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**Research Article**

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# Regulation of Human Eosinophil Degranulation and Activation by Endogenous Phospholipase A$_2$

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## Abstract

**The unique granular proteins of eosinophils may have a pathogenetic role in asthma and in the defense against parasitic infestations. However, the mechanisms regulating eosinophil degranulation are largely unknown. We examined the hypothesis that release of these proteins is regulated by endogenous activation of phospholipase A$_2$. Human eosinophils (HE) were isolated from the peripheral blood of 42 subjects either by Percoll density separation or by negative-selection immunomagnetic fractionation. Eosinophil activation was initiated in vitro with $10^{-6}$ M FMLP and 5 $\mu$g/ml cytochalasin B and was assessed by measurement of eosinophil peroxidase (EPO), leukotriene C$_4$ (LTC$_4$) and superoxide radical ($^{\bullet}$O$_2^-$) secretion. Treatment of HE with 100 $\mu$M mepacrine before activation blocked EPO release (2.0±0.2 vs 10.2±2.1% cell content for activated HE, $P$ < 0.004, $n$ = 9), $^{\bullet}$O$_2^-$ generation (2.6±0.9 vs 44.2±10.8 nmol/ml per $10^6$ HE, $P$ < 0.002, $n$ = 5), and LTC$_4$ secretion (68.2±32.2 vs 1,125.2±526.8 pg/ml per $10^6$ HE, $P$ < 0.04, $n$ = 8). Pretreatment of HE with 100 $\mu$M 4-bromophenacyl bromide before activation similarly blocked EPO release, $^{\bullet}$O$_2^-$ generation and LTC$_4$ secretion. Addition of AA to HE after treatment with 100 $\mu$M mepacrine and before subsequent activation reversed the inhibition of both EPO (10.4±2.2% with 1 $\mu$M AA vs 2.0±0.2% for mepacrine, $n$ = 5, $P$ < 0.02) and LTC$_4$ secretion (695.1±412.9 with 10 $\mu$M AA vs 68.2±32.2 pg/ml per $10^6$ HE for mepacrine, $n$ = 8, $P$ < 0.04), but did not reverse inhibition of $^{\bullet}$O$_2^-$ generation by mepacrine. We demonstrate that secretion of preformed cytotoxic proteins and $^{\bullet}$O$_2^-$ by eosinophils is regulated endogenously by phospholipase A$_2$. (*J. Clin. Invest.* 1993. 91:2118–2125.) Key words: eosinophils • phospholipase A$_2$ • eosinophil peroxidase • leukotrienes • mepacrine**

## Introduction

Human eosinophils may have a prominent role in the defense against parasitic infection and in the alterations of airway function in asthma (1). Eosinophil infiltration of airways is a characteristic feature of asthma, and the presence of blood, bronchoalveolar lavage fluid, and tissue eosinophilia correlates to the severity of the disease (2–5). Eosinophil activation and secretion of both lipid and protein inflammatory mediators, such as platelet-activating factor (6), leukotriene C$_4$ (LTC$_4$)[1] (7, 8), major basic protein (9, 10), eosinophil cationic protein (11), eosinophil-derived neurotoxin (11), and eosinophil peroxidase (EPO) (11, 12), have profound effects on the structure and function of airway cells, including the alteration of airway smooth muscle tone and damage to airway epithelial cells. However, the mechanisms by which eosinophils degranulate and release preformed protein mediators into the external environment has not been defined completely.

Phospholipase A$_2$ (EC3.1.1.4, PLA$_2$), a lipolytic enzyme that catalyzes the hydrolysis of a glycerol phosphatide to yield a lysophosphatide and AA, is an ubiquitous enzyme that exists in both cell membranes (13) and lysosomes (14, 15). Recently, PLA$_2$ release from eosinophils has been described in association with release of cationic protein granules from eosinophils (16), suggesting that this enzyme may be a part of either the granular matrix or the membrane of the granule. However, the function of PLA$_2$ in the granule is unknown, and the role of this enzyme in the regulation of eosinophil degranulation has not been defined.

The objective of this study was to determine if inhibition of endogenous eosinophil PLA$_2$ alters the release of granular protein mediators from activated eosinophils. We used two different inhibitors of PLA$_2$ function: mepacrine, which forms stable complexes of drug and phospholipid substrate (17, 18), or 4-bromophenacyl bromide (BPB), which modifies an active site histidine residue in PLA$_2$ (19). We isolated eosinophils from peripheral blood of normal volunteers and determined EPO release, LTC$_4$ secretion and superoxide radical ($^{\bullet}$O$_2^-$) generation after incubation with either mepacrine or BPB. We demonstrate that (*a*) inhibition of endogenous PLA$_2$ blocks eosinophil degranulation and release of EPO, secretion of LTC$_4$, generation of $^{\bullet}$O$_2^-$, and (*b*) addition of exogenous AA, a principal product of the hydrolytic reaction catalyzed by PLA$_2$, reverses the inhibition of eosinophil degranulation and secretion of LTC$_4$, suggesting that both processes may be dependent upon endogenous AA generated by PLA$_2$. These data suggest a mechanism by which products of PLA$_2$ may regulate degranulation and secretion of inflammatory mediators in human eosinophils.

## Methods

*Isolation of human eosinophils.* Human eosinophils were isolated from volunteers according to a protocol approved by the University of Chi-

1. *Abbreviations used in this paper:* BPB, 4-bromophenacyl bromide; CYB, cytochalasin B; EPO, eosinophil peroxidase; GEL, 0.1% gelatin in HBSS containing 1 mmol/liter Ca$^{2+}$; LTC$_4$, leukotriene C$_4$; PLA$_2$, phospholipase A$_2$.

cago Institutional Review Board. Informed consent was obtained from all volunteers in this study before participation. To ensure that the method of isolation was not a factor in these experiments, two different isolation methods were used.

In the first method, eosinophils were isolated by density fractionation centrifugation (20, 21). Whole blood (180 ml) was withdrawn from the antecubital vein of 17 human volunteers and placed into containers containing 2 ml of 1:1,000 heparin. Blood then was mixed 5:1 with 4.5% dextran in 0.9% NaCl and sedimented for 45 min at 22°C. The white blood cell layer was collected, divided into 30-ml aliquots, and layered over 10 ml Ficoll-Hypaque (density 1.077 g/ml) in 50-ml tubes. Cells then were centrifuged at 400 $g$ for 20 min (all centrifugations done at 22°C). The pellet containing leukocytes was resuspended in $Ca^{2+}$-free HBSS and twice washed and centrifuged at 400 $g$ for 10 min. Cells then were suspended in HBSS containing 1 mmol/liter $Ca^{2+}$ and 5% FCS and diluted to a concentration of 2 $\times$ $10^7$/ml. In separate 15-ml tubes discontinuous Percoll gradients were prepared (from top to bottom in g/ml [ml]: 1.080 [2.5]; 1.085 [2.5]; 1.090 [3.0]; 1.095 [3.0]; 1.100 [1.5], prepared in HBSS), over which 2 ml of cell suspension was layered. Gradients then were centrifuged at 700 $g$ for 20 min. The interface containing eosinophils (1.095–1.100 g/ml) was collected, diluted in 0.1% gelatin in HBSS containing 1 mmol/liter $Ca^{2+}$ (GEL) and then centrifuged at 400 $g$ for 10 min. Eosinophils then were washed, centrifuged at 400 $g$ for 10 min and resuspended in GEL. Eosinophils were counted and assessed for purity by Wright stain. Eosinophils were kept on ice until use and used within 1 h of final purification to prevent deterioration.

In the second method, eosinophils were isolated by negative-selection immunomagnetic fractionation using an antibody to CD16, a $FcR_{III}$ receptor found on neutrophils but not eosinophils (22). In 22 additional human volunteers, 60–120 ml whole blood was withdrawn from the antecubital vein and placed into containers containing 2 ml of 1:1,000 heparin. Blood was diluted 1:1 with $Ca^{2+}$-free HBSS, layered over 15 ml of 1.083 g/ml Percoll and centrifugated for 30 min at 900 $g$ (all centrifugations done at 4°C). Cells then were diluted 1:4 with an erythrocyte-lysing solution containing 155 mM $NH_4Cl$, 10 mM KHCO, and 0.1 mM EDTA for 15 min. The remaining granulocytes were centrifugated for 10 min at 400 $g$ and resuspended in 25 $\mu$l of 2% FCS in $Ca^{2+}$-free HBSS; 75 $\mu$l of anti–human CD-16 monoclonal antibody bound to 0.1-$\mu$m magnetic beads (Miltenyi Biotec GmbH, Bergisch-Gladbach, Federal Republic of Germany) then was added. Granulocytes were incubated at 4°C for 45 min and then resuspended in 4 ml of 2% FCS in $Ca^{2+}$-free HBSS. Granulocytes then were passed through a 1 $\times$ 10 cm column packed with steel wool and held within a 0.6 Tesla MACS magnet (Becton Dickinson, Mountain View, CA) over 30 min. Neutrophils binding the antibody-magnetic bead were retained in the magnetized steel wool, while eosinophils passing through the column were collected, washed, and resuspended in GEL. Counts and purity were assessed as above. Cells were kept on ice until use and used within 1 h of final purification as above.

*Determination of eosinophil peroxidase concentrations in eosinophils and eosinophil-conditioned media.* The EPO concentration in cells and conditioned media was measured as the velocity of maximal reaction of oxidation of o-phenylenediamine using a kinetic assay that we have developed previously for these studies (20). Eosinophil cell suspensions, eosinophil-conditioned medium, and purified EPO (mol wt $\sim$ 70,000) were suspended in GEL and frozen at $-70$°C until immediately before use. For the assay, 50 $\mu$l of sample or EPO standard were combined with 75 $\mu$l of substrate (final concentrations: 0.01% hydrogen peroxide, 6 mM o-phenylenediamine, and 0.06% Triton X-100 dissolved in 60 mM Tris, pH 8.0) in a polystyrene 96-well microplate and placed into a thermoregulating microplate absorbance spectrophotometer (Thermomax; Molecular Devices Corp., Menlo Park, CA) at 37°C. Absorbance at 492 nm was measured every 6 s for 3 min; the maximal velocity of the reaction was calculated by interpolation between successive three points (18 s) using customized software (Softmax v2.01; Molecular Devices Corp.) on a Macintosh computer, and compared to EPO standards generated at the same time. Standards

were assayed in triplicate; suspended cell and conditioned media samples were assayed in duplicate. Eosinophil cell suspensions were diluted 1:4 with HBSS containing 1 mmol/liter $Ca^{2+}$ immediately before assay. Final EPO concentrations then were calculated from standard curves fitted by four-parameter (iterative log-logit) analysis, and expressed finally as percent total EPO content of the eosinophils (medium/[medium + cell pellet]) in conditioned media (20).

*Determination of $LTC_4$ concentrations in eosinophil-conditioned media by enzyme-linked immunosorbent assay.* This technique offers exceptional sensitivity ($<$ 20 pg/ml at 80% $B/B_0$) and specificity (46% cross-reactivity with leukotriene $D_4$ and $<$ 2% cross-reactivity for other leukotrienes) in biologic fluids. This assay is particularly suitable for these studies because eosinophils do not synthesize leukotriene $D_4$ (23, 24). Microplates were prepared by coating with mouse monoclonal anti–rabbit IgG (Cayman Chemical Co. Inc., Ann Arbor, MI) in phosphate buffer and incubated for 18 h at 20°C. Plates then were saturated with buffer (300 mg/liter sodium azide and 3 g/liter BSA in phosphate buffer) for 18 h at 4°C. 50 $\mu$l of either standard or sample was added to each well, followed by 50 $\mu$l of $LTC_4$-specific acetylcholinesterase tracer and 50 $\mu$l of anti-$LTC_4$ rabbit antiserum. Plates were incubated for 18 h at 22°C, rinsed with buffer five to six times, and developed with 200 $\mu$l Ellman's reagent (14.6 mM NaCl, 12.5 mM $K_2HPO_4$, 0.5 mM acetylthiocholine, and 300 mg/liter 5,5'-dithio-bis-[2-nitrobenzoic acid] in $H_2O$ at pH 7.4) as substrate. Absorbance was measured with a microplate spectrophotometer at 412 nm and compared to a standard curve generated at the same time on the same plate. All measurements were made in duplicate.

*Determination of eosinophil viability after inhibition of phospholipase $A_2$ with mepacrine or BPB.* To determine if either mepacrine or BPB decreased eosinophil viability, trypan blue exclusion was determined in eosinophils incubated with either inhibitor. Aliquots of $10^5$ eosinophils isolated by immunomagnetic fractionation were incubated for 30 min at 37°C in microfuge tubes containing 0, 1, 3, 10, 30, 100, or 300 $\mu$M mepacrine ($n$ = 5, isolated by percoll separation) or BPB ($n$ = 3, isolated by magnetic fractionation) in GEL. Eosinophils then were centrifugated at 400 $g$ and pellets were resuspended in 200 $\mu$l GEL. An equal volume of 0.01% trypan blue was added, and viable eosinophils were counted in a hemacytometer.

*Determination of eosinophil release of EPO after inhibition of phospholipase $A_2$.* In these experiments, EPO secretion in stimulated eosinophils after $PLA_2$ inhibition was measured. Aliquots of 5 $\times$ $10^5$ eosinophils isolated by percoll separation from 11 donors were incubated in separate microfuge tubes containing 3, 10, 30, 100, or 300 $\mu$M mepacrine, or 1, 3, 10, 30, 100, or 300 $\mu$M BPB, in GEL for 30 min at 37°C. Eosinophils then were activated with $10^{-6}$ M FMLP plus 5 $\mu$g/ml cytochalasin B (CYB) (final volume 200 $\mu$l) for 30 min at 37°C and then centrifugated at 4°C at 400 $g$. Eosinophil pellets diluted to 1 ml with GEL and conditioned medium (undiluted) were stored separately at $-70$°C for determination of EPO concentrations. Separate control experiments were performed in the same manner for each experiment, in which eosinophils were pretreated with either 100 $\mu$M mepacrine or 300 $\mu$M BPB but not activated, or activated with FMLP and CYB but not pretreated with either $PLA_2$ antagonist.

*Determination of eosinophil $^•O_2^-$ generation after inhibition of phospholipase $A_2$.* In these experiments, $^•O_2^-$ generation in stimulated eosinophils after $PLA_2$ inhibition was measured. The method of Pick and Mizel (25) as modified by Sedgwick et al. (21) was used for this assay. Aliquots of $10^5$ eosinophils were incubated for 30 min at 37°C in microplate wells containing 0, 1, 3, 10, 30, 100, or 300 $\mu$M mepacrine ($n$ = 5, isolated by percoll separation) or BPB ($n$ = 5, isolated by magnetic fractionation), 100 $\mu$mol/liter of cytochrome c, $10^{-6}$ M FMLP and 5 $\mu$g/ml CYB in GEL (final volume of reaction, 200 $\mu$l). Absorbance at 550 nm was determined immediately and then every 5 min for 60 min in a microplate spectrophotometer. Identical control reactions were performed at the same time with the addition of 20 $\mu$g/ml SOD to each well. $^•O_2^-$ generation was calculated with an extinction coefficient of 21.1 $\times$ $10^3$ mol $\cdot$ liter$^{-1}$ $\cdot$ cm$^{-1}$ (26) and expressed finally as the nmol/ml per $10^6$ eosinophils minus SOD control.

**A**                    **B**                    **C**



Control Activated  Control Activated
Percoll isolation  Bead isolation

Control Activated  Control Activated
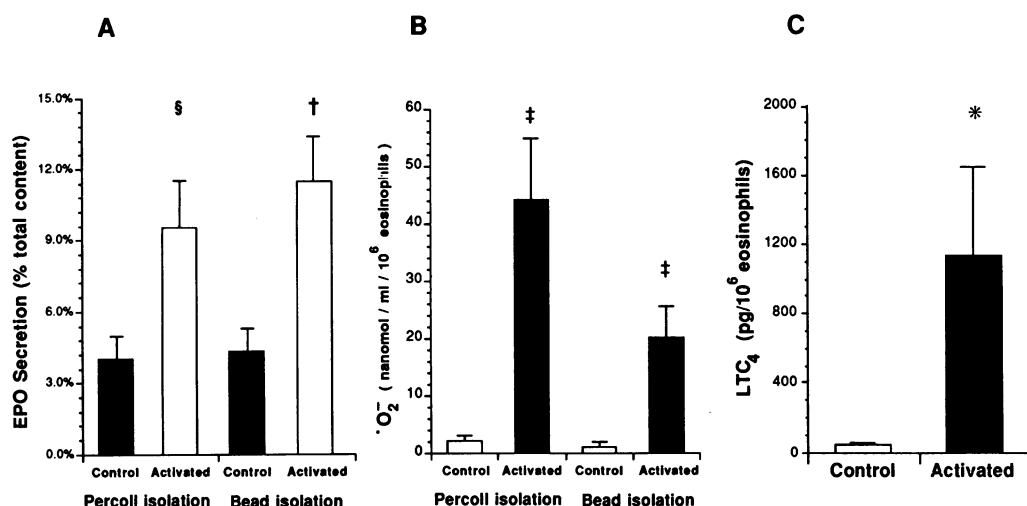Percoll isolation  Bead isolation

Control   Activated

*Figure 1.* Activation of eosinophils by *n*-formyl-met-leu-phe and cytochalasin B. Activation causes substantial release of (*A*) EPO, (*B*) generation of $^{\bullet}O_2^-$, and (*C*) secretion of $LTC_4$. Eosinophils were separated by immunomagnetic fractionation for (*C*) (*n* = 8), and both Percoll density separation and immunomagnetic fractionation for (*A*) (*n* = 11 and 8, respectively) and (*B*) (*n* = 5 and 7, respectively). *$P$ < 0.04; $^{\S}P$ < 0.02; $^{\ddagger}P$ < 0.005; $^{\dagger}P$ < 0.002 vs control.

*Reversal of mepacrine-induced inhibition of EPO release, $LTC_4$ secretion and $^{\bullet}O_2^-$ generation by arachidonic acid.* To determine the specificity of mepacrine in inhibiting eosinophil activation and degranulation, EPO release and $LTC_4$ secretion were measured in activated eosinophils after incubation with AA. In 13 additional experiments, aliquots of $5 \times 10^5$ eosinophils isolated by immunomagnetic fractionation were incubated for 15 min at 37°C with 100 μM mepacrine and then for an additional 15 min at 37°C with either 1 or 10 μM AA in GEL. Eosinophils then were incubated for 30 min at 37°C with $10^{-6}$ M FMLP plus 5 μg/ml CYB (final volume 300 μl). Eosinophils were centrifugated at 400 *g* for 10 min at 4°C, and both media and cell pellets were stored at −70°C for analysis of either EPO (*n* = five experiments) or $LTC_4$ (*n* = eight experiments) concentrations. Control experiments with either no mepacrine, no AA, or neither agent were done at the same time using identical numbers of eosinophils.

In three additional experiments the potential ability of AA to reverse the inhibition of $^{\bullet}O_2^-$ generation by mepacrine was tested. Eosinophils isolated by immunomagnetic fractionation were incubated in 100



control   CYB   fMLP   Both

*Figure 2.* Eosinophil secretion of EPO 30 min after activation with either 5 μg/ml CYB alone, $10^{-6}$ M FMLP alone, or the same concentrations of both CYB and FMLP (*n* = 3 for each). Neither CYB nor FMLP alone elicit significant EPO secretion compared to both FMLP + CYB together, or compared to control (neither FMLP nor CYB). Eosinophils were separated by immunomagnetic fractionation.

μM mepacrine for 15 min followed by either 0, 1, or 10 μM AA in GEL for an additional 15 min at 37°C. Activation with FMLP and CYB, and measurement of $^{\bullet}O_2^-$ generation, were then done as above.

*Materials.* Percoll, Tris (as 99.9% Trisma base), Triton X-100, $H_2O_2$, *o*-phenylenediamine, cytochalasin-B, FMLP, superoxide dismutase, ferricytochrome c, arachidonic acid, and gelatin were obtained from Sigma Chemical Co., St. Louis, MO. Mepacrine and 4-bromophenacyl bromide were obtained from Lilly Research Laboratories, Indianapolis, IN. All reagents were molecular biology grade or higher. Purified EPO was a gift of Dr. Gerald J. Gleich, Mayo Clinic, Rochester, MN, and was isolated from an eosinophil-rich suspension obtained by leukapheresis of patients with hypereosinophilic syndromes (27).

*Data analysis.* All data are expressed as mean±SEM. Comparisons of eosinophil secretion of EPO, $LTC_4$, and $^{\bullet}O_2^-$ between several groups were made by repeated-measures analysis of variance. When significant differences were found, additional comparisons were made with Fisher's protected least significant difference test. Comparisons between two groups were made with Student's *t* test; paired comparisons were made when appropriate. Due to the significance variance in secretion of $LTC_4$ after activation, a normal distribution of $LTC_4$ secretion was not assumed. Differences in $LTC_4$ secretion between treatment groups were compared by the Friedman statistic. When significant differences were found, additional comparisons were made with the Wilcoxon signed-rank test. Corrections for multiple comparisons were made as necessary with the Bonferroni correction (28). Significance was claimed when $P$ < 0.05.

## Results

*Activation of human eosinophils.* Isolation of eosinophils from peripheral blood by either centrifugation through discontinuous Percoll gradients or negative selection immunomagnetic fractionation yielded eosinophils of high purity (92.6±0.8% and 99.1±0.8%, respectively). Stimulation of eosinophils with $10^{-6}$ M FMLP and 5 μg/ml CYB elicited release of EPO, secretion of $LTC_4$, and generation of $^{\bullet}O_2^-$ in each experiment (Fig. 1). Treatment with 5 μg/ml CYB alone in three additional experiments did not cause either secretion of EPO (Fig. 2) or generation of $^{\bullet}O_2^-$ (data not shown). Treatment with $10^{-6}$ M FMLP alone in these experiments also did not cause eosinophil degranulation and release of EPO (Fig. 2).

There were no differences in the release of EPO in FMLP-activated eosinophils isolated by immunomagnetic fractionation compared to eosinophils isolated by Percoll density centrifugation. EPO release in eosinophils isolated by Percoll sepa-
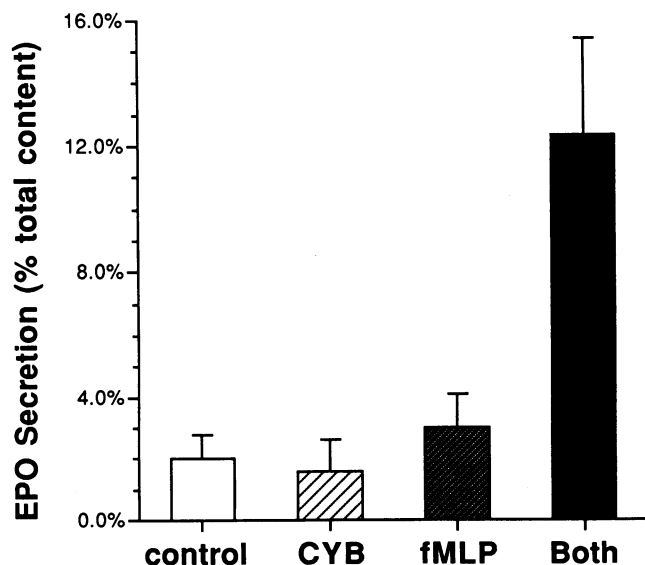
ration was 9.5±2.0% of total cell content; whereas EPO release in eosinophils isolated by immunomagnetic fractionation was 11.0±2.6% of total cell content ($P$ = 0.48) (Fig. 1). Eosinophils isolated by immunomagnetic fractionation secreted less $^\bullet O_2^-$ compared to eosinophils isolated by Percoll density centrifugation. Superoxide ion production stimulated by $10^{-6}$ M FMLP and 5 $\mu$g/ml CYB in Percoll-isolated eosinophils was 44.2±10.8 nmol/ml per $10^6$ eosinophils in five experiments vs 20.4±5.2 nmol/ml per $10^6$ eosinophils for magnetic-isolated eosinophils in eight other experiments ($P$ = 0.07). Because of potential differences in mediator secretion, the same isolation method was used throughout the series for each series of experiments.

*Determination of eosinophil viability after incubation with mepacrine or BPB.* Incubation of isolated eosinophils with either mepacrine or BPB in concentrations ≤ 100 $\mu$M did not alter significantly the ability of eosinophils to exclude trypan blue. In five experiments using mepacrine, control eosinophils were 95.2±1.4% viable compared to 94.4±1.8% viability in eosinophils incubated for 30 min with 30 $\mu$M mepacrine ($P$ = NS). Viability was > 88% of control in eosinophils incubated with 100 $\mu$M mepacrine. In three additional experiments using BPB, control eosinophils were 99.4±0.2% viable compared to 99.3±0.4% viability in eosinophils incubated for 30 min with 100 $\mu$M BPB ($P$ = NS).

*Inhibition of eosinophil release of EPO after inhibition of phospholipase $A_2$.* Incubation with either mepacrine or BPB inhibited the subsequent activation of eosinophils by FMLP + CYB. Pretreatment with 100 $\mu$M mepacrine decreased subsequent eosinophil release of EPO from 10.2±2.1% to 2.0±0.2% of total cell content ($P$ < 0.004, $n$ = nine experiments, Fig. 3). Pretreatment with 100 $\mu$M BPB decreased subsequent eosinophil release of EPO from 9.4±2.6% to 2.5±0.8% of total cell content ($P$ < 0.04, $n$ = seven experiments, Fig. 4). Inhibition of eosinophil degranulation was caused by preincubation with ≥ 3 $\mu$M of either antagonist (Figs. 3 and 4).

Incubation of nonactivated eosinophils with either mepacrine or BPB did not alter the baseline release of EPO. Incubation of nonactivated eosinophils with 300 $\mu$M mepacrine caused EPO release of 4.1±1.3% total cell content vs 4.8±1.0% total cell content for control eosinophils ($P$ = NS). Incubation of nonactivated eosinophils with 300 $\mu$M BPB caused EPO



*Figure 4.* Eosinophil secretion of EPO after inhibition of phospholipase $A_2$ by 4-bromophenacyl bromide. Incubation of human eosinophils ($n$ = seven experiments) with bromophenacyl bromide blocked the subsequent release of EPO after stimulation with FMLP and CYB. *$P$ < 0.04 vs control. Eosinophils were separated by Percoll density gradients.

release of 3.8±0.8% total cell content vs 4.4±1.2% total cell content for control eosinophils ($P$ = NS).

*Determination of eosinophil $^\bullet O_2^-$ generation after inhibition of phospholipase $A_2$.* Coincubation with mepacrine inhibited the generation of $^\bullet O_2^-$ in eosinophils activated with FMLP + CYB. In five experiments, treatment of eosinophils with 100 $\mu$M mepacrine during activation caused $^\bullet O_2^-$ generation of 1.9±0.9 nmol/ml per $10^6$ eosinophils after 30 min vs 44.2±10.8 nmol/ml per $10^6$ eosinophils for activated eosinophils not treated with mepacrine ($P$ < 0.002) (Fig. 5). Superoxide radical generation was inhibited in eosinophils after incubation with all concentrations of mepacrine ≥ 1 $\mu$M (Fig. 5). Mepacrine did not cause $^\bullet O_2^-$ generation in nonactivated eosinophils. After 30 min, nonactivated eosinophils treated with 100 $\mu$M mepacrine generated $^\bullet O_2^-$ equal to 2.2±1.2 nmol/ml per $10^6$ eosinophils vs 0.1±0.7 nmol/ml per $10^6$ eosinophils for control ($P$ = NS).

Coincubation with BPB also inhibited the generation of $^\bullet O_2^-$ in eosinophils activated with FMLP + CYB. In five additional experiments, treatment of eosinophils with 100 $\mu$M BPB during activation caused $^\bullet O_2^-$ generation of 1.2±0.5 nmol/ml per $10^6$ eosinophils after 30 min vs 26.3±5.2 nmol/ml per $10^6$ eosinophils for activated eosinophils not treated with BPB ($P$ < 0.005) (Fig. 6). Superoxide radical generation was inhibited in eosinophils after incubation with all concentrations of BPB ≥ 10 $\mu$M (Fig. 6).
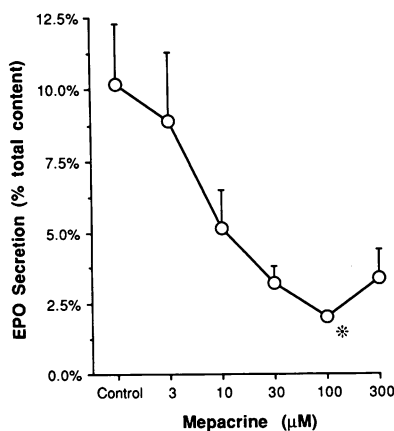


*Figure 3.* Eosinophil secretion of EPO after inhibition of phospholipase $A_2$ by mepacrine. Incubation of human eosinophils ($n$ = nine experiments) with mepacrine blocked the subsequent release of EPO after stimulation with FMLP and CYB. *$P$ < 0.004 vs control. Some standard error bars are contained within the point symbols. Comparison is made at 100 $\mu$M mepacrine, the point of maximal inhibition, in these and all subsequent experiments (see below). Eosinophils were separated by percoll density gradients.
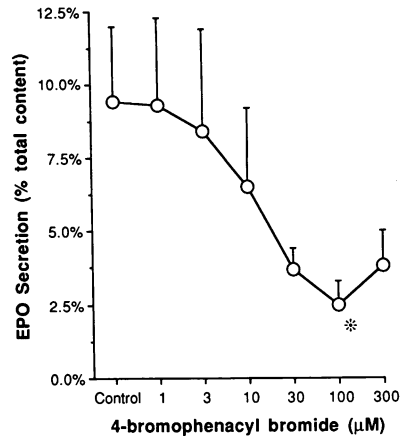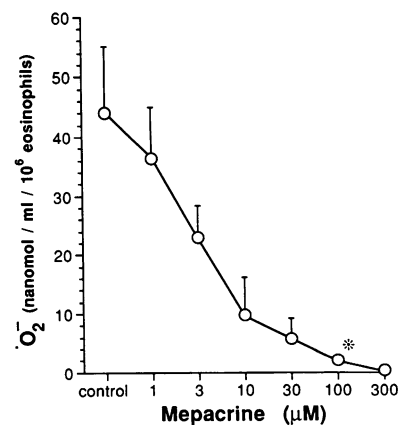


*Figure 5.* Eosinophil generation of $^\bullet O_2^-$ after inhibition of phospholipase $A_2$ by mepacrine. Incubation of human eosinophils ($n$ = five experiments) with mepacrine blocked the generation of $^\bullet O_2^-$ after stimulation with FMLP and CYB. *$P$ < 0.002 vs control. Some standard error bars are contained within the point symbols. Comparison is made at 100 $\mu$M mepacrine as above. Eosinophils were separated by Percoll density gradients.
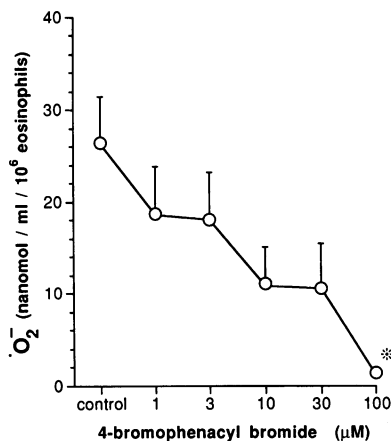
Figure 6. Eosinophil generation of $^{\bullet}O_2^-$ after inhibition of phospholipase $A_2$ by BPB. Incubation of human eosinophils ($n$ = five experiments) with BPB blocked the generation of $^{\bullet}O_2^-$ after stimulation with FMLP and CYB. *$P$ < 0.005 vs control. Some standard error bars are contained within the point symbols. Comparison is made at 100 $\mu$M BPB as above. Eosinophils were separated by immunomagnetic fractionation.
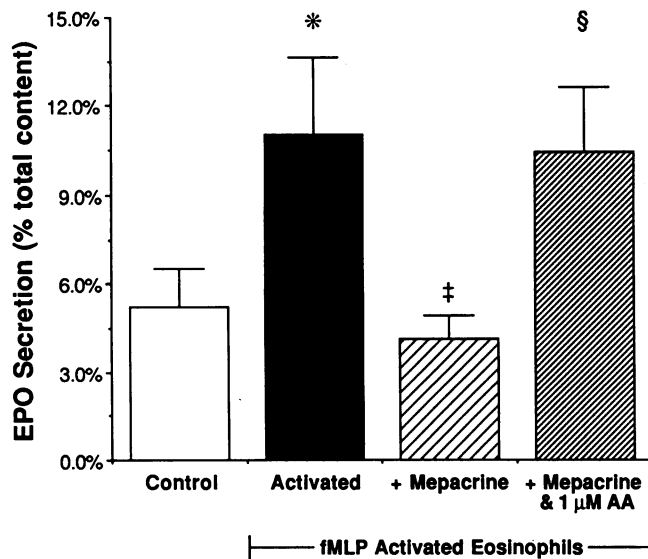


Figure 7. Reversal of mepacrine-induced inhibition of eosinophil secretion of EPO by AA. Incubation of human eosinophils ($n$ = five experiments) with 1 $\mu$M AA after pretreatment with 100 $\mu$M mepacrine reversed the inhibition of activated eosinophil EPO secretion noted with mepacrine alone. *$P$ < 0.05 vs control; $^{\ddagger}P$ < 0.02 vs activated eosinophils; $^{\S}P$ < 0.02 vs mepacrine-treated but not AA-treated eosinophils. Eosinophils were separated by immunomagnetic fractionation.

*Reversal of mepacrine-induced inhibition of EPO release, LTC$_4$ secretion, and $^{\bullet}O_2^-$ generation by arachidonic acid.* To determine the specificity of mepacrine in inhibiting eosinophil degranulation and leukotriene production, experiments were performed measuring EPO release, LTC$_4$ secretion, and $^{\bullet}O_2^-$ generation in activated eosinophils after treatment with exogenous AA. Eosinophils in these experiments were isolated by immunomagnetic separation.

Treatment of eosinophils with AA reversed substantially the inhibitory effects of mepacrine on both degranulation and secretion of LTC$_4$. In five experiments, eosinophils treated with both 100 $\mu$M mepacrine and 1 $\mu$M AA and then activated with FMLP and CYB released EPO at a concentration 10.4±2.2% total cell content vs 4.1±0.8% total cell content for eosinophils treated with mepacrine but not AA ($P$ < 0.02) (Fig. 7). Treatment with AA did not alter subsequent EPO secretion caused by FMLP and CYB: EPO secretion after both AA and FMLP/CYB was 93.9±21.0% of secretion caused by FMLP/CYB alone ($P$ = NS). Treatment with AA did not alter EPO release in nonactivated, quiescent eosinophils or cells treated with mepacrine (Table I).

In eight additional experiments, activation of eosinophils with FMLP + CYB caused LTC$_4$ secretion of 1,125±527 pg/ml per $10^6$ eosinophils after 30 min, vs 44.2±24.0 pg/ml per $10^6$ eosinophils after 30 min for nonactivated eosinophils ($P$ < 0.04) (Fig. 1). Incubation with 100 $\mu$M mepacrine before activation with FMLP + CYB almost completely inhibited LTC$_4$ secretion (68.2±32.2 vs 1,125±527 pg/ml per $10^6$ eosinophils, $P$ < 0.04) (Fig. 8). In eosinophils treated with 100 $\mu$M mepacrine, incubation with 10 $\mu$M AA before activation with FMLP + CYB restored LTC$_4$ secretion to 695±413 pg/ml per $10^6$ eosinophils ($P$ < 0.04 vs activated eosinophils treated with mepacrine but not AA; $P$ = NS vs activated eosinophils not treated with mepacrine) (Fig. 8). Treatment with AA did not alter LTC$_4$ secretion in nonactivated, quiescent eosinophils or cells treated with mepacrine (Table I).

Treatment of eosinophils with AA did not reverse the inhibition of $^{\bullet}O_2^-$ generation elicited by 100 $\mu$M mepacrine. In three additional experiments, eosinophils pretreated with both 100 $\mu$M mepacrine and 10 $\mu$M AA, $^{\bullet}O_2^-$ generation 30 min after activation with FMLP + CYB was 1.7±1.1 nmol/ml per $10^6$

cells vs 1.4±0.8 nmol/ml per $10^6$ cells for activated eosinophils pretreated with mepacrine but not AA. Treatment of activated eosinophils with 10 $\mu$M AA alone did not alter $^{\bullet}O_2^-$ generation, which after 30 min was 99.5±9.1% of activated eosinophils not treated with AA.

## Discussion

These data demonstrate that blockade of endogenous phospholipase $A_2$ causes inhibition of degranulation and release of EPO in activated human eosinophils. We found that eosinophil secretion of LTC$_4$ and generation of $^{\bullet}O_2^-$ also was blocked by inhibition of endogenous PLA$_2$. Both inhibition of EPO release and $^{\bullet}O_2^-$ generation by PLA$_2$ blockade were dependent upon

Table I. Release of EPO and Secretion of LTC$_4$ by Nonactivated (Quiescent) Human Eosinophils after Incubation with AA and Mepacrine

| Group | EPO | LTC$_4$ |
|---|---|---|
| | % total content* | pg/ml per $10^6$ cells |
| Control | 5.2±1.3$^{\ddagger}$ | 44.2±24.0 |
| Mepacrine alone | 3.5±0.6 | 0.5±0.1 |
| AA alone | 6.2±1.8 | 3.0±0.6 |
| Both mepacrine and AA | 5.3±1.0 | 16.1±8.6 |

Eosinophils were incubated with 100 $\mu$M mepacrine fro 15 min followed by 1 $\mu$M (for EPO experiments) or 10 $\mu$M (for LTC$_4$ experiments) AA for an additional 15 min at 37°C and compared to control eosinophils under same conditions. There are no differences in either EPO release or LTC$_4$ secretion between groups. * $n$ = 5 for EPO experiments and 8 for LTC$_4$ experiments. $^{\ddagger}$ Values represent mean±SE.
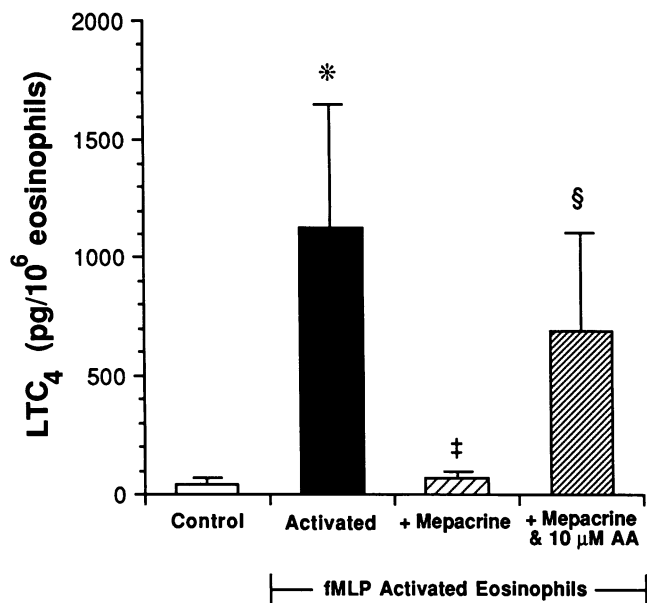
*Figure 8.* Eosinophil generation of $LTC_4$ after inhibition of phospholipase $A_2$ by mepacrine. Incubation of human eosinophils ($n$ = eight experiments) with 100 $\mu$M mepacrine blocked completely the secretion of $LTC_4$ after activation with FMLP and CYB. Incubation with 10 $\mu$M AA after mepacrine pretreatment and before activation significantly reversed the inhibitory effect of mepacrine. $*P < 0.04$ vs control; $\ddagger P < 0.04$ vs activated eosinophils; $\S P < 0.04$ vs mepacrine-treated eosinophils. Eosinophils were separated by immunomagnetic fractionation.

the concentration of the $PLA_2$ inhibitor. In these studies, inhibition of both EPO release and $LTC_4$ secretion was reversed by addition of arachidonic acid. We demonstrate that endogenous $PLA_2$ may regulate mediator secretion and degranulation in eosinophils.

Eosinophil activation and degranulation may be caused by a number of agents, such as platelet-activating factor, FMLP, and secretory immunoglobulin A (29–31). In these experiments, we used FMLP plus CYB as an activator for eosinophil degranulation. FMLP activates eosinophils by binding to specific membrane receptors (30), an action which is augmented substantially in the presence of CYB (20, 21). We have demonstrated previously that FMLP plus CYB causes eosinophil release of EPO that is measured readily in biologic fluids (20). Eosinophil peroxidase release and $LTC_4$ secretion were readily elicited by FMLP plus CYB in our experiments (Fig. 1). Superoxide radical generation was less readily elicited in eosinophils isolated by immunomagnetic separation compared to Percoll separation (Fig. 1). In several experiments using eosinophils isolated by immunomagnetic separation, $^{\bullet}O_2^-$ generation was not substantially above baseline, and these experiments were excluded from analysis. Sufficient $^{\bullet}O_2^-$ was generated after eosinophil activation with FMLP and CYB in each series of experiments to determine the effects of both mepacrine and BPB (Figs. 5 and 6), and the effects of exogenous AA on inhibition elicited by mepacrine (see above).

Both mepacrine and BPB inhibited subsequent degranulation and $^{\bullet}O_2^-$ generation by activated eosinophils, and mepacrine inhibited subsequent $LTC_4$ secretion. Each antagonist inhibits $PLA_2$ by a distinct mechanism. Mepacrine inhibits $PLA_2$

competitively either by direct interaction (17) or by formation of stable complexes of drug and phospholipid substrate (18). Bromophenacyl bromide inhibits $PLA_2$ noncompetitively by modification of an active site histidine residue (19). Concentrations and activity of endogenous eosinophil $PLA_2$ were not determined in this study. However, the similar actions of two specific agents with different modes of antagonism, and the bypass of the blockade of $PLA_2$ with exogenous arachidonic acid, suggest strongly that the inhibition of eosinophil activation and degranulation in these experiments is caused by inhibition of $PLA_2$ and is not a nonspecific, cytotoxic effect of either mepacrine or BPB.

The inhibition of $PLA_2$ elicited by mepacrine, and the subsequent inhibition of both EPO and $LTC_4$ secretion, was reversed by treating eosinophils with AA before activation with FMLP. The concentrations of AA used, 1–10 $\mu$M, were sufficient to permit both EPO release and $LTC_4$ secretion that was similar to that obtained by activated eosinophils not treated with mepacrine (Figs. 7 and 8). While pretreatment of eosinophils with either mepacrine or BPB blocked subsequent $^{\bullet}O_2^-$ generation, treatment with AA did not reverse inhibition elicited by mepacrine and did not restore $^{\bullet}O_2^-$ generation after stimulation with FMLP + CYB. Phospholipase $A_2$ activity previously has been demonstrated in neutrophils (32–35) and causes release of eicosanoid mediators and $^{\bullet}O_2^-$ (36–38); this can be blocked by either BPB (34) or mepacrine (35). Phospholipase $A_2$ stimulates $^{\bullet}O_2^-$ generation in neutrophils by releasing AA which activates NADPH oxidase (38); $^{\bullet}O_2^-$ generation can be blocked by inhibition of $PLA_2$ and restored by addition of exogenous AA. However, suppression of eicosanoid release and $^{\bullet}O_2^-$ generation by $PLA_2$ inhibition in macrophages may be mutually independent (39). In the present study, while both EPO release and $LTC_4$ secretion could be restored by addition of exogenous AA, inhibition of $^{\bullet}O_2^-$ generation by mepacrine was not reversed by exogenous AA. These data suggest that eicosanoid production in eosinophils, as in neutrophils, depends upon activation of $PLA_2$. Our data suggest further that in eosinophils, unlike neutrophils, $^{\bullet}O_2^-$ generation in eosinophils may not be linked to AA metabolism; however, we do not consider our data to be conclusive on this point.

Eosinophil peroxidase is a constituent of the matrix of cationic eosinophil granules (1, 27). This protein is synthesized during eosinopoiesis and are not further synthesized after release of the cell from the bone marrow (40, 41). Release of EPO and other eosinophil granular proteins involves solubilization of granular proteins into vacuoles followed by extrusion of the vacoular contents from the eosinophil into the surrounding environment (42). Therefore, determination of EPO concentrations in eosinophil-conditioned media is a marker for degranulation (20, 43). Eosinophil degranulation, and the inhibition of degranulation by mepacrine and BPB, were demonstrated consistently by this assay (Figs. 1, 3, 4, and 7).

In these studies, eosinophils were isolated in these experiments either by separation through discontinuous Percoll density gradients or by negative-selection immunomagnetic bead fractionation. The later method has been developed recently (22) and isolates eosinophils in greater number from equivalent volumes of blood compared with density gradient methods. Each method isolated eosinophils in sufficient purity (> 92 and > 98%, respectively) to exclude substantial numbers of neutrophils. In these studies, both the purity of isolated cells

and subsequent EPO release after incubation with FMLP + CYB by either method were comparable.

Phospholipase $A_2$ can mediate a variety of actions within cells either directly or via subsequent transformation of its products: arachidonic acid metabolized into prostaglandins, leukotrienes, thromboxanes, and lipoxins; and lysophospholipids metabolized into platelet activating factor (44). Each of these lipid mediators has been implicated in a number of inflammatory processes and tissue injury, and many may have roles as second messenger (45). Phospholipase $A_2$ activation also can modify cell membrane dynamics. Phospholipid turnover in cell membranes may represent both replacement of oxidized phospholipids (46) and also reflect fundamental signalling mechanisms by which a cell respond to its environment (44, 45). We did not delineate which of these activities of $PLA_2$ was responsible for eosinophil degranulation and $^\bullet O_2^-$ generation.

In summary, we demonstrate that inhibition of endogenous eosinophil $PLA_2$ inhibits release of EPO, $LTC_4$ secretion, and $^\bullet O_2^-$ generation from isolated, human eosinophils. Inhibition is specific to a product of the hydrolytic reaction catalyzed by $PLA_2$ and can be reversed by addition of exogenous arachidonic acid. These data demonstrate the potential of $PLA_2$ to regulate eosinophil secretory activity that pertains to inflammatory states such as human asthma.

## Acknowledgments

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