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Hormonal Control of Lysosomal Enzyme Release from Human Neutrophils: Elevation of Cyclic Nucleotide Levels by Autonomic Neurohormones

(guanosine 3′:5′-monophosphate/adenosine 3′:5′-monophosphate/lysosome membrane/acetylcholine/epinephrine)

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ABSTRACT **The influence of autonomic neurohormones on the immunologic release of β-glucuronidase (EC 3.2.1.31) from, and the cyclic nucleotide levels in, human neutrophils was determined. Interaction of neutrophils with rheumatoid arthritic, serum-treated zymosan particles in a neutral balanced salt solution at 37° resulted in the extracellular discharge of β-glucuronidase without any loss of cell viability, as indicated by the failure of incubated cells to take up eosin Y or to release cytoplasmic lactate dehydrogenase (EC 1.1.1.27). Epinephrine reduced the release of β-glucuronidase from neutrophils in the presence of zymosan during 2–30 min of incubation and elicited a concomitant elevation of adenosine 3′:5′-monophosphate levels. Propranolol, a β-adrenergic receptor antagonist, but not phentolamine, an α-adrenergic receptor antagonist, blocked both actions of epinephrine. Acetylcholine stimulated the release of β-glucuronidase, but not lactate dehydrogenase, and provoked a concomitant elevation of guanosine 3′:5′-monophosphate levels. Atropine, a muscarinic receptor antagonist, but not hexamethonium, a ganglionic blocker, inhibited both actions of acetylcholine. Interaction of neutrophils and zymosan particles resulted in an elevation of guanosine 3′:5′-monophosphate levels within 2 min. These data suggest that intracellular guanosine 3′:5′-monophosphate may be involved in mediating the immunologic release of lysosomal enzymes from human neutrophils whereas adenosine 3′:5′-monophosphate may inhibit enzyme release. Moreover, autonomic neurohormones appear to be capable of modulating lysosomal enzyme release by virtue of their capacity to elevate neutrophil cyclic nucleotide levels.**

Lysosomal enzymes are prominent mediators of acute inflammation (1, 2) and cartilage degradation (3, 4), and are selectively discharged from polymorphonuclear leukocytes during phagocytosis of, or cell-surface contact with, various immunologic reactants (5–8). In fact, the connective-tissue injury that results from the accumulation of granulocytes is probably a direct consequence of the extracellular release of lysosome granule constituents (9, 10). Recent studies indicate that contact of human granulocytes with altered immunoglobulin G-treated cartilage results in the extrusion of lysosomal neutral proteases and consequent degradation of the cartilage proteoglycan matrix (8).

In view of the potential destructive capacity of human granulocyte lysosomal contents, inhibition of the immunologic discharge of such deleterious substances is of fundamental importance in attenuating inflammation and connective tissue destruction. Several agents, including colchicine, vinblastine, prostaglandin E_1, and adenosine 3′:5′-monophosphate (cyclic AMP) have been reported to reduce lysosomal enzyme release from granulocytes (11). Recent studies in this laboratory indicate that certain catecholamines such as epinephrine inhibit, whereas cholinergic agents such as acetylcholine stimulate, the immunologic release of lysosomal enzymes from human mixed leukocytes (12, 13). These findings suggest that autonomic neurohormones possess the capacity to influence the course of the inflammatory process.

In the light of our previous findings that certain autonomic neurohormones and cyclic nucleotides are capable of influencing lysosomal enzyme release from leukocytes (12, 13), studies were designed to elucidate the relationship between the cellular actions of autonomic agents and the intracellular concentrations of cyclic nucleotides. The expression of cellular actions of certain adrenergic and cholinergic agents by cyclic AMP and guanosine 3′:5′-monophosphate (cyclic GMP), respectively, is well documented (14–16). Therefore, concentrations of cyclic AMP and cyclic GMP in purified human neutrophils were monitored during cell contact with phagocytosable particles, and the time course of changes in both β-glucuronidase release and cyclic nucleotide levels in the response of neutrophils to epinephrine and acetylcholine was determined.

MATERIALS AND METHODS

Isolation of Human Neutrophils. Human neutrophils were isolated from fresh heparinized venous blood drawn from healthy volunteers by a modification of the glass bead column chromatographic procedure described by Rabinowitz (17). Briefly, leukocyte-rich plasma, obtained after erythrocyte sedimentation, was incubated in a column of siliconized glass beads at 37° for 90 min. Neutrophils were eluted from the column with 0.1 M sodium phosphate, pH 7.3, after elution of lymphocytes, erythrocytes, and platelets with buffered autologous plasma. The final neutrophil suspension (5×10^6 cells per ml in Hanks' balanced salt solution, pH 7.4, containing 0.1% w/v glucose) consisted of 97–99% neutrophils and 0–2% mononuclear cells. Erythrocytes and platelets were absent.

Preparation of Zymosan Particles. Zymosan particles (Sigma Chemical Co., St. Louis, Mo.), 0.5- to 3-μm diameter, were suspended in Hanks' balanced salt solution (10 mg/ml), boiled, washed twice, resuspended in rheumatoid arthritic (RA) serum (1:10,240 titer of rheumatoid factors by agglutination test) at 10 mg/ml, and incubated at 37° for 30 min. Particles were then washed twice with saline, and resuspended

Abbreviations: cyclic AMP, adenosine 3′:5′-monophosphate; cyclic GMP, guanosine 3′:5′-monophosphate; RA, rheumatoid arthritic.

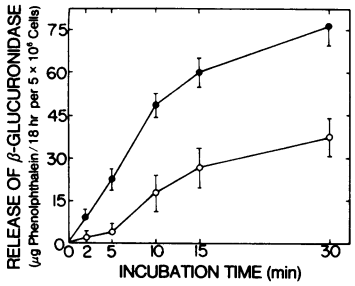

FIG. 1. Effect of epinephrine on immunologic release of β-glucuronidase from human neutrophils. Human neutrophils (5×10^6) were incubated with or without 1 μM epinephrine for 5 min at 37° before addition of rheumatoid arthritic serum-treated zymosan particles, and then incubated for an additional 2–30 min at 37°. Data represent the mean ± SEM of four separate determinations. Symbols are as follows: ●, neutrophils + zymosan; ○, neutrophils + 1 μM epinephrine + zymosan. Total β-glucuronidase activity, determined after freeze-thawing the cells, was 254 ± 14.8 μg of phenolphthalein/18 hr per 5×10^6 cells. Each point for neutrophils + zymosan + epinephrine (○) is significantly different ($P < 0.01$ by Student's t-test) from that for the corresponding neutrophils + zymosan (●). Neutrophils incubated either alone or with epinephrine in the absence of zymosan, for 0–30 min, released very small amounts of β-glucuronidase (less than 5 μg of phenolphthalein/18 hr per 5×10^6 neutrophils).

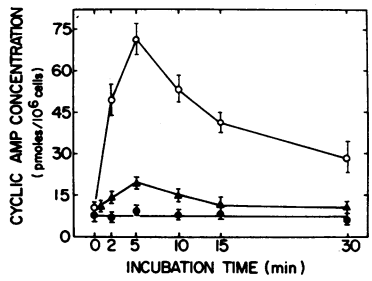

FIG. 2. Effect of epinephrine on adenosine 3′:5′-monophosphate levels in human neutrophils. Human neutrophils (5×10^6) were incubated with or without 1 μM epinephrine for 5 min at 37° before addition of either rheumatoid arthritic serum-treated zymosan particles or buffer, and then incubated for an additional 2–30 min at 37°. Data represent the mean ± SEM of four to six separate determinations. Symbols are as follows: ●, neutrophils + zymosan; ▲, neutrophils + 1 μM epinephrine but without zymosan; ○, neutrophils + 1 μM epinephrine + zymosan. Each point for neutrophils + epinephrine + zymosan (○) from 2 to 30 min is significantly different ($P < 0.001$ by Student's t-test) from that for the corresponding neutrophils + zymosan (●). Neutrophils incubated alone for 0–30 min showed no changes in cyclic AMP levels (6.8–10.2 pmoles/10^6 cells).

in Hanks' solution containing 0.1% glucose at 10 mg/ml. All particle suspensions were completely homogeneous and absent of particle aggregation, as ascertained by phase-contrast microscopy.

Enzyme Assays. Beta-glucuronidase (EC 3.2.1.31) activity was measured with phenolphthalein glucuronide as substrate (18). Data are expressed as μg of phenolphthalein liberated after 18 hr of incubation at 37°. Lactate dehydrogenase (EC 1.1.1.27) activity was measured according to the method of Bergmeyer *et al.* (19), and data are expressed as either the change (Δ) in absorbancy at 366 nm/min or percent of total activity.

Cyclic Nucleotide Assays. Cyclic AMP levels in neutrophils were measured according to a protein-binding procedure described by Gilman (20). Levels of cyclic GMP were measured essentially according to the radioimmunoassay of Steiner *et al.* (21). Neutrophil samples, which were processed after rapid freezing of individual incubation mixtures containing the cells, were acidified and extracted with ether as described (16). Cyclic nucleotide concentrations in entire incubation mixtures were determined, and the data are expressed as pmoles of either cyclic AMP or cyclic GMP per 10^6 neutrophils.

Compounds. Solutions of epinephrine contained 0.01% w/v sodium metabisulfite to prevent spontaneous oxidation of the former. Solutions of all compounds were prepared and used immediately. Acetylcholine chloride, atropine sulfate, hexamethonium bromide, *l*-epinephrine bitartrate, theophylline, adenosine 3′:5′-cyclic monophosphate sodium, $N^6,O^{2'}$-dibutyryl adenosine 3′:5′-cyclic monophosphate sodium, guanosine 3′:5′-cyclic monophosphate sodium, and $N^2,O^{2'}$-dibutyryl guanosine 3′:5′-cyclic monophosphate sodium were purchased from Sigma Chemical Co., St. Louis, Mo. Eight-bromoguanosine 3′:5′-cyclic monophosphate was purchased from ICN Corp., Irvine, Calif. Phentolamine methanesulfonate and *dl*-propranolol hydrochloride, respectively, were provided by CIBA-GEIGY Corp., Ardsley, N.Y. and Ayerst Laboratories, New York, N.Y.

RESULTS

Response of Human Neutrophils to Epinephrine and Cyclic AMP. Human neutrophils released β-glucuronidase, a lysosomal marker enzyme, but not lactate dehydrogenase, a cytoplasmic marker enzyme, into the extracellular medium during incubation of cells with phagocytosable zymosan particles. The selective discharge of lysosome granule contents from

TABLE 1. *Effect of propranolol on inhibition of human neutrophil enzyme release and increase in adenosine 3′:5′-monophosphate levels by epinephrine*

Experimental condition*	Release of β-glucuronidase activity†	Cyclic AMP concentration (pmoles/10^6 cells)
Neutrophils (N) + zymosan-RA serum (Z)	41.7 ± 3.5	13.4 + 0.9
N + Z + 1 μM epinephrine (E)	18.7 ± 1.4‡	43.0 ± 2.1‡
N + Z + E + 1 μM propranolol	40.5 ± 2.2§	18.5 ± 2.7§
N + Z + E + 1 μM phentolamine	14.6 ± 1.1§	73.1 ± 5.8§

* Human neutrophils (5×10^6) were incubated with or without compound(s) for 5 min at 37° before addition of rheumatoid arthritic serum-treated zymosan particles, and then incubated for an additional 10 min at 37°. Data represent the mean ± SEM of four to six separate determinations.

† Expressed as μg of phenolphthalein/18 hr per 5×10^6 neutrophils; total activity was 249 ± 18.2.

‡ Significantly different ($P < 0.001$) from corresponding values in group N + Z, by Student's t-test.

§ Significantly different ($P < 0.05$) from corresponding values in group N + Z + E, by Student's t-test.

granulocytes during cell contact with phagocytosable (5–7, 12, 13) and nonphagocytosable (6–8) immune reactants has been reported. Epinephrine (1 μM) markedly reduced the immunologic release of β-glucuronidase activity from human neutrophils by zymosan particles during 2–30 min of incubation at 37° (Fig. 1). Previous studies indicated that exogenous cyclic AMP can inhibit lysosomal enzyme release from human phagocytic leukocytes (5, 12). Therefore, in view of the knowledge that epinephrine is capable of stimulating the formation of cyclic AMP in diverse tissues, measurements were made of neutrophil levels of cyclic AMP in the absence and presence of epinephrine. Epinephrine elicited a time-dependent elevation of cyclic AMP levels in neutrophils during cell contact with zymosan (Fig. 2). Incubation of cells with zymosan alone did not alter cyclic AMP levels. In the absence of phagocytosable particles, epinephrine produced only a small increase in cyclic AMP levels.

These data illustrate a close temporal correlation between inhibition of lysosomal enzyme release and elevation of cyclic AMP levels by epinephrine in human neutrophils during cell interaction with immunologic reactant. The association between these two cellular events is even more evident with the finding that propranolol (1 μM), a β-adrenergic receptor antagonist, blocked the actions of epinephrine on both enzyme release and cyclic AMP levels (Table 1). Phenotolamine (1 μM), an α-adrenergic receptor antagonist, did not block but, in fact, enhanced both effects of epinephrine. All of these findings suggest that cyclic AMP is capable of mediating the actions of epinephrine in neutrophils. This view is supported by the observations that cyclic AMP and its dibutyryl analog (10–0.1 μM) reduced the immunologic release of β-glucuronidase (Table 2).

Response of Human Neutrophils to Acetylcholine and Cyclic GMP. Acetylcholine (1 μM) markedly increased the discharge of β-glucuronidase, but not lactate dehydrogenase, from human neutrophils during 2–30 min of incubation at 37° with serum-treated zymosan particles (Fig. 3). In the absence of zymosan no significant enzyme release was produced by acetylcholine. In view of previous observations that added cyclic GMP can accelerate lysosomal enzyme release from human mixed leukocytes (12) and purified neutrophils (13),

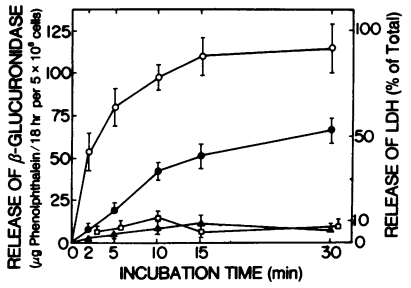

FIG. 3. Effect of acetylcholine on immunologic release of β-glucuronidase from human neutrophils. Human neutrophils (5 × 10^6) were incubated with or without 1 μM acetylcholine for 5 min at 37° before addition of either rheumatoid arthritic serum-treated zymosan particles or buffer, and then incubated for an additional 2–30 min at 37°. Data represent the mean ± SEM of four separate determinations. Symbols are as follows: ●, β-glucuronidase release from neutrophils + zymosan; ○, β-glucuronidase release from neutrophils + 1 μM acetylcholine + zymosan; ▲, β-glucuronidase release from neutrophils + 1 μM acetylcholine but without zymosan; □, lactate dehydrogenase (*LDH*) release from neutrophils + 1 μM acetylcholine + zymosan. Total enzyme activities, determined after the cells were freeze-thawed, were 291 ± 17.7 μg of phenolphthalein/18 hr per 5 × 10^6 neutrophils (β-glucuronidase) and 0.113 ± 0.007 Δ absorbancy (366 nm)/min per 5 × 10^6 neutrophils (lactate dehydrogenase). Each point for neutrophils + acetylcholine + zymosan (○) from 2 to 30 min is significantly different ($P < 0.001$ by Student's *t*-test) from that for the corresponding neutrophils + zymosan (●). Less than 10% of total lactate dehydrogenase activity was released from neutrophils that were incubated alone, with zymosan, or with acetylcholine in the absence of zymosan.

and that acetylcholine can stimulate tissue formation of cyclic GMP (15, 16), the effect of acetylcholine on neutrophil levels of cyclic GMP was studied. Acetylcholine provoked a significant elevation of cyclic GMP levels in neutrophils during cell contact with zymosan (Fig. 4). In the absence of acetylcholine the zymosan particles elicited a time-dependent elevation of neutrophil cyclic GMP.

The concomitant increase in enzyme release and cyclic GMP levels in neutrophils elicited by acetylcholine indicates that a close association of these two cellular events exists.

TABLE 2. *Opposing actions of adenosine 3′:5′-monophosphate and guanosine 3′:5′-monophosphate on release of β-glucuronidase from human neutrophils*

Experimental condition*	Release of β-glucuronidase activity (μg of phenolphthalein/18 hr per 5 × 10^6 cells)		
	Concentration of compound		
	10 μM	1.0 μM	0.1 μM
Control release		(52.2 ± 3.1)†	
Total enzyme activity		(257.4 ± 15.8)†	
Cyclic AMP	33.4 ± 4.8‡	45.9 ± 4.0	58.6 ± 4.4
Cyclic AMP + 1 μM theophylline	23.5 ± 2.6‡	31.8 ± 3.1‡	40.7 ± 2.8‡
Dibutyryl cyclic AMP	20.9 ± 1.3‡	29.8 ± 3.6‡	39.7 ± 3.8‡
Cyclic GMP	—	119.2 ± 12.4‡	91.4 ± 7.2‡
Dibutyryl cyclic GMP	—	139.4 ± 15.0‡	103.4 ± 6.6‡
8-Bromocyclic GMP	—	160.8 ± 12.6‡	122.1 ± 9.8‡

* Human neutrophils (5 × 10^6) were incubated with or without compound(s) for 5 min at 37° before addition of rheumatoid arthritic serum-treated zymosan particles, and then incubated for an additional 15 min at 37°. Data represent the mean ± SEM of four separate determinations.

† No compound was added.

‡ Significantly different ($P < 0.01$) from control enzyme release, by Student's *t*-test.

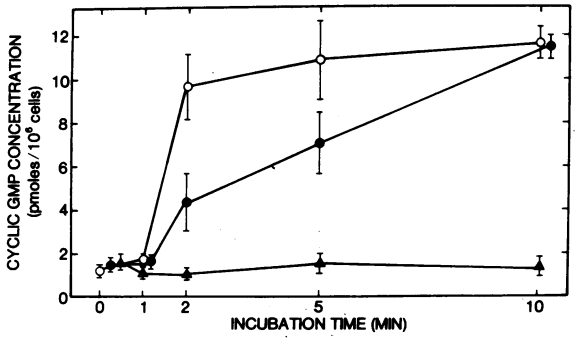

FIG. 4. Effect of acetylcholine and zymosan on guanosine 3′:5′-monophosphate levels in human neutrophils. Human neutrophils (5×10^6) were incubated with or without 1 μM acetylcholine for 5 min at 37° before addition of rheumatoid arthritic serum-treated zymosan particles, and then incubated for an additional 1–10 min at 37°. Data represent the mean ± SEM of three to five separate determinations. Symbols are as follows: ●, neutrophils + zymosan; ○, neutrophils + 1 μM acetylcholine + zymosan; ▲, neutrophils alone. The 2- and 5-min points for neutrophils + acetylcholine + zymosan (○) are significantly different ($P < 0.05$ by Student's t-test) from those for the corresponding neutrophils + zymosan (●). The 2-, 5-, and 10-min points for both neutrophils + acetylcholine + zymosan (○) and neutrophils + zymosan (●) are significantly different ($P < 0.001$) from those for the corresponding neutrophils alone (▲). Neutrophils incubated with acetylcholine in the absence of zymosan, for 0–10 min, showed no changes in cyclic GMP levels (0.9–1.7 pmoles/10^6 cells).

Atropine (1 μM), a muscarinic receptor antagonist, but not hexamethonium (1 μM), a nicotinic blocker, inhibited the actions of acetylcholine on both enzyme release (12, 13) and cyclic GMP levels (cyclic GMP levels were reduced from 10.7 ± 1.5 to 5.6 ± 0.8 pmoles/10^6 cells at 2 min of incubation). Atropine did not affect zymosan-induced enzyme release or elevation of cyclic GMP at 2 and 5 min of incubation in the absence of acetylcholine. These data suggest that cyclic GMP is capable of mediating these actions of acetylcholine on neutrophils. Indeed, exogenous cyclic GMP and two of its analogs (10–0.1 μM) enhanced the immunologic discharge of β-glucuronidase from human neutrophils (Table 2).

DISCUSSION

The close temporal relationship between inhibition of extracellular release of β-glucuronidase and elevation of cyclic AMP levels by epinephrine in human neutrophils during cell contact with phagocytosable zymosan particles suggests that the inhibitory action of the catecholamine is mediated by intracellular cyclic AMP. Additional evidence in support of this hypothesis derives from the findings that β-adrenergic receptor blockade markedly reduces the capacity of epinephrine to both inhibit granule enzyme discharge and elevate cyclic AMP levels, and that exogenous cyclic AMP or its dibutyryl analog inhibits enzyme release. This inhibitory action of cyclic AMP and certain agents, such as theophylline and prostaglandin E_1, that elevate cyclic AMP levels in tissues other than leukocytes, has been reported (11–13).

In most mammalian biologic systems, acetylcholine elicits pharmacologic effects that are often opposite in action to those of epinephrine. Such a relationship is also evident in human neutrophils with regard to the immunologic release of lysosomal enzymes. Acetylcholine stimulates whereas epinephrine inhibits enzyme release. The finding that acetylcholine elevates neutrophil levels of cyclic GMP implies that this nucleotide may mediate the stimulatory action of acetylcholine on phagocytic enzyme release. Indeed, when atropine was used to inhibit the elevation of cyclic GMP levels by acetylcholine, the enhancing effect of acetylcholine on β-glucuronidase release was also blocked. Further, cyclic GMP and some of its structural analogs stimulate release of lysosomal enzymes from human neutrophils. We have demonstrated previously the capacity of cyclic GMP to stimulate the release of lysosome granule enzymes from human mixed granulocytes (12).

The presence of both cyclic AMP and cyclic GMP, as well as the cellular machinery for elevating the levels of either nucleotide, in human neutrophils endows this cell type with the capacity to exercise a bidirectional control of lysosomal enzyme release. The opposing actions of cyclic AMP and cyclic GMP on phagocytic enzyme release represent the first clear demonstration of a bioregulatory concept of "Yin Yang" as originally proposed by Goldberg (22), whereby these two cyclic nucleotides directly elicit oppositional effects. In view of the concomitant elevation of cyclic GMP levels and stimulation of β-glucuronidase release during contact of neutrophils with immunologic reactant, it appears reasonable to consider that cyclic GMP is involved intimately in triggering the immunologically provoked extracellular discharge of lysosomal enzymes from human neutrophils.

At this time the precise intracellular mechanism by which cyclic nucleotides modulate enzyme release is unknown. Cyclic AMP and cyclic GMP, however, have been shown to influence markedly the permeability of the lysosome membrane to enzyme proteins (23–25). Moreover, cyclic nucleotides have been shown many times to bind to and activate nucleotide-dependent protein kinases. Therefore, activation of specific protein kinases by cyclic AMP or cyclic GMP, resulting in the phosphorylation of one or more cellular components, may play a part in regulating lysosomal enzyme release. Such phosphorylation reactions could alter the physical and/or functional properties of lysosomes and thereby interfere with specific functions of these organelles, such as peripheral migration or fusion of granules with heterophagic vacuoles or the plasma membrane.

The data in this report suggest that the immunologic discharge of lysosomal enzymes from human neutrophils is mediated by neutrophilic cyclic GMP, and that autonomic neurohormones are capable of modulating enzyme release via the selective elevation of intracellular levels of the appropriate cyclic nucleotide.

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