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This information is current as of October 14, 2019.

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J Immunol 2007; 178:416-427; ;
doi: 10.4049/jimmunol.178.1.416
<http://www.jimmunol.org/content/178/1/416>

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Regulation of Leukocyte Degranulation by cGMP-Dependent Protein Kinase and Phosphoinositide 3-Kinase: Potential Roles in Phosphorylation of Target Membrane SNARE Complex Proteins in Rat Mast Cells¹

Masakatsu Nanamori, Jia Chen, Xiaoping Du, and Richard D. Ye²

We examined the roles of cGMP-dependent protein kinase (PKG) and PI3K in degranulation induced by fMLF and by FcεRI cross-linking. In rat basophilic leukemia-2H3 cells expressing formyl peptide receptor, the PKG inhibitors KT5823 and Rp-8-Br-PET-cGMP, as well as the PI3K inhibitor LY294002, reduced agonist-stimulated β-hexosaminidase release in a dose-dependent manner. These inhibitors also abolished vesicular fusion with the plasma membrane, as evidenced by diminished annexin V staining. Agonist-induced degranulation was completely blocked when LY294002 was applied together with one of the PKG inhibitors, suggesting an additive and possibly synergistic effect. In contrast, the PKG inhibitors did not affect fMLF-induced intracellular calcium mobilization and Akt phosphorylation. Likewise, LY294002 did not alter fMLF-induced elevation of intracellular cGMP concentration, and the inhibitory effect of LY294002 was not reversed by a cell-permeable analog of cGMP. Treatment with fMLF induced phosphorylation of soluble N-ethylmaleimide-sensitive factor-attachment protein (SNAP)-23, syntaxins 2, 4, and 6, and Munc18-3. The induced phosphorylation of SNAP-23 and syntaxins 2 and 4 was blocked by Rp-8-Br-PET-cGMP and LY294002. However, LY294002 was less effective in inhibiting Munc18-3 phosphorylation. The induced phosphorylation of syntaxin 6 was not effectively blocked by either Rp-8-Br-PET-cGMP or LY294002. Treatment of human neutrophils with the PKG inhibitors and LY294002 reduced enzyme release from primary, secondary, and tertiary granules. These results suggest that PKG and PI3K are involved in degranulation, possibly through phosphorylation of target membrane SNAP receptor proteins and their binding proteins. *The Journal of Immunology*, 2007, 178: 416–427.

Leukocyte degranulation is an important mechanism of host defense against invading microorganisms and a major contributing factor for allergic reaction. In mast cells, cross-linking of FcεRI with allergen-specific IgE leads to release of inflammatory mediators such as histamine and serotonin, which are responsible for various allergic conditions including contact dermatitis and hypersensitivity (1). In neutrophils, several different types of granules are formed during differentiation and maturation of myeloid cells (2–4). The content of these granules is released in response to chemoattractant stimulation and to cross-linking of FcγRs as seen in phagocytosis. An understanding of the regulatory mechanism of degranulation will help to promote innate immunity and reduce unwanted allergic reactions.

Research conducted thus far has led to the identification of several important regulatory mechanisms for leukocyte degranulation, including elevation of intracellular Ca²⁺ concentration, activation

of the cytoskeletal contractile apparatus, and phosphorylation of the soluble N-ethylmaleimide-sensitive factor-attachment protein (SNAP)³ receptor proteins (SNAREs). SNAREs, which were initially identified in neuronal cells for their function in membrane fusion (5), include vesicular SNAREs such as vesicle-associated membrane proteins, target membrane SNAREs (t-SNAREs) such as syntaxins, and the Sec1/Munc18 family of proteins that serve as binding partners of t-SNAREs. SNAP-23 is a nonneuronal homolog of SNAP-25, which is present in leukocytes such as mast cells (6) and neutrophils (7). At resting state, Munc18-bound syntaxins assume a closed conformation that prevent SNARE core complex formation (8, 9). Phosphorylation of Munc18-1 in neuronal cells (10, 11) and Munc18-3 and syntaxin 4 in endothelial cells (12) promotes Munc18-syntaxin dissociation, thereby facilitating vesicular fusion. Phosphorylation of syntaxins, Munc18, and SNAP-23 is catalyzed by kinases such as protein kinase C (PKC) (12–14). In mast cells, a mechanism of degranulation induced by FcεRI cross-linking involves recruitment and activation of the tyrosine kinases Lyn and Syk, phosphorylation of several tyrosine residues in FcεRI, activation of phospholipase Cγ (PLCγ), and generation of the second messengers diacylglycerol and inositol trisphosphate (1, 15). These second messengers are responsible for calcium flux and

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Received for publication January 24, 2006. Accepted for publication October 17, 2006.

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¹ This work was supported by Grants AI033503 and HL068819 from the National Institutes of Health (NIH). M.N. was supported by a National Research Service Award/NIH Institutional T32 Training Grant, "Training Program in Signal Transduction and Cellular Endocrinology" (T32 DK07739) and is currently a recipient of the American Heart Association Midwest Affiliate Predoctoral Fellowship.

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³ Abbreviations used in this paper: SNAP, soluble N-ethylmaleimide-sensitive factor-attachment protein; SNARE, SNAP receptor protein; t-SNARE, target membrane SNARE; PLC, phospholipase C; PKC, protein kinase C; PKG, cGMP-dependent protein kinase; sGC, soluble guanylyl cyclase; RBL, rat basophilic leukemia; FPR, formyl peptide receptor; DN, dominant negative; LTF, lactoferrin; MMP, matrix metalloproteinase; IBMX, 3-isobutyl-1-methylxanthine; NOS, NO synthase; eNOS, endothelial NOS; fMLF, formyl-methionyl-leucyl-phenylalanine.

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PKC activation that lead to phosphorylation of the SNARE proteins and granule release.

Activation of several chemoattractant receptors results in leukocyte degranulation. However, it is unclear whether chemoattractant-induced degranulation uses the same mechanisms as those triggered by Fc ϵ RI cross-linking. Leukocyte chemoattractant receptors functionally couple to heterotrimeric G proteins and can trigger PLC β activation either directly (through the Gq family of G α proteins) or via the released G $\beta\gamma$ proteins (16). Chemoattractant-induced activation of PLC β leads to accumulation of diacylglycerol and inositol trisphosphate, thereby contributing to PKC activation and calcium flux (17). In addition to PKC, published reports indicate that leukocyte degranulation also involves PI3K, p38MAPK, and cGMP-dependent protein kinase (PKG) (18–21). Pharmacological inhibitors of these kinases effectively block chemoattractant-induced degranulation in neutrophils, eosinophils, and basophils, indicating the involvement of these proteins in leukocyte degranulation. It has been well-established that leukocyte degranulation requires a higher chemoattractant concentration than chemotaxis, which may reflect the need for activation of multiple signaling pathways for degranulation. However, it is presently unclear how these signaling pathways coordinate and what are the sites of regulation by these protein kinases with respect to leukocyte degranulation.

The current study focuses on PKG for its role in regulating leukocyte degranulation. cGMP is generally regarded as a positive mediator of leukocyte functions while cAMP is considered a negative regulator (22, 23). Exposure of neutrophils to cell-permeable cGMP or cGMP-elevating agents promotes β -glucuronidase release, whereas an increase in cellular cAMP concentration inhibits enzyme release (23). However, although a role of cGMP in blood vessel relaxation has been well-established, the mechanism by which a rise of cGMP concentration regulates leukocyte degranulation remains largely uncharacterized. PKG is a major effector of the cGMP pathway, which includes NO as an upstream activator of soluble guanylyl cyclase (sGC). It was reported that upon stimulation with formyl-methionyl-leucyl-phenylalanine (fMLF), PKG was activated and redistributed to subcellular compartments with intermediate filaments (24). However, published reports also indicate that several NO donors could inhibit neutrophil functions (25–27), raising the question of whether PKG positively or negatively regulates leukocyte degranulation. To clarify this issue and identify the signaling pathways used by PKG for regulating degranulation, we examined the rat mast cell line rat basophilic leukemia (RBL)-2H3 and a formyl peptide receptor (FPR)-expressing RBL cell line, using pharmacological inhibitors that block PKG. We determined enzyme release as well as the phosphorylation status of t-SNARE and Munc18 proteins. In parallel studies, we examined cells treated with LY294002, an inhibitor of PI3K that is critical to leukocyte degranulation (18, 28). Because it is unclear whether PKG plays a role in Fc ϵ RI-mediated degranulation, we also conducted degranulation assays with Fc ϵ RI cross-linking and compared the results with those obtained from fMLP-stimulated cells. Our experimental data indicate that activation of PKG is required for degranulation in RBL and RBL-FPR cells that are stimulated with either fMLF or Fc ϵ RI cross-linking. In neutrophils, enzyme release from primary, secondary, and tertiary granules are all susceptible to PKG inhibition. Furthermore, inhibition of PKG or PI3K effectively reduces phosphorylation of selected t-SNAREs and Munc18 proteins in RBL-FPR cells. These results indicate that PKG may regulate t-SNARE phosphorylation and the PKG-dependent pathway is used for exocytosis of different types of granules in response to multiple extracellular stimuli.

Materials and Methods

Materials

The chemotactic peptide fMLF, *p*-nitrophenyl-*N*-acetyl- β -D-glucosamide, cytochalasin B, and anti-SNAP-23 Ab were obtained from Sigma-Aldrich. WKYMVM was synthesized at Macromolecular Resources. Rp-8-Br-PET-cGMP was purchased from Biolog Life Science Institute. FITC-conjugated annexin V and anti-syntaxin-6 Ab were obtained from BD Biosciences. The Phosphoprotein Purification kit was obtained from Qiagen. The anti-syntaxin 2, 3, and 4 and Munc18-2 Abs were obtained from Calbiochem/EMD Bioscience. The anti-Munc18-3 and anti-PKG (cGK I α /I β) Abs were purchased from Santa Cruz Biotechnology.

Cell preparation and culture

Peripheral blood was drawn from healthy donors using a protocol approved by the Institutional Review Board at the University of Illinois (Chicago, IL). Neutrophils were isolated with Percoll (Amersham Biosciences) gradient centrifugation as described (29). The preparation gave ~97% neutrophils with a viability of ~98%. The cells were kept in HBSS containing 20 mM HEPES (pH 7.4) and 0.2% BSA (HBSS-HB) and used within a few hours of purification. Generation and maintenance of the RBL-2H3 cells expressing human FPR (RBL-FPR) were described previously (30, 31). Transient transfection of RBL-2H3 cells with FPR and a dominant-negative PKG (DN-PKG), consisting of amino acids 1–342 of the human PKG I α in the pcDNA3.1-zeo vector (Invitrogen Life Technologies), was conducted using a nucleofection kit from Amaxa Biosystems according to the manufacturer's specifications. Briefly, 2×10^6 cells per sample were nucleofected with 4 μ g of plasmid DNA (2 μ g of the FPR expression vector or pcDNA3.1-zeo, together with 2 μ g of the DN-PKG I α expression vector or pcDNA3.1-zeo). Solution R, supplied with the kit, was added to the cell-DNA mixture that was subjected to nucleofection using the T-020 program. Nucleofected cells were plated for 12 h before assay.

Isolation of total RNA and RT-PCR

Total cellular RNA was extracted from RBL cells or freshly isolated human neutrophils using TRIzol reagent (Invitrogen Life Technologies). First-strand cDNA was synthesized from 5 μ g of total RNA in a 20- μ l reaction volume containing 200 U of SuperScript II reverse transcriptase (Invitrogen Life Technologies). PCR amplifications were performed with 0.25 μ g of cDNA (corresponding to 5% of the total RNA input), 0.2 μ M primers, 0.8 mM dNTPs, 1.5 mM MgCl $_2$, and 1.25 U of TaqDNA polymerase in a 50- μ l reaction volume, for 35 cycles with denaturation at 94°C for 30 s, annealing at 55°C for 1 min and extension at 72°C for 1 min. The presence of rat and human PKG isoforms was determined by PCR using specific primers to human PKG I α (5'-GGAGGAGAGGATCAAAA GAG-3' and 5'-CCATGACATACACCAGTGAC-3'), human PKG I β (5'-ACAGTACGCGCTCCAGGAGAAGATC-3' and 5'-TTGGGGGTAGAAG GGCAGGGTCAAC-3'), human PKG II (5'-GGACCTATGACCTGAACA AAC-3' and 5'-CTCGCCTCTCTAATGATG-3'), rat PKG I α (5'-AAAA TGAGCGAACTGGAGGAAGAC-3' and 5'-GATTTAGTGAACCTCCG GAACGCC-3'), rat PKG I β (5'-GCGGACTGGGATGCTCAGAAG CC-3' and 5'-TGGGCTCTGGGGTGAAGGGCAG-3') and rat PKG II (5'-GTGTTCTCCCTCATCGT-3' and 5'-GTCGCCTGTTATCTTTT TA-3'). The specificities of the obtained PCR products were confirmed by parallel PCR using human PKG I α , I β , and II cDNA and rat normal brain tissue cDNA (Bio Chain Institute). Control PCR assays were performed using specific primers for rat GAPDH and human β -actin as external standards. The amplified products were separated by electrophoresis on a 1.5% agarose gel.

Annexin V binding

Vesicular fusion to plasma membrane was visualized with FITC-annexin V, as described previously (32). Briefly, RBL-FPR cells were grown on a glass coverslip for 48 h, washed once with HBSS, and incubated with 10 μ M cytochalasin B and a 1/20 dilution of FITC-annexin V in HBSS-HB for 15 min on ice and then 15 min at 37°C. The cells were stimulated with 100 nM fMLF for 15 min at 37°C in the presence of cytochalasin B and FITC-annexin V. After stimulation, the cells were washed with ice-cold PBS, fixed with 3% paraformaldehyde in PBS for 15 min at 23°C, and mounted with VectaShield mounting medium containing 4,6-diamidino-2-phenylindole (Vector Laboratories). A fluorescence image was taken with a Nikon TE300 inverted epifluorescence microscope.

Degranulation assays

For release of β -hexosaminidase with Fc ϵ RI cross-linking, RBL-FPR cells were grown for 48 h and incubated with monoclonal anti-DNP IgE (clone

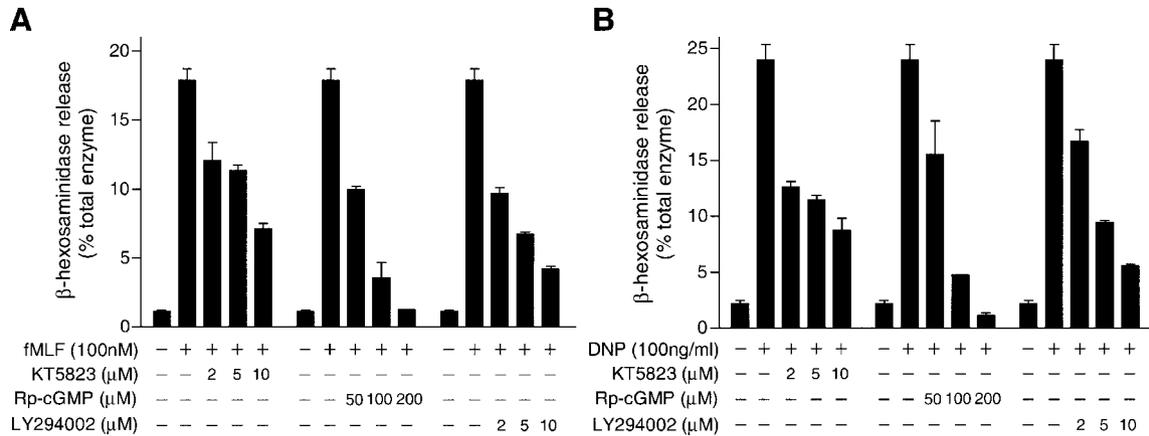


FIGURE 1. Attenuation of FPR and Fc ϵ RI-mediated β -hexosaminidase release by PKG and PI3K inhibitors. RBL-FPR (A) and RBL-2H3 (B) were plated for 48 h before stimulation. For Fc ϵ RI cross-linking, cells were incubated with monoclonal anti-DNP IgE (clone SPE-7, 1/5000 or 0.2 μ g/ml) during the final 16 h. Cytochalasin B (10 μ M) was added and cells were incubated for 15 min on ice and 15 min at 37°C. The indicated amounts of inhibitors were then added and cells were incubated for 15 min at 37°C before stimulation with fMLF (A) or DNP (DNP-BSA; B) at 37°C for 10 min; Rp-cGMP, Rp-Br-PET-cGMP. Degranulation reaction was terminated with rapid cooling of samples on ice. The amount of secreted β -hexosaminidase was quantified as described in *Materials and Methods*. Data from three to four repeating experiments were averaged and presented as percent of total β -hexosaminidase associated with the cells.

SPE-7 from Sigma-Aldrich; 1/5000 or 0.2 μ g/ml) for 16 h. Following a brief wash with HBSS-HB, cells were preincubated with 10 μ M cytochalasin B for 15 min on ice and then 15 min at 37°C. For inhibitor treatment, the indicated amount of inhibitors was added for 15 min at 37°C before cell stimulation with DNP-BSA for 10 min at 37°C. The degranulation reaction was terminated by placing samples on ice. The amount of secreted β -hexosaminidase was quantified by incubating 20 μ l of supernatant with 10 μ l of 1 mM *p*-nitrophenyl-*N*-acetyl- β -D-glucosamide in 0.1 M sodium citrate buffer (pH 4.5) at 37°C for 1 h in a 96-well plate. The reaction was terminated by the addition of 200 μ l of 0.1 M Na₂CO₃ and 0.1 M NaHCO₃ (pH 10), and absorbance at 405 nm was determined in a SpectroMax 340 96-well plate reader (Molecular Devices). Total cellular β -hexosaminidase was determined with cell lysate in 0.1% Triton X-100. Data are presented as percent of total β -hexosaminidase released.

For β -glucuronidase release, human blood neutrophils were preincubated with 10 μ M cytochalasin B and then with the inhibitors as described above. The cells were then stimulated with fMLF for 10 min at 37°C. The degranulation reaction was terminated by placing samples on ice. Cells were separated from supernatant through centrifugation. The amount of released β -glucuronidase was quantified by incubating 20 μ l of supernatant with 20 μ l of 10 mM 4-methylumbelliferyl β -D-glucuronide hydrate in 0.1 M sodium acetate (pH 4.0) and 0.1% Triton X-100 at 37°C for 15 min. The reaction was terminated by the addition of 300 μ l of Stop solution (containing 50 mM glycine and 5 mM EDTA (pH 10.4)). Fluorescence was measured immediately with a spectrofluorometer (Photon Technology International), with an excitation wavelength at 365 nm and an emission wavelength at 460 nm.

The secretion of the secondary granule enzyme lactoferrin (LTF) and tertiary granule enzyme matrix metalloproteinase-9 (MMP-9) was determined using competitive ELISA kits from Calbiochem/EMD Bioscience per manufacturer's instructions.

Measurement of intracellular cGMP concentration

Intracellular cGMP concentration was measured using competitive ELISA that selectively detects cGMP (<0.001% cross-reactivity with cAMP; BIOMOL). RBL cells were grown in 6-well plate for 48 h before assay. Cells were washed and preincubated with 0.5 mM 3-isobutyl-1-methylxanthine (IBMX) in HBSS-HB for 15 min at 37°C. The cells were stimulated with 0, 30, and 100 nM fMLF for 15 min and reaction was terminated by addition of 250 μ l of ice-cold lysis buffer (0.1 M HCl with 0.1% Triton X-100). For determination of cGMP concentration in human neutrophils, cells were preincubated with 0.5 mM IBMX for 3 min before stimulation with 100 nM fMLF for 5 min. Cellular cGMP contents were extracted with 0.1% HCl containing 0.1% Triton X-100. Collected RBL and neutrophil samples were cleared of cell debris by centrifugation before measurements with ELISA. Absorbance at 405 nm was determined in a SpectraMax 340 96-well microplate reader. Intracellular cGMP was quantified against a standard curve generated in parallel.

Calcium mobilization assay

Agonist-induced calcium flux was measured in Indo-1/AM-loaded RBL-FPR cells and isolated human neutrophils as described previously (33). Three minutes before fMLF stimulation, cells were incubated with Rp-Br-PET-cGMP or with vehicle that contained the same amount of organic solvent. To chelate extracellular Ca²⁺, 5 mM EDTA was added before stimulation. Intracellular Ca²⁺ level was expressed as relative fluorescence, calculated based on the ratio of Indo-1 fluorescence at 405 and 485 nm.

Protein phosphorylation assays

Phosphorylation of Akt, a downstream effector of PI3K, was assessed with an Ab detecting a phosphorylated Ser⁴⁷³ (Cell Signaling Technology) at a 1/1000 dilution. Phosphorylation of p38 MAPK was similarly detected using an Ab detecting phosphorylated Thr¹⁸⁰ and Tyr¹⁸² (Cell Signaling Technology). HRP-conjugated anti-rabbit Ab was used as secondary Ab. The resulting immunocomplex was visualized by SuperSignal West Pico Chemiluminescence kit (Pierce) according to the manufacturer's instruction. The Western blot result was quantified with ImageQuant software (version 3.3; Molecular Devices).

Enrichment and separation of phosphorylated proteins from cell lysates was performed using a Phosphoprotein Purification kit from Qiagen, according to the manufacturer's instruction. Briefly, cells were grown at a density of 1 \times 10⁷ per 90-mm diameter tissue-culture dish. Cells were washed with HBSS-HB, pretreated with or without inhibitors for 15 min, and stimulated with 100 nM fMLF for 5 min at 37°C. Cells were quickly washed with ice-cold HEPES-buffered saline (20 mM HEPES (pH 7.4) and 155 mM NaCl) and harvested with 2 ml of lysis buffer containing 0.25% CHAPS. Cells were collected and incubated for 30 min at 4°C before centrifugation. Cleared lysates were loaded onto equilibrated phosphoprotein purification columns supplied with the kit and washed with 6 ml of lysis buffer. The phosphorylated proteins were eluted with 2 ml of elution buffer containing 0.25% CHAPS. Flow-through, containing nonphosphorylated proteins, and eluate, containing phosphorylated proteins, were collected for determination of protein concentration.

For detection of the eluted proteins, 12 μ g of the above flow-through or eluates were analyzed by SDS-PAGE and Western blotting with Abs against SNAP-23 (1/1000), syntaxin 2 (1/750 dilution), syntaxin 3 (1/1000), syntaxin 4 (1/1000), syntaxin 6 (1/1000), Munc18-2 (1/2000), and Munc18-3 (1/1000). As positive controls, phosphorylation of p38 MAPK and Akt was determined with anti-phosphoantibodies for these two kinases at 1/2000 and 1/1000, respectively. HRP-conjugated anti-rabbit or anti-goat Abs (for Munc18-3) were used as secondary Abs. The resulting immunocomplex was visualized by SuperSignal West Pico Chemiluminescence kit (Pierce) according to the manufacturer's instruction.

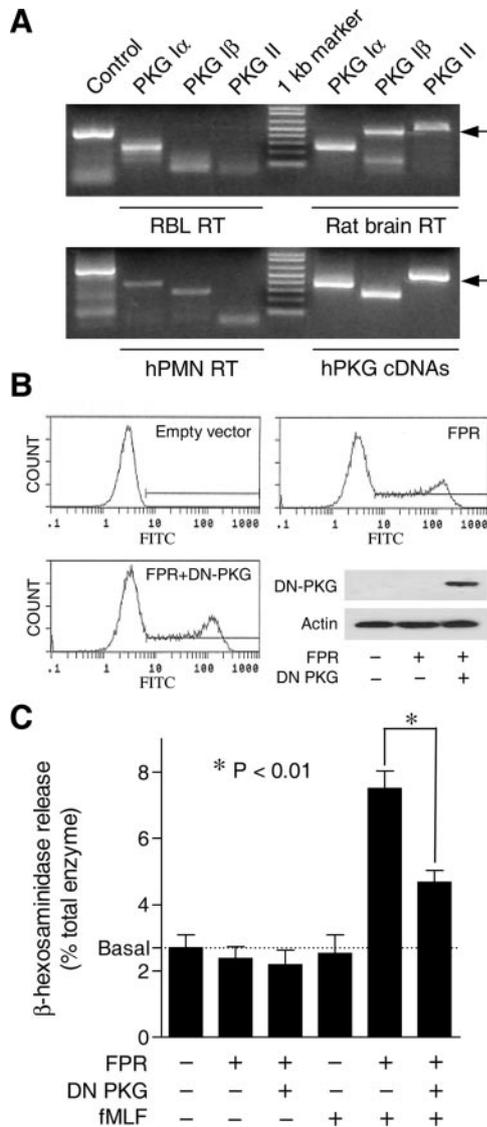


FIGURE 2. Expression of PKG isoforms in RBL and neutrophils and the effect of a dominant-negative PKG mutant on β -hexosaminidase release. *A*, Total RNA isolated from RBL-2H3 cells (RBL, upper panel) and human neutrophils (PMN, lower panel) were subjected to RT-PCR as described in *Materials and Methods*. RT-PCR products were analyzed by agarose gel electrophoresis to determine the presence of transcripts for the three isoforms of PKG. Rat brain reverse transcription products and human cDNAs for the three PKG isoforms were used as templates for positive controls, which produced DNA fragments of 262 bp (rat PKG I α), 474 bp (rat PKG I β), 511 bp (rat PKG II), 386 bp (human PKG I α), 282 bp (human PKG I β), and 560 bp (human PKG II). The arrowheads on the right mark the position of 500 bp. The minor bands in the test lanes (left to the 1-kb marker), which are absent from the corresponding control lanes (right to the 1-kb marker), are PCR primers or nonspecific PCR products. The first lane in each panel contains either a rat GAPDH cDNA fragment (451 bp, upper panel) or a human β -actin cDNA fragment (616 bp, lower panel), which was used as external controls for PCR. *B*, RBL-2H3 cells were transiently transfected with either an empty vector (pcDNA3.1-zeo), an expression construct of FPR, or the FPR construct plus an expression vector for a human DN PKG I α in pcDNA3.1-zeo, using the Amaxa nucleofection procedure as described in *Materials and Methods*. Twelve hours after transfection, cell surface expression of FPR was analyzed using FITC-conjugated formyl-Nle-Leu-Phe-Nle-Tyr-Lys (Molecular Probes) and flow cytometry. Approximately 40–45% of the transfected cells expressed FPR based on mean fluorescence intensity. The expression of the DN-PKG (~3.8 kDa) was determined with Western blotting using an Ab against human PKG I α . The endogenous PKG I α in RBL cells was also detected by the Ab as a species with higher m.w. (data not shown). The same blot

Results

To examine the role of PKG in leukocyte degranulation, we measured β -hexosaminidase release from fMLF-stimulated RBL-FPR cells in the absence or presence of two PKG inhibitors: KT5823 (34, 35) and Rp-8-bromo-PET-cGMP (Rp-Br-PET-cGMP) (36). RBL-FPR cells responded to fMLF (0.1 nM–10 μ M) with a maximal release of 19.4% of the total extractable β -hexosaminidase and an EC₅₀ of 112 nM. To test the effect of the PKG inhibitors in enzyme release, 100 nM fMLF was used that produced a 5-fold increase in β -hexosaminidase release over basal level. KT5823 and Rp-Br-PET-cGMP did not significantly alter the basal level of enzyme release, but dose-dependently reduced fMLF-stimulated release of β -hexosaminidase (Fig. 1). A parallel study was conducted using LY294002, a PI3K inhibitor (37), which showed dose-dependent inhibition of the fMLF-stimulated enzyme release.

Cross-linking of Fc ϵ RI, a high-affinity IgE receptor expressed in RBL cells, activates a classical pathway involving protein tyrosine kinases and PLC γ , leading to release of allergic mediators and lysosomal enzymes such as β -hexosaminidase (1). To investigate whether PKG is a degranulation signal produced by different agonists, we conducted cross-linking of Fc ϵ RI in RBL cells sensitized to an IgE specific for DNP with and without the PKG inhibitors. Addition of DNP-BSA (100 ng/ml) to the sensitized cells resulted in the release of 18.6% of the total extractable β -hexosaminidase, which was equivalent to a 6-fold increase over the basal level. When the sensitized RBL cells were treated with the two PKG inhibitors or with LY294002 before Fc ϵ RI cross-linking, inhibition comparable to that from fMLF-stimulated RBL-FPR cells were observed (Fig. 1*B*). These results indicate that degranulation mediated by the structurally different FPR and Fc ϵ RI shares pathways that are regulated by PKG and PI3K.

KT5823 and Rp-Br-PET-cGMP are structurally different PKG inhibitors. KT5823 is a staurosporine analog that inhibits PKG by interfering ATP binding to the catalytic domain (34, 35). Rp-Br-PET-cGMP is a cell-permeable cGMP diastereomer that competes with cGMP for binding to the regulatory domain (36). The observation that both inhibitors produced similar effects in degranulation by fMLF and Fc ϵ RI cross-linking indicates that the inhibitory effect was specifically targeted at PKG. This notion was supported by additional experimental data obtained with a DN construct of PKG. Using RT-PCR, we found that RBL cells express PKG I α but not PKG I β or PKG II, whereas human neutrophils express both PKG I α and PKG I β (Fig. 2*A*). Based on the expression profile, a DN construct consisting of the regulatory domain of PKG I α was transiently expressed in RBL cells along with a human FPR cDNA construct, using nucleofection as described in *Materials and Methods* (Fig. 2*B*). As expected, release of β -hexosaminidase in fMLF-stimulated cells was significantly reduced in the presence of the DN PKG I α (Fig. 2*C*).

Vesicular fusion with the plasma membrane, a final step of degranulation, is regulated by interactions between SNAREs and their binding proteins (38–40). We used FITC-conjugated annexin V to visualize granule fusion with the plasma membrane taking advantage of its ability to bind phosphatidylserine that is exposed to the outer leaflet of the plasma membrane during exocytosis (32, 41, 42). This approach was used in a previous study to demonstrate

was stripped and probed with an Ab against β -actin as a measure for protein loading and transfer (lower panel). *C*, The transiently transfected RBL cells were stimulated with 100 nM fMLF and the released β -hexosaminidase was determined. Data shown are means \pm SEM from three independent experiments and are expressed as percentage of total enzymes associated with the cells.

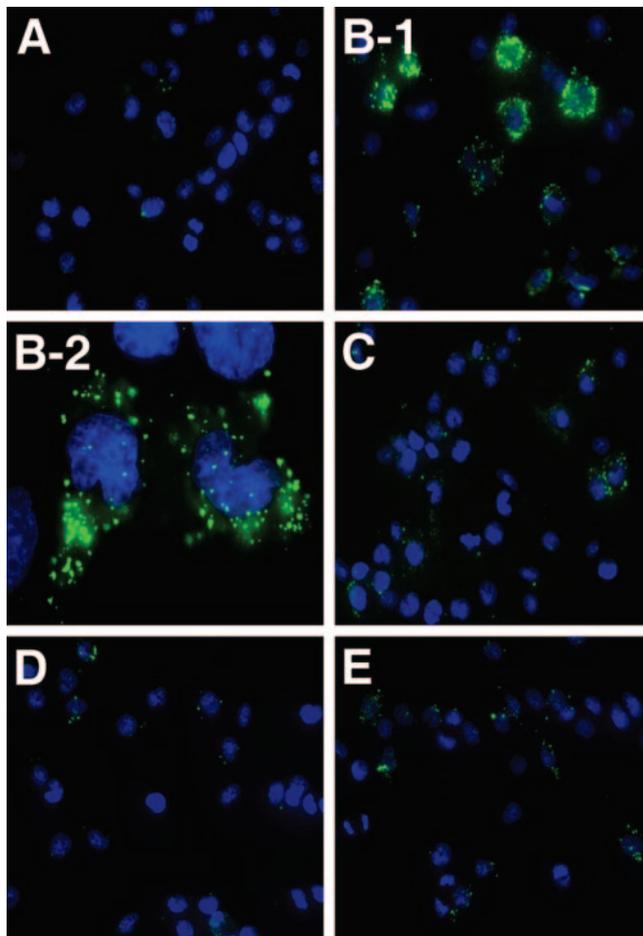


FIGURE 3. Inhibition of vesicular fusion to plasma membrane as detected with FITC-annexin V. Fusion of the granules with plasma membrane was visualized with annexin V binding to externalized phosphatidylserine. RBL-FPR cells were cultured on coverslip for 48 h. Before stimulation, cells were washed and preincubated with 10 μ M cytochalasin B and a 1/20 dilution of FITC-annexin V in HBSS-HB. Cells were then stimulated with 100 nM fMLF for 15 min at 37°C in the presence of cytochalasin B and FITC-annexin V. The reaction was terminated, cells were fixed with 3% paraformaldehyde, and fluorescence images ($\times 200$) were taken. *A*, Non-stimulated cells. *B*, fMLF-stimulated cells without inhibitors (*B-2*, at $\times 600$ magnification). Some samples were preincubated with 10 μ M KT5823 (*C*), 100 μ M Rp-Br-PET-cGMP (*D*), or 10 μ M LY294002 (*E*) before fMLF stimulation. A set of representative images, taken from three experiments, is shown.

colocalization of annexin V with SNARE proteins after vesicular fusion with the plasma membrane (41). To investigate a potential role of PKG in vesicle fusion, RBL-FPR cells were preincubated with FITC-conjugated annexin V before stimulation with fMLF (100 nM) or vehicle (buffer solution) for 15 min. As shown in Fig. 3, fMLF induced a significant increase in FITC-annexin V staining (Fig. 3*B-1*) over basal level (Fig. 3*A*). The punctate-staining pattern in degranulating RBL-FPR cells was drastically different from a more uniform staining of blebs in apoptotic cells (43). The PKG inhibitors KT5823 (Fig. 3*C*) and Rp-Br-PET-cGMP (Fig. 3*D*) markedly reduced fluorescent staining, suggesting inhibition of vesicular fusion with the plasma membrane. LY294002, a PI3K inhibitor known to block degranulation in RBL-2H3 cells (18, 44), reduced fluorescent staining to a similar extent (Fig. 3*E*).

In the above experiments, inhibition of either PKG or PI3K could down-regulate β -hexosaminidase release in RBL and RBL-FPR cells. The combined effect of the PKG and PI3K inhibitors on

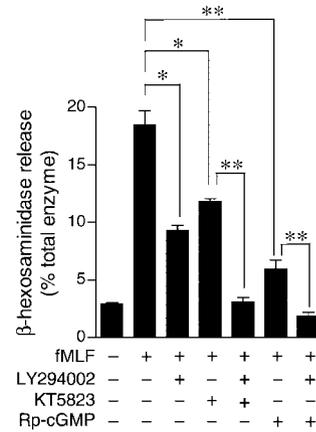


FIGURE 4. Combined effects of PKG and PI3K inhibitors on degranulation. RBL-FPR cells that were preincubated with KT5823 (10 μ M), Rp-Br-PET-cGMP (75 μ M) and LY294002 (5 μ M), either alone or in combinations. The combined use of the PKG inhibitors with LY294002 reduced fMLF-mediated β -hexosaminidase release to basal level. The amount of β -hexosaminidase was quantified and expressed similarly as in Fig. 1.

enzyme release was examined. RBL-FPR cells were incubated with KT5823 (10 μ M) or Rp-Br-PET-cGMP (75 μ M) either alone or in combination with LY294002 (5 μ M). As shown in Fig. 4, the combined use of a PKG inhibitor with LY294002 caused further inhibition and nearly completely blocked fMLF-induced β -hexosaminidase release. It is possible that the two types of enzyme inhibitors act either in tandem or in parallel to produce additional inhibitory effect.

Akt, a serine/threonine kinase downstream of PI3K, is known to directly phosphorylate endothelial NO synthase (eNOS) on a serine residue, causing increased production of NO (45–47). Therefore, PI3K possibly can regulate PKG activation through the NO-sGC-cGMP pathway. To determine whether PI3K indeed regulates PKG in RBL mast cells, we examined the effect of PI3K inhibition on cGMP accumulation. When RBL-FPR cells were stimulated with 30 and 100 nM fMLF, intracellular cGMP concentrations were increased from 1.0 pM/ml to 1.6 pM/ml and 1.9 pM/ml, respectively (Fig. 5*A*). The relatively small but significant increase in intracellular cGMP is thought to contribute to neutrophil activation, as a much larger increase in cGMP could inhibit neutrophil functions (48). However, treatment with the PI3K inhibitor LY294002 did not significantly alter the fMLF-stimulated cGMP elevation (filled bars, Fig. 5*A*). We postulated that if inhibition of PI3K could contribute to diminished degranulation through reduction in PKG activity, then the inhibitory effect would be reversed by treatment with a cell-permeable cGMP analog. Apparently, this was not the case, as 8-bromo-PET-cGMP, a cell-permeable cGMP analog, did not restore the inhibition of β -hexosaminidase release in LY294002-treated RBL-FPR cells (Fig. 5*B*), suggesting that the inhibition by LY294002 was not due to a reduction in intracellular cGMP concentration. We also examined whether the PKG inhibitors could alter PI3K-mediated functions using Akt phosphorylation as readout. As shown in Fig. 6, the fMLF-stimulated Akt phosphorylation in RBL-FPR cells was effectively blocked by the PI3K inhibitor LY294002 (compare Fig. 6*A* with 6*B*). In comparison, the effect of KT5823 and Rp-Br-PET-cGMP on Akt phosphorylation was small. KT5823 treatment reduced the basal level of Akt phosphorylation, but not fMLF-stimulated Akt phosphorylation (Fig. 6*C*). Rp-Br-PET-cGMP did not affect basal Akt phosphorylation or its peak induction by fMLF at 7 min, but slightly reduced the rise of Akt phosphorylation (at 3

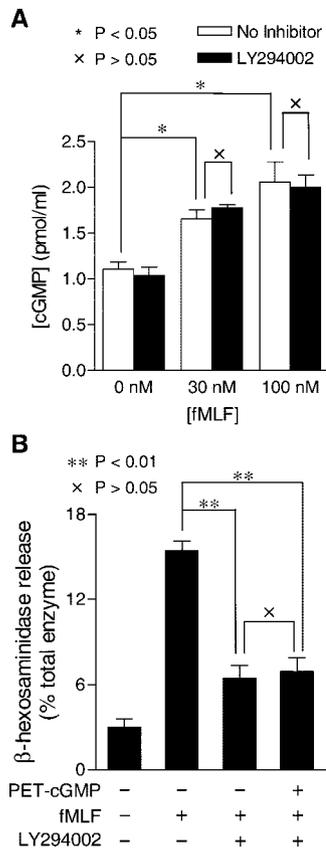


FIGURE 5. Effect of LY294002 on intracellular cGMP concentration and cGMP-independent inhibition of enzyme release. RBL-FPR cells were plated for 48 h before stimulation with fMLF (30 and 100 nM). Some samples were pretreated with LY294002 (10 μ M). *A*, After stimulation, the accumulation of cGMP was measured with a competitive enzyme immunoassay kit in the presence of IBMX (0.5 mM) as detailed in *Materials and Methods*. Intracellular cGMP was quantified against a standard curve generated in parallel. Data presented are means \pm SEM from three independent experiments. *B*, Application of an exogenous, cell-permeable cGMP analog did not rescue LY294002-inhibited β -hexosaminidase release. RBL-FPR cells were preincubated with 5 μ M LY294002 for 15 min and then with or without 100 μ M 8-bromo-PET-cGMP (PET-cGMP), a cell-permeable cGMP analog, for 5 min before stimulation with 100 nM fMLF. The released β -hexosaminidase was quantified as described in *Materials and Methods*. Data are expressed as percent release of total cellular enzyme and are means \pm SEM from three independent experiments.

min) and accelerated its return to baseline (at 15 min). These results suggest that the two PKG inhibitors can slightly alter Akt phosphorylation in different manners, although the mechanism underlying the difference remains unknown.

Elevation of intracellular calcium concentration is critical to exocytosis in RBL cells, whereas in neutrophils, secretion from the three different granule populations is differentially regulated by intracellular calcium concentration (49). We examined whether PKG inhibition could compromise calcium mobilization and thereby inhibit degranulation. RBL-FPR cells were stimulated with fMLF in the presence or absence of EDTA, allowing a distinction between intracellular calcium mobilization vs calcium influx. At 50 μ M, Rp-Br-PET-cGMP did not alter calcium mobilization from intracellular stores or calcium influx (Fig. 7). At a higher concentration (100 μ M), a small reduction in intracellular calcium mobilization was observed but the difference was statistically insignificant. These results exclude the possibility that inhibition of intracellular calcium mobilization or calcium influx is responsible for reduction of degranulation by the PKG inhibitor.

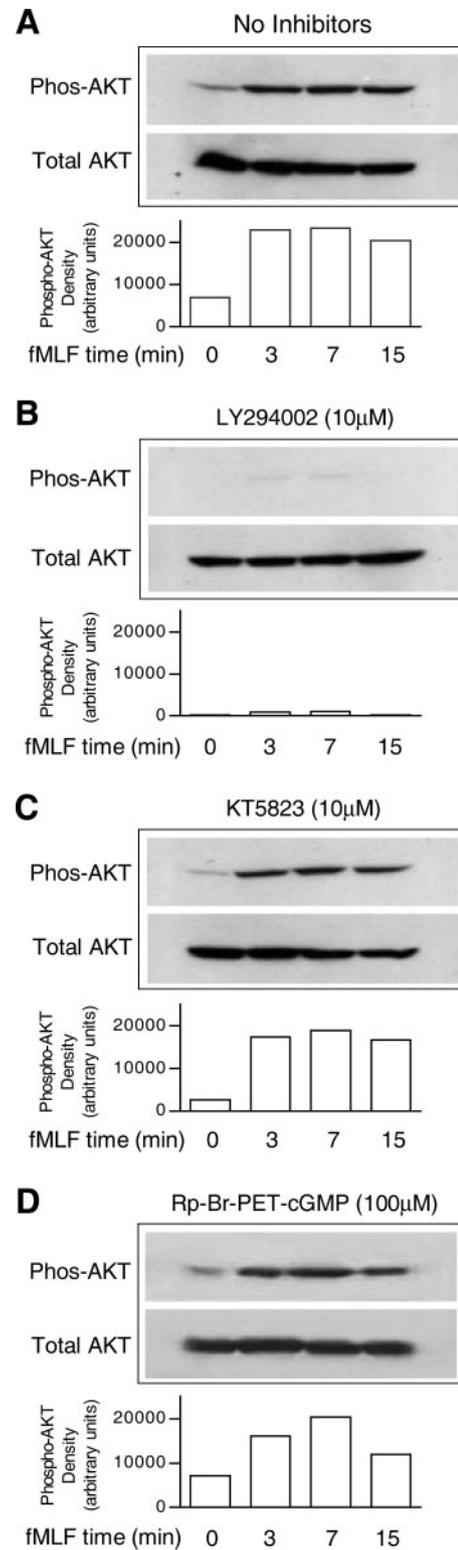


FIGURE 6. Effects of the PKG inhibitors on Akt phosphorylation. RBL-FPR cells were cultured in 12-well plates and serum-starved overnight. Cells were pretreated for 15 min with inhibitors (*B–D*) or vehicle (*A*) as indicated and then stimulated with 100 nM fMLF for the indicated time periods. The reaction was terminated by addition of ice-cold SDS-PAGE loading buffer. After a brief sonication and boiling, samples were analyzed by SDS-PAGE and Western blotting using anti-Akt and anti-phospho-Akt Abs (1/1000 dilution) and HRP-conjugated anti-rabbit Ab as the secondary Ab. The resulting immunocomplex was visualized by chemiluminescence. Semiquantitative analysis of the Western blots was conducted with the ImageQuant software based on the density of each band. A set of blots, representative of three independent experiments, is shown.

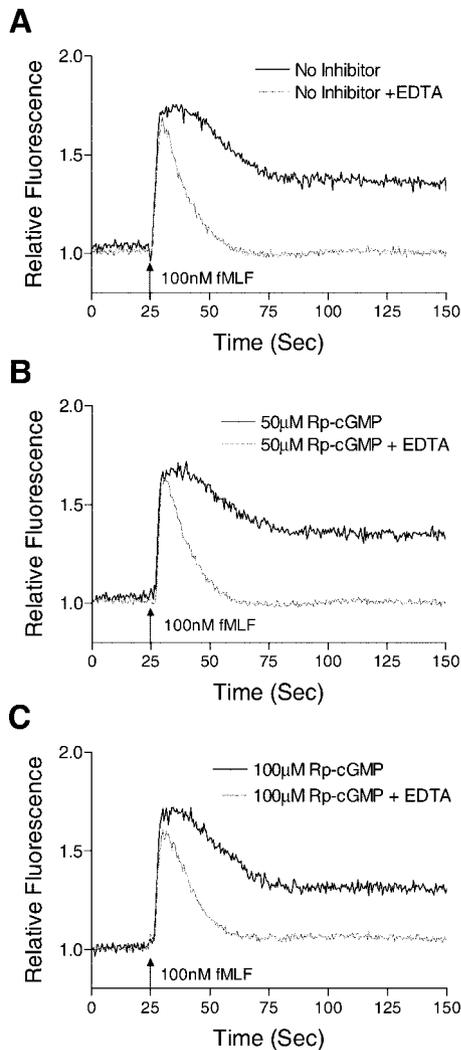


FIGURE 7. Inhibition of PKG has no significant effect on fMLF-stimulated intracellular calcium mobilization. Agonist-induced intracellular calcium elevation was measured in Indo-1/AM-loaded RBL-FPR cells as described in *Materials and Methods*. Three minutes before fMLF stimulation, cells were incubated with Rp-Br-PET-cGMP at two different concentrations (B–D) or with vehicle (A). For chelation of extracellular Ca^{2+} , 5 mM EDTA was added before stimulation. Representative tracings collected from several experiments were shown.

RBL cells express syntaxins 2, 3, 4, SNAP-23, Munc18-2 and 18-3, but not the neuronal syntaxin 1 and Munc18-1 (6, 14, 42). Our result indicates that RBL cells also express syntaxin 6 (see Fig. 9E). Previous studies have shown that overexpression of syntaxin 4 (6) or Munc18-2 (42) leads to inhibition of $\text{Fc}\epsilon\text{RI}$ -induced degranulation from RBL cells, implying the involvement of these proteins in mast cell exocytosis. Published reports also suggest phosphorylation of syntaxins and Munc18 proteins as a mechanism for vesicular fusion (10–14). We investigated a possible role of PKG in the phosphorylation of these proteins. Because specific anti-phosphoantibodies against these proteins are not available, we used a phosphoprotein affinity purification approach to enrich the phosphorylated proteins in RBL-FPR cells. The proteins of interest were subsequently identified through Western blotting as described in *Materials and Methods*. The feasibility of this approach was shown in Fig. 8, in which fMLF-induced p38 MAPK phosphorylation was detected in RBL-FPR cells. The phosphorylated p38 MAPK was successfully retained in the affinity column, eluted, and detected by Western blotting using an anti-p38 Ab

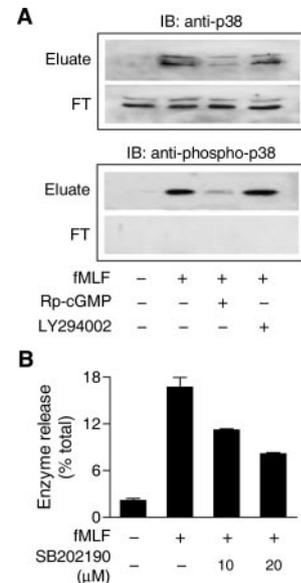
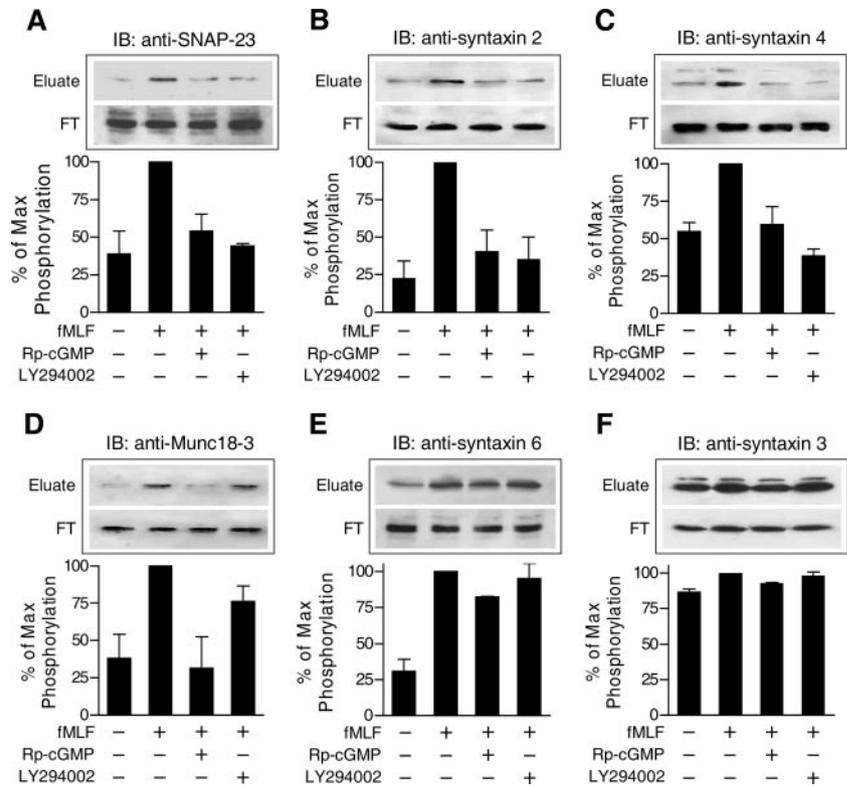


FIGURE 8. Phosphorylation of p38 MAPK in fMLF-stimulated cells and its role in degranulation. *A*, Inhibition of PKG attenuated FPR-mediated p38MAPK phosphorylation. Separation of phosphorylated proteins from RBL-FPR cell lysates was performed with a phosphoprotein purification kit (Qiagen), as described in *Materials and Methods*. Both the eluate and the flow-through fractions were collected and analyzed on SDS-PAGE and by Western blotting. On the *top panels*, both phosphorylated (eluate) and nonphosphorylated p38 MAPK (flow-through) were detected with an anti-p38 Ab. On the *bottom panel*, phosphorylated proteins were detected with an anti-phospho-p38 MAPK Ab only in eluate fraction, indicating the ability of the purification system to enrich phosphorylated proteins. Preincubation with Rp-Br-PET-cGMP (100 μM , 15 min) before fMLF stimulation markedly reduced phosphorylation of p38 MAPK; however, LY294002 (10 μM) had no significant effect. *B*, The p38 MAPK inhibitor SB202190 dose-dependently suppressed fMLF-induced degranulation. RBL-FPR cells were preincubated with SB202190 at two different concentrations for 15 min before fMLF stimulation (100 nM, 10 min). The released β -hexosaminidase was determined as described in *Materials and Methods*. Data shown are means \pm SEM from three independent experiments and are expressed as percentage of total enzyme associated with the cells.

(Fig. 8A, *upper panel*). The result was comparable to Western blotting showing the presence of phosphorylated p38 MAPK using an anti-phospho-p38 Ab (Fig. 8A, *lower panel*). A similar purification result was obtained with phosphorylated Akt (data not shown). Treatment with the PKG inhibitor Rp-Br-PET-cGMP reduced fMLF-stimulated phosphorylation of p38 MAPK, whereas LY294002 had no significant effect. This result is consistent with a previous report showing that activation of p38 MAPK is regulated by cGMP and is downstream of PKG (50). Likewise, treatment of RBL-FPR cells with the p38 MAPK inhibitor SB202190 reduced fMLF-stimulated β -hexosaminidase release (Fig. 8B).

Using the affinity purification approach, we determined the effects of PKG and PI3K inhibition in the phosphorylation status of SNARE proteins. As shown in Fig. 9, fMLF stimulated phosphorylation of SNAP-23, syntaxin 2, syntaxin 4, syntaxin 6, and Munc18-3 in RBL-FPR cells. Except syntaxin 6, the induced phosphorylation of all five proteins was inhibited by Rp-Br-PET-cGMP, given before fMLF stimulation. In comparison, LY294002 potently inhibited fMLF-stimulated phosphorylation of SNAP-23, syntaxin 2, and syntaxin 4, but had little inhibitory effect on the phosphorylation of Munc18-3 and syntaxin 6. These results suggest differential regulation of phosphorylation of t-SNAREs and Munc18 proteins by the PKG and PI3K inhibitors. Constitutive

FIGURE 9. Effects of the PKG and PI3K inhibitors on fMLF-induced phosphorylation of SNAREs and Munc18 proteins. Phosphorylation of selected SNAREs and Munc18 proteins was examined using the same procedure described in Fig. 8. The extent of protein phosphorylation was analyzed by SDS-PAGE and Western blotting with the following Abs: anti-SNAP-23 (A), anti-syntaxin 2 (B), anti-syntaxin 4 (C), anti-Munc18-3 (D), anti-syntaxin 6 (E), anti-syntaxin 3 (F), and anti-Munc18-2 (data not shown). An HRP-conjugated anti-rabbit Ab, or an anti-goat Ab for Munc18-3, were used as secondary Abs. The resulting immunocomplex was visualized by ECL. No phosphorylation of Munc18-2 was detected in either unstimulated or stimulated cells (data not shown). Stimulation with 100 nM fMLF led to phosphorylation of SNAP-23, syntaxins 2, 4 and 6, and Munc18-3. Pretreatment with Rp-Br-PET-cGMP (100 μ M) inhibited phosphorylation of all these proteins except that the inhibition on syntaxin 6 was minimal. LY294002 (10 μ M) was effective in preventing the induced phosphorylation of SNAP-23, syntaxin 2, and syntaxin 4, but ineffective in the inhibition of phosphorylation of Munc18-3 and syntaxin 6 ($p > 0.05$). Western blot images were representative of a typical experiment, from a total of three experiments. Western blot results were quantified with the ImageQuant software and shown in bar graphs as means \pm SEM of three independent experiments.



phosphorylation was detected with syntaxin 3 and was not affected by either Rp-Br-PET-cGMP or LY294002. In contrast, Munc18-2 phosphorylation was undetected in either unstimulated or fMLF-stimulated cells, although the Ab could readily identify Munc18-2 in the flow-through fraction confirming its expression in RBL-FPR cells (data not shown).

Stimulation of neutrophils with fMLF results in the release of enzymes from all major types of granules, which are formed during different phases of granulocyte maturation (2-4). We compared neutrophil degranulation with that of RBL cells with respect to PKG involvement and its regulatory mechanism. As shown in Fig. 10A, fMLF induced a small but significant increase in neutrophil

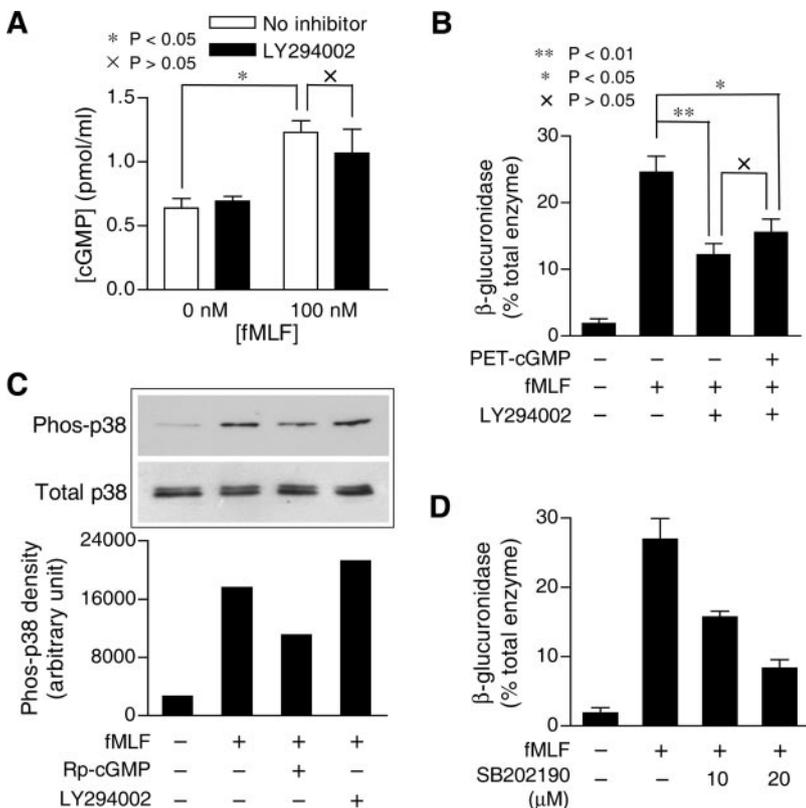


FIGURE 10. Regulation of neutrophil degranulation through interference of the PKG and PI3K pathways. A, The effect of LY294002 (10 μ M) on fMLF (100 nM) induced cGMP accumulation was determined in human blood neutrophils. The same procedure in Fig. 5A was used for this analysis. Data presented are means \pm SEM from three independent experiments. B, Application of an exogenous, cell-permeable cGMP analog (8-bromo-PET-cGMP, 100 μ M) did not significantly reverse the inhibitory effect of LY294002 (5 μ M) on the release of β -glucuronidase from fMLF-stimulated neutrophils. C, Rp-Br-PET-cGMP (100 μ M, 15 min) produced an inhibitory effect on fMLF (100 nM) induced p38 MAPK phosphorylation. On the contrary, LY294002 (10 μ M) slightly increased p38 MAPK phosphorylation. A representative Western blot from a total of three experiments is shown. D, Inhibition of p38 MAPK by SB202190 caused a dose-dependent suppression of fMLF (100 nM) induced release of β -glucuronidase. Shown in the figure are means \pm SEM from three independent experiments.

Table I. Effects of PKG and PI3K inhibitors on the release of enzymes from three different classes of neutrophil granules^a

% Inhibition	Rp-8-Br-PET-cGMP		LY294002	
	50 μ M	100 μ M	5 μ M	10 μ M
β -Glucuronidase	15.8 \pm 4.1	47.2 \pm 5.2	27.8 \pm 4.1	53.8 \pm 4.4
LTF	23.7 \pm 0.2	28.6 \pm 3.4	15.7 \pm 1.1	32.7 \pm 1.3
MMP-9	27.6 \pm 4.3	32.8 \pm 2.6	38.9 \pm 4.8	48.6 \pm 3.8

^a Peripheral blood neutrophils were purified as described in *Materials and Methods*. Isolated human neutrophils were pre-incubated with 10 μ M cytochalasin B with or without inhibitors. The cells were then stimulated with 100 nM fMLF for 10 min at 37°C, and the amount of released enzymes was determined based on enzymatic cleavage of fluorogenic substrate (for β -glucuronidase) or by ELISA (for LTF and MMP-9), as detailed in *Materials and Methods*. Data presented are percentage of inhibition and are means \pm SEM from three independent experiments.

cGMP concentration. The induced increase in cGMP concentration was slightly affected by the PI3K inhibitory LY294002, but the change was statistically insignificant. Likewise, inhibition of β -glucuronidase release by LY294002 could not be restored by the cell-permeable cGMP analog, 8-bromo-PET-cGMP (Fig. 10B). Among other features shared with the RBL cells, the cGMP inhibitor Rp-Br-PET-cGMP partially inhibited fMLF-induced p38 MAPK phosphorylation, and the p38 MAPK inhibitor SB202190 dose-dependently reduced fMLF-induced β -glucuronidase release (Fig. 10, C and D; compare with Fig. 8). Rp-Br-PET-cGMP did not affect fMLF-induced calcium mobilization in neutrophils (data not shown). We were unable to measure phosphorylation of syntaxins and Munc-18 proteins using the phosphoprotein affinity purification procedure owing to the abundance of proteases and phosphatases in neutrophils that diminish binding of phosphorylated proteins to the column.

Exocytosis of different granule populations is thought to involve distinctive regulatory mechanisms based on a hierarchical release kinetics ranging from the fastest for tertiary granule to the slowest for primary granule (51). To determine whether PKG plays a role in the hierarchical release of these granules, we measured enzymes released from primary granules (β -glucuronidase), secondary granules (LTF), and tertiary granules (MMP-9), in the presence or absence of Rp-Br-PET-cGMP and LY294002. The results are summarized in Table I. Stimulation with 100 nM fMLF caused the release of 16.1 \pm 0.77% of total extractable β -glucuronidase, a 11-fold increase over basal level. Rp-Br-PET-cGMP and LY294002 reduced fMLF-stimulated β -glucuronidase release with maximal inhibition of 47.2 \pm 5.2% and 53.8 \pm 4.4%, respectively. The same sets of samples were used for measurements of LTF and MMP-9 release. fMLF stimulation resulted in a 7-fold increases over basal level for both lactoferrin and MMP-9. Release of these enzymes was inhibited by Rp-Br-PET-cGMP and LY294002, with maximal inhibition of 28.6 \pm 3.4% and 32.7 \pm 1.3% for lactoferrin, and 32.8 \pm 2.6% and 48.6 \pm 3.8% for MMP-9, respectively. The PKG inhibitor KT5823 (10 μ M) produced inhibitory effects comparable to that of Rp-Br-PET-cGMP (data not shown). Our results indicate that exocytosis of all three types of neutrophil granules was affected by inhibition of PKG and PI3K, suggesting that these kinases are common effector proteins for degranulation in RBL cells and in neutrophils.

Discussion

In this study, we examined the effects of PKG and PI3K inhibition in degranulation from RBL and FPR-expressing RBL cells. Remarkably, similar results were obtained when these cells were stimulated with either fMLF or cross-linking of Fc ϵ RI. Both Rp-Br-PET-cGMP and KT5823 produced inhibitory effects in the release of β -hexosaminidase. Furthermore, a DN mutant of PKG I α

reduced fMLF-stimulated enzyme release in transiently transfected RBL cells, confirming a role of PKG I α , the only form of PKG detected in RBL cells, in agonist-induced degranulation. Although LY294002 produced similar inhibitory effects, it seems to target a regulatory mechanism parallel to the PKG pathway, as LY294002 did not significantly affect the induced elevation of intracellular cGMP concentration and inhibition of degranulation by LY294002 could not be reversed by a cell-permeable cGMP analog. KT5823 and Rp-Br-PET-cGMP each had an effect on fMLF-induced phosphorylation of Akt, an effector of PI3K, but the inhibitory effect was small and did not affect peak Akt phosphorylation detected after 3 min of agonist stimulation. Both the PKG inhibitors and LY294002 produced inhibitory effects in the induced phosphorylation of t-SNAREs including syntaxin 2 and 4, and SNAP-23, while neither inhibitors could block syntaxin 6 phosphorylation. Inhibition of the two types of kinases produced different results in the phosphorylation of Munc18-3, with LY294002 being much less effective than Rp-Br-PET-cGMP. Taken together, these results demonstrated that PKG and PI3K regulate degranulation by targeting the signaling pathways downstream of both a G protein-coupled receptor and a tyrosine kinase-coupled receptor. The two kinase pathways most likely operate in parallel, but can converge to produce additive effect in degranulation. This notion was supported by the observation of additive effect when the PI3K inhibitor was used with either one of the PKG inhibitors. The presence of a distinct regulatory mechanism is suggested by the inability of LY294002 to effectively block the induced phosphorylation of Munc18-3. Due to technical limitations, we were unable to determine the effects of these inhibitors on the phosphorylation of neutrophil SNARE proteins. Data from a preliminary study of neutrophil degranulation suggest that release of enzymes from all three major classes of neutrophil granules is subjected to inhibition of PKG and PI3K (Table I). Recently, Li et al. (52) reported that PKG plays an important role in aggregation-dependent release of dense granule and α -granule in platelets. Thus, PKG may be involved in degranulation triggered by multiple extracellular stimuli in different types of cells.

It has been well-documented that cGMP and PKG regulate leukocyte functions including chemotaxis and degranulation (22, 23, 53). However, the mechanism for chemoattractant-induced activation of PKG remains largely unknown. Leukocyte chemoattractants such as fMLF and C5a may increase intracellular cGMP concentration through induction of NOS and production of NO (54, 55), which in turn activates sGC. NO is a ubiquitous messenger that plays a role in neutrophils as well as in other types of cells. For example, fMLF-mediated chemotaxis was decreased by an inhibitor of NOS, *N*-monomethyl-L-arginine, and this inhibition was reversed by exogenous application of cGMP (56). However, early studies using a number of NO donors such as SIN-1 also revealed

inhibition of leukocyte functions including chemotaxis and superoxide generation, especially when these NO donors were used at higher concentrations (25–27). With respect to degranulation, published reports indicate a biphasic dose response in which NO and cGMP enhance exocytosis at low concentrations and inhibits exocytosis at higher concentrations (48, 57–59). The inhibitory effect in leukocyte functions may be attributed to the ability of several NO donors and cGMP analogs to stimulate cAMP accumulation when used at high concentrations (60). Similar phenomenon has been observed in platelets, in which cAMP-dependent protein kinase (PKA) is largely responsible for the inhibition of platelet activation while PKG plays a stimulatory role under several conditions, both in vitro and in vivo (61, 62). In neutrophils, fMLF induces a moderate increase in NO and intracellular cGMP concentration as compared with the increases invoked by most NO donors (48), most likely because of the low NOS expression level in the terminally differentiated granulocytes (63, 64). The small increase in intracellular cGMP is associated with enhanced neutrophil functions such as degranulation and chemotaxis (48).

A recent report showed that targeted deletion of mouse PKG α (cGKI α) led to a complex phenotype characterized by a loss of cGMP-dependent decrease in C5a-induced calcium response and an increase in C5a- and IL-8-induced neutrophil chemotaxis (65). Release of lysozyme, which is contained in primary, secondary, and tertiary granules (2), was reduced from 90% of the total enzyme in wild-type neutrophils to 63% in PKG $\alpha^{-/-}$ neutrophils stimulated with fMLF. The observed reduction (~30%) in enzyme release is consistent with data derived from our study using pharmacological inhibitors of PKG. Together, these findings indicate a positive regulatory role of PKG in leukocyte degranulation. Interestingly, both the total extractable myeloperoxidase and its release were increased in the PKG $\alpha^{-/-}$ neutrophils elicited from peritoneal cavity (65). The reason for this change remains unclear, as results from the same report demonstrated the presence of two pathways: while PMA failed to stimulate myeloperoxidase release, it was able to induce exocytosis of secondary and tertiary granules evidenced by the release of lysozyme in both wild-type and PKG $\alpha^{-/-}$ neutrophils. Because a fraction of the enzymes were retained in the stimulated cells, it is possible that these enzymes are packed in a population of granules not subject to regulation by PKG. The nature of this granule population remains undefined and heterogeneity of neutrophils granules may contribute to the observed discrepancy in enzyme release. It would be interesting to determine whether release of other primary granule enzymes, such as β -glucuronidase, is affected in PKG $\alpha^{-/-}$ neutrophils. Also, it would be helpful to know whether an additional inhibitory effect could be produced with PKG inhibitors in the PKG $\alpha^{-/-}$ neutrophils as compensatory mechanisms may be present in the knockout neutrophils.

Because multiple kinases are involved in leukocyte degranulation, we sought to determine whether these kinases have distinct or overlapping roles in the degranulation process. PI3K is a lipid kinase and its function in receptor-mediated signaling has been well established (66). Therefore, inhibition of PI3K possibly can affect signaling pathways upstream of granule exocytosis. A role of PKG in regulating the proximal (immediately downstream of receptor activation) signaling pathway is not well-defined, although this kinase is required for LPS-induced p38 MAPK activation (50). Chemoattractants such as fMLF may use one or more of the available pathways to activate PKG. The classical NOS-NO-sGC-cGMP pathway has been established in fMLF-stimulated neutrophils (55, 67). Because low level expression of both inducible NOS and eNOS has been detected in neutrophils (64, 68) and PI3K-dependent activation of Akt is known to phosphorylate and

activate eNOS, activation of PKG may be controlled by PI3K as seen in endothelial cells (45–47). This possibility was investigated in the current study. Our results showed no significant effect of PI3K inhibition in fMLF-induced elevation of the cGMP level (Fig. 5A). This finding suggests the presence of an alternative pathway for PKG activation. A cGMP-independent mechanism for PKG activation, that involves PKC-mediated PKG phosphorylation, was reported recently (69). This activation mechanism may be triggered by chemoattractants such as fMLF, which stimulates PLC β and causes accumulation of the second messengers necessary for PKC activation (70). Fc ϵ RI cross-linking leads to activation of PLC γ and production of the second messenger inositol trisphosphate and diacylglycerol through hydrolysis of membrane-bound phosphatidylinositol (4, 5)-bisphosphate, all being contributing factors for PKC activation (71–73). Therefore, PKC-dependent activation of PKG may play a role in PKG regulation of degranulation in IgE-stimulated mast cells. The lack of PI3K regulation in PKG activation, combined with the inability of a cell-permeable cGMP analogs to reverse the inhibitory effect of LY294002, suggest independent regulation of the PI3K-Akt pathway and the PKG activation pathway downstream of the two receptors studied in this work.

To address the possible mechanisms of PKG and PI3K in regulating vesicular fusion, we examined phosphorylation of t-SNAREs and the Munc18 proteins that constitute a family of regulatory molecules for vesicle interaction with the plasma membrane (8–12). The seven proteins studied in RBL-FPR cells can be categorized into four classes based on the different patterns of their phosphorylation: 1) phosphorylation induced by agonist stimulation and subjected to inhibition by both Rp-Br-PET-cGMP and LY294002. The proteins exhibiting this pattern include SNAP-23, syntaxin 2, and syntaxin 4. 2) Phosphorylation induced by agonist stimulation and inhibited by one but not the other inhibitors. Munc18-3 belongs to this group as Rp-Br-PET-cGMP but not LY294002 effectively reduced its phosphorylation. 3) Phosphorylation induced by agonist stimulation but not effectively inhibited by either type of inhibitors. Syntaxin 6 exhibits this property. 4) Phosphorylation status unchanged by either agonist stimulation or treatment with the inhibitors. Syntaxin 3 (constitutively phosphorylated) and Munc18-2 (no detectable phosphorylation) are proteins in this class. These patterns of phosphorylation are intriguing and probably suggest that maximal degranulation requires phosphorylation on multiple SNARE proteins, but inhibition of phosphorylation in one or two of these proteins can be sufficient to compromise degranulation. Published studies indicate that phosphorylation of SNAP-23 and syntaxin 4 are essential for degranulation in RBL cells and platelets (13, 14). Furthermore, expression of a phosphorylation-deficient mutant in RBL cells inhibited degranulation (14). Thus, negative regulation of degranulation by Rp-Br-PET-cGMP and LY294002 may be attributed to their inhibitory effect on the phosphorylation of SNARE proteins.

Phosphorylation of the SNARE proteins may be accomplished through a direct interaction with PKG, or through an indirect action via another protein kinase such as p38 MAPK (74). Due to the paucity of PKG-specific substrates, it is presently difficult to determine whether PKG can directly phosphorylate one or more SNARE proteins. Using a phosphorylation site prediction tool (www.cbs.dtu.dk/services/NetPhosK), we found that the SNAREs and their binding proteins that are phosphorylated upon fMLF stimulation and regulated by the PKG inhibitors contain potential phosphorylation sites for either PKG (syntaxin 2, Munc18-3) or p38 MAPK (syntaxin 4, Munc18-3). The t-SNAREs that are not subject to regulation by the PKG inhibitor (syntaxin 3, syntaxin 6) also do not contain potential phosphorylation sites for PKG and

p38 MAPK. These findings suggest the possibility that PKG and p38 MAPK, which is downstream of the NO-cGMP-PKG pathway (74), may directly phosphorylate these SNARE and Munc18 proteins. A more detailed biochemical analysis will be necessary to determine whether the above proteins are substrates for PKG and p38 MAPK using *in vitro* kinase assays. Consistent with published reports on PKC-mediated phosphorylation of SNARE proteins (14, 32), all the proteins examined in this study contain multiple sites for PKC phosphorylation.

In conclusion, results from this study suggest that PKG and PI3K are involved in degranulation triggered by multiple stimuli in RBL cells, and that phosphorylation of selected SNAREs and their binding proteins may be a mechanism of regulation by these kinases. Under the experimental conditions used in this study, PKG and PI3K represent parallel signaling pathways with minimal cross-talk, but the two pathways can converge to produce additive effect on degranulation. Our preliminary results also suggest that PKG and PI3K are necessary for enzyme release from the three major classes of neutrophil granules. It remains to be determined whether inhibition of PKG and PI3K can affect phosphorylation of SNAREs in neutrophils.

Acknowledgments

We thank Dr. Zhenyu Li for providing the DN-PKG construct and Drs. Jian Fu and Rong He for helpful discussions.

Disclosures

The authors have no financial conflict of interest.

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