Biochemical Pharmacology, Vol. 40, No. 4, pp. 699–707, 1990.
Printed in Great Britain.

0006-2952/90 $3.00 + 0.00

DIFFERENTIAL INHIBITION OF HUMAN NEUTROPHIL FUNCTIONS

ROLE OF CYCLIC AMP-SPECIFIC, CYCLIC GMP-INSENSITIVE PHOSPHODIESTERASE

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(*Received* 23 *June* 1989; *accepted* 17 *January* 1990)

Abstract—Multiple molecular forms of cyclic nucleotide phosphodiesterase have been characterized in various tissues and cells according to their substrate specificity, intracellular location, and calmodulin dependence. The purpose of this study was to evaluate the possible involvement of different molecular forms of phosphodiesterase in regulating the respiratory burst and lysosomal enzyme release responses of human neutrophils. Treatment with the selective cyclic AMP-specific, cyclic GMP-insensitive phosphodiesterase inhibitors Ro 20-1724 or rolipram, or the nonselective phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine (IBMX), resulted in inhibition of respiratory burst stimulated by the chemoattractants formyl-L-methionyl-L-leucyl-L-phenylalanine (FMLP) (IC_{50} values: 0.71–17 μM) and complement fragment C5a (IC_{50} values: 61–93 μM), but did not inhibit phagocytosis-stimulated respiratory burst (<10% inhibition at 100 μM). Selective inhibitors of calmodulin-dependent phosphodiesterase (ICI 74,917), calmodulin-insensitive, cyclic GMP-specific phosphodiesterase (M&B 22,948), cyclic GMP-stimulated phosphodiesterase (AR-L 57), or cyclic AMP-specific, cyclic GMP-inhibited phosphodiesterase (amrinone and cilostamide) exhibited little or no inhibitory effect on FMLP- or phagocytosis-stimulated respiratory burst (0–42% inhibition at 100 μM). Regulation of neutrophil activation by phosphodiesterase was also response specific, as Ro 20-1724, rolipram and IBMX were less potent inhibitors of FMLP-induced lysosomal enzyme release (0–14% inhibition at 100 μM). Analysis of human neutrophil preparations confirmed the existence of a cyclic AMP-specific, cyclic GMP-insensitive phosphodiesterase, which was associated with the particulate fraction of the cell. These results demonstrate a role for the cyclic AMP-specific, cyclic GMP-insensitive phosphodiesterase in the regulation of human neutrophil functions, which appears to be both stimulus specific and response specific.

The role of cyclic nucleotides in modulating signal transduction of human neutrophils has been examined in several previous studies. Increases in intracellular cyclic AMP levels in neutrophils are associated with a decrease in several neutrophil functions, including chemotaxis, respiratory burst, and lysosomal enzyme release [1–8]. Chemoattractants such as complement component C5a and formyl-L-methionyl-L-leucyl-L-phenylalanine (FMLP) have been shown to produce a rapid, transient rise in cyclic AMP levels in neutrophils [3, 9]. Although FMLP does not directly stimulate adenylate cyclase, it has been shown to indirectly cause an increase in cyclic AMP by a mechanism requiring Ca^{2+} mobilization [9]. In contrast to chemotactic stimuli, β-adrenergic receptor agonists and prostaglandin E_1 (PGE_1) produce sustained increases in cyclic AMP by receptor-mediated stimulation of adenylate cyclase [3, 5, 10–12]. The differential activation of adenylate cyclase is illustrated further by the ability of FMLP to potentiate PGE_1-induced cyclic AMP production by a Ca^{2+} and calmodulin-dependent process [13].

Cyclic nucleotide levels in neutrophils may be regulated by either their rates of production or degradation. For example, β-adrenergic agonists and PGE_1 stimulate sustained increases in cyclic AMP levels by receptor-mediated stimulation of adenylate cyclase [3, 5, 10–12]. In contrast, neutrophils contain a cyclic nucleotide phosphodiesterase which mediates cyclic AMP degradation [14–17].

Studies in various tissues and cells have demonstrated the existence of several distinct molecular forms of cyclic nucleotide phosphodiesterase, which vary with regard to substrate specificity (cyclic AMP vs cyclic GMP), intracellular localization (soluble vs membrane-bound), and response to calmodulin [18–21]. In addition selective inhibitors of several of the different phosphodiesterases have been identified recently [21]. These agents have proven useful in characterizing the involvement of the different molecular forms of phosphodiesterases in regulating several physiological events, including myocardial contractility and lipolysis [22, 23].

In the present series of experiments, the involvement of the different forms of cyclic nucleotide phosphodiesterase in regulating human neutrophil functions was investigated by (i) evaluating the ability of various selective and nonselective phosphodiesterase inhibitors to inhibit neutrophil activation, and (ii) characterizing the phosphodiesterase activity

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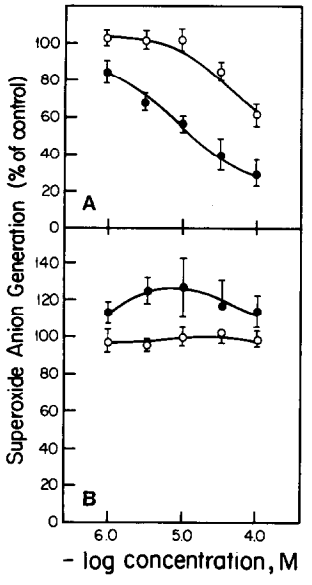

Fig. 1. Effects of nonselective phosphodiesterase inhibitors IBMX (—●—) and aminophylline (—○—) on the respiratory burst of human neutrophils in response to formyl-L-methionyl-L-leucyl-L-phenylalanine (FMLP) (A) or serum-opsonized zymosan (SOZ) (B). For control cells, FMLP and SOZ stimulated superoxide anion generation at a rate of 8.0 ± 0.99 nmol/10^6 cells/10 min and 12.5 ± 3.6 nmol/10^6 cells/30 min respectively. Each symbol is the mean ± SE of 3–6 separate duplicate determinations.

present in neutrophils. The results of these experiments demonstrate that human neutrophils possess a cyclic AMP-specific, cyclic GMP-insensitive form of phosphodiesterase, which appears to play an important role in regulating certain components of neutrophil activation.

EXPERIMENTAL PROCEDURES

Isolation of human neutrophils. Neutrophils were isolated from anticoagulant-treated venous blood drawn from healthy human volunteers according to the procedure of Ferrante and Thong [24]. The cell preparations obtained consisted of greater than 98% neutrophils as determined microscopically by differential staining. Dulbecco's phosphate-buffered saline was used as the cell suspension medium throughout this study.

Assays of neutrophil functions. Both particulate and soluble stimuli were used to induce the respiratory and secretory functions of human neutrophils. The particulate stimulus serum-opsonized zymosan was prepared as described by Wardlaw and Pillemer [25], and used at a final concentration of 1.25 mg/mL. FMLP was used at a final concentration of 1.0 μM to stimulate the respiratory burst. To stimulate lysosomal enzyme release, FMLP was used at a final concentration of 100 μM following a 10-min preincubation with 10 μM cytochalasin B. The human complement fragment C5a, prepared according to the procedure of Fernandez and Hugli [26], was used at a final concentration of 1 μg/mL following a similar preincubation with cytochalasin B.

The respiratory burst of human neutrophils was measured as superoxide dismutase inhibitable reduction of cytochrome *c* by superoxide anion [27]. The particulate stimulus serum-opsonized zymosan or soluble stimulus, C5a or FMLP, were incubated at 37° with 1×10^6 neutrophils in a final volume of 1.0 mL. Following a 30-min incubation with serum-opsonized zymosan or C5a, or a 10-min incubation with FMLP, the respiratory burst was stopped by placing the reaction tubes in ice water. Cells were removed by centrifugation, and superoxide generation was quantitated spectrophotometrically at an absorbance of 550 nm. The potential of the phosphodiesterase inhibitors to act as oxygen radical scavengers was determined using acellular xanthine oxidase-dependent generation of superoxide anion [28].

Lysosomal enzyme release from human neutrophils was evaluated using myeloperoxidase as a primary granule marker and lysozyme as a non-specific secondary granule marker [29]. Stimuli were incubated with 2×10^6 neutrophils in a final volume of 1.0 mL. Following a 30-min incubation at 37°, the reaction was terminated by rapid cooling in an ice bath. Following centrifugation, the cell supernatant fractions were assayed for lysosomal enzyme activities. Myeloperoxidase was assayed according to the procedure of Baggiolini *et al.* [30]. Lysozyme was assayed according to the procedure of Shugar [31]. In addition, the cytoplasmic enzyme lactate dehydrogenase (LDH) was assayed to detect possible drug-induced cytotoxicity using the procedure of Wacker *et al.* [32]. Enzyme release in response to the various stimuli was quantitated as a percentage of the total cellular enzyme content. Enzyme content was measured in cell sonicates, prepared by lysing cells at 4° with three 10-sec bursts using a Branson sonicator (setting of 7), which resulted in maximal solubilization of enzymes [33].

The inhibitory effects of test compounds on human neutrophils were evaluated in duplicate at concentrations ranging from 1 to 100 μM. Linear regression analysis of the means of the inhibition values for neutrophils from at least two donors was performed to determine the drug concentration required for 50% inhibition (IC_{50}) of the responses.

Cyclic AMP quantitation. Neutrophils were stimulated with FMLP or serum-opsonized zymosan as previously described for stimulation of the respiratory burst. After a 10- or 30-min incubation at 37° with FMLP or serum-opsonized zymosan, respectively, the cellular responses were stopped by placing the reaction tubes in ice water. After centrifugation, the cell pellets were extracted with 1 mL of acidic ethanol (1:100 1 N HCl:absolute ethanol) with 4 mM EDTA. Cyclic AMP concentrations in the extracts were quantitated using a commercially available radioimmunoassay kit obtained from the Amersham Corp. (Arlington Heights, IL).

Procedures for isolating neutrophil phosphodiesterase. Soluble and membrane-bound fractions of the human neutrophils were prepared using a modification of the isolation procedure

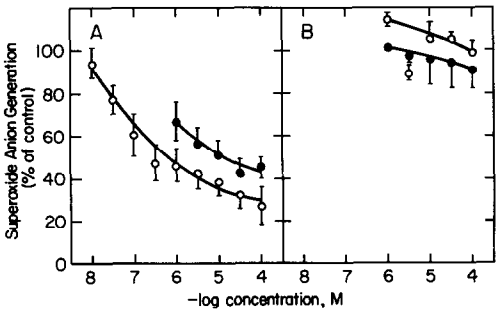

Fig. 2. Effects of the selective cyclic AMP-specific, cyclic GMP-insensitive phosphodiesterase inhibitors Ro 20-1724 (—●—) and rolipram(—○—) on the respiratory burst of human neutrophils in response to formyl-L-methionyl-L-leucyl-L-phenylalanine (FMLP) (A) or serum-opsonized zymosan (SOZ) (B). For control cells, FMLP and SOZ stimulated superoxide anion generation at a rate of 6.2 ± 0.65 nmol/10^6 cells/10 min and 10.5 ± 2.4 nmol/10^6 cells/30 min respectively. Each symbol is the mean ± SE of 3–6 separate duplicate determinations.

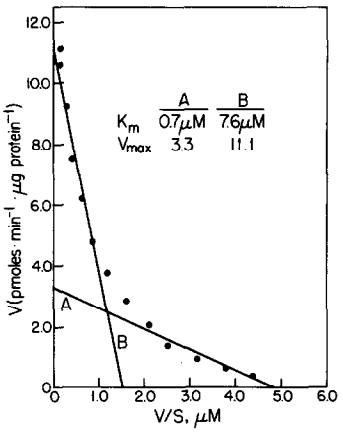

Fig. 3. Determination of K_m and V_{max} values for cyclic AMP hydrolysis by the particulate fraction from human neutrophils. For this evaluation, phosphodiesterase activity was measured at substrate concentrations ranging from 0.1 to 100 μM according to the method of Hofstee [35].

described by Elks and Manganiello [23]. Approximately 2.0 mL of packed neutrophils were homogenized in 10 vol. of a buffer containing 0.25 M sucrose, 10 mM Tris–HCl (pH 7.8), 5 mM $MgCl_2$ and 0.2 mM ethyleneglycolbis(aminoethylether)-tetra-acetate (EGTA). The peptidase inhibitors leupeptin, pepstatin A, and phenylmethylsulfonylflouride (PMSF) were also included in this homogenizing buffer, each at a final concentration of 100 nM. The cells were homogenized with a Dounce hand-held homogenizer (4 passes, 10 strokes per pass with a type B pestle, and a 30-sec rest period between each pass). This and all subsequent procedures were performed at 4°. The homogenate was then centrifuged at 100,000 *g* for 40 min after which the supernatant fraction was decanted. The pellet was resuspended in 10 vol. of homogenizing buffer and centrifuged as before. The resulting supernatant fraction was discarded, and the pellet was resuspended in 10 vol. of the homogenizing buffer.

Assaying phosphodiesterase activity. Cyclic AMP and cyclic GMP phosphodiesterase activity was measured in the crude homogenate, the initial supernatant fraction, and the resuspended pellet as previously described [34]. The measurements were made in the presence and absence of saturating levels of calcium and calmodulin [34]. K_m and V_{max} values for hydrolysis of cyclic AMP by the resuspended pellet were determined using the procedure of Hofstee [35]. Kinetic studies were not performed using cyclic GMP as substrate due to insufficient cyclic GMP phosphodiesterase activity in the resuspended pellet. All studies with selective and nonselective phosphodiesterase inhibitors were conducted using the resuspended pellet.

All phosphodiesterase inhibitors examined were dissolved in dimethyl sulfoxide (DMSO) at a final concentration of 2.5% in the reaction medium. This DMSO concentration inhibited enzyme activity by approximately 10%. The IC_{50} values for the inhibitors examined were determined from concentration–response curves, in which concentrations ranged from 0.1 to 100 μM for the more potent inhibitors and 10 to 1000 μM for the less potent inhibitors (half-log increments). Two to three such curves were generated for each agent.

Materials. All reagents used were of the highest obtainable commercial purity. Dulbecco's phosphate-buffered saline was obtained from GIBCO Laboratories (Grand Island, NY). FMLP was obtained from Behring Diagnostics (La Jolla, CA). Cytochalasin B and 3-isobutyl-l-methylxanthine (IBMX) were purchased from the Aldrich Chemical Co. (Milwaukee, WI). Aminophylline, theophylline, histamine, and the LDH reagent kit were obtained from the Sigma Chemical Co. (St. Louis, MO). Rolipram was obtained from Schering AG (Berlin, F.R.G.). Ro 20-1724 was obtained from Hoffmann-LaRoche (Nutley, NJ). Amrinone, AR-L 57, cilostamide, ICI 74,917, and M&B 22,948 were prepared by the Chemistry Department of the Parke-Davis Pharmaceutical Research Division, Warner-Lambert Co.

RESULTS

Effects of nonselective phosphodiesterase inhibitors on the respiratory burst of human neutrophils. The nonselective phosphodiesterase inhibitor IBMX inhibited respiratory burst in human neutrophils (measured as the generation of superoxide anion) stimulated with the synthetic chemotactic peptide FMLP with an IC_{50} of 16.6 μM (Fig. 1A) (Table 1). The nonselective inhibitor aminophylline also inhibited this response (38% inhibition at 100 μM, which was its solubility limit). In contrast, at concentrations up to 100 μM, neither IBMX nor aminophylline inhibited respiratory burst when neutrophils were exposed to the phagocytic stimulus serum-opsonized zymosan (Fig. 1B). In addition, neither compound inhibited acellular generation of superoxide anion by xanthine oxidase at concentrations as high as 100 μM, indicating that the compounds were not oxygen radical scavengers (data not shown).

Table 1. Effects of nonselective and selective phosphodiesterase inhibitors on superoxide anion generation by human neutrophils in response to FMLP or SOZ

Inhibitor	Phosphodiesterase selectivity*	IC_{50}†, μM (or % Inhibition at 100 μM)	
		Stimuli for superoxide anion generation	
		FMLP‡	SOZ§
IBMX	Nonselective	16.6	⩾100 (<10%)
Aminophylline	Nonselective	>100 (38%)	⩾100 (<10%)
ICI 74,917	Calmodulin-dependent phosphodiesterase	⩾100 (<10%)	⩾100 (<10%)
M&B 22,948	Cyclic GMP-specific phosphodiesterase	>100 (27%)	⩾100 (<10%)
AR-L 57	Cyclic GMP-stimulated phosphodiesterase	>100 (42%)	⩾100 (11%)
Dipyridamole	Cyclic GMP-stimulated phosphodiesterase	⩾100 (<10%)	⩾100 (<10%)
Amrinone	Cyclic AMP-specific, cyclic GMP-inhibited phosphodiesterase	>100 (28%)	⩾100 (<10%)
Cilostamide	Cyclic AMP-specific, cyclic GMP-inhibited	⩾100 (<10%)	⩾100 (16%)
Ro 20-1724	Cyclic AMP-specific, cyclic GMP-insensitive phosphodiesterase	16.8	⩾100 (<10%)
Rolipram	Cyclic AMP-specific cyclic GMP-insensitive phosphodiesterase	0.71	⩾100 (<10%)

* The selectivity of the inhibitors was described previously in Refs. 20 and 21.

† Each IC_{50} value is the mean of 2–3 separate experiments.

‡ FMLP, formyl-L-methionyl-L-leucyl-L-phenylalanine (10^{-6} M). Mean superoxide anion production = 8.0 ± 1.2 nmol/10^6 cells/10 min.

§ SOZ, serum-opsonized zymosan (1.25 mg/mL). Mean superoxide anion production = 9.9 ± 1.4 nmol/10^6 cells/30 min.

These results indicate a stimulus-specific involvement of phosphodiesterase in regulating the respiratory burst of human neutrophils.

Effects of selective phosphodiesterase inhibitors on the respiratory burst of human neutrophils. The involvement of phosphodiesterase in the regulation of the neutrophil respiratory burst was characterized further using selective inhibitors of the various phosphodiesterase subclasses. The selectivity of the inhibitors was assessed by their ability to inhibit one type of phosphodiesterase with a potency one to three orders of magnitude greater than for the other types [21]. As shown in Table 1, inhibitors of the calmodulin-dependent phosphodiesterase (ICI 74,917), the cyclic GMP-specific phosphodiesterase (M&B 22,948), the cyclic GMP-stimulated phosphodiesterase (AR-L 57), and the cyclic AMP-specific, cyclic GMP-inhibited phosphodiesterase (amrinone and cilostamide) had little or no inhibitory effect on the generation of superoxide anion by neutrophils stimulated with either FMLP or serum-opsonized zymosan. In contrast, selective inhibitors of the cyclic AMP-specific, cyclic GMP-insensitive subclass of phosphodiesterase (rolipram and Ro 20-1724) were potent inhibitors of the FMLP-stimulated respiratory burst (Fig. 2A). The IC_{50} value for Ro 20-1724 was 16.8 μM, and the IC_{50} value for rolipram was 0.71 μM (Table 1). Ro 20-1724 and rolipram exhibited similar inhibitory effects against superoxide anion generation in response to FMLP plus cytochalasin B under conditions used to stimulate lysosomal enzyme release. As with the nonspecific phosphodiesterase inhibitors IBMX and aminophylline, both Ro 20-1724 and rolipram had little inhibitory effect on phagocytosis-stimulated oxygen radical production (<10% inhibition at 100 μM) (Fig. 2B). Incubation of Ro 20-1724 or rolipram with serum-opsonized zymosan for 30 min at 37° had no effect on the subsequent inhibitory activities of the compounds against the FMLP-stimulated respiratory burst, indicating that the stimulus-specific inhibitory effects of these compouds were not the result of nonspecific removal of the compounds from solution by the zymosan particles. In addition, neither rolipram nor Ro 20-1724 inhibited acellular generation of superoxide anion by xanthine oxidase, indicating that their effects on superoxide anion generation were not due to scavenging of oxygen free radicals (data not shown).

The selective cyclic AMP-specific, cyclic GMP-insensitive phosphodiesterase inhibitors rolipram and Ro 20-1724, and the nonselective inhibitor IBMX, also inhibited superoxide anion generation by neutrophils stimulated by the complement fragment C5a (in the presence of cytochalasin B) (Table 2). However, the concentrations required for such inhibition were higher than for inhibition of FMLP-stimulated superoxide generation. Inhibitors of the other phosphodiesterase subclasses blocked the C5a-induced respiratory burst by 0–18% at concentrations as high as 100 μM.

Table 2. Effects of nonselective and selective phosphodiesterase inhibitors on C5a-stimulated respiratory burst of human neutrophils*

Inhibitor	IC_{50}† (μM)
IBMX	92.6
Ro 20-1724	60.8
Rolipram	81.1

* C5a (1 μg/mL) + cytochalasin B (10 μM).
Mean superoxide anion production = 18.1 ± 0.3 nmol/10^6 cells/30 min.
† Each IC_{50} value is the mean of 2–3 separate experiments.

Effects of phosphodiesterase inhibitors on lysosomal enzyme release from human neutrophils. The role of cyclic nucleotide phosphodiesterase in the activation of human neutrophils was characterized further by examining the effects of selective and nonselective phosphodiesterase inhibitors on lysosomal enzyme release (Table 3). The selective cyclic AMP-specific, cyclic GMP-insensitive phosphodiesterase inhibitors (Ro 20-1724 and rolipram) and the nonselective phosphodiesterase inhibitors (IBMX and aminophylline) were weak inhibitors of release of primary (myeloperoxidase) or secondary (lysozyme) lysosomal granule enzymes following stimulation of neutrophils by either FMLP or serum-opsonized zymosan. In contrast to the potent inhibitory effects of these compounds on the FMLP-stimulated respiratory burst, the compounds exerted only a 10–15% inhibitory effect on lysosomal enzyme release at concentrations as high as 100 μM. Likewise, the other selective phosphodiesterase inhibitors did not exert a profound inhibitory effect on the release of lysosomal enzymes from human neutrophils in response to either stimulus. These results demonstrate that FMLP-induced lysosomal enzyme release from human neutrophils is less sensitive to inhibitors of cyclic AMP-specific, cyclic GMP-insensitive phosphodiesterase than the generation of oxygen radicals.

Effects of phosphodiesterase inhibitors on intracellular cAMP levels in stimulated human neutrophils. The nonselective phosphodiesterase inhibitor IBMX and the cyclic AMP-specific, cyclic GMP-insensitive phosphodiesterase inhibitors Ro 20-1724 and rolipram were examined for their effects on intracellular cAMP levels in stimulated human neutrophils (Table 4). Under conditions used to stimulate the respiratory burst, neutrophils incubated with FMLP or serum-opsonized zymosan had cAMP levels of 0.95 ± 0.15 pmol/2 × 10^6 cells and 0.87 ± 0.16 pmol/2 × 10^6 cells respectively. In the presence of 100 μM IBMX, Ro 20-1724, or rolipram, similar increases in cAMP levels were observed following stimulation with FMLP or serum-opsonized zymosan. These results demonstrate a stimulus-independent increase in cAMP by these phosphodiesterase inhibitors. These results contrast with the selective inhibitory effects of these agents for the FMLP-induced respiratory burst. At the same concentration, inhibitors of the other phosphodiesterase subtypes caused only a 0–17% increase of cAMP in response to FMLP or serum-opsonized zymosan. These results indicate that increases in cAMP alone do not account for the stimulus-specific inhibition of the respiratory burst.

Table 3. Effects of nonselective and selective phosphodiesterase inhibitors on lysozomal enzyme release by human neutrophils in response to FMLP or SOZ

	IC_{50}†, μM (or % Inhibition at 100 μM)			
	Stimuli for lysosomal enzyme release			
	FMLP + CB‡		SOZ§	
Inhibitor*	Myeloperoxidase	Lysozyme	Myeloperoxidase	Lysozyme
IBMX	NA‖	>100 (14%)	>100 (10%)	NA
Aminophylline	>100 (17%)	>100 (15%)	NA	NA
ICI 74,917	⩾100 (<10%)	NA	⩾100 (<10%)	>100 (15%)
M&B 22,948	NA	⩾100 (<10%)	>100 (49%)	NA
Dipyridamole	⩾100 (<10%)	NA	>100 (26%)	NA
AR-L 57	>100 (10%)	NA	>100 (46%)	>100 (20%)
Amrinone	63.2	>100 (11%)	>100 (19%)	NA
Cilostamide	NA	>100 (20%)	>100 (34%)	⩾100 (<10%)
Ro 20-1724	NA	⩾100 (<10%)	⩾100 (<10%)	>100 (24%)
Rolipram	>100 (14%)	>100 (11%)	⩾100 (<10%)	NA

* The selectivities of the inhibitors are summarized in Table 1.
† Each IC_{50} value is the mean of 2–4 separate experiments.
‡ FMLP + CB, formyl-L-methionyl-L-leucyl-L-phenylalanine (1×10^{-4} M) + cytochalasin B (1×10^{-5} M). Mean myeloperoxidase release = 30.3 ± 2.1% of total cellular enzyme; mean lysozyme release = 56.9 ± 2.8% of total cellular enzyme.
§ SOZ, serum-opsonized zymosan (1.25 mg/mL). Mean myeloperoxidase release = 3.6 ± 1.0% of total cellular enzyme; mean lysozyme release = 24.4 ± 1.3% of total cellular enzyme.
‖ NA, no inhibitory activity at a 100 μM concentration.

Table 4. Effects of nonselective and selective phosphodiesterase inhibitors on cAMP levels in neutrophils in response to FMLP or SOZ

Inhibitor	% of Control at 100 μM	
	FMLP*	SOZ†
IBMX	166.2 ± 22.6‡	163.0 ± 21.1‡
ICI 74,917	107.8 ± 53.8	110.0 ± 19.0
M&B 22,948	114.3 ± 5.7	107.3 ± 7.4
Dipyridamole	109.7 ± 8.0	97.3 ± 5.8
AR-L 57	117.3 ± 9.6	92.3 ± 8.3
Amrinone	109.7 ± 22.7	112.3 ± 9.0
Cilostamide	110.3 ± 21.7	114.0 ± 5.3
Ro 20-1724	196.2 ± 31.6‡	200.8 ± 40.6‡
Rolipram	212.1 ± 25.5‡	198.3 ± 37.4‡

Values are the means ± SEM of 3–9 separate experiments. Control cAMP levels in neutrophils incubated with FMLP or SOZ were 0.95 ± 0.15 and 0.87 ± 0.16 pmol/2×10^6 cells respectively.

* FMLP, formyl-L-methionyl-L-leucyl-L-phenylalanine (10^{-6} M).

† SOZ, serum-opsonized zymosan (1.25 mg/mL).

‡ $P < 0.05$ vs control.

Table 5. Effect of adenylate cyclase stimulating agents on superoxide anion production by human neutrophils

Agent	IC_{50}*, μM (or % Inhibition at 100 μM)		
	Stimuli		
	FMLP†	SOZ‡	C5a§
Adenosine	>100 (48%)	⩾100 (<10%)	>100 (54%)
Histamine	>100 (47%)	⩾100 (<10%)	>100 (42%)
PGE_1	3.8	>100 (23%)	>100 (40%)

* Each IC_{50} value is the mean of 2–3 separate experiments.

† FMLP, formyl-L-methionyl-L-leucyl-L-phenylalanine (10^{-6} M).

‡ SOZ, serum-opsonized zymosan (1.25 mg/mL).

§ C5a (1 μg/mL) + cytochalasin B (10 μM).

Effects of agents that stimulate cyclic AMP production on activation of human neutrophils. The regulatory role of cAMP was evaluated further by comparing the effects of agents that promote cAMP production (i.e. adenosine, histamine, and PGE_1) against the neutrophil respiratory burst (Table 5). These agents exhibited greater inhibitory effects against superoxide anion production stimulated by FMLP or C5a than against the response stimulated by serum-opsonized zymosan. In addition, none of these agents inhibited lysosomal enzyme release by greater than 25% at concentrations as high as 100 μM (data not shown). These results demonstrate that agents which stimulate cAMP production exhibit a stimulus- and response-selective inhibitory profile similar to that observed for IBMX or inhibitors of the cyclic AMP-specific, cyclic GMP-insensitive class of phosphodiesterase.

Biochemical characterization of human neutrophil phosphodiesterases. Crude homogenates of human neutrophils were examined for their ability to hydrolyze cyclic AMP and cyclic GMP. As shown in Table 6, the cyclic AMP hydrolytic activity of the homogenate was greater than the cyclic GMP hydrolytic activity. Cyclic nucleotide phosphodiesterase activity was observed in both the soluble (initial supernatant) and particulate (washed, resuspended pellet) fractions. Like the crude homogenate, the particulate fraction, constituting 80% of the total phosphodiesterase activity, preferentially hydrolyzed cyclic AMP. In contrast, the soluble fraction exhibited comparable hydrolytic activity for cyclic AMP and cyclic GMP. These preliminary observations suggest that human neutrophils contain more than one form of cyclic nucleotide phosphodiesterase, localized in different cellular compartments.

Based on the cyclic AMP specificity of the particulate fraction and phosphodiesterase subclass specificity of Ro 20-1724 and rolipram, subsequent kinetic and inhibitor studies were focused on the particulate enzyme. Cyclic AMP hydrolytic activity in the particulate fraction was not stimulated by calmodulin (in the presence of calcium). In addition, cyclic AMP hydrolysis by the membrane-bound fraction was not stimulated by low concentrations of cyclic GMP, and was inhibited only by high concentrations of cyclic GMP ($IC_{50} \sim 1.0$ mM). Kinetic studies revealed two hydrolytic sites for cyclic AMP, with K_m values of 0.7 and 7.6 μM (Fig. 3). These values are comparable to those previously reported for cyclic AMP-specific phosphodiesterase from cardiac and vascular smooth muscle [19, 34]. The minimal cyclic GMP hydrolytic activity of the particulate fraction precluded kinetic analysis with this substrate.

The effects of various selective and nonselective phosphodiesterase inhibitors on the particulate cyclic AMP-specific, cyclic GMP-insensitive phosphodiesterase are shown in Table 7. The nonselective inhibitor IBMX potently inhibited particulate phosphodiesterase activity ($IC_{50} = 23.0$ μM). The cyclic AMP-specific, cyclic GMP-insensitive phosphodiesterase inhibitors Ro 20-1724 and rolipram were also potent inhibitors of the particulate activity (IC_{50} values = 10.0 and 1.7 μM respectively). Selective inhibitors of the other phosphodiesterase subtypes were less active against the particulate fraction enzyme activity, with IC_{50} values greater than those required for inhibition of the phosphodiesterase subtypes for which they exhibit specificity [21, 22, 34]. These results demonstrate that the human neutrophil particulate fraction contains a cyclic AMP-specific, cyclic GMP-insensitive phosphodiesterase, whose pharmacologic modulation is similar to that previously reported for this phosphodiesterase subclass [21, 22].

DISCUSSION

Activation of neutrophils by a variety of physiological stimuli has been shown to utilize a common mechanism in which stimulus and response are linked by a phospholipid-mediated metabolic process [36–40]. Interaction of agonists with specific cell

Table 6. Cyclic AMP and cyclic GMP hydrolytic activity of human neutrophils

Preparation	Cyclic AMP hydrolytic activity (pmol/min/mg protein)	Cyclic GMP hydrolytic activity (pmol/min/mg protein)	Cyclic AMP / Cyclic GMP
Crude homogenate	2.78	0.62	4.5
Soluble fraction (initial supernatant)	4.22	3.51	1.2
Particulate fraction (washed, resuspended pellet)	2.11	0.30	7.0

Table 7. Effects of nonselective and selective phosphodiesterase inhibitors on particulate phosphodiesterase activity from human neutrophils

Inhibitor	Phosphodiesterase selectivity*	IC_{50}† (μM)
IBMX	Nonselective	23.0
ICI 74,917	Calmodulin-dependent phosphodiesterase	52.0
M&B 22,948	Cyclic GMP-specific phosphodiesterase	70.0
AR-L 57	Cyclic GMP-stimulated phosphodiesterase	75.0
Amrinone	Cyclic AMP-specific, cyclic GMP-inhibited phosphodiesterase	250
Cilostamide	Cyclic AMP-specific, cyclic GMP-inhibited phosphodiesterase	55.0
Ro 20-1724	Cyclic AMP-specific cyclic GMP-insensitive phosphodiesterase	10.0
Rolipram	Cyclic AMP-specific, cyclic GMP-insensitive phosphodiesterase	1.7

* The inhibitory selectivity for the agents examined was described previously in Refs. 20 and 21.

† Each IC_{50} value is the mean of 2–3 separate determinations.

membrane receptors on human neutrophils stimulates phospholipase C through a guanine nucleotide binding protein [41, 42], leading to production of the second messenger molecules inositol 1,4,5-trisphosphate and 1,2-diacylglycerol. Activated neutrophils subsequently exhibit a variety of responses, including the release of enzymes from lysosomal granules and generation of superoxide anion [27, 43].

Although induction of functional responses in human neutrophils requires a common signal transduction mechanism, the results of the present study demonstrate both stimulus-specific and response-specific regulation of neutrophil activation by cyclic AMP. The differential involvement of cyclic nucleotides was suggested by the observation that IBMX, a nonselective phosphodiesterase inhibitor, was a more potent inhibitor of the respiratory burst of neutrophils stimulated by the chemoattractants FMLP and C5a than of the response induced by phagocytosis of serum-opsonized zymozan. The inhibitory effect of IBMX was also shown to be response specific, with FMLP-stimulated superoxide anion generation being more sensitive to inhibition than lysosomal enzyme release in response to the same stimulus. Similar results have been reported by Lad *et al.* [5] and Schmeichel and Thomas [7]. IBMX also failed to inhibit lysosomal enzyme release in response to serum-opsonized zymosan.

Since IBMX inhibits both cyclic AMP- and cyclic GMP-hydrolyzing phosphodiesterases [21], the abilities of selective inhibitors of the various molecular forms of cyclic nucleotide phosphodiesterase to mimic the stimulus- and response-specific effects of IBMX on neutrophil activation were evaluated. These experiments utilized selective inhibitors of the calmodulin-stimulated phosphodiesterase (ICI 74,917), the calmodulin-insensitive, cyclic GMP-specific phosphodiesterase (M&B 22,948), the cyclic GMP-stimulated phosphodiesterase (AR-L 57), the cyclic AMP-specific, cyclic GMP-inhibited phosphodiesterase (amrinone and cilostamide), and the cyclic AMP-specific, cyclic GMP-insensitive phosphodiesterase (Ro 20-1724 and rolipram) [21]. The results of these experiments suggest that the cyclic AMP-specific, cyclic GMP-insensitive form of phosphodiesterase plays an important role in modulating neutrophil activation, since only the selective inhibitors rolipram and Ro 20-1724 exerted an effect on neutrophil function. As with the nonselective phosphodiesterase inhibitors IBMX and aminophylline, rolipram and Ro 20-1724 inhibited the respiratory burst stimulated by FMLP, but did not inhibit the respiratory burst stimulated by phagocytosis. At concentrations up to 100 μM, these two selective phosphodiesterase inhibitors also failed to inhibit lysosomal enzyme release in response to chemoattractant (FMLP) or phagocytic (serum-opsonized zymosan) stimulus. These observations therefore suggest that the cyclic GMP-insensitive subclass of cyclic AMP-specific phosphodiesterase plays an important role in modulating activation of neutrophils.

To characterize further the stimulus-specific inhibitory effects of IBMX, Ro 20-1724, and rolipram on the respiratory burst, the compounds were examined for their effects on intracellular cAMP concentrations in neutrophils stimulated with either FMLP or opsonized zymosan. As summarized in Table 4, similar increases in cAMP were obtained regardless of the stimulus used, suggesting that regulation of the respiratory burst is the result of a cAMP-dependent process(es) specific for cells stimulated by the chemotactic stimulus. These results are con-

sistent with the observation of Nielson [8] that β-adrenergic agonist-mediated inhibition of the neutrophil respiratory burst is stimulus dependent, whereas activation of adenylate cyclase is not.

The involvement of this subclass of phosphodiesterase in modulating neutrophil activation is also supported by the identification of a cyclic AMP-specific, cyclic GMP-insensitive form of phosphodiesterase which is associated with the particulate fraction in human neutrophils. The kinetic characteristics and inhibitor sensitivity of this enzyme are identical to the cyclic AMP-specific, cyclic GMP-insensitive phosphodiesterase present in calf liver [44] and canine ventricle [22]. However, while the canine ventricular enzyme was isolated from the soluble fraction of tissue homogenates, the cyclic AMP-specific, cyclic GMP-insensitive phosphodiesterase present in human neutrophils is membrane-bound. The importance of this difference in intracellular localization of this subclass of cyclic AMP-specific phosphodiesterase is unclear at this time.

The results of this study suggest that cyclic AMP has a negative regulatory role for the respiratory burst of neutrophils stimulated with chemotactic stimuli such as FMLP and C5a. Inhibition of phosphodiesterase-dependent hydrolysis of cyclic AMP should result in an increased intracellular concentration of the cyclic nucleotide which then selectively inhibits the FMLP or C5a-stimulated respiratory burst. Sedgwick *et al.* [45] have shown previously that receptor-mediated stimulation of adenylate cyclase by PGE_1 causes a similar selective inhibition of FMLP-stimulated oxygen radical generation. FMLP-stimulated respiratory burst is also inhibited by the β-receptor agonist isoproterenol [8].

These phosphodiesterase inhibitors exhibit greater inhibitory activity against the respiratory burst stimulated by FMLP than by C5a. Neutrophils produced superoxide anion at similar rates in response to these stimuli, though addition of cytochalasin B was required for the C5a response. In addition, PGE_1 was also shown to be a more potent inhibitor of the FMLP response. These results may indicate additional mechanistic differences in the regulation of cellular responses to these stimuli.

The stimulus-specific regulation of the respiratory burst by a cyclic AMP-dependent phosphodiesterase may indicate differential regulation of the activation of NADPH oxidase. Such differential regulation has been suggested previously by the observation that stimulation of neutrophils with soluble stimuli results in activation of NADPH oxidase over the entire surface of the cell [46], whereas phagocytosis-induced stimulation results in activation of NADPH oxidase only at the site of interaction between the phagocyte and the particulate stimulus [47]. It is possible that phagocytic stimulation bypasses the cyclic AMP-dependent regulatory step for the respiratory burst.

In conclusion, the present study demonstrates that human neutrophils possess a cyclic AMP-specific, cyclic GMP-insensitive form of phosphodiesterase, which is sensitive to the selective phosphodiesterase inhibitors rolipram and Ro 20-1724. Additional studies indicate that this enzyme is comparable to the rolipram-sensitive form of phosphodiesterase previously characterized in canine left ventricle [22], with the exception that in the human neutrophil the enzyme is membrane-bound. Experiments with various selective and nonselective phosphodiesterase inhibitors have provided evidence to indicate that this form of phosphodiesterase plays an important role in neutrophil activation, and that this role is both stimulus and response specific. Further studies with this enzyme may provide insight into the metabolic events that regulate the response of neutrophils to various stimuli, and the exact role of cyclic AMP in modulating this response.

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