

β -Adrenergic regulation of the eosinophil respiratory burst as detected by lucigenin-dependent luminescence

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Because β -adrenoceptor agonists are commonly used in the treatment of disease states associated with eosinophil activation, β -adrenergic regulation of the eosinophil respiratory burst (as monitored with lucigenin-dependent luminescence) was evaluated. Normodense, nonprimed eosinophils from healthy volunteer subjects were potently inhibited by very low concentrations of isoproterenol. The inhibitory concentration of 50% for isoproterenol was approximately 2 nmol/L. The β -agonist was able to inhibit the eosinophil respiratory burst induced by receptor-mediated (chemotactic peptide) and nonreceptor-mediated (calcium ionophore and phorbol ester) stimuli. Thus β -agonist inhibition was unlikely to be isolated to an effect at the receptor or G protein linkage. To determine whether cyclic adenosine 3',5' monophosphate (cAMP) may mediate β -agonist effects, studies were performed with the type IV cAMP phosphodiesterase inhibitor Ro-201724. β -Agonist inhibition of the respiratory burst was clearly synergistic with effects of Ro-201724. We conclude that β -adrenoceptor agonists can regulate the eosinophil respiratory burst at least partially through an effect mediated by cAMP. Because regulation of the eosinophil by isoproterenol was observed at very low concentrations, these results may be relevant to pharmacologic effects of β -agonists in disease states complicated by eosinophil activation during asthma. (J ALLERGY CLIN IMMUNOL 1995; 95:735-41.)

Key words: Isoproterenol, eosinophil, respiratory burst

Eosinophils are important in the pathogenesis of a diversity of atopic and respiratory diseases.¹⁻³ Although the eosinophil may have an important role in host defense against parasitic disease, eosinophil activation with release of granules, arachidonic acid derivatives, and superoxide anion can induce major host injury.^{4,5} Consequently, regulation of eosinophil activation by endogenous medi-

Abbreviations used

BSA:	Bovine serum albumin
[Ca ⁺⁺]:	Intracellular free calcium
cAMP:	Cyclic adenosine 3',5'-monophosphate
cps:	counts per second
DPBS:	Dulbecco's phosphate-buffered saline
fMLP:	N-formyl-methionyl-leucyl-phenylalanine
PBS:	Phosphate-buffered saline
PMA:	Phorbol, 12-myristyl, 13-acetate

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ators and pharmacologic agents is of clinical importance.

Eosinophil infiltration and activation may be particularly relevant to the pathogenesis of asthma.^{3,6} Eosinophil infiltration has been associated with bronchial hyperresponsiveness and the late asthmatic response.^{7,8} Mediators generated by eosinophils, including the sulfidyl peptide leukotrienes and superoxide anion, cause bronchial con-

striction and edema.^{5,9} It may be very relevant that effective therapeutic intervention in asthma including β -adrenoceptor agonists and glucocorticoids can inhibit eosinophil mobilization and activation.¹⁰⁻¹⁴

Although eosinophil regulation may be of pathophysiologic importance, the mechanisms of eosinophil regulation are not well understood, and few studies have characterized the actions of β -agonists or cyclic adenosine 3',5' monophosphate (cAMP) on the eosinophil respiratory burst.^{15,16} β -Agonists had no effect on superoxide anion generation in a single study of primed eosinophils in which high concentrations of phorbol ester were used to induce degranulation and the respiratory burst.¹⁵ However, cAMP and selective type IV cAMP phosphodiesterase inhibitors have been demonstrated to inhibit the eosinophil.¹⁶ Because our studies with neutrophils suggest that regulation of the respiratory burst may be very dependent on the degree and mechanism of stimulation, primed eosinophils stimulated with high concentrations of phorbol ester may not be responsive to any usual mechanism of cell regulation. Using lucigenin-dependent luminescence, a very sensitive means of detecting superoxide anion generation during the respiratory burst, we have evaluated β -adrenergic regulation of the respiratory burst induced by chemotactic peptide, calcium ionophore, and low concentrations of phorbol ester. Under these conditions, eosinophils are potentially regulated by β -adrenoceptor agonists.

METHODS

Hansel stain was obtained from Lide Labs, Inc. (Floissant, Mo.). Ro-201724 was purchased from BIOMOL Research Laboratory, Inc. (Plymouth Meeting, Pa.). All other chemicals were obtained from Sigma Chemical Company (St. Louis, Mo.).

Eosinophils were isolated from healthy subjects by phlebotomy. Subjects gave informed consent to a study protocol approved by the University of Washington–Seattle, Office of Research, Human Subjects Division. Percoll density gradient centrifugation was used to separate eosinophils from other leukocytes. Briefly, 45 ml of blood was obtained by venipuncture and anticoagulated with 7.5 ml of ethylenediaminetetraacetic acid (10 mg/ml) in saline solution. After the addition of 9 ml of 4.5% dextran in saline solution, blood was sedimented (1 g) for 45 minutes at room temperature (23°C). Leukocyte-rich plasma was collected, diluted 1:1 (vol/vol) with phosphate-buffered saline–sodium citrate–bovine serum albumin (PBS-citrate-BSA) buffer. The PBS-citrate-BSA buffer consisted of NaCl (136 mmol/L), KCl (2.7 mmol/L), Na_2PO_4 (8.1 mmol/L), KH_2PO_4 (1.47

mmol/L), sodium citrate (13 mmol/L), BSA (0.5% wt/vol); adjusted to pH 7.4, 290 mOsm/kg, 1.012 gm/ml.

After centrifugation (10 minutes at 200 g) of the diluted leukocyte-rich plasma, the supernatant was decanted, and the cell pellet was resuspended in 4 ml of PBS-citrate-BSA (25 to 30×10^6 cells/ml). Cell suspension (2 ml) was gently layered atop a multiple, discontinuous, Percoll density gradient consisting of the following volumes and densities (top to bottom): 2 ml at 1.077 gm/ml, 2 ml at 1.0825 gm/ml, 2 ml at 1.085 gm/ml, 2 ml at 1.0875 gm/ml, 2 ml at 1.090 gm/ml, 1 ml at 1.0925 gm/ml, and 1 ml at 1.095 gm/ml. Percoll was mixed with 10-fold concentrated PBS to obtain a stock Percoll solution of 1.12 gm/ml and 290 mOsm/kg, pH 7.4. This stock Percoll solution was then mixed with appropriate volumes of PBS-citrate-BSA to obtain solutions of the desired density. All solutions were adjusted to pH 7.4, 290 mOsm/kg. Gradient fractions were collected after centrifugation for 30 minutes at 1000 g by piercing the bottom of the polypropylene centrifuge tube with an 18-gauge needle and collecting fractions. After washing and hypotonic lysis of erythrocytes, gradient fractions were suspended in ice-cold Dulbecco's phosphate-buffered saline (DPBS) containing glucose (4.44 mmol/L) and 0.1% BSA. Cells were stored on ice until they were used. Cell counts in each fraction were determined with a hemocytometer, and percentage of eosinophils in each fraction was evaluated by means of differential counts of Hansel-stained cytospin slides. The percentage of eosinophils in the peripheral blood from normal subjects used for these studies averaged to 2.4%. The yield of eosinophils (total from all fractions) averaged to 0.110×10^6 cells per milliliter of blood obtained by phlebotomy. Assuming a total leukocyte count of 7×10^6 cells/ml, this is an eosinophil yield of about 64%. Fractions containing purified eosinophils (>95% eosinophils in fractions of density ≥ 1.0875) were used for luminometry studies.

Eosinophils were washed with DPBS and resuspended in DPBS with calcium (1 mmol/L), glucose (4.44 mmol/L), and 0.02% BSA (both buffers at room temperature). Cells were counted and adjusted to 1.25×10^5 cells/ml, and 0.2 ml of adjusted cell suspension was injected into tubes containing 0.8 ml buffer (with and without treatments). In the studies with Ro-201724, eosinophils were preincubated for 15 minutes at 37°C with Ro 20-1724 (10 nmol/L) or isoproterenol (0.3 nmol/L) before stimulation with N-formyl-methionyl-leucyl-phenylalanine (fMLP). Eosinophils were incubated at 37°C, and lucigenin-dependent luminescence from each tube was monitored in a Packard Picolite luminometer (Packard Instrument Co., Meriden, Conn.) for 1 second at 2-minute intervals for 26 minutes. Incubations were continuously agitated and mixed by the tube-carousel mechanism of the luminometer. Incubation tubes contained lucigenin (10 $\mu\text{mol/L}$) for detection of superoxide anion.¹⁷ Lucigenin-dependent luminescence was linearly related to superoxide anion generated from the degradation of xanthine by xanthine oxidase (1 to 10×10^{-3}

units/ml xanthine oxidase and 100 $\mu\text{mol/L}$ xanthine for 26 minutes at 37° C with 10 $\mu\text{mol/L}$ lucigenin; linear regression $R^2 = 0.97$). This range of superoxide anion generation was similar to that generated by stimulated eosinophils (2.5×10^4 cells/ml). Isoproterenol (0.1 $\mu\text{mol/L}$) did not interfere with lucigenin-dependent luminescence from superoxide anion generated by xanthine and xanthine oxidase. As other investigators have reported,¹⁷ our preliminary studies demonstrated that under equivalent experimental conditions and cell densities, the respiratory burst from eosinophils was four to five times greater than that from neutrophils. Preliminary studies demonstrated that low doses of phorbol, 12-myristyl, 13-acetate (PMA) (30 to 100 pmol/L) elicited a respiratory burst from eosinophils comparable to that produced by fMLP (1 $\mu\text{mol/L}$).

Each experiment was conducted in duplicate, and the means from N experiments were used in the analyses. Values for N are given in the figure legends. Statistical analysis was conducted with Minitab Statistical Software, Version 9 (State College, Pa.). Treatments were compared with controls by a paired *t* test or, for multiple comparisons, by one-way analysis of variance and Dunnett's test (family error rate = 5%).

RESULTS

The chemotactic peptide (fMLP)-induced eosinophil respiratory burst, monitored as lucigenin-dependent luminescence, has a biphasic pattern (Fig. 1), similar to that described for neutrophils.¹⁸ An increase in lucigenin-dependent chemiluminescence from fMLP-stimulated eosinophils was detected at 2 minutes. Subsequently, after a short period in which chemiluminescence decreased from its peak value, a sustained increase in chemiluminescence occurred. Other agonists for eosinophils produced chemiluminescence responses with different kinetics. Calcium ionophore stimulated a predominantly early burst, whereas phorbol ester (PMA) induced a late response.

The lucigenin-dependent luminescence from eosinophils stimulated with fMLP was inhibited in a dose-dependent manner by isoproterenol (Fig. 2). Isoproterenol inhibition was significant at concentrations as low as 3 nmol/L (Dunnett's test, $p < 0.05$). Maximal inhibition by isoproterenol was observed when early phases (0 to 4 minutes) of the eosinophil response were analyzed (Fig. 2, A). The inhibitory concentration of 50% for isoproterenol inhibition of the early chemiluminescence response (0 to 4 minutes) was 2.2 nmol/L. Isoproterenol also inhibited later phases of the eosinophil chemiluminescence response (Fig. 2, B), but the early phase of the fMLP stimulation is more sensitive to β -agonist inhibition than the later phase.

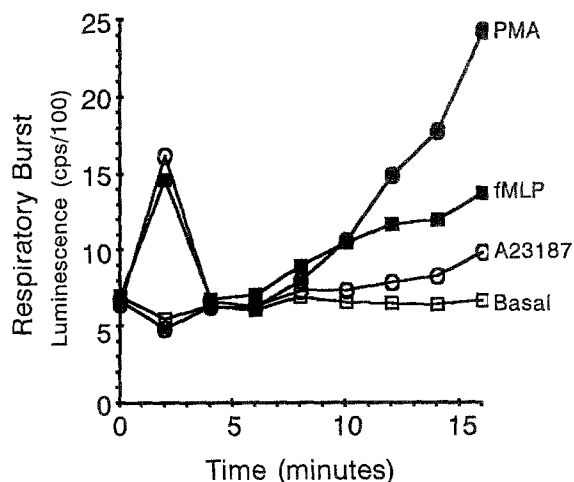


FIG. 1. Kinetics of eosinophil respiratory burst (as monitored by lucigenin-dependent chemiluminescence) in response to various stimuli. Eosinophils (2.5×10^4 cells) were incubated at 37° C in DPBS (1 ml) containing vehicle, fMLP (1 $\mu\text{mol/L}$), A23187 (0.2 $\mu\text{mol/L}$), or PMA (0.1 nmol/L) as described in the Methods section. Respiratory burst was detected by chemiluminescence from lucigenin (10 $\mu\text{mol/L}$). Chemiluminescence (counts per second [cps]) versus time (min) is depicted. Means from a single experiment conducted in duplicate, which is representative of three experiments, are shown.

To determine whether β -agonist inhibition was mediated at the chemotactic receptor or at coupling of the chemotactic peptide receptor, as has been reported in neutrophils,¹⁹ effects of isoproterenol on the nonreceptor-mediated stimuli, calcium ionophore and phorbol ester, were studied. Stimulation of eosinophil degranulation by fMLP occurs through phospholipase C activation with subsequent generation of both diacylglycerol and inositol phosphates and increases in intracellular free calcium levels, $[\text{Ca}^{++}]_i$.^{20, 21} Calcium ionophore causes an increase of $[\text{Ca}^{++}]_i$, whereas PMA is a ligand of protein kinase C and stimulates the activity of this kinase. Because PMA has minimal ability to increase lucigenin-dependent chemiluminescence from eosinophils before 4 minutes, A23187 primarily stimulates eosinophil lucigenin-dependent chemiluminescence during the first 5 minutes, and fMLP induces a biphasic response; total chemiluminescence was measured over 15 minutes to allow comparison of the respiratory bursts induced by each of the agents. Isoproterenol dose-dependently inhibited the chemiluminescence response elicited by nonreceptor- and receptor-mediated agonists (Fig. 3). Inhibition was significant at concentrations of isoproterenol greater than or equal to 10 nmol/L (Dunnett's test, $p < 0.05$).

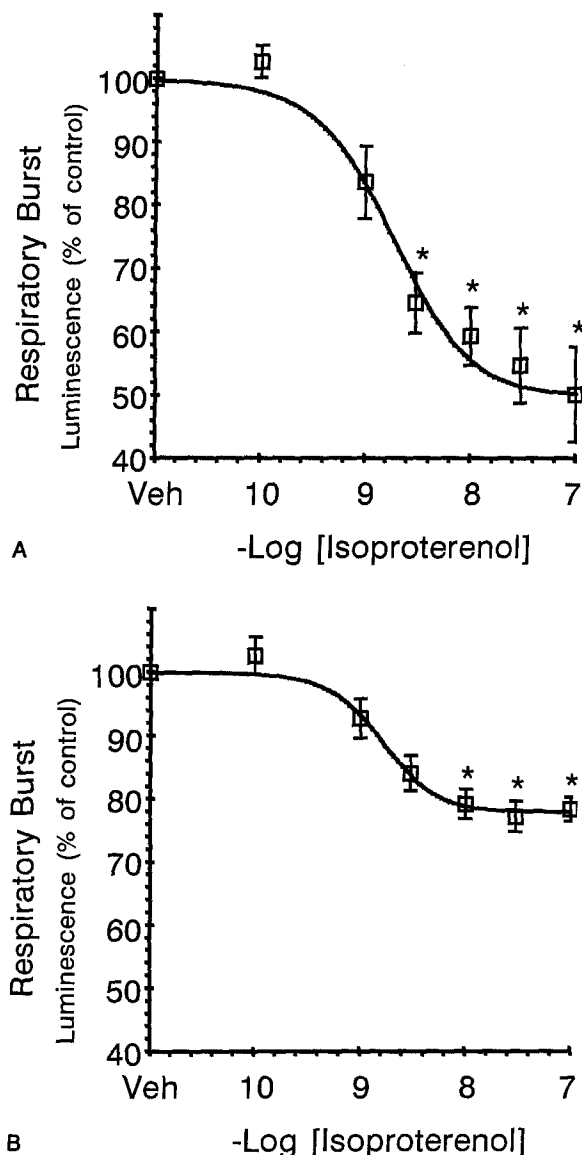


FIG. 2. Isoproterenol dose-dependently inhibits the eosinophil chemiluminescence response induced by fMLP. Eosinophils (2.5×10^4 cells) were incubated at 37°C in DPBS (1 ml) containing fMLP ($1 \mu\text{mol/L}$) and varying concentrations of isoproterenol as described in the Methods section. Respiratory burst was detected by chemiluminescence from lucigenin ($10 \mu\text{mol/L}$). Chemiluminescence integrated from 0 to 4 minutes after exposure of eosinophils to fMLP and isoproterenol is shown in **A**, and 0 to 15-minute data are shown in **B**. Data represent percent of control stimulation (cell sample stimulated by fMLP without isoproterenol). Means $\pm$ SEM ($n = 7$) are shown. Treatments significantly different from control are marked by an asterisk (Dunnett's test, $p < 0.05$).

To determine whether β -agonist inhibition of the respiratory burst, as detected by lucigenin-dependent chemiluminescence, may be mediated by cAMP, the effects of a selective type IV phos-

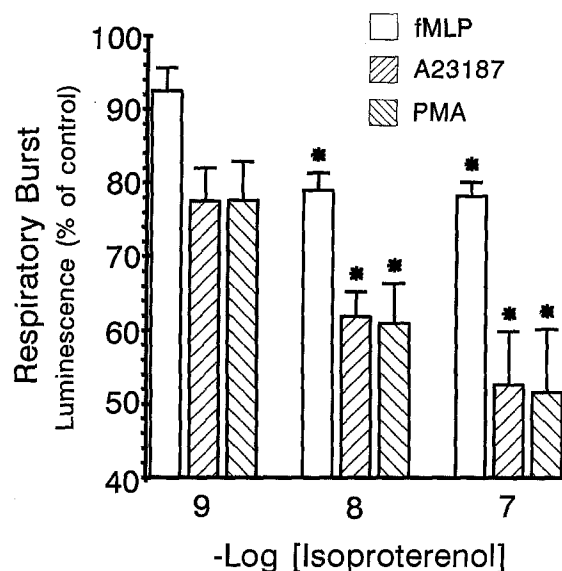


FIG. 3. Isoproterenol inhibition of the eosinophil chemiluminescence response induced by receptor (fMLP) and nonreceptor-mediated agonists (ionophore, PMA). Eosinophils (2.5×10^4 cells) were incubated at 37°C in DPBS (1 ml) containing fMLP ($1 \mu\text{mol/L}$), A23187 ($0.2 \mu\text{mol/L}$), or PMA (0.1 nmol/L) and varying concentrations of isoproterenol as described in the Methods section. Respiratory burst was detected by chemiluminescence from lucigenin ($10 \mu\text{mol/L}$). Chemiluminescence (cps) was integrated from 0 to 15 minutes after exposure of eosinophils to agonists and isoproterenol. Data represent percent of control stimulation (cell sample stimulated by agonist without isoproterenol). Means $\pm$ SEM ($n = 7$) are shown. Integrated chemiluminescence averaged 5392 cps, 21,213 cps, 14,604 cps, and 20,312 cps for vehicle, fMLP, A23187, and PMA, respectively. Treatments significantly different from control are marked by an asterisk (Dunnett's test, $p < 0.05$).

phodiesterase inhibitor, Ro-201724, were studied. In the absence of isoproterenol, Ro-201724 inhibited the later phase of the eosinophil chemiluminescence response induced by fMLP but had less effect on the early response (Fig. 4). Although preincubation increased early inhibition slightly, inhibition by Ro-20724 remained less than isoproterenol. In contrast, Ro-201724, when introduced with isoproterenol at the time of fMLP activation, markedly enhanced early and late eosinophil inhibition by a suboptimal concentration of isoproterenol (Fig. 4). In both cases the effects of isoproterenol and Ro-201724 were synergistic. During the early phase, Ro-201724 had minimal effect but markedly enhanced isoproterenol inhibition, and during the late phase, isoproterenol had minimal effect but markedly enhanced Ro-201724 inhibition. The synergistic effects of the combination were very apparent when eosinophils were prein-

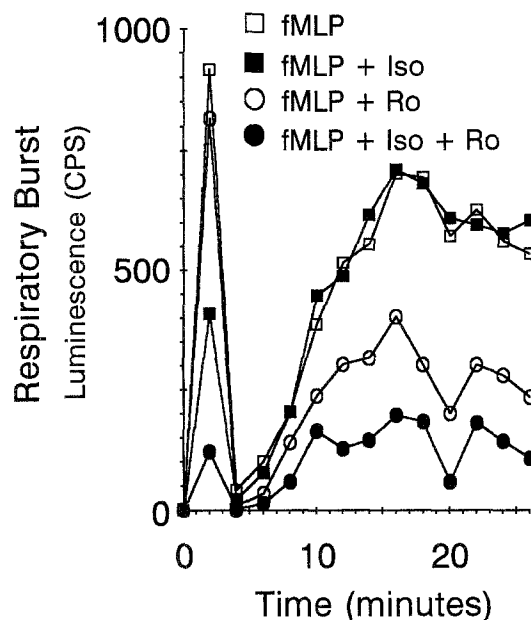


FIG. 4. Kinetics of the eosinophil chemiluminescence response induced by fMLP in the presence of a suboptimal concentration of isoproterenol or Ro-201724. Eosinophils (2.5×10^4 cells) were incubated at 37°C in DPBS (1 ml) containing fMLP ($1 \mu\text{mol/L}$) and isoproterenol (1 nmol/L) and/or Ro-201724 (100 nmol) as described in the Methods section. Respiratory burst was detected by chemiluminescence from lucigenin ($10 \mu\text{mol/L}$). Chemiluminescence (cps) versus time (min) is depicted. Means from a single experiment conducted in duplicate, which is representative of three experiments, are shown with basal chemiluminescence subtracted.

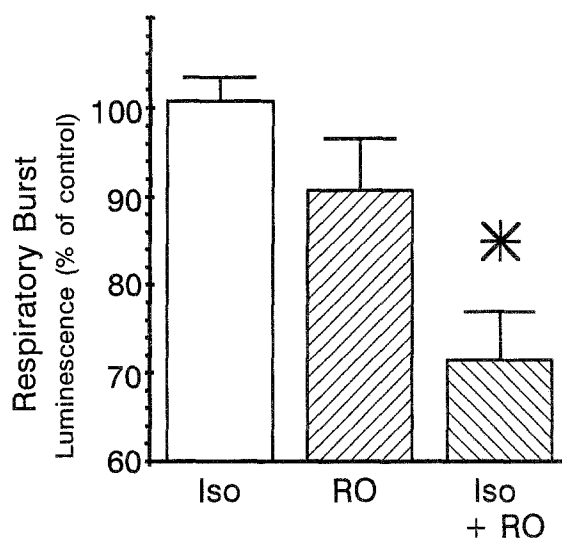


FIG. 5. Synergistic effect of type IV phosphodiesterase inhibitor (Ro-201724) and suboptimal concentration of isoproterenol on inhibition of the eosinophil chemiluminescence response. Eosinophils (2.5×10^4 cells) were preincubated at 37°C in DPBS (1 ml) for 15 minutes with isoproterenol (0.3 nmol/L) and/or Ro-201724 (10 nmol/L) before stimulation by fMLP ($1 \mu\text{mol/L}$) as described in the Methods section. Respiratory burst was detected by chemiluminescence from lucigenin ($10 \mu\text{mol/L}$). Chemiluminescence (cps) was integrated from 0 to 15 minutes after exposure of eosinophils to fMLP. Data represent percent of control stimulation (cell sample stimulated by fMLP without isoproterenol and/or Ro-201724). Means $\pm$ SEM ($n = 4$) are shown. Treatments significantly different from control are marked by an asterisk (Dunnett's test, $p < 0.05$).

cubated with suboptimal concentrations of Ro-201724 and isoproterenol before stimulation with fMLP (Fig. 5). Separately, suboptimal concentrations of the phosphodiesterase inhibitor and β -agonist did not significantly inhibit the chemiluminescence response induced by fMLP, whereas significant inhibition was observed when they were used together.

To determine whether agents used therapeutically for treatment of asthma could inhibit eosinophil activation, studies were also conducted with the β_2 -agonist, albuterol. The eosinophil chemiluminescence response was inhibited by albuterol (inhibitory concentration of 50% = 30 nmol/L) at a concentration similar to that which may be achieved in vivo (Fig. 6).^{18, 22}

DISCUSSION

Isoproterenol was a potent inhibitor of the fMLP-stimulated respiratory burst from eosinophils as detected with lucigenin-dependent luminescence. Although β -adrenoceptors have been identified on eosinophils,¹⁵ their functional signif-

icance is unclear.^{10, 15} At low cell concentrations our results demonstrate that the eosinophil respiratory burst, as detected with lucigenin, can be inhibited by the β -adrenoceptor agonists, isoproterenol and albuterol. Because actions of β -adrenoceptor agonist and selective phosphodiesterase inhibitor were synergistic, inhibitory actions may be mediated by cAMP.

The methods used in this study clearly distinguish the results from those of a previous report, which did not identify any effect of β -agonist on the respiratory burst.¹⁵ In that report eosinophils at a high concentration from patients with eosinophilia were evaluated after stimulation with high ($>10 \text{ nmol/L}$) PMA concentrations. In contrast, we have used lucigenin detection of superoxide anion, a very sensitive assay allowing the study of unprimed eosinophils at low densities (2.5×10^4 cells/ml) stimulated with low concentrations of respiratory burst agonists. Studies of low cell densities may be of value because small numbers of eosinophils are available from a healthy subject's

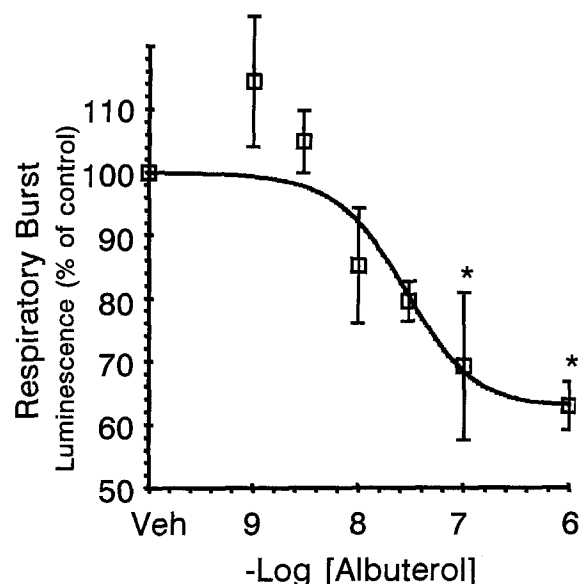


FIG. 6. Inhibition of the eosinophil fMLP-induced, chemiluminescence response by albuterol. Eosinophils (2.5×10^4 cells) were incubated at 37°C in DPBS (1 ml) containing fMLP ($1 \mu\text{mol/L}$) with and without albuterol as described in the Methods section. Respiratory burst was detected by chemiluminescence from lucigenin ($10 \mu\text{mol/L}$). Chemiluminescence integrated from 0 to 4 minutes after exposure of eosinophils to fMLP and salbutamol is shown. Data represent percent of control stimulation (cell sample stimulated by fMLP without albuterol). Means $\pm$ SEM ($n = 5$) are shown. Treatments significantly different from control are marked by an asterisk (Dunnett's test, $p < 0.05$).

blood sample and because low cell densities minimize effects of mediators generated by the cells. Eosinophils generate platelet activating factor,²³ a mediator established to prime cells in the immediate proximity,^{17, 24} which may alter the signal transduction mechanism of eosinophils.²⁴ In addition, at high cell densities the accumulation of cell metabolites, such as adenosine (an agent which affects adenylyl cyclase in many cells), may complicate interpretation of effects mediated by cAMP. Consequently, the results of this study are distinguished by methods that use low cell concentrations and may more accurately demonstrate cell responses. Additionally, another eosinophil response, granule secretion, is inhibited by β -adrenergic agents.²⁵ Additionally, another eosinophil response, granule secretion, is inhibited by β -adrenergic agents.²⁵ Although primed eosinophils may have different responses, eosinophils from normal subjects, when studied at low concentrations, are sensitive to inhibition by β -adrenergic agents.

Isoproterenol inhibited stimulation of the eo-

sinophil chemiluminescence response by nonreceptor-mediated and receptor-mediated agonists. Although actions of β -agonists to inhibit receptor coupling have been suggested to occur in neutrophils,^{18, 26} the observed inhibition of eosinophils stimulated by non-surface receptor-mediated stimuli suggests that inhibition of the eosinophil by β -agonist is not mediated by an isolated action at the fMLP receptor or on fMLP receptor coupling. Because β -adrenoceptor agonist and selective cAMP phosphodiesterase inhibitors caused synergistic inhibition of the respiratory burst detected with lucigenin-dependent luminescence, it is likely that cAMP mediated eosinophil inhibition. Consistent with this conclusion, phosphodiesterase inhibitors and dibutyl cAMP have previously been shown to inhibit the eosinophil respiratory burst.¹⁶

The respiratory burst, which was induced by chemotactic peptide and detected with lucigenin, had biphasic temporal characteristics. Isoproterenol appeared to cause greatest inhibition of the initial phase of the chemiluminescence response. Although the mechanism of reduced subsequent activity is not established, β -receptor desensitization, changes in signal transduction mechanisms, or increased cAMP degradation during later phases of the respiratory burst could contribute.^{24, 27} Because a cAMP phosphodiesterase inhibitor and β -agonist synergistically inhibited the later phase of the eosinophil chemiluminescence response, it appears that cAMP generation was still induced by β -agonist and that cAMP-mediated inhibition was still effective. If increased phosphodiesterase activity contributes to reduced β -agonist activity, then pharmacologic intervention to inhibit phosphodiesterase activity could have anti-inflammatory value. In contrast to actions of β -agonist, phosphodiesterase inhibitor caused the greatest decrease in the later phases of the eosinophil chemiluminescence response. If cAMP is generated during the eosinophil respiratory burst as occurs in neutrophils,²⁸⁻³⁰ the later effects of phosphodiesterase inhibitor may represent cAMP accumulation. Because combinations of phosphodiesterase inhibitor and β -agonist could have anti-inflammatory actions, further study of the mechanisms of eosinophil inhibition may be of value.

Inhibition of eosinophil function by β -agonists and phosphodiesterase inhibitors may be of clinical importance because β -agonists and agents including theophylline, which potentially affect eosinophil cAMP, are commonly used in the treatment of asthma. Although the mechanisms of cAMP action

remain unclear, effects of commonly used therapeutic agents to inhibit the eosinophil may be of clinical relevance and require further investigation.

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