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Bidirectional Regulation of Lysosomal Enzyme Secretion and Phagocytosis in Human Neutrophils by Guanosine 3′,5′-Monophosphate and Adenosine 3′,5′-Monophosphate[1] (39232)

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Recent reports from this laboratory (1–3) and from other laboratories (4–6) indicated that autonomic neurohormones and cyclic nucleotides influence the immunologic secretion of lysosomal enzymes from human neutrophils. For example, cholinergic agonists and guanosine 3′,5′-monophosphate (cyclic GMP) stimulate, whereas epinephrine and adenosine 3′,5′-monophosphate (cyclic AMP) inhibit lysosomal enzyme secretion. Further studies revealed that the immunologic secretion of lysosomal contents is accompanied by the accumulation of cyclic GMP, but not of cyclic AMP, in neutrophils (7). Moreover, the stimulatory action of acetylcholine on enzyme secretion is associated with a further accumulation of cyclic GMP (7, 8). The inhibitory action of epinephrine on lysosomal enzyme secretion is accompanied by cyclic AMP accumulation (6, 9) without any effect on cyclic GMP accumulation (8). A clear-cut effect of cyclic nucleotides and neurohormones on phagocytosis of particulate material by neutrophils has not yet been demonstrated. Cyclic AMP and certain catecholamines have been found to retard particle ingestion by neutrophils, although no effect was obtained with cyclic GMP and certain cholinergic agonists (10). The absence of an effect, especially a stimulatory one, by the latter pharmacologic agents may have been due to experimental design. For example, the particle/cell ratio in our phagocytosis experiments was very high. Thus, maximal rates of phagocytosis may have prevailed, and we were unable to observe any potential increases in phagocytic rate in the presence of cyclic GMP or cholinergic agonists.

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The principal objective of the present study was to examine in greater detail the actions of immunologic reactants and autonomic neurohormones on lysosomal enzyme secretion and cyclic nucleotide accumulation. In addition, a more careful analysis of the effects of these agents on phagocytosis was conducted.

Materials and methods. Human neutrophils were isolated and purified by a glass bead column chromatographic procedure as described previously (10). Cells were suspended (5×10^6 cells per ml) in Hanks' balanced salt solution, pH 7.4, containing 0.1% w/v glucose (Hanks'-glucose). Final cell suspensions consisted of 96–99% neutrophils; platelets and erythrocytes were absent. Over 99% of the neutrophils were viable as determined by dye exclusion and lack of lactate dehydrogenase extrusion (10).

Neutrophils (5×10^6) were incubated in Hanks' glucose at 37° in a final volume of 1.0–1.2 ml. Cells were preincubated either alone or with pharmacologic agents for 5 min at 37° before addition of immunologic reactant, and then further incubated at 37° for various time intervals. In those experiments in which β-glucuronidase secretion was measured, incubations were terminated by centrifugation and the supernatants were assayed for β-glucuronidase and lactate dehydrogenase activities (10). Phagocytosis of zymosan particles by neutrophils was quantitated as described previously (10).

Heat-aggregated human immunoglobulin G (agg.IgG) (2, 3) and serum-treated zymosan particles (10) were prepared and utilized as described.

Cyclic GMP and cyclic AMP concentrations, respectively, were measured according to the procedures described by Steiner *et al.* (11) and Gilman (12). Cyclic nucleotide concentrations in entire incubation media (cells + media) were measured. Incubations

were terminated by rapid freezing of samples in either a Dry Ice, acetone mixture or liquid nitrogen. Incubation samples were then acidified and extracted with ether as described earlier (13). In each experiment authentic standards of cyclic GMP and cyclic AMP were added to samples of incubation mixtures in order to determine losses of measurable quantities of cyclic nucleotides. Cyclic AMP was recovered quantitatively, that is, 90–100% of added cyclic AMP was detected, whereas 40–60% of added cyclic GMP was detected. The latter is due to our findings that inorganic salts interfere somewhat with the radioimmunoassay for cyclic GMP. However, the loss of measurable cyclic GMP is constant from sample to sample and from experiment to experiment. All values of cyclic GMP concentration have been corrected in order to account for losses. Additional experiments in which all inorganic salts were removed, and cyclic GMP and cyclic AMP were separated from one another and from other nucleotides by column chromatography (using first neutral alumina and then a Dowex anion exchange resin), indicated that our routine measurements of cyclic GMP and cyclic AMP concentrations were accurate. Both cyclic nucleotides were susceptible to hydrolysis by phosphodiesterase (from beef heart).

Solutions of epinephrine contained 0.01% sodium metabisulfite to prevent spontaneous oxidation and were utilized within 10 min of preparation. All other solutions were used within 15 min of preparation. The following agents were purchased from Sigma Chemical Co.: acetylcholine chloride, pilocarpine hydrochloride, choline chloride, atropine sulfate, hexamethonium bromide, *l*-epinephrine bitartate, *l*-metanephrine HCl, cyclic 3′,5′-guanosine monophosphate sodium, guanosine 5′-monophosphate sodium, cyclic 3′,5′-adenosine monophosphate sodium, N^6,$O^{2'}$-dibutyryl cyclic 3′,5′-adenosine monophosphate sodium, adenosine 5′-monophosphate sodium. Ciba-Geigy Corp. supplied phentolamine methanesulfonate and Ayerst provided *dl*-propranolol hydrochloride.

Results and discussion. Incubation of neutrophils with agg.IgG or serum-treated zymosan particles in a balanced salt solution at 37° results in the secretion of β-glucuronidase activity into the extracellular environment (Table I). This secretory process is selective for lysosomal contents, requires extracellular calcium and is not accompanied by any deterioration of cell viability (7, 8, 10, 14). Cholinergic agents such as acetylcholine and pilocarpine enhance the immunologic secretion of β-glucuronidase from neutrophils (Table I). This stimulatory ef-

TABLE I. EFFECTS OF AUTONOMIC AGENTS AND CYCLIC NUCLEOTIDES ON THE IMMUNOLOGIC SECRETION OF β-GLUCURONIDASE FROM HUMAN NEUTROPHILS.

Experimental condition[a]	β-Glucuronidase secretion (μg phenolphthalein/18 hr/5 × 10^6 cells)		
	Neutrophils	Neutrophils + agg. IgG	Neutrophils + serum-zymosan
Control	5.2 ± 0.4	39 ± 2.6	42 ± 3.1
Acetylcholine 1 μM (A)	6.8 ± 0.8	68 ± 5.9[b]	81 ± 7.8[b]
Pilocarpine 1 μM	7.6 ± 1.2	77 ± 6.1[b]	97 ± 9.7[b]
Choline 1 μM	5.0 ± 0.5	37 ± 2.4	44 ± 3.9
A + Atropine 1 μM	–	46 ± 4.3	48 ± 5.4
A + Hexamethonium 1 μM	–	72 ± 8.5[b]	77 ± 8.0[b]
Cyclic GMP 0.1 μM	6.2 ± 0.7	75 ± 6.6[b]	72 ± 7.5[b]
Epinephrine 1 μM (E)	5.1 ± 0.4	17 ± 2.3[b]	16 ± 2.6[b]
Metanephrine 1 μM	6.0 ± 0.6	41 ± 3.8	46 ± 5.8
E + Propranolol 1 μM	–	36 ± 4.7	35 ± 4.2
E + Phentolamine 1 μM	–	12 ± 2.4[b]	13 ± 1.5[b]
Cyclic AMP 10 μM	4.8 ± 0.5	22 ± 1.8[b]	25 ± 4.4[b]
Dibutyryl Cyclic AMP 1 μM	5.2 ± 0.6	18 ± 2.6[b]	15 ± 2.2[b]

[a] Human neutrophils (5 × 10^6) were preincubated with or without compounds for 5 min at 37° before addition of immune reactant, and then further incubated for 15 min at 37°. Total β-glucuronidase activity amounted to 229 ± 26 μg of phenolphthalein/18 hr/5 × 10^6 cells. Data represent the mean ± SEM of three to five separate experiments.

[b] Significantly different ($P < 0.05$) from control.

fect appears to be specific for agonists of the muscarinic type as atropine, but neither hexamethonium nor *d*-tubocurarine (10), blocks the effect. Moreover, choline, a precursor in the biosynthesis of acetylcholine, does not influence enzyme secretion. Many of the pharmacologic effects of cholinergic agonists of the muscarinic type are mimicked and perhaps mediated by cyclic GMP (15–20). Similarly, the stimulatory action of cholinergic agents on lysosomal enzyme secretion is mimicked by cyclic GMP (Table I).

Catecholamines, which stimulate β-adrenergic receptors, inhibit the immunologic secretion of β-glucuronidase from neutrophils. Such agents include epinephrine (Table I) and isoproterenol (1). The inhibitory action of epinephrine appears to be specific for β-adrenergic receptors as propranolol, but not phentolamine, blocks the action. Metanephrine, a metabolite of epinephrine, does not influence enzyme discharge. Cyclic AMP and dibutyryl cyclic AMP inhibit the immunologic secretion of β-glucuronidase from human neutrophils (Table I). The effects of epinephrine and cyclic AMP are opposite in direction to those of cholinergic agents and cyclic GMP.

The data in Table II illustrate the effects of various agents on phagocytosis by human neutrophils. Particle uptake at two different particle/cell ratios was studied. Particle/cell ratios of 5 and 60, respectively, are indices of submaximal (about 50% of maximal) and maximal rates of particle ingestion (10). Each of the agents studied elicits effects on phagocytosis that are qualitatively similar to those on lysosomal enzyme secretion. Thus, cholinergic agents such as acetylcholine and pilocarpine enhance, whereas epinephrine inhibits, phagocytosis (Table II). Similarly, cyclic GMP enhances where dibutyryl cyclic AMP inhibits phagocytosis. Enhancement of particle ingestion by cholinergic agents and cyclic GMP is observed at low but not high particle/cell ratios. Presumably, this is because maximum or near-maximum rates of phagocytosis occur at the high particle/cell ratio and further enhancement of phagocytosis by chemical agents is not easily achievable. Inhibition of phagocytosis by epinephrine and dibutytyl cyclic AMP, on the other hand, is observed at both low and high particle/cell ratios. Preliminary studies in our laboratory indicate that phagocytosis (of serum-treated zymosan particles), like lysosomal enzyme secretion, requires the presence of extracellular calcium.

The stimulatory effect of cholinergic agents on phagocytosis, as on lysosomal enzyme secretion, appears to be the result of an interaction with muscarinic receptors since atropine, but not hexamethonium, blocks the effect (Table II). Choline does not influence phagocytosis. The inhibitory effect of epinephrine on particle ingestion is presumably the result of interaction with β-adrenergic receptors as propranolol, but not phentolamine, blocks the effect. Metanephrine does not influence particle uptake.

Interaction of neutrophils and either agg.IgG or serum-treated zymosan results in the accumulation of cyclic GMP but not of cyclic AMP (Table III). Cyclic GMP accumulation requires the presence of calcium

TABLE II. EFFECTS OF AUTONOMIC AGENTS AND CYCLIC NUCLEOTIDES ON PHAGOCYTOSIS OF PARTICLES BY HUMAN NEUTROPHILS.

Experimental condition[a]	Particle uptake (number of particles/100 cells)	
	Particle/Cell ratio	
	5	60
Control	170 ± 12	428 ± 38
Acetylcholine 1 μM (A)	274 ± 35[b]	466 ± 52
Pilocarpine 1 μM	296 ± 44[b]	476 ± 58
Choline 1 μM	158 ± 20	414 ± 30
A + Atropine 1 μM	192 ± 24	–
A + Hexamethonium 1 μM	251 ± 24[b]	–
Cyclic GMP 0.1 μM	286 ± 36[b]	448 ± 32
Epinephrine 1 μM (E)	94 ± 8.4[b]	322 ± 31[b]
Metanephrine 1 μM	–	418 ± 42
E + Propranolol 1 μM	154 ± 16	412 ± 56
E + Phentolamine 1 μM	70 ± 9.8[b]	304 ± 40[b]
Dibutyryl Cyclic AMP 1 μM	66 ± 8.2[b]	288 ± 32[b]

[a] Human neutrophils (5×10^6) were preincubated with or without compounds for 5 min at 37° before addition of serum-treated zymosan particles, and then further incubated for 15 min at 37°. Data represent the mean ± SEM of three to six separate determinations.

[b] Significantly different ($P < 0.05$) from control.

TABLE III. EFFECTS OF IMMUNOLOGIC REACTANTS AND AUTONOMIC AGENTS ON THE ACCUMULATION OF CYCLIC GMP AND CYCLIC AMP IN HUMAN NEUTROPHILS.

Experimental condition[a]	Concentration (pmole/10^6 cells)	
	Cyclic GMP	Cyclic AMP
Neutrophils (N)	0.29 ± 0.14	5.7 ± 0.8
N + Acetylcholine 1 μM	1.1 ± 0.4	6.9 ± 1.3
N + Epinephrine 1 μM	0.52 ± 0.12	7.1 ± 1.6
N + Agg·IgG	11.6 ± 2.5[b]	4.3 ± 0.7
N + Serum-Zymosan	9.8 ± 2.1[b]	4.9 ± 1.2
N + Agg·IgG + Acetylcholine 1 μM	34.7 ± 8.0[c]	5.5 ± 1.4
N + Agg·Igg + Epinephrine 1 μM	10.1 ± 2.7[b]	31.8 ± 6.2[b]
N + Serum-Zymosan + Acetylcholine 1 μM	38.3 ± 7.4[c]	6.1 ± 1.9
N + Serum-Zymosan + Epinephrine 1 μM	10.8 ± 1.8[b]	40.4 ± 7.5[b]

[a] Human neutrophils (5×10^6) were preincubated with or without compounds for 5 min at 37° before addition of immune reactant, and then further incubated for 5 min at 37°. Entire incubation mixtures were assayed for cyclic nucleotide levels. Data represent the mean ± SEM of four to six separate determinations.

[b] Significantly different ($P < 0.01$) from neutrophils alone.

[c] Significantly different ($P < 0.05$) from neutrophils (N) + immune reactant.

(8). Acetylcholine stimulates further the accumulation of cyclic GMP without affecting the cellular levels of cyclic AMP (Table III). Thus, the immunologic as well as the pharmacologic enhancement of lysosomal enzyme secretion from neutrophils is associated with a concomitant elevation of cyclic GMP levels. Cyclic AMP appears to play a role in the pharmacologic inhibition of lysosomal enzyme secretion. Thus, epinephrine inhibits β-glucuronidase release and elevates, concomitantly, the cellular levels of cyclic AMP but not cyclic GMP (Table III). It is of interest that the pharmacologic enhancement of either cyclic GMP or cyclic AMP levels in human neutrophils requires the presence of an active immunologic reactant, that is, one capable of provoking lysosomal enzyme secretion. The reason for this requirement is unknown at the present time. Perhaps divalent cation transport processes at the neutrophil plasma membrane are important, since divalent cation ionophores can substitute for immune reactants in the expression of the effects of autonomic neurohormones on lysosomal enzyme secretion.

The data suggest that cyclic GMP, and perhaps calcium, plays a role in mediating, whereas cyclic AMP signals the inhibition of the immunologic secretion of lysosomal enzymes from human neutrophils. The existence of specific receptor sites, which respond to autonomic neurohormones possessing opposing influences on neutrophil function, affords this polymorphonuclear leukocyte with the capacity to regulate the secretion of lysosomal contents. Whether neutrophils actually come in contact with sympathomimetic or parasympathomimetic hormones *in vivo* during normal or diseased states is presently unknown, although there is reason to believe that such is the case (21). In support of the present data, direct opposing influences of cyclic GMP and cyclic AMP on lysosomal enzyme secretion from neutrophils under a variety of experimental conditions have been reported from this laboratory (1-3, 7-10, 21), and by Zurier *et al.* (6). Particle ingestion by neutrophils can also be influenced in opposing directions by cyclic GMP and acetylcholine, on the one hand, and cyclic AMP and epinephrine, on the other hand. Thus, the cellular processes of phagocytosis and extrusion of lysosomal contents appear to be closely related. Indeed, both neutrophil functions require calcium, are associated with a concomitant accumulation of cyclic GMP, and are inhibited by cyclic AMP and agents that elevate the levels of cyclic AMP.

The opposing influences of cyclic GMP and cyclic AMP on human neutrophil function represent a clear example of the "Yin Yang" concept of the bioregulation of cell function as proposd originally by Goldberg *et al.* (17), whereby the two cyclic nucleotides elicit directly opposing effects on cellular processes. We propose that cyclic GMP accumulation, in the presence of extracellular calcium, signals phagocytosis and the immunologic secretion of lysosomal enzymes from human neutrophils, whereas cyclic

AMP accumulation controls both processes by mediating an opposing and, therefore, inhibitory effect on neutrophil function. Thus, human neutrophils possess the cellular mechanisms required for the bidirectional regulation of at least two primary physiologic functions associated with this cell type.

Summary. The biologic roles of guanosine 3′,5′-monophosphate (cyclic GMP) and adenosine 3′,5′-monophosphate (cyclic AMP) in the secretion of lysosomal enzymes from, and in phagocytosis by, human neutrophils were studied. Contact between neutrophils and particulate immunologic reactants results in both phagocytosis of the particles and secretion of lysosomal enzymes. These cellular events are accompanied by the accumulation of cyclic GMP and require the presence of extracellular calcium. Acetylcholine, pilocarpine, and cyclic GMP enhance, whereas epinephrine, cyclic AMP, and/or dibutyryl cyclic AMP inhibit, both phagocytosis and lysosomal enzyme secretion. The stimulatory action of cholinergic agents and the inhibitory action of epinephrine are accompanied by the accumulation of cyclic GMP and cyclic AMP, respectively, in human neutrophils. The data suggest that cyclic GMP mediates whereas cyclic AMP inhibits the major functions of human neutrophils. Moreover, by virtue of their effects on cyclic nucleotide accumulation, autonomic neurohormones are capable of modulating human neutrophil function.

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