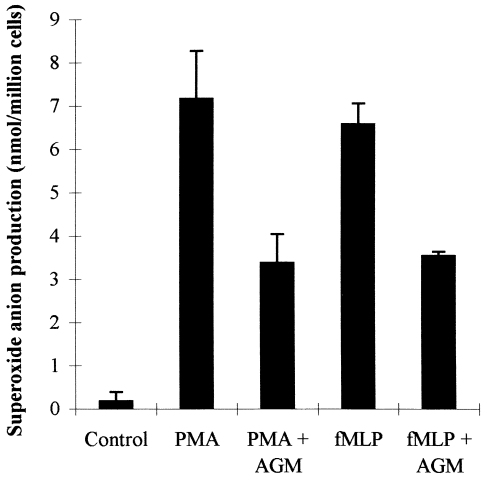


**FIG. 7. Effect of AGM-1470 (5 μM) on PMA- (0.5 μM) and fMLP- (1 μM) induced neutrophil superoxide anion generation (5-min incubation). Values are means ± SD of three experiments.**

MAPK in the induction of neutrophil activation-associated responses. PD98059, a specific inhibitor of MEK (and therefore MAPK), was found to partially inhibit CPPD(T) crystal- and fMLP-induced neutrophil respiratory burst (unpublished data). This study demonstrates an association

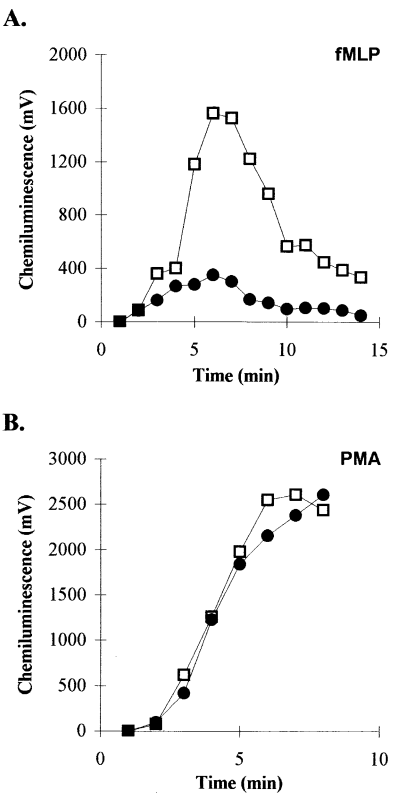


**FIG. 8. Effect of wortmannin (10 nM) on (A) fMLP- (1 μM) and (B) PMA- (0.5 μM) induced chemiluminescence. Luminol-enhanced chemiluminescence was monitored in neutrophils ($5 \times 10^6$/mL) incubated with (A) fMLP (1 μM, 1 min) with (●) and without (□) wortmannin (10 nM) for periods of 1–15 min, and with (B) PMA (0.5 μM) with and without wortmannin for 1–8 min. Values are the means of two experiments.**

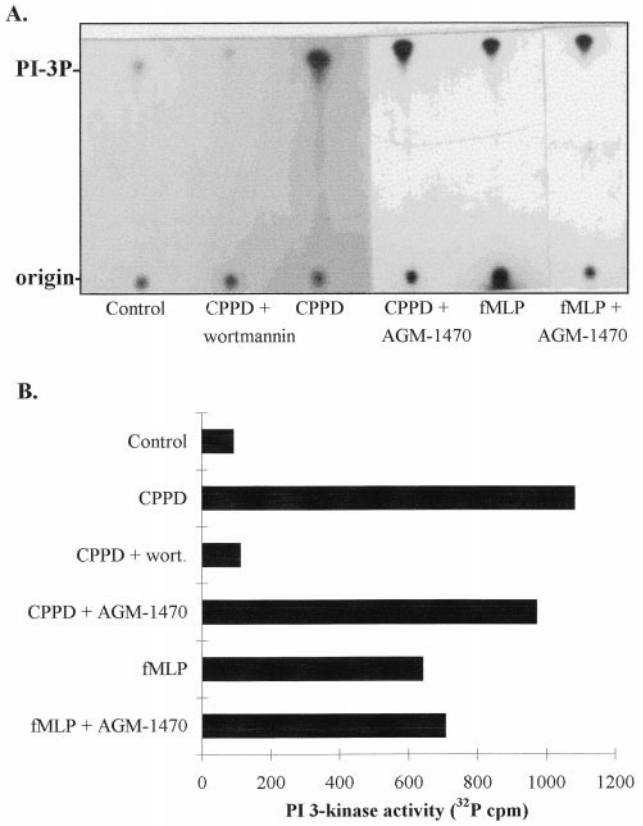


**FIG. 9. Effect of wortmannin (10 nM) or AGM-1470 (5 μM) on PI 3-kinase activity in CPPD(T)- (50 mg/mL) and fMLP- (1 μM) treated neutrophils. (A) TLC of [γ-$^{32}$P]ATP-labeled phosphorylated phosphatidylinositol substrate from crude neutrophil extracts. (B) Scintillation counting of PI 3-phosphate spots from TLC. These results are representative of two individual experiments.**

between agonist-dependent [CPPD(T), fMLP, or PMA] MAPK activation and stimulation of neutrophil oxidase responses. Figures 3 and 4 show that although MAPK was activated in response to each agonist analyzed in this report, only fMLP and PMA induction, but not CPPD(T) crystal induction, of MAPK was inhibited by AGM-1470 pretreatment. This correlation between inhibition of MAPK activation and abrogation of neutrophil oxidative response by AGM-1470 indicates that MAPK may be involved in a signal transduction pathway leading to neutrophil activation induced by fMLP and PMA. The observation that both MAPK and neutrophil activation associated with CPPD(T) crystal treatment were not affected by AGM-1470 pretreatment indicates that the function of MAPK in the signaling pathway leading to oxidative response is dependent upon the agonist. MAPK activation in response to CPPD(T) crystals may contribute to neutrophil oxidative responses, but this cannot be confirmed by these observations. Since AGM-1470 inhibited CPPD(T) crystal-induced PKC activity, but not neutrophil activation, we propose that it is possible that both PKC and MAPK activity are necessary for oxidative responses, and that AGM-1470 fails to inhibit CPPD(T) crystal-induced neutrophil activation because it does not inhibit a mediator of the MAPK pathway.

fMLP-induced activation of MAPK has been described previously where it was suggested that fMLP-induced oxidase activation is stimulated through the MAPK pathway [23, 24]. Therefore, we speculated that taxol inhibition of fMLP-induced neutrophil responses [11] may be a result of inhibition of MAPK activity by this drug. Our initial studies, however, indicated that taxol inhibits fMLP-induced chemiluminescence by 40% and PMA-induced chemiluminescence by only 10% [12]. The data presented in this manuscript demonstrated that AGM-1470 (5 μM) inhibited fMLP- and PMA-induced chemiluminescence and superoxide anion generation by approximately 46 and 54%, respectively. Even at higher concentrations of AGM-1470 (10 μM), slightly below the concentration where cell death was detectable (50 μM), neutrophil activation was not inhibited any further. These observations indicate that neutrophil responses such as oxidase activation are mediated by distinct pathways that each contribute to the total response. Furthermore, we propose that one of these pathways may be mediated through MAPK.

Recently, we demonstrated that PI 3-kinase is activated in crystal-stimulated neutrophils [12], and that two specific inhibitors of PI 3-kinase, wortmannin and LY294002, were shown to completely suppress plasma-coated CPPD(T) crystal-incubated neutrophil activation at concentrations lower than the known $IC_{50}$ of these inhibitors for PI 3-kinase. Additionally, the role of PI 3-kinase activity in chemoattractant-stimulated neutrophils has been reported previously [27, 28], and PI 3-kinase has also been implicated in the activation of MAPK [29, 30]. Since we demonstrated that wortmannin inhibited fMLP- but not PMA-induced chemiluminescence, and that AGM-1470 did not inhibit fMLP- or CPPD(T) crystal-induced PI 3-kinase activity, we suggest that AGM-1470 probably inhibits the signal transduction pathway leading to neutrophil activation in a manner that is independent of PI 3-kinase. We previously reported that the PKC pathway, leading to the activation of $p70^{S6K}$, is distinct from the PI 3-kinase pathway in neutrophils [20]. Therefore, any contribution of PKC towards neutrophil activation may be independent of PI 3-kinase. Our results indicate that the neutrophil oxidative response mediated by PI 3-kinase is independent of MAPK. It has been observed that in other cell lines, MAPK can be activated independent of PI 3-kinase activation [31–36]. We propose that neutrophil oxidative responses are regulated by multiple and distinct pathways that utilize MAPK and PI 3-kinase, and that AGM-1470 functions to inhibit neutrophil activation in a MAPK-dependent, PI 3-kinase-independent manner that requires PKC activity.

The signaling pathways leading to neutrophil activation have not been elucidated completely. The use of various agents as pharmacological probes has provided novel insights into the biochemical nature of signal transduction molecules and pathways that lead to neutrophil activation.

These agents also have been utilized to identify putative pharmacological targets for therapeutic intervention against leukocytes and other cells. This study has utilized the anti-angiogenesis agent AGM-1470 to further dissect the pathways leading to neutrophil activation in response to various agonists. We have demonstrated that AGM-1470 suppressed fMLP- and PMA-stimulated neutrophil activation and further established this drug as a prototypic agent for a new class of potential anti-arthritic therapy. We also have demonstrated that AGM-1470 inhibited PKC activation in response to CPPD(T) crystals and fMLP, and inhibited fMLP- and PMA-induced MAPK activity effectively without affecting PI 3-kinase activity. From these results we propose that at least two pathways exist leading to the activation of neutrophil oxidative responses. One is mediated by PI 3-kinase as has been proposed elsewhere, whereas the other may be mediated in parallel with MAPK, where a downstream effector probably also requires the activity of PKC. Until very recently, the mechanisms by which AGM-1470 inhibits neovascularization, endothelial cell proliferation, collagen-induced arthritis pannus formation, and other arthritis-like symptoms, have remained elusive [15, 16, 37–40]. The data presented here provide further insight into the mechanism of action of AGM-1470 and the molecular signaling in stimulated neutrophils that leads to respiratory burst activation by various agents.

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## References

1. Unkeless JC, Boros P and Fein M, Structure, signaling, and function of $Fc_{\epsilon}R$. In: *Inflammation: Basic Principles and Clinical Correlates* (Eds. Gallin JI, Goldsten RM and Snyderman R), pp. 497–510. Raven Press, New York, 1992.
2. Edwards SW, Cell signaling by integrins and immunoglobulin receptors in primed neutrophils. *Trends Biochem Sci* **20**: 363–367, 1995.
3. Bokoch GM, Chemoattractant signaling and leukocyte activation. *Blood* **86**: 1649–1660, 1995.
4. McCarthy DJ, Pathogenesis and treatment of crystal induced inflammation. In: *Arthritis and Allied Conditions* (Ed. McCarthy DJ), pp. 1495–1514. Lea & Febiger, Philadelphia, 1985.
5. Burt HM, Jackson JK, Dryden P and Salari H, Crystal-induced protein tyrosine phosphorylation in neutrophils and the effect of a tyrosine kinase inhibitor on neutrophil responses. *Mol Pharmacol* **43:** 30–36, 1993.
6. Winternitz CI, Jackson JK and Burt HM, The interaction of monoclinic calcium pyrophosphate dihydrate crystals with neutrophils. *Rheumatol Int* **16:** 101–107, 1996.
7. Kozin F, Ginsberg MH and Skosey JL, Polymorphonuclear leukocyte responses to monosodium urate crystals: Modification of absorbed serum proteins. *J Rheumatol* **6:** 519–526, 1979.
8. Nagase M, Baker DG and Schumacher HR Jr, Immunoglobulin G coating on crystals and ceramics enhances polymorphonuclear cell superoxide production: Correlation with immunoglobulin G absorbed. *J Rheumatol* **16:** 971–976, 1989.
9. Worthen GS, Avdi N, Buhl AM, Suzuki N and Johnson GL, FMLP activates Ras and Raf in human neutrophils. Potential role in activation of MAP kinase. *J Clin Invest* **94:** 815–823, 1994.
10. Krump E, Sanghera JS, Pelech SL, Furuya W and Grinstein S, Chemotactic peptide *N*-formyl-Met-Leu-Phe activation of p38 mitogen-activated protein kinase (MAPK) and MAPK-activated protein kinase-2 in human neutrophils. *J Biol Chem* **272:** 937–944, 1997.
11. Jackson JK, Tudan C, Salh B, Pelech SL and Burt HM, Calcium pyrophosphate dihydrate crystals activate MAP in human neutrophils: Inhibition of MAP kinase, oxidase activation and degranulation responses of neutrophils by taxol. *Immunology* **90:** 502–510, 1997.
12. Jackson JK, Lauener R, Duronio V and Burt HM, The involvement of phosphatidylinositol 3-kinase in crystal induced human neutrophil activation. *J Rheumatol* **24:** 341–348, 1997.
13. Peacock DJ, Banquerigo ML and Brahn E, Angiogenesis inhibition suppresses collagen arthritis. *J Exp Med* **175:** 1135–1138, 1992.
14. Oliver SJ, Banquerigo ML and Brahn E, Suppression of collagen-induced arthritis using an angiogenesis inhibitor, AGM-1470, and a microtubule stabilizer, taxol. *Cell Immunol* **157:** 291–299, 1994.
15. Oliver SJ, Cheng TP, Banquerigo ML and Brahn E, Suppression of collagen-induced arthritis by an angiogenesis inhibitor, AGM-1470, in combination with cyclosporin: Reduction of vascular endothelial growth factor (VEGF). *Cell Immunol* **166:** 196–206, 1995.
16. Peacock DJ, Banquerigo ML and Brahn E, A novel angiogenesis inhibitor suppresses rat adjuvant arthritis. *Cell Immunol* **160:** 178–184, 1995.
17. Burt HM and Jackson JK, Characterization and membranolytic effects of triclinic calcium pyrophosphate dihydrate crystals. *J Rheumatol* **14:** 968–973, 1987.
18. Malviya AN and Block C, Nuclear protein kinase C and signal transduction. *Receptor* **3:** 257–275, 1993.
19. Nishizuka Y, Protein kinase C and lipid signaling for sustained cellular responses. *FASEB J* **9:** 484–496, 1995.
20. Tudan C, Jackson JK, Charlton L, Pelech SL, Salh B and Burt HM, Activation of S6 kinase in human neutrophils by calcium pyrophosphate dihydrate crystals: Protein kinase C-dependent and phosphatidylinositol 3-kinase-independent pathways. *Biochem J* **331:** 531–537, 1998.
21. Verma S, Bhanot S, Arikawa E, Yao L and McNeill JH, Direct vasodepressor effects of pioglitazone on spontaneously hypertensive rats. *Pharmacology* **56:** 7–16, 1998.
22. Hori A, Ikeyama S and Sudo K, Suppression of cyclin D1 mRNA expression by the angiogenesis inhibitor TNP-470 (AGM-1470) in vascular endothelial cells. *Biochem Biophys Res Commun* **204:** 1067–1073, 1994.
23. Grinstein S and Furuya W, Chemoattractant-induced tyrosine phosphorylation and activation of microtubule-associated protein kinase in human neutrophils. *J Biol Chem* **267:** 18122–18125, 1992.
24. Torres M, Hall FL and O'Neill K, Stimulation of human neutrophils with formyl-methionyl-leucyl-phenylalanine induces tyrosine phosphorylation and activation of two distinct mitogen-activated protein-kinases. *J Immunol* **150:** 1563–1577, 1993.
25. Arcaro A and Wymann MP, Wortmannin is a potent phosphatidylinositol 3-kinase inhibitor: The role of phosphatidyl-

inositol 3,4,5-trisphosphate in neutrophil responses. *Biochem J* **296:** 297–301, 1993.
26. Mayer AM, Brenic SS and Glaser KB, Pharmacological targeting of signaling pathways in protein kinase C-stimulated superoxide generation in neutrophil-like HL-60 cells: Effect of phorbol ester, arachidonic acid and inhibitors of kinase(s), phosphatase(s) and phospholipase $A_2$. *J Pharmacol Exp Ther* **279:** 633–644, 1996.
27. Knall C, Young S, Nick JA, Buhl AM, Worthen GS and Johnson GL, Interleukin-8 regulation of the Ras/Raf/mitogen-activated protein kinase pathway in human neutrophils. *J Biol Chem* **271:** 2832–2838, 1996.
28. Okada T, Sakuma L, Fukui Y, Hazeki O and Ui M, Blockage of chemotactic peptide-induced stimulation of neutrophils by wortmannin as a result of selective inhibition of phosphatidylinositol 3-kinase. *J Biol Chem* **269:** 3563–3567, 1994.
29. Nishioka N, Hirai S-I, Mizuno K, Osada S-I, Suzuki A, Kosaka K and Ohno S, Wortmannin inhibits the activation of MAP kinase following vasopressin V1 receptor stimulation. *FEBS Lett* **377:** 393–398, 1995.
30. Karnitz LM, Burns LA, Sutor SL, Blenis J and Abraham RT, Interleukin-2 triggers a novel phosphatidylinositol 3-kinase-dependent MEK activation pathway. *Mol Cell Biol* **15:** 3049–3057, 1995.
31. Blenis J, Signal transduction via the MAP kinases: Proceed at your own RSK. *Proc Natl Acad Sci USA* **90:** 5889–5892, 1993.
32. Gardner AM, Vaillancourt RR and Johnson GL, Activation of mitogen-activated protein kinase/extracellular signal-regulated kinase kinase by G protein and tyrosine kinase oncoproteins. *J Biol Chem* **268:** 17896–17901, 1993.
33. Crespo P, Xu N, Simonds WF and Gutkind SJ, Ras-dependent activation of MAP kinase pathway mediated by G-protein $\beta\gamma$ subunits. *Nature* **369:** 418–420, 1994.
34. Cobb MH and Goldsmith EJ, How MAP kinases are regulated. *J Biol Chem* **270:** 14843–14846, 1995.
35. Duff JL, Marrero MB, Paxton WG, Schieffer B, Bernstein KE and Berk BC, Angiotensin II signal transduction and the mitogen-activated protein kinase pathway. *Cardiovasc Res* **30:** 511–517, 1995.
36. Stein B and Anderson D, The MAP kinase family: New 'MAPs' for signal transduction pathways and novel targets for drug discovery. *Annu Rep Med Chem* **31:** 289–292, 1996.
37. Yamaoka M, Yamamoto T, Ikeyama S, Sudo K and Fujita T, Angiogenesis inhibitor TNP-470 (AGM-1470) potently inhibits the tumor growth of hormone-independent human breast and prostate carcinoma cell lines. *Cancer Res* **53:** 5233–5236, 1993.
38. Kusaka M, Sudo K, Matsutani E, Kozai Y, Marui S, Fujita T, Ingber D and Folkman J, Cytostatic inhibition of endothelial cell growth by the angiogenesis inhibitor TNP-470 (AGM-1470). *Br J Cancer* **69:** 212–216, 1994.
39. Antoine N, Greimers R, De Roanne C, Kusaka M, Heinen E, Simar LJ and Castronovo V, AGM-1470, a potent angiogenesis inhibitor, prevents the entry of normal but not transformed endothelial cells into the $G_1$ phase of the cell cycle. *Cancer Res* **54:** 2073–2076, 1994.
40. Antoine N, Daukandt M, Locigno R, Heinen E, Simar LJ and Castronovo V, The potent angioinhibin AGM-1470 stimulates normal but not human tumoral lymphocytes. *Tumori* **82:** 27–30, 1996.