

Tyrosine phosphorylation and inositol phosphate production: Are early events in human eosinophil activation stimulated by immobilized secretory IgA and IgG?

Hirohito Kita, MD, Masahiko Kato, MD, Gerald J. Gleich, MD, and Robert T. Abraham, PhD Rochester, Minn.

Eosinophils play a major role in host immunity to parasite infections and allergic reactions such as bronchial asthma.¹ During such inflammatory reactions, soluble mediators released by T cells, macrophages, and other inflammatory cells induce migration of eosinophils from the blood into tissue. Subsequently, appropriate stimuli (e.g., Ig-coated helminths) trigger the eosinophils to degranulate, which results in the local release of a series of cytotoxic and proinflammatory proteins such as major basic protein, eosinophil peroxidase, eosinophil cationic protein, and eosinophil-derived neurotoxin (EDN).^{1, 2} Deposition of released granule proteins has been shown in various diseases, including bronchial asthma and parasite infections.¹ Accumulating evidence that eosinophil granule proteins are implicated in tissue damage associated with these diseases has focused interest on the regulation of eosinophil degranulation. However, at present, both the actual physiologic stimuli for eosinophil degranulation and the transmembrane signaling events for triggering degranulation from eosinophils are poorly understood.

In numerous receptor systems, ligand binding triggers a rapid increase in phosphoinositide hydrolysis catalyzed by receptor-coupled phospholipase C (PLC) activity.³ Phosphoinositide breakdown generates at least two second-messenger molecules, inositol 1,4,5-triphosphate and 1,2-diacylglycerol, that in turn increase $[Ca^{2+}]_i$ and activate protein kinase C, respectively.⁴ In mast cells, platelets, and neutrophils, exocytotic events

Abbreviations used

ADP:	Adenosine diphosphate
DCS:	Defined calf serum
EDN:	Eosinophil-derived neurotoxin
FMLP:	N-formyl-methionyl-leucyl-phenylalanine
NAD:	Nicotinamide adenine dinucleotide
PAF:	Platelet-activating factor
PLC:	Phospholipase C
PMA:	Phorbol myristate acetate
PTK:	Protein tyrosine kinase
PTX:	Pertussis toxin
sIgA:	Secretory IgA
SDS-PAGE:	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis

are preceded by phosphoinositide turnover.⁵⁻⁷ Recent studies have defined transmembrane receptors that regulate various second messenger-generating enzymes through intermediate guanine nucleotide-binding (G) proteins.⁸ The G proteins implicated in receptor-mediated signaling are membrane-bound $\alpha\beta\gamma$ -heterotrimers. Certain α -subunits of G proteins (G_α -subunit) serve as a relatively specific substrate for adenosine diphosphate (ADP)-ribosylation by cholera toxin or pertussis toxin (PTX).⁹ ADP-ribosylation of the G_α -subunit catalyzed by these bacterial toxins often modifies G protein-effector coupling. For example, PTX pretreatment profoundly inhibits the coupling of N-formyl-methionyl-leucyl-phenylalanine (FMLP) receptor to PLC activity in neutrophils.¹⁰ The major substrate for ADP-ribosylation by PTX in neutrophil membranes is a 41 kd protein that was recently identified as the G_{12} α -subunit.¹¹

After ligation of growth factor receptors containing intrinsic tyrosine kinase domains, and after activation of other receptors that lack such

From the Departments of Immunology and Internal Medicine, Mayo Clinic and Mayo Foundation.

Reprint requests: Hirohito Kita, MD, Mayo Clinic, 200 First St. S.W., Rochester, MN 55905.

J ALLERGY CLIN IMMUNOL 1994;94:1272-81.

Copyright © 1994 by Mosby-Year Book, Inc.

0091-6749/94 \$3.00 + 0 1/0/60691

TABLE I. Eosinophil degranulation induced by various stimuli

Study 1			Study 2		
Stimulus	Released EDN		Stimulus	Released EDN	
	ng/5 × 10 ⁵ cells (n)	% total		ng/5 × 10 ⁵ cells (n)	% total
HSA beads	<15 (20)	<1.2	None	26.1 ± 3.9 (22)	1.5 ± 0.3
IgG beads	110 ± 9 (22)	8.6 ± 0.5	OVA beads	27.3 ± 6.6 (8)	1.5 ± 0.3
IgE beads	<15 (11)	<1.2	sIgA beads	349.4 ± 20.4 (18)	20.7 ± 1.4
IgM beads	<15 (9)	<1.2	IgG beads	86.7 ± 14.7 (18)	5.2 ± 0.4
IgD beads	<15 (9)	<1.2	A23187, 1 μmol/L	239.0 ± 37.4 (9)	14.5 ± 2.1
IgA beads	60 ± 4 (7)	4.7 ± 0.3	FMLP, 1 μmol/L	218.9 ± 24.1 (5)	142 ± 3.0
Total EDN	1276 ± 42	100	PMA, 10 ng/ml	88.9 ± 12.2 (8)	5.2 ± 0.7
			Total EDN	1704.4 ± 56.9 (24)	100

From Abu-Ghazaleh RI, et al. *J Immunol* 1989;142:2393-2400 and Kita H, et al. *J Immunol* 1991;147:3466-73. Copyright 1989 and 1991, The Journal of Immunology.

*n, Number of experiments.

domains, such as the T-cell receptor, the phosphorylation of tyrosine residues in proteins is a well-characterized event.¹² Until recently, the role of tyrosine phosphorylation for signal transduction mediated by Fc receptors has been less well characterized. However, recent evidence suggests that increased tyrosine phosphorylation accompanies activation of Fc receptors in neutrophils¹³ and platelets¹⁴ and is required for Fc receptor-mediated cytotoxicity in natural killer cells,¹⁵ which suggests a role of tyrosine kinase for the Fc receptor-mediated activation of leukocytes. Various transmembrane receptors can be coupled to PLC through tyrosine kinase. For example, growth factor receptors with intrinsic protein tyrosine kinase (PTK) activity, such as the epidermal growth factor receptor and the platelet-derived growth factor receptor, can directly phosphorylate tyrosine members of the PLC-γ family.¹⁶ Activation of the T-cell receptor, which does not contain an intrinsic PTK domain, has also been shown to induce the tyrosine phosphorylation of PLC-γ1.¹⁷ Recently it was shown that Fcγ receptor-initiated phosphoinositide turnover in natural killer cells is regulated by tyrosine phosphorylation of PLC-γ1 and PLC-γ2.¹⁸

Our earlier studies demonstrated that secretory IgA (sIgA) or IgG bound to Sepharose beads triggered the degranulation of human normodense eosinophils in vitro.¹⁹ Others have reported degranulation of eosinophils and hypodense eosinophils can be induced in an IgE-dependent manner.²⁰ In spite of these observations, little is known about the transmembrane signaling events in eosinophil activation induced by Ig receptors. In this report we have studied the signal trans-

duction of human peripheral blood eosinophils with use of sIgA and IgG beads as stimuli. We have focused on early signaling events, especially the role of G proteins, phosphoinositide turnover, and tyrosine kinase activation.

MATERIAL AND METHODS

Reagents

Calcium ionophore A23187, ionomycin, PMA, FMLP, PTX, and genistein were obtained from Calbiochem Corp. (San Diego, Calif.). Nicotinamide adenine [adenylate-³²P] dinucleotide ([³²P] NAD) (specific activity 250 Ci/mmol) was purchased from ICN Biomedicals, Inc. (Costa Mesa, Calif.). Anti-PLC-γ1 antiserum was produced by immunizing New Zealand white rabbits with a PLC-γ2 peptide (bovine PLC-γ1 amino acid 1249-1262) chemically coupled to KLH (Calbiochem). Similarly, PLC-γ2 antiserum was produced by immunizing New Zealand white rabbits with a PLC-γ1 peptide (human PLC-γ2 amino acid 461-481).¹⁸ The anti-PLC-γ1 and anti-PLC-γ2 sera immunoprecipitated the predicted 150 and 140 kd isoforms, respectively. The murine antiphosphotyrosine antibody (4G10) was obtained from Upstate Biotechnology, Inc. (Lake Placid, N.Y.), and the peroxidase conjugated rabbit anti-mouse IgG antibody (p260) was obtained from Dako Corp. (Carpenteria, Calif.).

Eosinophil isolation

The magnetic cell separation system (Becton Dickinson Immunocytometry Systems, San Jose, Calif.) was used with minor modifications of the method described previously.²¹ Heparinized blood was obtained from normal volunteers and diluted 1:1 with phosphate-buffered saline solution. Blood was overlaid on an isotonic Percoll solution (density 1.082 gm/ml) in 50 ml Falcon plastic tubes (Becton Dickinson Labware, Lincoln Park, N.J.), and centrifuged for 30 minutes, 1000 g at 4° C.

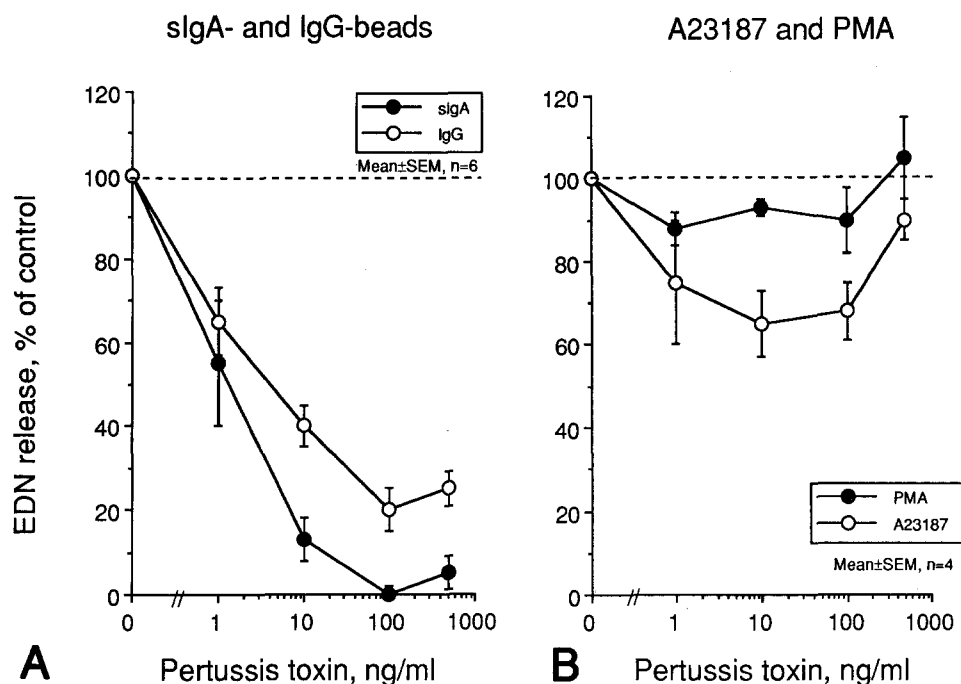


FIG. 1. Effects of PTX on eosinophil degranulation induced by Ig-coated beads and soluble stimuli. Eosinophils were pretreated with the indicated concentrations of PTX for 2 hours and stimulated for 2 hours with Ig-coated beads (**A**) or soluble stimuli (**B**). Results are presented as EDN release, normalized to those obtained with cells pretreated with medium only (no PTX). Data and error bars represent mean $\pm$ SEM for the number of experiments indicated in the figure. (From Kita H, et al. *J Immunol* 1991;147:3466-73. Copyright 1991, The Journal of Immunology.)

The supernatant and the mononuclear cells at the interface were carefully aspirated and the inside wall of the tube was wiped with a sterile gauze to remove mononuclear cells attached to the wall. Erythrocytes in the sediment were lysed twice with water. Isolated granulocytes were washed twice with Pipes buffer (25 mmol/L Pipes, 50 mmol/L NaCl, 5 mmol/L KCl, 25 mmol/L NaOH, 5.4 mmol/L glucose, pH 7.4) with 1% defined calf serum (DCS), and an approximately equal volume of anti-CD16 antibody conjugated with magnetic particles (Miltenyi Biotec, Sunnyvale, Calif.) was added to the cell pellet. After 60 minutes of incubation on ice, 5 ml of Pipes buffer with 1% DCS was added to the cell/antibody mixture. Resuspended cells were loaded onto the separation column positioned in the magnetic cell separation system strong magnetic field. Cells were eluted three times with 5 ml of Pipes buffer with 1% DCS. The purity of eosinophils counted by Randolph's stain was greater than 98%.

Eosinophil degranulation

Various immunoglobulins coupled to cyanogen bromide-activated Sepharose 4B beads (Sigma Diagnostics, St. Louis, Mo.) were used as stimuli for eosinophil degranulation.¹⁹ The beads were coupled to human sIgA (Accurate Chemical & Scientific Corp., Westbury, N.Y.) or human serum IgG (Organon Teknika Corp., Durham, N.C.) at a concentration of 5 mg protein per

milliliter packed beads as described previously. Purified eosinophils were washed twice with Hybri-Care medium containing 50 μ g/ml gentamicin and 0.1% human serum albumin. The washed cells were resuspended at 5×10^5 cells per milliliter in the same medium, and aliquots (100 μ l) of cell suspension were incubated in 96-well round bottom tissue culture plates (Costar Corp., Cambridge, Mass.) with 50 μ l of Hybri-Care medium containing PTX, genistein, or vehicle control for the indicated times. Cellular degranulation was initiated by addition of 50 μ l of medium only, Ig-coated Sepharose bead suspension, or the indicated soluble stimuli. All of the assays were performed in duplicate. After incubation for 2 hours at 37° C in a 5% CO₂ atmosphere, the plates were centrifuged at 1200 g for 10 minutes at 4° C. Cell-free supernatants from each well were collected and frozen at -20° C. EDN released into the cell-free supernatants was quantitated by radioimmunoassay.²² Total cellular EDN contents were measured in parallel supernatants from cells lysed with 0.5% NP-40 detergent.

PLC- γ immunoprecipitation and immunoblotting

Aliquots of eosinophils (1×10^7 cells/sample) were lysed with 1 ml of ice-cold lysis buffer containing 20 mmol/L Tris-HCl, 40 mmol/L NaCl, 5 mmol/L ethylenediamine tetraacetic acid, 50 mmol/L NaF, 30

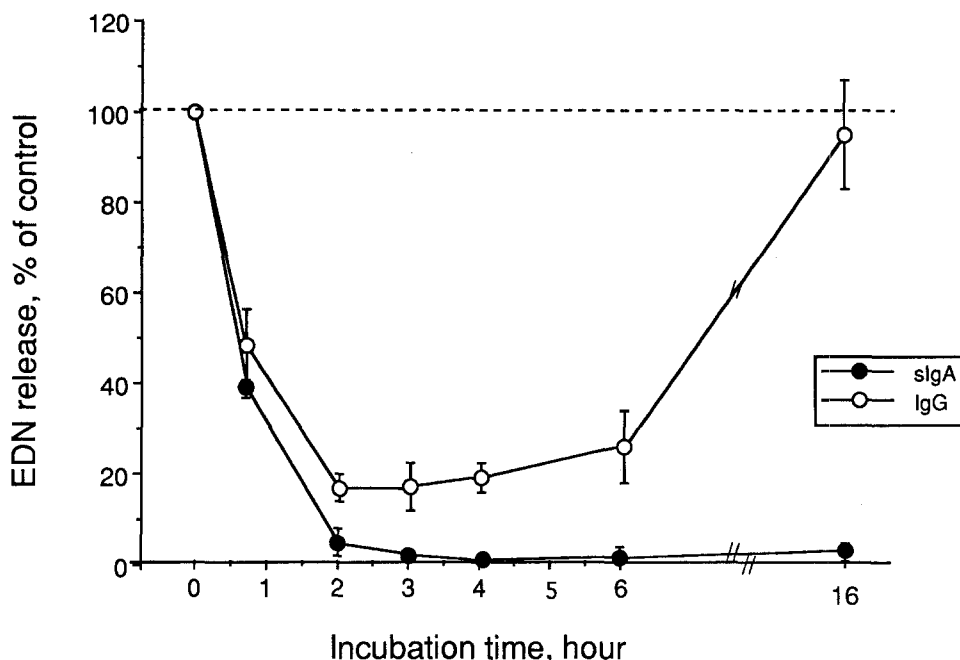


FIG. 2. Kinetic effects of PTX on eosinophil degranulation. Eosinophils were incubated with 100 ng/ml of PTX for the indicated period and stimulated for 2 hours with slgA- (closed circle) or IgG- (open circle) coated beads. Results are presented as EDN release, normalized to those obtained in parallel samples pretreated with medium only (no PTX) before stimulation. Data and error bars represent mean $\pm$ SEM from four experiments. (From Kita H, et al. *J Immunol* 1991;147:3466-73. Copyright 1991, The Journal of Immunology.)

mmol/L $\text{Na}_4\text{P}_2\text{O}_7$, 500 mmol/L Na_3VO_4 , 1 mmol/L phenylmethylsulfonyl fluoride, 5 $\mu\text{g}/\text{ml}$ aprotinin, 10 $\mu\text{g}/\text{ml}$ leupeptin, and 0.5% Triton X-100, pH 7.4. After 10 minutes at 4° C, the samples were centrifuged (15,000 g for 5 minutes) to remove nuclear and cellular debris. The resulting supernatants were immunoprecipitated for 1 hour at 4° C with PLC- γ 1- or PLC- γ 2-specific antisera, and for another hour with protein A-Sepharose beads. The immunoprecipitates were washed three times, and bound proteins were eluted with 50 μl of sodium dodecyl sulfate (SDS) sample buffer and resolved by SDS-polyacrylamide gel electrophoresis (PAGE). The proteins on the SDS-PAGE gels were electronically transferred to nitrocellulose membranes. Nitrocellulose membranes were blocked overnight in Tris-buffered saline solution containing 4% bovine serum albumin, 0.5% Tween 20, and 0.05% NaN_3 , and were then probed for 2 hours with PLC- γ 1- or PLC- γ 2-specific antisera. The membranes were washed three times with 0.2% Tween 20 in Tris-buffered saline solution and immunoreactive proteins were detected with protein A-horseradish peroxidase (Pierce Chemical Company, Rockford, Ill.) and the ECL detection system from Amersham Corp. (Arlington Heights, Ill.).

Inositol phosphate production

Isolated eosinophils (3 to 5×10^6 cells/ml) were cultured overnight in labeling medium (inositol-free RPMI 1640 medium supplemented with 10% DCS)

that contained 40 $\mu\text{Ci}/\text{ml}$ *myo*-[2- ^3H] inositol (NEN Research Products, Boston, Mass.). The cells were treated with PTX for the indicated times before termination of metabolic labeling. The labeled cells were washed and suspended at 1 to 2×10^6 cells per milliliter in labeling medium that contained 10 mmol/L LiCl. When the effect of genistein was studied, cells were preincubated for 1 hour in the presence or absence of genistein. Samples (2×10^5 cells/sample) were preincubated for 15 minutes at 37° C and stimulated for 30 minutes with Ig-coated beads. The final reaction volume was 200 μl . Reactions were terminated with 0.6 ml chloroform:methanol:HCl (1:2:0.02), and water-soluble inositol phosphates were extracted by addition of 0.2 ml of chloroform and 0.2 ml 1 mol/L NaCl. The aqueous phases were removed and diluted with 10 ml of aqueous 30 mmol/L sodium formate–2.5 mmol/L sodium tetraborate. Radiolabeled inositol phosphates were isolated by anion exchange chromatography as described previously.²³ Total inositol phosphates (inositol mono-, bis-, and trisphosphates) were eluted from the anion exchange columns with 2.5 ml of 1.2 mol/L ammonium formate–0.1 mol/L formic acid. ^3H -labeled inositol phosphates were assayed by mixing the column eluates with 20 ml of scintillation cocktail, followed by liquid scintillation counting.

ADP-ribosylation assays

Purified eosinophils (4×10^6 cells/sample) were suspended in assay medium and treated with PTX (10^{-7}

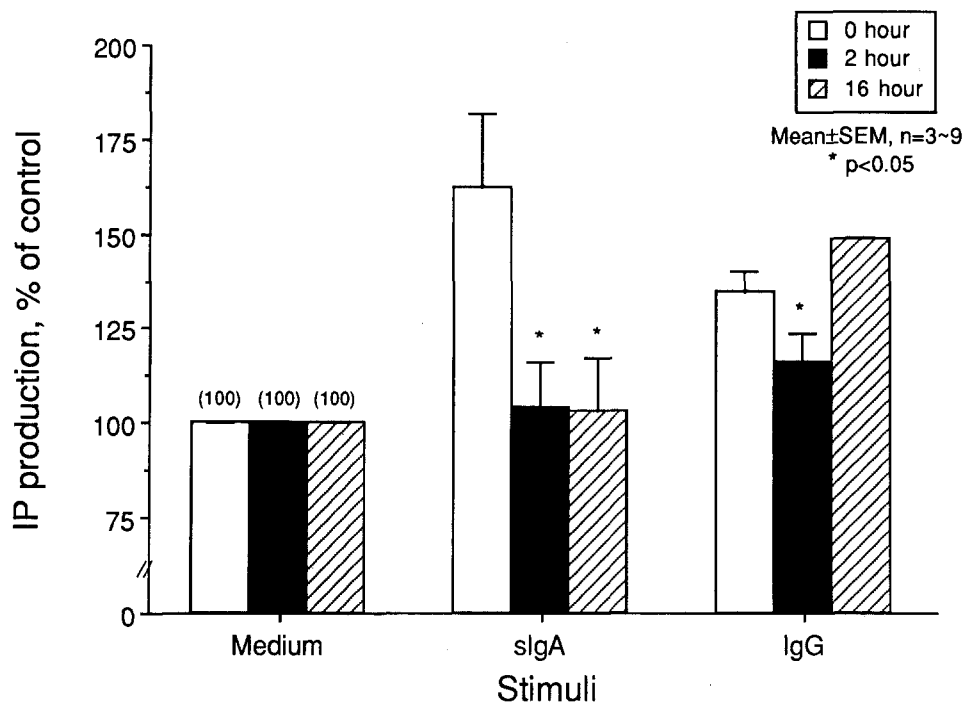


FIG. 3. Effects of PTX on eosinophil inositol phosphate (IP) production. Eosinophils were preincubated with 100 ng/ml of PTX for 0, 2 or 16 hours and stimulated for 30 minutes with medium alone or Ig-coated beads. Data are normalized to the [^{3} H]inositol phosphate cpm measured in unstimulated cells from each pretreatment group: no PTX, 398 ± 118 cpm; 2 hours PTX, 357 ± 114 cpm; and 16 hours PTX, 262 ± 39 cpm. Results from three to nine experiments were presented as mean $\pm$ SEM. Asterisk indicates significant difference ($p < 0.05$) from value obtained with non-PTX treated cells exposed to the same stimulus.

g/ml) or medium for 2 hours. After two washes with phosphate-buffered saline solution containing 0.5 mmol/L ethylenediamine tetraacetic acid (pH 7.2), the cells were suspended in 0.4 ml reaction buffer (100 mmol/L Tris-HCl, pH 7.5, 2 mmol/L $MgCl_2$, 15 mmol/L thymidine, 1 mmol/L adenosine triphosphate, 1 mmol/L guanosine triphosphate, and protease inhibitors).²⁴ The cells were lysed with two 10 second bursts from a sonicator. ADP-ribosylation reactions were initiated by adding 50 μ l of reaction buffer containing 40 μ g/ml preactivated PTX (20 minutes incubation at 37° C in 20 mmol/L dithiothreitol) and 5 mmol/L [^{32} P]NAD (specific activity 20 mCi/mmol) to each sample. PTX was preactivated by a 20-minute incubation (37° C) in 20 mmol/L dithiothreitol. Samples were incubated at 37° C for 30 minutes and reactions were terminated with 0.5 ml of ice-cold 20% (wt/vol) TCA. Denatured proteins were precipitated by centrifugation at 4° C, and the protein pellets were washed with cold 10 mmol/L KH_2PO_4 buffer (pH 2.0). After solubilization in 30 μ l of boiling Laemmli sample buffer, the proteins were separated by SDS-PAGE. Radiolabeled proteins were detected by autoradiography at -70° C.

Antiphosphotyrosine immunoblotting

Aliquots of eosinophils (2×10^6 cells/sample) were resuspended in 50 μ l of RPMI 1640 medium containing

25 mmol/L Hepes, pH 7.2. Cells were stimulated by adding 50 μ l of Ig-coated beads (5×10^4 /sample) at 37° C in a microcentrifuge tube. After the indicated time, cells were centrifuged briefly (15,000 g for 15 seconds), and cell pellets were lysed by the addition of 75 μ l of ice-cold lysis buffer (see previous description of PLC- γ immunoprecipitation and immunoblotting). After 10 minutes at 4° C, the samples were centrifuged (15,000 g for 5 minutes) to remove nuclear and cellular debris. Postnuclear supernatants (60 μ l) were mixed with 4 $\times$ Laemmli sample buffer and resolved by SDS-PAGE. Immunoblotting was performed as described earlier except that antiphosphotyrosine monoclonal antibody (4CG10) and peroxidase conjugated rabbit anti-mouse IgG (p260) were used as first and second antibody, respectively.

RESULTS

Stimulus-dependent eosinophil degranulation

We reported previously that sIgA or IgG coupled to Sepharose beads were effective stimuli for the release of EDN from human eosinophils (Table I). The degranulation response induced by sIgA beads was nearly fourfold higher than that evoked by IgG beads. IgA beads also caused

eosinophil degranulation, although the amount of released EDN was less than that released by IgG beads. In contrast, beads coated with IgE, IgM, or IgD did not induce eosinophil degranulation. Calcium ionophore A23187 and the protein kinase C activator PMA also triggered cellular EDN release. Calcium ionophore, ionomycin, and PMA synergistically induced both EDN release and leukotriene C4 production from eosinophils, suggesting important roles for increased $[Ca^{2+}]_i$ and increased activity of protein kinase C in the degranulation of eosinophils (data not shown).

Effect of PTX on eosinophil degranulation

PTX catalyzes the ADP-ribosylation of certain G protein α -subunits, resulting in the uncoupling of these G proteins from receptors. To examine the potential involvement of a PTX-sensitive G protein(s) in eosinophil degranulation, eosinophils were pretreated with PTX for 2 hours before stimulation with sIgA- or IgG-coated beads. As shown in Fig. 1, *A*, eosinophil responsiveness to sIgA beads was abolished by pretreatment of cells with PTX. Eosinophil degranulation induced by IgG was also inhibited by PTX pretreatment, although the degree of inhibition was at most 80%. Calcium ionophore A23187 and PMA increase $[Ca^{2+}]_i$ and activate protein kinase C, respectively, independent of cell surface receptor stimulation. Hence the eosinophil degranulation induced by A23187 and PMA should not exhibit sensitivity to G protein modification by PTX. As shown in Fig. 1, *B*, degranulation induced by PMA was unaffected by pretreatment of eosinophils with PTX. On the other hand, A23187, which potently induced eosinophil degranulation (Table 1), consistently showed partial sensitivity to PTX (Fig. 1, *B*). Although the underlying mechanism remains unclear, we speculate that A23187-induced eosinophil degranulation may be partially stimulated by de novo synthesized mediators, such as platelet-activating factor (PAF). It is known that eosinophils can synthesize PAF in response to calcium ionophore,²⁵ and that the PAF receptor is coupled to PTX-sensitive G protein.²⁶

The eosinophil response to PTX shows interesting kinetics. As shown in Fig. 2, the degranulation response to sIgA-coated beads was progressively reduced as the treatment with PTX was increased from 0.5 to 3 hours. The decreased degranulation with longer PTX treatment was consistent with the cumulative modification and inactivation of a critical intermediate G protein(s)

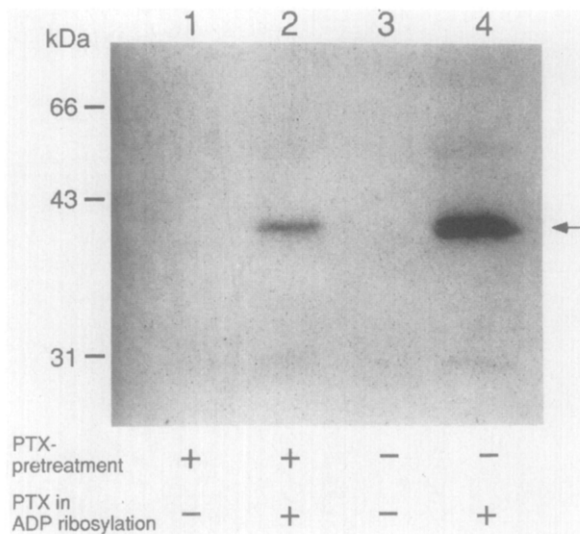


FIG. 4. ADP-ribosylation of eosinophil membrane proteins catalyzed by PTX. Eosinophils were pretreated with medium alone or 100 ng/ml of PTX for 2 hours. After the cell lysis, cellular proteins were ADP-ribosylated in vitro in the presence or absence of activated PTX and $[^{32}P]$ NAD (see description in Material and Methods section). Samples were analyzed with SDS-PAGE, and ^{32}P -labeled protein was visualized by autoradiography. Arrow denotes position of 41 kd PTX substrate.

by the ADP-ribosyltransferase activity of the PTX catalytic subunit. Eosinophils remained nonresponsive to sIgA beads after continuous exposure to PTX for 16 hours. The release of EDN induced by IgG-coated beads was also markedly reduced after 0.5 to 2 hours of PTX treatment (Fig. 2). However, this early inhibitory effect of PTX treatment was reversible, because the eosinophils gradually recovered their responsiveness to IgG beads during the subsequent 14 hours of continuous exposure to PTX.

Stimulation of phosphoinositide hydrolysis by Ig-coated beads

In many cell types, stimulus-dependent exocytotic responses are accompanied by increases in PLC activity and phosphoinositide turnover.³⁻⁵ To investigate whether Ig-dependent stimulation of eosinophils activates PLC activity, we first examined whether PLC- γ 1 or its closely related isoform PLC- γ 2 are expressed in human eosinophils. Immunoprecipitation and immunoblotting with anti-PLC- γ 1 and anti-PLC- γ 2 detected the 140 kd form of PLC- γ 2, but not PLC- γ 1, from eosinophil lysates (data not shown). These same antibodies could detect large amounts of PLC- γ 1 and lesser amounts of PLC- γ 2 from Jurkat cells (a human leukemic T cell line).¹⁸ As shown in Fig. 3, stimula-

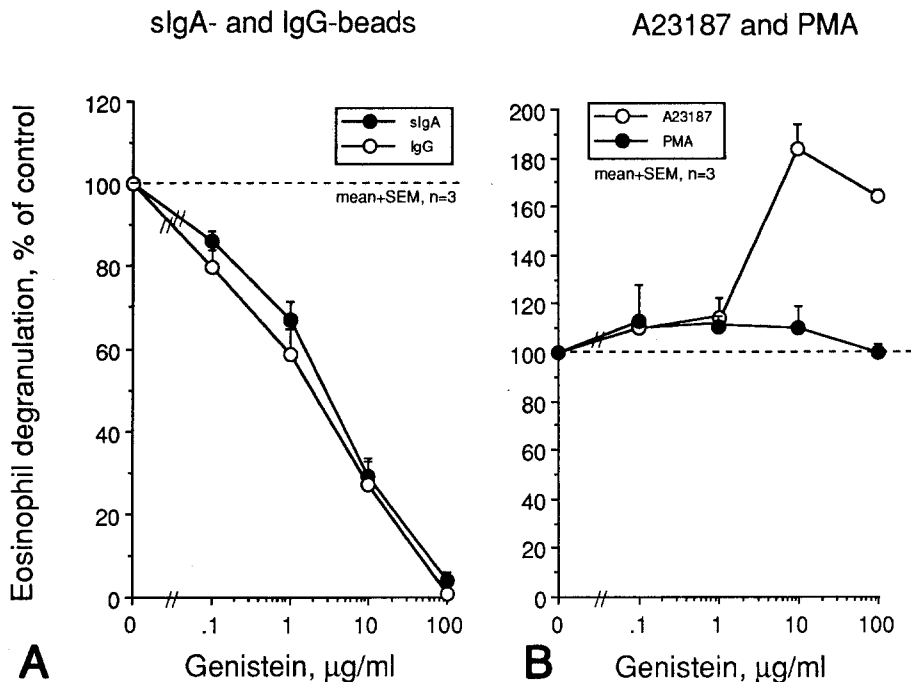


FIG. 5. Effects of genistein on eosinophil degranulation induced by Ig-coated beads and soluble stimuli. Eosinophils were pretreated with the indicated concentrations of genistein for 1 hour and stimulated for 2 hours with Ig-coated beads (**A**) or soluble stimuli (**B**). Results are presented as EDN release, normalized to those obtained with cells pretreated with medium only (no genistein) before stimulation: medium, 12.3 ± 5.0 ; sIgA beads, 256.0 ± 56.3 ; IgG beads, 115.4 ± 23.4 ; A23187, 342.6 ± 87.5 ; and PMA, 201.8 ± 116.9 ng/ 2.5×10^5 cells (mean $\pm$ SEM, $n = 3$). Data and error bars represent mean $\pm$ SEM from three experiments.

tion of *myo*[^3H]inositol-labeled eosinophils with sIgA or IgG beads for 30 minutes induced significant increases in total inositol phosphate accumulation, which suggests that Ig-mediated signals, at least in part, are coupled to phosphoinositide-specific PLC activity. Pretreatment of eosinophils with PTX for 2 hours or 16 hours virtually abolished the production of inositol phosphate induced by sIgA-coated beads. The increase in inositol phosphate production stimulated by IgG-coated beads was inhibited in eosinophils exposed to PTX for 2 hours. However, the cells completely recovered responsiveness to immobilized IgG during the 16 hours of continuous exposure to PTX.

PTX-mediated ADP-ribosylation of eosinophil proteins

PTX exhibits ADP-ribosyltransferase activity toward the α -subunit of certain G proteins.⁹ To define the PTX substrate on eosinophils, cell lysates were incubated with activated PTX in the presence of [^{32}P]NAD. As shown in Fig. 4, exposure of eosinophil lysates to activated PTX in vitro resulted in the transfer of [^{32}P]ADP-ribose to a prominent 41 kd protein substrate. The

ADP-ribosylation of this protein was absolutely dependent on the presence of PTX in the in vitro reaction mixture. Pretreatment of intact eosinophils with PTX for 2 hours before ADP-ribosylation of cellular proteins in vitro led to inhibition of the radiolabeling of the 41 kd protein. These findings suggest that this 41 kd protein is the major substrate for PTX in human eosinophils.

Stimulation of eosinophils with Ig beads increases the tyrosine phosphorylation of proteins: This effect was blocked by genistein

In many cell types, occupancy of receptor by ligand induces activation of tyrosine kinases and subsequent tyrosine phosphorylation of various proteins.¹²⁻¹⁵ To investigate whether Ig-dependent stimulation of eosinophils activates PTK, we stimulated eosinophils with Ig beads, and eosinophil lysates were analyzed by immunoblot with use of antiphosphotyrosine antibody. When eosinophils were stimulated by sIgA beads, a rapid increase (9 minutes) in protein tyrosine phosphorylation was detected. The proteins of 115, 100, 70, 65, and 50 kd were most distinct. The continuous

exposure of eosinophils to sIgA beads resulted in dephosphorylation of these proteins. In contrast, when eosinophils were stimulated with IgG beads, the progress of tyrosine phosphorylation was much slower (maximum at 30 minutes) than that induced by sIgA beads. Stimulation of sIgA and IgG produced similar numbers and molecular weights of tyrosine phosphorylated proteins (results not shown).

We next examined the sensitivity of Ig-stimulated tyrosine phosphorylation to an inhibitor of PTK, genistein.¹⁸ Genistein is a tyrosine-specific kinase inhibitor with an ID₅₀ of 6 to 7 µg/ml; genistein scarcely inhibits the enzymatic activities of serine- and threonine-specific protein kinases, such as cyclic adenosine monophosphate-dependent protein kinase and protein kinase C.²⁷ The pretreatment of eosinophils for 1 hour with genistein completely blocked tyrosine phosphorylation of various proteins in eosinophils elicited by sIgA and IgG beads. The pretreatment of eosinophils with genistein did not affect cell viability and left the non-PTK-dependent cellular response intact (see discussion to follow).

Inhibition of tyrosine phosphorylation blocks Ig-stimulated inositol phosphate production and degranulation of eosinophils

Recent studies suggested that receptors can be coupled to PLC via PTK-dependent pathways.¹⁶ Because stimulation of eosinophils with Ig beads resulted in the tyrosine phosphorylation of eosinophil cellular proteins, we proceeded to evaluate whether these signaling events influenced PLC activation. Eosinophils were preincubated with genistein for 1 hour, and inositol phosphate production was then quantitated after Ig stimulation. Whereas both sIgA and IgG beads increased inositol phosphate production from eosinophils, this Ig-stimulated inositol phosphate production was completely inhibited by 50 µg/ml of genistein to the base levels.

To further evaluate the role of protein tyrosine phosphorylation in the effector function of eosinophils, we examined the effects of genistein on EDN release from eosinophils. Pretreatment of eosinophils with genistein inhibited both sIgA- and IgG-induced degranulation in a concentration-dependent manner (Fig. 5). The ID₅₀ of genistein for Ig-induced degranulation was 3 µg/ml, which is a comparable ID₅₀ value of genistein required to inhibit tyrosine-specific protein kinases. An experiment in which another tyrosine kinase inhibitor, herbimycin A, was used also demonstrated concentration dependent inhi-

bition of Ig-induced eosinophil degranulation (data not shown). In contrast, when PMA was used to stimulate eosinophils, the degranulation was not affected by genistein. These findings suggest genistein specifically inhibited Ig-mediated degranulation of eosinophils and that the action of genistein is not the result of nonspecific inhibition of eosinophil function.

DISCUSSION

PTX pretreatment markedly reduced the responsiveness of human eosinophils to sIgA beads. The increase in phosphoinositide hydrolysis and subsequent EDN release were inhibited by PTX in a concentration-dependent manner. A patch-clamp study by Nüsse et al.²⁸ demonstrates that a nonhydrolyzable guanosine triphosphate analog stimulated fusion of cytoplasmic granules with plasma membranes of guinea pig eosinophils, which suggests that a G protein-dependent mechanism is involved in the degranulation mechanism of eosinophils. Collectively, these data are consistent with the possibility that ADP-ribosylation of G protein(s) by PTX inactivates the coupling of sIgA receptors and the degranulation mechanism. The ADP-ribosyltransferase activity of PTX is relatively specific to the α -subunit of G_i or G_o heterotrimers.^{9, 10} The modification of these G proteins by PTX is known to disrupt the coupling of cell surface receptors to intracellular effector enzymes, including PLC.¹⁰ Compelling evidence suggests that the neutrophil FMLP receptor is coupled to PLC through a G_i-like protein.¹⁰ The 41 kd protein is the major PTX substrate in neutrophils and is identified as the α -subunit of the G_{i2} heterotrimer.¹¹ In the present study, we also detected a 41 kd major PTX substrate in human eosinophils. The biochemical and effector functions of eosinophils, including inositol phosphate production and degranulation, were also progressively inhibited by PTX. Therefore, we propose that a 41 kd substrate in eosinophils represents a PTX-sensitive G protein α -subunit (possibly G_{i2}- α) involved in the transduction of the activation signal from sIgA receptors on eosinophils.

Unexpectedly, pretreatment of eosinophils with PTX for various periods revealed a biphasic alteration in eosinophil responsiveness to IgG beads. Maximal inhibitions of IgG-induced degranulation and inositol phosphate production were observed with cells treated with PTX for 2 hours. When the time of PTX treatment was extended to 16 hours, both degranulation and inositol phosphate production induced by IgG beads returned to levels seen in untreated cells.

The reversibility of the inhibitory effect of PTX on cellular responsiveness to IgG beads clearly differentiated the postreceptor signaling mechanism initiated by sIgA beads. The most tenable explanation for these results is that receptors for IgG on eosinophils transduce signals through a PTX-sensitive G protein distinct from that used for sIgA signaling. This hypothesis is supported by the *in vitro* ADP-ribosylation experiments. Eosinophils contained a minor 44 kD PTX substrate in addition to the major 41 kD substrate.²⁹ The 44 kD protein could be particularly important for IgG signaling because treatment of intact eosinophils with PTX resulted in a biphasic change in the unmodified protein. Prior exposure of eosinophils to PTX for 2 hours virtually abolished the *in vitro* ADP-ribosylation of the 44 kD protein; the expression of unmodified 44 kD protein and *in vitro* ADP-ribosylation of this protein recovered with longer exposure of eosinophils to PTX.²⁹ The decline and recovery of this unmodified 44 kD protein closely parallel the cellular responsiveness to IgG beads, as shown by inositol phosphate production, and EDN release. Although definitive biochemical evidence is not available, these results are compatible with the hypothesis that the 44 kD PTX substrate represents an α -subunit of heterotrimeric G proteins responsible for signal transduction induced by IgG beads. However, we need to keep in mind that the present data are also compatible with the potential involvement of both PTX-sensitive and PTX-nonsensitive G proteins in the eosinophil response to IgG beads.

Treatment of eosinophils with a protein kinase inhibitor markedly reduced the responsiveness of human eosinophils to sIgA and IgG beads. The increase in phosphoinositide hydrolysis and subsequent EDN release were inhibited by genistein in a concentration-dependent manner. The ID₅₀ of genistein required for the inhibition of Ig response was comparable with the reported ID₅₀ value of genistein as a tyrosine-specific kinase inhibitor. Although little is known about the role of tyrosine kinases for the activation of human eosinophils, we could detect several tyrosine phosphorylated proteins after *in vitro* stimulation of eosinophils with sIgA and IgG beads. Tyrosine phosphorylation of these proteins was also inhibited by genistein. Collectively, these data are consistent with the possibility that inhibition of tyrosine phosphorylation by genistein inactivates the coupling of Ig receptors and the degranulation mechanism.

The present study provides a tentative model for the sequence of events that culminate in the

degranulation mechanisms of Ig activated eosinophils. sIgA or IgG bind to distinct surface receptors expressed by eosinophils. Ligation of these receptors activates at least two receptor-coupled G proteins that, in turn, stimulate phosphoinositide-specific PLC activity and EDN release from eosinophils. Tyrosine kinases are also probably involved in this receptor-PLC coupling. Because the effects of PTX and genistein on PLC activation induced by sIgA beads are complete, we can speculate that G protein and protein kinase exist in series, but not in parallel, for signal relay from the membrane receptor to PLC activity. Studies with other types of cells suggest that the enzymes included in inositol phospholipid metabolism, which is highly regulated by G proteins, can be targets of phosphorylation by activated src gene products.³⁰ Although a more detailed understanding of the stimulus-response coupling mechanisms involved in eosinophil activation is clearly required, our study indicates important roles for PLC-coupled G protein and for tyrosine kinases for the degranulation of human eosinophils.

SUMMARY

Eosinophils are implicated as major inflammatory cells in parasite infection and allergic reactions. Among various mediators, eosinophil granule cationic proteins play an important role in the pathophysiology of diseases. However, little is known about the actual physiologic stimuli for eosinophil degranulation and the signaling events for triggering eosinophil degranulation. A series of *in vivo* and *in vitro* studies suggest that the interaction between antibody coated parasites and Fc receptors on eosinophils is one of the most effective triggers for eosinophil degranulation. Similarly, eosinophil degranulation can be induced *in vitro* by Sepharose beads coated with human sIgA or IgG. Eosinophil degranulation induced by these stimuli is mediated by PTX-sensitive membrane-bound heterotrimeric G protein(s), and is accompanied by the rapid turnover of inositol phosphates. The production of inositol phosphates is inhibited by PTX. Eosinophil activation by sIgA and IgG also involves tyrosine phosphorylation of several proteins and is inhibited by tyrosine kinase inhibitors. Thus phospholipase C-coupled G protein(s) and tyrosine kinases are key molecules in early signal transduction of eosinophil activation induced by sIgA and IgG. Although further studies are needed to identify which tyrosine kinase(s) is specifically involved in the eosinophil degranulation mecha-

nism, these molecules could be a target for therapies of human diseases where eosinophils are involved.

REFERENCES

- Gleich GJ, Adolphson CR, Leiferman KM. The eosinophil. In: Gallin JI, Goldstein IM, Snyderman R, eds. *Inflammation: Basic principles and clinical correlates*. 2nd ed. New York: Raven Press, 1992:663-700.
- Butterworth AE, Remold HG, Houba V, et al. Antibody-dependent eosinophil-mediated damage to ^{51}Cr -labeled schistosomula of *Schistosoma mansoni*: mediation by IgG, and inhibition by antigen-antibody complexes. *J Immunol* 1977;118:2230-6.
- Cockcroft S. G-protein-regulated phospholipases C, D and A_2 -mediated signaling in neutrophils. *Biochim Biophys Acta* 1992;1113:135-60.
- Abdel-Latif AA. Calcium-mobilizing receptors, polyphosphoinositides, and the generation of second messengers. *Pharmacol Rev* 1986;38:227-72.
- Ishizaka T. Role of GTP-binding protein in histamine release from mast cells. *Clin Immunol Immunopathol* 1989;50:20-9.
- Sha'afi RI, Molski TFP. Activation of the neutrophil. *Prog Allergy* 1988;42:1-64.
- Lapetina EG. Inositide-dependent and independent mechanisms in platelet activation. In: Putney JW Jr., ed. *Phosphoinositide and receptor mechanisms*. New York: Alan R. Liss, 1986:271-86.
- Gilman AG. G proteins: transducers of receptor-generated signals. *Ann Rev Biochem* 1987;56:615-49.
- Bokoch GM, Katada T, Northup JK, Hewlett EL, Gilman AG. Identification of the predominant substrate for ADP-ribosylation by islet activating protein. *J Biol Chem* 1983;258:2072-5.
- Okajima F, Katada T, Ui M. Coupling of the guanine nucleotide regulatory protein to chemotactic peptide receptors in neutrophil membranes and its uncoupling by islet-activating protein, pertussis toxin: a possible role of the toxin substrate in Ca^{2+} -mobilizing receptor-mediated signal transduction. *J Biol Chem* 1985;260:6761-8.
- Snyderman R, Perianin A, Evans T, Polakis P, Didsbury J. G proteins and neutrophil function. In: Moss J, Vaughan M, eds. *ADP-ribosylating toxin and G-proteins*. Washington, D.C.: American Society for Microbiology, 1990:295-323.
- Klausner RD, Samuelson LE. T cell antigen receptor activation pathways: the tyrosine kinase connection. *Cell* 1991;64:875-8.
- Connelly PA, Farrell CA, Merenda JM, Conklyn MJ, Showell HJ. Tyrosine phosphorylation is an early signaling event common to Fc receptor crosslinking in human neutrophils and rat basophilic leukemia cells (RBL-2H3). *Biochem Biophys Res Commun* 1991;177:192-201.
- Huang M-M, Indik Z, Brass LF, Hoxie JA, Schreiber AD, Brugge JS. Activation of $\text{Fc}\gamma\text{RII}$ induces tyrosine phosphorylation of multiple proteins including $\text{Fc}\gamma\text{II}$. *J Biol Chem* 1992;267:5467-73.
- Einspahr KJ, Abraham RT, Binstadt BA, Uehara Y, Leibson PJ. Tyrosine phosphorylation provides an early and requisite signal for the activation of natural killer cell cytotoxic function. *Proc Natl Acad Sci USA* 1991;88:6279-83.
- Meisenhelder J, Suh P-G, Rhee SG, Hunter T. Phospholipase C- γ is a substrate for the PDGF and EGF receptor protein-tyrosine kinases in vivo and in vitro. *Cell* 1989;57:1109-22.
- Weiss A, Koretzky G, Schatzman RC, Kadlecsek T. Functional activation of the T-cell antigen receptor induces tyrosine phosphorylation of phospholipase C- γ 1. *Proc Natl Acad Sci USA* 1991;88:5484-8.
- Ting AT, Karnitz LM, Schoon RA, Abraham RT, Leibson PF. $\text{Fc}\gamma$ receptor activation induces the tyrosine phosphorylation of both phospholipase C (PLC)- γ 1 and PLC- γ 2 in natural killer cells. *J Exp Med* 1992;176:1751-5.
- Abu-Ghazaleh RI, Fujisawa T, Mestecky J, Kyle RA, Gleich GJ. IgA-induced eosinophil degranulation. *J Immunol* 1989;142:2393-2400.
- Tomassini M, Tsiocopoulos A, Tai PC, et al. Release of granule proteins by eosinophils from allergic and nonallergic patients with eosinophilia on immunoglobulin-dependent activation. *J ALLERGY CLIN IMMUNOL* 1991;88:365-75.
- Ide M, Weiler D, Kita H, Gleich GJ. Ammonium chloride exposure inhibits cytokine-mediated eosinophil survival. *J Immunol Methods* 1994;168:187-96.
- Ackerman SJ, Loegering DA, Venge O, Olsson I, Harley JB, Fauci AS, Gleich GJ. Distinctive cationic proteins of the human eosinophil granule: major basic protein, eosinophil cationic protein, and eosinophil-derived neurotoxin. *J Immunol* 1983;131:2977-82.
- Downes CP, Michell RH. The polyphosphoinositide phosphodiesterase of erythrocyte membranes. *Biochem J* 1981;198:133-40.
- Milligan G. Techniques used in the identification and analysis of function of pertussis-toxin sensitive guanine nucleotide binding proteins. *Biochem J* 1988;255:1-13.
- Asmis R, Jorg A. Calcium ionophore-induced formation of platelet activating factor and leukotrienes by horse eosinophils: a comparative study. *Eur J Biochem* 1990;187:475-80.
- Honda Z-I, Nakamura M, Miki I, Minami M, Watanabe T, Seyama Y, Okado H, Toh H, Ito K, Miyamoto T, Shimazu T. Cloning by functional expression of platelet-activating factor receptor from guinea-pig lung. *Nature* 1991;349:342-6.
- Akiyama T, Ishida J, Nakagawa S, et al. Genistein, a specific inhibitor of tyrosine-specific protein kinases. *J Biol Chem* 1987;262:5592-5.
- Nüsse O, Lindau M, Cromwell O, Kay AB, Gomperts BD. Intracellular application of guanosine-5'-O-(3-thiotriphosphate) induces exocytotic granule fusion in guinea pig eosinophils. *J Exp Med* 1990;171:775-86.
- Kita H, Abu-Ghazaleh RI, Gleich GJ, Abraham RT. Role of pertussis toxin-sensitive G proteins in stimulus-dependent human eosinophil degranulation. *J Immunol* 1991;147:3466-73.
- Cantley LC, Augar KR, Carpenter C, et al. Oncogenes and signal transduction. *Cell* 1991;64:281-302.