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Eosinophil Major Basic Protein Stimulates Neutrophil Superoxide Production by a Class I_\text{A} Phosphoinositide 3-Kinase and Protein Kinase C-\zeta-Dependent Pathway\(^1\)

Neeta G. Shenoy,* Gerald J. Gleich,† and Larry L. Thomas\(^2\)*

Eosinophil major basic protein (MBP) is an effective stimulus for neutrophil superoxide (O\(_2^-\)) production, degranulation, and IL-8 production. In this study we evaluated the participation of phosphoinositide 3-kinase (PI3K) and PI3K-associated signaling events in neutrophil activation by MBP. Inhibition of PI3K activity blocked MBP-stimulated O\(_2^-\) production, but not degranulation or IL-8 production. Measurement of Akt phosphorylation at Ser\(^{73}\) and Thr\(^{308}\) confirmed that MBP stimulated PI3K activity and also demonstrated indirectly activation of phosphoinositide-dependent kinase-1 by MBP. Genistein and the Src kinase family inhibitor, 4-amino-5-(4-methyphenyl)-7-((t-butyl)pyrazolo[3,4-d]pyrimidine, inhibited MBP-stimulated phosphorylation of Akt. 4-Amino-5-(4-methyphenyl)-7-((t-butyl)pyrazolo[3,4-d]pyrimidine also inhibited MBP-stimulated O\(_2^-\) production. MBP stimulated phosphorylation and translocation of the p85 subunit of class I\(_\text{A}\) PI3K, but not translocation of the p110\(_\gamma\) subunit of class I\(_\text{B}\) PI3K, to the neutrophil membrane. Inhibition of protein kinase Cz (PKCz) inhibited MBP-stimulated O\(_2^-\) production. Measurement of phosphorylated PKCz (Thr\(^{410}\)) and PKCd (Thr\(^{505}\)) confirmed that PKCz, but not PKCd, is activated in MBP-stimulated neutrophils. The time courses for phosphorylation and translocation of the p85 subunit of class I\(_\text{A}\) PI3K, activation of Akt, and activation of PKCz were similar. Moreover, inhibition of PI3K activity inhibited MBP-induced activation of PKCz. We conclude that MBP stimulates a Src kinase-dependent activation of class I\(_\text{A}\) PI3K and, in turn, activation of PKCz in neutrophils, which contributes to the activation of NADPH oxidase and the resultant O\(_2^-\) production in response to MBP stimulation. The Journal of Immunology, 2003, 171: 3734–3741.

M ajor basic protein (MBP)\(^3\) is a 13.8-kDa, arginine-rich polypeptide present in eosinophil secondary granules (1). MBP is a functionally pleiotropic molecule that contributes to both the host defense role of eosinophils in helminth infection and the pathogenesis observed in allergic diseases and other diseases characterized by eosinophilia (1). The antiparasite function and some of the eosinophil-associated pathogenesis are attributed to the well-described cytotoxicity of MBP for parasite larvae and some mammalian cells in vitro (1). MBP, however, also contributes to eosinophil-associated inflammation through a variety of noncytotoxic actions, including stimulation of mediator release by a number of inflammatory cells (2). In this context we have shown previously that MBP is an effective stimulus for neutrophil superoxide (O\(_2^-\)) production as well as for neutrophil degranulation and IL-8 production (3–5).

Production of O\(_2^-\) and other reactive oxygen species, such as hydrogen peroxide, is important to the bactericidal function of neutrophils and also contributes to tissue damage at sites of neutrophil inflammation (6). Several signaling pathways, most notably phospholipase C-mediated generation of diacylglycerol and phospholipase D-mediated production of phosphatidic acid, are implicated in the regulation of NADPH oxidase (7–10). MBP, however, does not stimulate an increase in the diacylglycerol content in neutrophils (4) and stimulates only a small, biologically insignificant increase in phospholipase D-mediated phosphatidic acid production (4). Furthermore, MBP-stimulated O\(_2^-\) production by neutrophils is not sensitive to inhibition by pertussis toxin (4), thereby excluding participation of a pertussis toxin-sensitive G protein in the mechanism.

Activation of phosphoinositide 3-kinase (PI3K) is now established as an important step in numerous cellular functions (11), including neutrophil O\(_2^-\) production (11). The class I\(_\text{B}\) PI3K are composed of the regulatory subunit p85, p50, or p55 and are coupled to Src-like kinases (12). The class I\(_\text{A}\) PI3K is composed of the regulatory subunit p101 and the catalytic subunit p110\(\gamma\) and is functionally activated by a variety of signaling pathways, including Akt/protein kinase B, phosphoinositide-dependent kinase-1 (PDK1), and protein kinase C (PKC)z (12, 13). The importance of PI3K in neutrophil O\(_2^-\) production was first demonstrated by the finding that two PI3K inhibitors, wortmannin and LY294002 (14, 15), inhibit the response stimulated by fMLP. More recently, the finding that neutrophils from p110\(\gamma\) knockout mice display impaired chemotaxis and O\(_2^-\) production in response to fMLP stimulation directly implicated the class IB PI3K in these responses (16–18). In contrast, the class IA PI3K is implicated in Fc\(\gamma\)R-mediated neutrophil activation (19, 20). The present study was performed to evaluate the hypothesis that PI3K plays a role in neutrophil activation by MBP.
Materials and Methods

MBP was isolated from patients with hypereosinophilia as described previously (21). The purity of the MBP was established by SDS-PAGE and staining with Coomassie brilliant blue R. W. Wortmannin, L2934/002, GFT10920X, rottlerin, genistein, 4-amino-5-(4-methylphenyl)-7-(t-butyl)pyrazolo[3,4-d]pyrimidine (PPII), and human serum albumin were purchased from Calbiochem (La Jolla, CA). Lymphocyte separation medium was purchased from ICN Pharmaceutical (Costa Mesa, CA). HBSS, HEPES buffer solution, and RPMI 1640 were purchased from Life Technologies (Grand Island, NY). Polyonal IgG Abs were purchased as follows: anti-Akt, anti-phosphorylated (Ser473) Akt, anti-phosphorylated (Thr308) Akt, anti-phosphorylated (Thr410) PKCζ, anti-phosphorylated (Thr505) PKCα, HRP-conjugated anti-phosphotyrosine (Cell Signaling Technologies, Beverly, MA); anti-PKCζ (Upstate Biotechnologies, Lake Placid, NY); anti-PKCα, anti-p85 subunit of class I PI3K, and anti-p110α subunit of class I PI3K (Santa Cruz Biotechnology, Santa Cruz, CA); and anti-lactoferrin (BioDesign, Saco, ME). HRP-conjugated goat anti-mouse IgG and anti-rabbit IgG were purchased from Southern Biotechnology Associates (Birmingham, AL). A myristoylated peptide (Myr-SIYR-RARRWRRKLL-OH) corresponding to the N-terminal pseudosubstrate sequence of PKCζ (22) and the nonmyristoylated control peptide were purchased from BioSource International (Camarillo, CA). All other stimulants and reagents were purchased from Sigma-Aldrich (St. Louis, MO).

Measurement of O$_2^-$ production

Neutrophils were isolated from the venous blood of healthy adult volunteers by density gradient centrifugation as described previously (23). O$_2^-$ production was measured as described previously (4). Briefly, cells were suspended in HBSS supplemented with 10 mM HEPES (pH 7.4) and 30 μg/ml human serum albumin (HBSS buffer), and aliquots (2 × 10$^6$ cells) were added to 12 × 75-mm polystyrene tubes. The cells were preincubated in the presence or the absence of the pharmacological agents for the times indicated at 37°C. After addition of the stimulus and cytochrome c (80 μM), the mixtures (0.2 ml) were incubated for 30 min at 37°C in an oscillating water bath. The reaction was stopped by centrifugation of the mixtures at 400 g for 5 min at 4°C, and the A$_{560}$ of the supernatant was measured at 4°C. A standard curve was plotted in saline with known nanomoles of O$_2^-$ produced. The O$_2^-$ production was calculated using an extinction coefficient of 21.3 (mM/cm)$^2$ 1 M$^{-1}$. and the results are expressed as nanomoles of O$_2^-$ per 10$^6$ cells per 30 min.

Measurement of lysozyme release

Neutrophils (2 × 10$^6$ cells) were preincubated in HBSS buffer with or without wortmannin in polystyrene tubes for 10 min at 37°C. After addition of the stimuli, the cell mixtures (0.2 ml) were incubated for 1 h at 37°C in an oscillating water bath. The reaction was terminated by centrifugation of the mixture at 200 × g for 5 min at 4°C, and the supernatants were stored at −20°C. Lysozyme release was measured as a percentage of total lysozyme activity, which was measured in a cell aliquot lysed by incubation with 0.04% Triton X-100. Spontaneous lysozyme release was calculated as a percentage of total lysozyme activity, which was measured in a cell aliquot lysed by incubation with 0.04% Triton X-100. Spontaneous lysozyme release was measured in cells incubated in the absence of stimulus.

Measurement of IL-8 production

Neutrophils were isolated as described above under sterile conditions and were suspended in RPMI medium containing 100 U/ml penicillin, 100 μg/ml streptomycin, and 2 mM t-glutamine (5). Aliquots containing 10$^6$ cells were added to wells in a 24-well tissue culture plate, and the cells were preincubated with wortmannin for 10 min at 37°C in a 5% CO$_2$ atmosphere. After addition of stimuli, the cells were incubated overnight at 37°C in a 5% CO$_2$ atmosphere. The total reaction volume was 0.5 ml. The incubation was stopped by centrifugation at 400 × g for 10 min at 4°C, and the supernatants were stored at −20°C until use. The IL-8 content of the supernatants was measured by ELISA (BioSource International).

Immunoprecipitation and Western blot analysis

Cell lysates were obtained as described previously (4) with slight modifications. Briefly, neutrophils (2 × 10$^6$ cells) were incubated in quadruplicate in HBSS buffer in the presence or the absence of stimuli for the indicated times at 37°C in an oscillating water bath. Incubation volume was 0.2 ml. Where indicated, cells were preincubated with pharmacological inhibitors. The reactions were stopped by placing the cells on ice and then centrifugation at 200 × g for 15 s at 4°C. The cell pellets were suspended in ice-cold lysis buffer (1% Nonidet P-40, 50 mM Tris (pH 8.0), 2 mM EDTA, 150 mM NaCl, 2 mM Na$_2$VO$_4$, 50 mM NaF, 2 mM PMSF, 10 μg/ml apronin, 10 μg/ml leupeptin, 10 μg/ml peptatin, 2 mM (2-aminoethyl)-benzenesulfonyl fluoride, and 4 mM di-isopropyl fluorophosphate), and the cell suspension was placed on ice for 15 min. The cell lysates were centrifuged at 14,000 × g for 15 min at 4°C, and supernatants were pooled and placed on ice, while the protein content was measured by the bichinchorinic acid method (Pierce, Rockford, IL). Pooled lysates containing 100–120 μg of protein were then immediately incubated with polyclonal anti-Akt, anti-p85, anti-PKCζ, or anti-PKCδ Abs (1/100 dilutions) overnight at 4°C as recommended by the manufacturers. Sepharose-protein A beads (Pierce) were added to the lysates (15 μl of beads/250 μl of lysate), and the mixture was incubated for 3 h at 4°C with constant rocking. The protein A beads were washed three times in lysis buffer and boiled in SDS sample buffer (containing 200 mM DTT), and the proteins were resolved on 10% SDS-PAGE. The proteins were transferred electrophoretically to ECL Hybond nitrocellulose (Amersham Pharmacia Biotech, Piscataway, NJ). With two exceptions, membranes were incubated with 3% nonfat dry milk in TBST for 1 h at room temperature to reduce any nonspecific binding, followed by overnight incubation with the primary Ab diluted 1/1000 in 3% milk in TBST, as recommended by the manufacturers. After washing several times with TBST, the membranes were incubated with HRP-conjugated goat anti-rabbit or goat anti-mouse IgG in 3% milk in TBST and were washed again several times. For Western blot analysis with anti-phosphorylated PKCζ and the HRP-anti-phosphotyrosine Abs, 5% BSA was substituted for the 3% milk. Proteins were visualized using Supersignal West Pico Chemiluminescent substrate (Pierce), and the densities of the protein bands were quantified using a Personal Densitometer SI (Amersham Pharmacia Biotech).

Translocation of PI3K subunits to neutrophil membranes

Neutrophil membranes were isolated as described previously (24) with slight modifications. Briefly, neutrophils were suspended in MBBP, and reactions were stopped by centrifugation as described above. The cell pellets were suspended in ice-cold extraction buffer (50 mM Tris-HCl (pH 7.5), 2 mM EGTA, 50 mM 2-ME, 1 mM PMSF, 10 μg/ml apronin, 10 μg/ml leupeptin, 10 μg/ml pepstatin, 2 mM (2-aminoethyl)-benzenesulfonyl fluoride, and 4 mM di-isopropylfluorophosphate) at a concentration of 5 × 10$^6$ cells/ml and were sonicated twice for 12 s each time on ice. The sonicates were centrifuged at 150,000 × g for 90 min at 4°C (4). The pellet was suspended in extraction buffer by brief sonication on ice. The protein content was measured with a protein assay (Bio-Rad, Hercules, CA). Membrane fractions containing 40 μg of protein were boiled in SDS sample buffer and the proteins were separated by 10% SDS-PAGE. Western blot analysis was performed as described above using Abs specific for the p85 subunit of class I PI3K or for the p110γ subunit of class I PI3K diluted 1/200 in 3% milk in TBST. After Western blot analysis with anti-p85 or anti-p110γ Ab, the membrane was incubated with Re-Blot-Plus Mild (Chemicon International, Temecula, CA) for 15 min at room temperature. The membrane was then incubated with 3% gelatin in TBST for 1 h at 30°C, followed by incubation with 0.001 μg/ml anti-lactoferrin Ab in 3% gelatin in TBST for 1 h at 30°C. After thorough washing, the membrane was incubated with 0.2 μg/ml HRP-conjugated goat anti-rabbit in 3% gelatin in TBST for 1 h. Proteins were visualized using ECL.

Statistical analysis

All statistical analyses were performed using StatView 4.01 (Abacus Concepts, Berkeley, CA). Student’s paired t test was used to determine the level of significance, which was set at p < 0.05.

Results

Inhibition of PI3K activity selectively blocks MBBP-stimulated O$_2^-$ production

Involvement of PI3K in MBBP-stimulated O$_2^-$ production by neutrophils was first assessed using the PI3K inhibitor wortmannin. As shown in Fig. 1A, preincubating neutrophils with 1–30 nM wortmannin inhibited O$_2^-$ production stimulated by 1 μM MBBP in a concentration-dependent manner, with 10 nM wortmannin causing complete inhibition. Wortmannin caused similar inhibition of O$_3^-$ production stimulated by 1 μM MLP, the positive control, but had...
no effect on O$_2^-$ production stimulated by 2 ng/ml PMA, the negative control, in the same experiments (data not shown). Preincubating neutrophils with LY294002, a second inhibitor of PI3K, also inhibited MBP-stimulated neutrophil O$_2^-$ production in a concentration-dependent manner over the concentration range of 3–100 μM (Fig. 1A, inset). Wortmannin (1–30 nM), however, did not significantly inhibit either degranulation, as measured by lysozyme release, or IL-8 production (Fig. 1B) stimulated by 1 μM MBP. At the highest concentration tested, wortmannin inhibited MBP-stimulated degranulation and IL-8 production by neutrophils by <25 and 10%, respectively. Wortmannin also caused only minimal inhibition (<10%) of lysozyme release stimulated by 1 μM fMLP or 0.1 μg/ml of the calcium ionophore A23187 and minimal inhibition (<20%) of IL-8 production stimulated by 1 μM fMLP or 100 ng/ml LPS in the same experiments (results not shown).

**MBP stimulates phosphorylation of Akt**

To confirm activation of PI3K in MBP-stimulated neutrophils, activation of Akt, an established downstream target of PI3K (25), was assessed by phosphorylation of Akt at Ser$^{473}$ (16, 26) and Thr$^{308}$ (26) in Akt immunoprecipitates. The results in Fig. 2 show that 2 μM MBP stimulated phosphorylation of Akt at both Ser$^{473}$ (Fig. 2A) and Thr$^{308}$ (Fig. 2C) after 5 min of incubation, the earliest time point examined. The level of phosphorylated Akt increased further at 10 min and then decreased after 20 min of incubation. Densitometric analysis indicated that MBP stimulated 2.2-, 2.8-, and 1.4-fold increases (n = 3) in the level of phosphorylated (Ser$^{473}$) Akt (Fig. 2B) and 2.1-, 3.4-, and 1.3-fold increases (n = 3) in the level of phosphorylated (Thr$^{308}$) Akt (Fig. 2D) after 5, 10, and 20 min of incubation, respectively. Phosphorylation of Akt at Ser$^{473}$ and that of Thr$^{308}$ were examined in separate experiments.

**Genistein inhibits MBP-stimulated Akt activation**

We reported previously that the tyrosine kinase inhibitor, genistein, inhibits MBP-stimulated O$_2^-$ production by neutrophils (4). To determine whether a genistein-sensitive step is present upstream of MBP-stimulated PI3K activation, the effect of genistein on PI3K activation by MBP was examined using phosphorylation of Akt at Ser$^{473}$ as the index of PI3K activation. As shown in Fig. 3A, preincubating the neutrophils with 10 and 100 μM genistein produced a concentration-dependent inhibition of MBP-stimulated O$_2^-$ production. MBP stimulated phosphorylation of Akt. Neutrophils were incubated with or without 2 μM MBP for 5, 10, and 20 min at 37°C. Akt was immunoprecipitated from cell lysates, resolved by 10% SDS-PAGE, and subjected to Western blot analysis using Ab specific for Akt phosphorylated at Ser$^{473}$ (A) or at Thr$^{308}$ (C) and anti-Akt Ab (A and C). Positive bands were detected by ECL. The levels of phosphorylated Akt were quantified densitometrically and normalized to the levels of Akt in the same samples. The results from three independent experiments each for phosphorylated (Ser$^{473}$) Akt (B) and phosphorylated (Thr$^{308}$) Akt (D) are presented as fold increases in phosphorylated Akt in stimulated cells compared with unstimulated cells.
phosphorylation of Akt at Ser473. The higher concentration of genistein also inhibited spontaneous phosphorylation of Akt at Ser473. Densitometric analysis of results obtained in three experiments showed that a 2.1-fold increase in phosphorylated Akt in MBP-stimulated cells decreased to 0.9- and 0.7-fold after incubation with 10 and 100 μM genistein, respectively (Fig. 3B). Genistein caused ~10% inhibition of spontaneous phosphorylation of Akt at Ser473 in the same experiments (results not shown).

**MBP-stimulated O$_2^-$ production and Akt activation are Src kinase dependent**

Src kinases have been implicated in neutrophil activation (27, 28). To determine whether the inhibition by genistein reflected a role for Src kinase(s) in MBP-stimulated O$_2^-$ production and Akt activation, the effect of the Src kinase inhibitor, PP1, on the responses was assessed. Preincubating neutrophils with 1–30 μM PP1 inhibited MBP-stimulated O$_2^-$ production in a concentration-dependent manner, with the 10-μM concentration causing complete inhibition (Fig. 4A). In the same experiments PP1 also blocked O$_2^-$ production stimulated by fMLP, the positive control, but did not block O$_2^-$ production stimulated by PMA, the negative control (data not shown). Preincubating the neutrophils with 10 μM PP1 also inhibited MBP-stimulated phosphorylation of Akt at Ser473 (Fig. 4B). In three experiments, preincubation with 10 μM PP1 reduced the level of phosphorylated Akt in MBP-stimulated cells from a 1.9-fold increase to a 1.1-fold increase (Fig. 4C).

**MBP stimulates translocation and phosphorylation of p85 subunit of the class IA PI3K to neutrophil membranes**

The inhibition of MBP-stimulated O$_2^-$ production (4) and PI3K activation by genistein and the Src kinase inhibitor PP1 suggested participation of class IA PI3K in MBP-stimulated neutrophil O$_2^-$ production. To evaluate this conclusion, the ability of MBP to stimulate activation of class IA PI3K and class IB PI3K was determined by translocation of the p85 regulatory subunit of class IA PI3K and the p110γ catalytic subunit of class IB PI3K (29), respectively, to neutrophil membranes. The results in Fig. 5A show that 2 μM MBP stimulated translocation of the p85 subunit to neutrophil membranes after 5 min of incubation and that the amount of the translocated p85 subunit increased further after 10 min of incubation. In contrast, MBP did not stimulate translocation of the p110γ subunit of class IA PI3K in the same experiments (Fig. 5B). Reprobing the blot for membrane-associated lactoferrin (30) confirmed equal loading of the samples (Fig. 5A). Densitometric analysis of results obtained in three experiments indicated that MBP stimulated increases of 1.6- and 2-fold in the level of translocated p85 subunit (Fig. 5C) after 5 and 10 min of incubation, respectively. MBP did not stimulate translocation of 110γ in the same experiments (results not shown). Activation of class IA PI3K was also evaluated by phosphorylation of the p85 subunit. As shown in Fig. 5D, 2 μM MBP stimulated phosphorylation of the p85 subunit after 5 min of incubation, the earliest time point examined, and the amount of phosphorylation increased further after 10 min of incubation. Probing the membrane with anti-Akt Ab confirmed equal loading of the samples.
Inhibition of PKC partially inhibits MBP-stimulated \( \text{O}_2^- \) production

Our previous findings (4) have excluded involvement of the classical PKC isoforms (24, 31, 32) in MBP-stimulated \( \text{O}_2^- \) production. We, therefore, assessed pharmacologically the roles of PKC, a downstream target of PI3K (33–35) that is implicated in fMLP-stimulated superoxide production by neutrophils (36), and PKC (24) in MBP-stimulated \( \text{O}_2^- \) production. Preincubating neutrophils with 0.03–1 \( \mu \text{M} \) GF109203X, a general inhibitor of PKC isoforms (22, 37), inhibited MBP-stimulated \( \text{O}_2^- \) production by up to \( \sim45\% \) in a concentration-dependent fashion (Fig. 6A). The inhibition, however, occurred only at concentrations \( \geq0.3 \mu\text{M} \). In contrast, preincubation with 1–30 \( \mu\text{M} \) rottlerin, a preferential inhibitor of PKC (22), caused almost complete inhibition of MBP-stimulated \( \text{O}_2^- \) production, with an 50% inhibitory concentration (IC\(_{50}\)) value of \( \sim6 \mu\text{M} \) (Fig. 6B). GF109203X and rottlerin each inhibited \( \text{O}_2^- \) production stimulated by fMLP and PMA in the same experiments. Preincubating neutrophils with 0.3–3 \( \mu\text{M} \) of a myristoylated peptide corresponding to the N-terminal pseudosubstrate sequence of PKC (22) inhibited MBP-stimulated \( \text{O}_2^- \) release up to \( \sim70\% \) in a concentration-dependent manner (Fig. 6C). The PKC antagonist also inhibited \( \text{O}_2^- \) production stimulated by fMLP and PMA in the same experiments up to 35 and 30\%, respectively. The control nonmyristoylated peptide inhibited \( \text{O}_2^- \)
production stimulated by MBP or PMA by <5% and fMLP-stimulated 
production by <10% (results not shown).

**MBP stimulates PI3K-dependent activation of PKCζ, but not PKCδ**

The ability of MBP to activate PKCζ and PKCδ was examined directly by assessing the capacity of MBP to stimulate the phosphorylation of PKCζ at Thr410 and PKCδ at Thr505, respectively (38). The results in Fig. 7A show that MBP stimulated an increase in the level of phosphorylated (Thr410) PKCζ in PKCζ immunoprecipitates after 5 min of incubation. The level of phosphorylated PKCζ increased further after 10 min of incubation and subsequently declined after 20 min of incubation. Similar patterns of activation were obtained in three independent experiments, although densitometric analysis could not be conducted accurately due to the low amounts of immunoprecipitated PKCζ. In contrast, MBP did not stimulate any increase in the level of phosphorylated (Thr505) PKCδ in PKCδ immunoprecipitates over the same time frame (Fig. 7B). Similar results were obtained in two additional experiments. To determine whether the activation of PKCζ was PI3K dependent, the effect of wortmannin on PKCζ activation in MBP-stimulated neutrophils was examined in additional experiments. Pretreatment of the neutrophils with 30 nM wortmannin (Fig. 7C) or anti-phosphorylated (Thr505) PKCδ Abs (B) was confirmed by the finding that MBP stimulated the activation of Akt, a prominent downstream target of activated PI3K (12, 13). The activation of Akt was determined by the phosphorylation of Akt at Ser473 and Thr308, which are characteristic of and required for maximum Akt activation (25). The MBP-stimulated increase in Akt activity is transient, in that activation of Akt occurred within 5 min of stimulation, peaked after 10 min of stimulation, and returned to baseline or near baseline levels by 20 min. Because phosphorylation of Akt at Thr308 is catalyzed by PDK1 (25), the finding that MBP stimulated the phosphorylation of Akt at Thr308 demonstrates indirectly that MBP also stimulated activation of PDK1.

**Discussion**

The results presented here demonstrate that PI3K plays an essential role in MBP-stimulated O2− production by neutrophils. Inhibition of PI3K activity by wortmannin or LY294002 inhibited O2−-production stimulated by MBP, with the IC50 values for inhibition (-2 nM and 6 μM for wortmannin and LY294002, respectively) agreeing well with the reported IC50 values for inhibition of the PI3K enzymatic activity (-5 nM for wortmannin and 1–20 μM for LY294002) (25, 39). The activation of PI3K in MBP-stimulated neutrophils was confirmed by the finding that MBP stimulated the activation of Akt, a prominent downstream target of activated PI3K (12, 13). The activation of Akt was determined by the phosphorylation of Akt at Ser473 and Thr308, which are characteristic of and required for maximum Akt activation (25). The MBP-stimulated increase in Akt activity is transient, in that activation of Akt occurred within 5 min of stimulation, peaked after 10 min of stimulation, and returned to baseline or near baseline levels by 20 min. Because phosphorylation of Akt at Thr308 is catalyzed by PDK1 (25), the finding that MBP stimulated the phosphorylation of Akt at Thr308 demonstrates indirectly that MBP also stimulated activation of PDK1.

MBP also stimulates the release of secondary granule contents as well as IL-8 production by neutrophils (3, 5). Wortmannin, however, caused minimal inhibition of either response, thus indicating that PI3K activation is probably not required for these responses. Wortmannin had similar minimal effects on degranulation stimulated by fMLP or the calcium ionophore A23187 and on IL-8 production stimulated by fMLP or LPS. The possibility that neutrophil degranulation may bypass PI3K activation has also been suggested by earlier findings (40). Wortmannin inhibited O2− production stimulated by fMLP with an IC50 value consistent with inhibition of PI3K activity, but a 20-fold higher concentration (100 nM) of wortmannin inhibited fMLP-stimulated degranulation by only 55% (40). A similar difference has been observed in the sensitivity of the respiratory burst and degranulation responses to inhibition of Pyk2 and Syk kinase activity in TNF-α-stimulated neutrophils (41, 42). Together, these findings demonstrate the differential involvement of specific kinases, including Src-related kinases, in the two responses. It is interesting that IL-8 production by neutrophils, at least for the stimuli tested here, is not sensitive to inhibition of PI3K activity, although variables, such as timing and length of preincubation with the PI3K inhibitor, may influence the effect on the IL-8 response.

Neutrophils contain both class IA and class IB isoforms of PI3K (29, 43, 44). The class IA isoform, which is preferentially activated by βγ subunits of G protein-coupled receptors (12), is strongly implicated in neutrophil O2− production and chemotaxis by chemotactic. Specifically, neutrophils from P110γ knockout mice exhibit impaired chemotaxis and O2− production in response to fMLP. Conversely, the class IA isoforms, which are activated by receptor-associated tyrosine kinases or Src-like kinases (12), are implicated in FcγR-mediated neutrophil activation (19, 20). The results presented here demonstrate the preferential involvement of class IA PI3K in MBP-stimulated O2− production by neutrophils. Specifically, MBP stimulated translocation of the p85 subunit of class IA PI3K, but not translocation of the p110 subunit of class IA PI3K to the neutrophil membrane. Activation of class IA PI3K was further demonstrated by the finding that MBP also stimulated phosphorylation of the p85 subunit. Significantly, the time courses for translocation and phosphorylation of the p85 subunit paralleled the time course for Akt activation in the MBP-stimulated cells. The apparent absence of class IB PI3K activation is consistent with our previous finding (4) that pertussis toxin does
not inhibit MBP-stimulated O$_2^-$ production, thus excluding the participation of at least pertussis toxin-sensitive G-proteins in the mechanism. We previously showed that genistein inhibits MBP-stimulated O$_2^-$ production (4), thus implicating tyrosine kinase(s) in the response. As shown here, genistein in concentrations that blocked the O$_2^-$ response (4) also inhibited MBP-stimulated activation of Akt, thus indicating that at least one of the tyrosine kinase-dependent steps is upstream of PKCζ activation. Moreover, the finding here that the Src kinase family inhibitor, PP1, also inhibited both MBP-stimulated O$_2^-$ production and Akt phosphorylation indicates that tyrosine kinase-dependent events upstream of PKCζ in MBP-stimulated neutrophils include a member of the Src kinase family, a finding consistent with the activation of class Ia PKCζ (12).

PKCζ is a downstream target of PKCζ in various cells (33–35) and has been implicated (36) in activation of the NADPH oxidase. Specifically, PKCζ phosphorylates p47$^{phox}$ of the NADPH oxidase complex at Ser$^{308}$ and Ser$^{315}$ (36), and the peptide antagonist corresponding to the pseudosubstrate sequence of PKCζ (22) inhibits both phosphorylation of p47$^{phox}$ and O$_2^-$ production in fMLP-stimulated neutrophils (36). As shown here, the PKCζ antagonist (22) also inhibited MBP-stimulated O$_2^-$ production by neutrophils by up to ~70% in a concentration-dependent manner. The peptide antagonist also inhibited O$_2^-$ production stimulated by fMLP and PMA to a lesser degree. We, however, did not observe the difference reported previously (36) in the susceptibility of fMLP- and PMA-stimulated O$_2^-$ production to the PKCζ antagonist. Consistent with the pharmacological results, MBP stimulated the activation of PKCζ, as detected by phosphorylation of PKCζ at Thr$^{308}$ (38). Two findings indicate that the PKCζ activation is linked to activation of PKCζ. First, the time course for PKCζ activation paralleled the time courses for translocation and phosphorylation of the p85 subunit of class Ia PKC as well as the time course for activation of Akt. Second, inhibition of PKCζ activity by wortmannin blocked MBP-induced activation of PKCζ. The finding that MBP-stimulated phosphorylation of Akt at Thr$^{308}$, a reaction catalyzed by PDK1 (25), together with reports that PDK1 phosphorylates PKCζ at Thr$^{308}$ in a PI3K-dependent manner (38, 45) suggest that PDK1 mediates the activation of PKCζ in MBP-stimulated neutrophils.

Neutrophils also contain three classical PKC isoforms as well as the novel isoform PKCδ (24, 36, 46), each of which has been variously implicated in O$_2^-$ production (24, 36, 46, 47). We previously showed that MBP does not stimulate an increase in neutrophil diacylglycerol content (4), thus arguing against the involvement of the classical PKC isoforms (α, β, and δ) in neutrophil MBP-stimulated O$_2^-$ production. The failure of GF109203X to inhibit MBP-stimulated O$_2^-$ production at concentrations consistent with the IC$_{50}$ value (14–16 nM) for inhibition of classical PKC isoforms (22, 37) supports this conclusion. GF109203X likewise did not inhibit MBP-stimulated neutrophil O$_2^-$ production at concentrations consistent with the reported IC$_{50}$ value (210 nM) for inhibition of PKCζ activity (22, 37). The latter finding argues against participation of PKCζ in the response, a conclusion supported by the failure of GF to activate PKCζ, as measured by phosphorylation at Thr$^{308}$. Although rottlerin inhibited MBP-stimulated O$_2^-$ production, with an IC$_{50}$ value (~6 μM) that corresponds to the reported IC$_{50}$ value (3–6 μM) for inhibition of PKCζ activity (48), it is not clear that rottlerin is a selective inhibitor of PKCζ. Indeed, others have reported that rottlerin did not inhibit PKCζ activity in concentrations (10 or 20 μM) above the reported IC$_{50}$ value for inhibition (49, 50). Instead, rottlerin inhibited the activity of several other kinases (49) and also decreased ATP availability in cells by uncoupling mitochondrial respiration from oxidative metabolism (50), thus providing PKCζ-independent mechanisms for the inhibitory activity of rottlerin. Together, the above findings indicate that PKCζ is the primary PKC isoform involved in MBP-stimulated O$_2^-$ production.

In summary, although the identity of the MBP receptor remains undefined, the results presented here indicate that MBP stimulates the activation of class Ia PKCζ in neutrophils through activation of a Src-related kinase(s). The MBP-induced activation of PKCζ leads to the activation of Akt and PDK1. Activation of PKCζ, presumably by PDK1, is postulated to contribute, in turn, to the activation of the NADPH oxidase via phosphorylation of p47$^{phox}$ at Ser$^{304}$ and Ser$^{315}$ (36), a step implicated in membrane oxidase assembly (51). The failure of the PKCζ peptide antagonist to completely block MBP-stimulated O$_2^-$ production indicates, however, that other PI3K-mediated signaling events also contribute to NADPH oxidase activation in MBP-stimulated neutrophils. For example, PI3K-catalyzed lipid products bind p47$^{phox}$ and p40$^{phox}$ and thus are probably critical for assembly of the NADPH oxidase (52, 53). Nevertheless, the results of the present study identify class Ia PI3K-mediated activation of PKCζ as one link in the MBP-stimulated response.

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References


