MEDIATION OF IMMUNOLOGIC DISCHARGE OF LYSOSOMAL ENZYMES FROM HUMAN NEUTROPHILS BY GUANOSINE 3',5'-MONOPHOSPHATE

REQUIREMENT OF CALCIUM, AND INHIBITION BY ADENOSINE 3',5'-MONOPHOSPHATE

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The selective discharge of lysosome granule enzymes from human neutrophils by certain immunologic stimuli can be influenced in opposing directions by guanosine 3',5'-monophosphate (cyclic GMP) and adenosine 3',5'-monophosphate (cyclic AMP) (1-3). Similarly, the immunologic secretion of lysosomal enzymes can be enhanced or inhibited by specific autonomic neurohormones (1-3). In fact, certain experimental evidence suggests that the enhancing action of acetylcholine and the inhibitory action of epinephrine on enzyme release from purified neutrophils may be mediated by intracellular cyclic GMP and cyclic AMP, respectively (4). Expression of the cellular actions of certain cholinergic and adrenergic agents by cyclic GMP and cyclic AMP, respectively, is well documented (5-10). Moreover, convincing evidence has been accumulated by Goldberg and co-workers (7, 8) that the opposing influences of cyclic GMP and cyclic AMP on various cellular processes provides the basis for the bidirectional regulation of cell function.

The principal objective of the present investigation was to determine whether cyclic GMP plays a definitive role in mediating the immunologic discharge of lysosomal enzymes from human neutrophils. Recent reports from this laboratory indicated that cyclic GMP concentrations in neutrophils were elevated markedly during the lysosomal enzyme discharge that accompanied cell contact with serum-treated zymosan particles (4). In order to ascertain whether this direct relationship applies to all types of immunologic stimuli employed to provoke lysosomal enzyme release the neutrophil levels of cyclic GMP were moni-
tored during cell contact with a variety of immune reactants. In addition, the influences of acetylcholine and epinephrine on the time-course of lysosomal enzyme discharge and accumulation of cyclic nucleotides in neutrophils were determined. In view of numerous reports illustrating the requirement of calcium in secretory processes (11), the relationship of extracellular calcium to immunologically provoked discharge of lysosomal enzymes from, and elevation of cyclic GMP levels in, human neutrophils was studied. Further, an assessment was made of the requirement of extracellular calcium for the cholinergic enhancement of enzyme secretion and cyclic GMP accumulation.

The results of this investigation suggest that intracellular cyclic GMP mediates the immunologic discharge of lysosome granule contents from human neutrophils and that calcium is involved intimately in both the accumulation of cyclic GMP and the concomitant secretion of lysosomal contents. Preliminary reports of some of the present findings appeared earlier (12, 13).

Materials and Methods

Isolation of Human Neutrophils.—Human neutrophils were isolated from fresh heparinized venous blood drawn from healthy volunteers by methods described previously (4). The final cell suspension (97-99% neutrophils) was prepared in Hanks’ balanced salt solution containing 0.1% (wt/vol) glucose (Hanks’-glucose) and diluted to $5 \times 10^6$ cells/ml. In some experiments cells were suspended in calcium-free Hanks’-glucose. Viability of the neutrophils was always greater than 99% (trypan blue or eosin Y exclusion).

Preparation of Immunologic Reactants.—Untreated, serum-treated and rheumatoid arthritic (RA) serum-treated zymosan particles, and zymosan-treated serum were prepared by procedures described previously (4). Solutions of human IgG, suspensions of heat-aggregated (agg) IgG, suspensions of RA serum-treated agg IgG, and immobilized RA serum-treated agg IgG were prepared and utilized as described (2, 3).

Conditions of Incubations, Enzyme Assays, and Nucleotide Measurements.—Neutrophils ($5 \times 10^6$ in 1.0 ml of Hanks’-glucose) were preincubated and incubated at 37°C in a Dubnoff metabolic shaker while agitating at 120 excursions per min. Cells were preincubated either with pharmacologic agents or alone at 37°C for 10 min before the addition of a given immunologic reactant and then further incubated at 37°C for various time intervals. Incubations of cells with immobilized reactants were executed as described (3). After the incubations the samples were processed and measurements of $\beta$-glucuronidase and lactate dehydrogenase discharge from, and determinations of cyclic GMP and cyclic AMP concentrations in, neutrophils were made according to methods outlined previously (4). Recoveries of $\beta$-glucuronidase and lactate dehydrogenase activities from neutrophils and incubation media were determined in each of the experiments by procedures described previously (4). Complete recoveries (94-103%) were obtained in each experiment.

Drug Solutions and Sources.—Solutions of epinephrine contained 0.01% (wt/vol) sodium metabisulfite to prevent spontaneous oxidation and were utilized within 10 min of preparation. All other solutions of test agents were prepared fresh and used within 30 min. The complete names of all the chemical agents and drugs, including their salts, used in this study are indicated below. The following agents were purchased from Sigma Chemical Co., (St. Louis, Mo.): L-epinephrine bitartrate, acetylcholine chloride, N$^6$,O$^2$-dibutyl cyclic 3’,5’-adenosine monophosphate sodium, cyclic 3’,5’-guanosine monophosphate sodium, ethyleneglycol-bis (β-aminoethyl ether) N,N’-tetraacetic acid.
RESULTS

Effects of Zymosan, Acetylcholine, and Epinephrine on Concentrations of Cyclic GMP and Cyclic AMP in, and Discharge of β-Glucuronidase from, Human Neutrophils.—Neutrophils were examined for possible changes in the endogenous concentrations or levels of cyclic GMP and cyclic AMP during the lysosomal enzyme discharge that results from cell contact with phagocytosable, RA serum-treated zymosan particles. Cells (5 × 10⁶) were preincubated in 1.0 ml of Hanks'-glucose at 37°C for 10 min before the addition of 4 × 10⁸ zymosan particles and then further incubated at 37°C for 10 min. Under these experimental conditions phagocytosis of particles occurred and β-glucuronidase was discharged into the extracellular medium (4). Contact of cells with zymosan particles provoked a marked elevation of cyclic GMP levels but no change in cyclic AMP levels in neutrophils (Fig. 1). This increase in cyclic GMP levels was accompanied by a concomitant increment in β-glucuronidase discharge from neutrophils (Fig. 2, control data).

Previous reports from this laboratory indicated that acetylcholine enhanced whereas epinephrine inhibited the immunologic discharge of lysosomal enzymes from human mixed leukocytes (1) and purified neutrophils (3, 4). However, the precise time-course of release of β-glucuronidase under these conditions was not reported. This data is now depicted in Fig. 2 for the purpose of illustrating the close temporal relationships between β-glucuronidase extrusion and neutrophil levels of cyclic GMP and cyclic AMP, as influenced by either acetylcholine (Fig. 3) or epinephrine (Fig. 4). Acetylcholine provoked an increase in both cyclic GMP levels (Fig. 3) and β-glucuronidase discharge (Fig. 2) above those produced by zymosan alone, and these two cellular events were closely associated temporally. Neutrophil concentrations of cyclic AMP were not affected by either zymosan (Fig. 1) or zymosan plus acetylcholine (Fig. 3). In opposition to the actions of acetylcholine, epinephrine inhibited the immunologic release of

Fig. 1. Cyclic GMP and cyclic AMP concentrations in human neutrophils during cell contact with phagocytosable zymosan particles. Neutrophils were preincubated at 37°C for 10 min before addition of RA serum-treated zymosan particles and then further incubated at 37°C for 1-10 min. Data represent the Mean ± SEM of six separate determinations.
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Fig. 2. Effects of acetylcholine and epinephrine on the immunologic discharge of \( \beta \)-glucuronidase from human neutrophils. Neutrophils were preincubated with either acetylcholine or epinephrine at 37°C for 10 min before addition of RA serum-treated zymosan particles and then further incubated at 37°C for 2-30 min. Data represent the Mean ± SEM of five separate determinations.

\[ \text{INCUBATION TIME (min)} \]

\[ \beta\text{-GLUCURONIDASE RELEASE} \]

\[ \text{Acetylcholine } 10^{-6} \text{ M} \]

\[ \text{Control} \]

\[ \text{Epinephrine } 10^{-6} \text{ M} \]

\[ \text{No Zymosan} \]

Fig. 3. Effects of acetylcholine on cyclic GMP and cyclic AMP concentrations in human neutrophils during cell contact with phagocytosable zymosan particles. Neutrophils were preincubated with 10\(^{-6}\) M acetylcholine at 37°C for 10 min before addition of RA serum-treated zymosan particles and then further incubated at 37°C for 1-10 min. Data represent the Mean ± SEM of three to five separate determinations.

\[ \text{INCUBATION TIME (min)} \]

\[ \text{CYCLIC GMP CONCENTRATION} \]

\[ \text{Cyclic GMP (acetylcholine)} \]

\[ \text{Cyclic GMP (no acetylcholine)} \]

\[ \text{CYCLIC AMP CONCENTRATION} \]

\[ \text{Cyclic AMP (acetylcholine)} \]

\[ \text{Cyclic AMP (no acetylcholine)} \]

\[ \text{INCUBATION TIME (min)} \]

\( \beta \)-glucuronidase from neutrophils (Fig. 2) and this was associated with a concomitant elevation of the levels of cyclic AMP (Fig. 4). Epinephrine did not alter the increase in cyclic GMP levels provoked by the zymosan particles.

Influence of Autonomic Neurohormones and Cyclic Nucleotides on the Response of Human Neutrophils to Various Immunologic Reactants.—Discharge of \( \beta \)-glucuronidase from human neutrophils was provoked by several different immunologic reactants (Table I). Zymosan particles treated with either normal or RA serum, agg IgG used by itself or as a particulate or immobile complex with constituents from RA serum, and zymosan-treated normal serum all stimulated the neutrophils to release \( \beta \)-glucuronidase into the extracellular medium.
Untreated zymosan, unaltered IgG, and normal serum did not cause enzyme release. Acetylcholine and cyclic GMP enhanced the discharge of lysosomal enzyme from neutrophils in contact with each of the immune reactants that elicited enzyme release by themselves (Table I). These two chemical agents did not cause discharge of β-glucuronidase from cells incubated either alone or in the presence of untreated zymosan, unaltered IgG or normal serum. In opposi-
tion to the actions of acetylcholine and cyclic GMP, epinephrine and the
dibutyryl analog of cyclic AMP inhibited β-glucuronidase release provoked by
each of the immune reactants (Table I).

Stimulation of lysosomal enzyme (β-glucuronidase) discharge from human
neutrophils by either immunologic reactants alone or reactants plus acetyl-
choline or cyclic GMP proceeded in the absence of a concomitant release of
cytoplasmic constituents. Evidence for this is presented in Table II, the data
from which indicate that cytoplasmic lactate dehydrogenase was not discharged
by immune reactants or combinations of reactants and other agents. Further,
the essential lack of uptake of eosin Y by neutrophils during immunologic dis-
charge of β-glucuronidase indicates that cell viability was maintained through-
out the incubations of cells with reactants and chemical agents (Table II).

Complete recoveries (94–103%) of β-glucuronidase and lactate dehydro-
genase activities from cells and incubation media containing test agents were
obtained in each experiment. Therefore, all enzyme activities were accounted
for after all incubations and the presence of immune reactants and test agents
did not alter enzyme activities appreciably.

### TABLE II

**Effect of Immunologic Reactants, Acetylcholine and Cyclic GMP on Viability of Human Neutrophils**

| Experimental condition | Discharge of lactate dehydrogenase§ | Neutrophils with eosin Y uptake |
|------------------------|-------------------------------------|--------------------------------|
|                         | %                                   |                                |
| 5 x 10⁶ Neutrophils (N) | 0.0028 ± 0.0003  0.8 ± 0.2          |                                |
| N + serum-treated zymosan (Z) | 0.0026 ± 0.0002  0.7 ± 0.1          |                                |
| N + RA serum-treated Z | 0.0034 ± 0.0004  1.5 ± 0.4          |                                |
| N + RA serum-treated Z + 10⁻⁶ M acetylcholine | 0.0036 ± 0.0005  1.2 ± 0.3          |                                |
| N + RA serum-treated Z + 10⁻⁷ M cyclic GMP | 0.0030 ± 0.0003  1.6 ± 0.3          |                                |
| N + agg IgG | 0.0038 ± 0.0006  0.9 ± 0.2          |                                |
| N + RA serum-treated agg IgG | 0.0036 ± 0.0005  1.3 ± 0.5          |                                |
| N + RA serum-treated agg IgG + 10⁻⁶ M acetylcholine | 0.0033 ± 0.0006  1.4 ± 0.4          |                                |
| N + RA serum-treated agg IgG + 10⁻⁷ M cyclic GMP | 0.0038 ± 0.0007  1.6 ± 0.5          |                                |
| N + imm. RA serum-treated agg IgG | 0.0029 ± 0.0004  —                |                                |
| N + imm. RA serum-treated agg IgG + 10⁻⁶ M acetylcholine | 0.0037 ± 0.0008  —                |                                |
| N + Z-treated serum | 0.0026 ± 0.0003  0.7 ± 0.2          |                                |
| N + Z-treated serum + 10⁻⁶ M acetylcholine | 0.0034 ± 0.0006  0.9 ± 0.4          |                                |
| N + Z-treated serum + 10⁻⁷ M cyclic GMP | 0.0031 ± 0.0004  1.2 ± 0.5          |                                |

* Data represent the Mean ± SEM of four-six separate determinations.
† Neutrophils were preincubated with pharmacologic agents at 37°C for 10 min before
addition of immunologic reactants, and then further incubated at 37°C for 15 min.
§ Expressed as Δ absorbancy (366 nm)/min/5 x 10⁶ neutrophils; value for total cell
enzyme activity was 0.141 ± 0.010.
Effects of Various Immunologic Reactants and Autonomic Neurohormones on Concentrations of Cyclic GMP and Cyclic AMP in Human Neutrophils.—Each of the immunologic reactants that stimulated lysosomal enzyme discharge (Table I) also provoked a marked elevation of cyclic GMP levels in neutrophils (Table III). These reactants include zymosan particles treated with either normal or RA serum, agg IgG used by itself or as a particulate or immobile complex with constituents from RA serum, and zymosan-treated normal serum. Cyclic AMP levels were not altered by these reactants. Untreated zymosan, unaltered IgG, and normal serum did not elevate the concentrations of cyclic GMP in neutrophils. These data are consistent with the failure of the latter three substances to provoke the discharge of β-glucuronidase (Table I).

Acetylcholine enhanced the effects of the immune reactants not only on β-glucuronidase release (Table I) but also on the increase in neutrophil levels of cyclic GMP (Table III). In the absence of a suitable reactant, that is, in the absence of immunologically induced lysosomal enzyme release, acetylcholine failed to elevate cyclic GMP levels (Table III) or to promote β-glucuronidase discharge (Table I). For example, no cholinergic stimulation of neutrophil function was observed when cells contacted untreated zymosan, unaltered IgG, or normal serum. Thus, once again the discharge of β-glucuronidase from neutrophils was associated with a concomitant increase in the cellular concentrations of cyclic GMP. Neither the reactants alone (Table III) nor reactants plus acetylcholine (Fig. 4) affected the neutrophil concentrations of cyclic AMP.

Epinephrine inhibited the immunologic discharge of β-glucuronidase from

| Experimental condition | Nucleotide concentrations (pmol/10⁶ neutrophils) |
|------------------------|-----------------------------------------------|
|                        | Cyclic GMP                                     | Cyclic AMP                                    |
|                        | Control                                       | Acetylcholine 10⁻⁶ M                          | Control                                       | Epinephrine 10⁻⁶ M |
| 5 X 10⁶ Neutrophils (N) | 1.34 ± 0.12                                   | 1.61 ± 0.22                                   | 7.34 ± 1.06                                   | 13.6 ± 2.32       |
| N + zymosan (Z)        | 2.10 ± 0.43                                   | 1.94 ± 0.30                                   | 8.96 ± 0.92                                   | 12.1 ± 1.96       |
| N + serum-treated Z    | 12.4 ± 2.65                                   | 34.8 ± 4.33§                                  | 9.32 ± 1.74                                   | 40.5 ± 2.18§      |
| N + RA serum-treated Z | 22.3 ± 3.44                                   | 68.5 ± 8.14§                                  | 7.94 ± 0.87                                   | 55.1 ± 3.40§      |
| N + IgG                | 1.82 ± 0.84                                   | —                                            | 6.74 ± 1.02                                   | —                |
| N + agg IgG            | 12.7 ± 1.60                                   | 30.4 ± 5.73§                                  | 9.40 ± 1.39                                   | 49.7 ± 4.02§      |
| N + RA serum-treated agg IgG | 41.1 ± 5.32                                   | 93.8 ± 10.44§                                | 9.32 ± 1.24                                   | 59.8 ± 4.11§      |
| N + immobilized RA serum-treated agg IgG | 56.3 ± 7.13                                   | 89.7 ± 6.34§                                  | 8.76 ± 2.19                                   | 53.3 ± 5.41§      |
| N + serum              | 0.91 ± 0.37                                   | —                                            | 8.06 ± 1.18                                   | —                |
| N + Z-treated serum    | 44.8 ± 6.07                                   | 98.1 ± 8.80§                                  | 7.88 ± 1.62                                   | 42.6 ± 5.70§      |

* Data represent the Mean ± SEM of three-five separate determinations.
† Neutrophils were preincubated with either acetylcholine or epinephrine at 37°C for 10 min before addition of the reactants indicated in the left hand column, and then further incubated at 37°C for 10 min.
‡ Significantly different (P < 0.01) from corresponding control.
neutrophils (Table I) but did not lower the elevated levels of cyclic GMP that were associated with enzyme release (Fig. 3). Instead, epinephrine provoked a concomitant elevation of neutrophil levels of cyclic AMP, and these elevated cyclic AMP levels were associated with a reduction in the release of β-glucuronidase (Table III). The enzyme release data illustrated in Table I have been compared with and related to the nucleotide data in Table III, although the incubation times of the reactions conducted for Table III were 10 min whereas those for Table I (β-glucuronidase discharge) were 15 min. The reason for this was that changes in nucleotide levels may have preceded the associated changes in enzyme release by up to 5 min (preliminary findings).

**Requirement of Extracellular Calcium for Both Immunologic Discharge of β-Glucuronidase from and Elevation of Cyclic GMP in Human Neutrophils**.— The requirement of extracellular calcium for numerous secretory processes is well documented (11). Likewise, the data in Table IV reveal the possible importance of calcium in mediating the immunologic release of lysosomal enzymes from neutrophils. Basal levels of cyclic GMP in neutrophils were not altered

| Experimental condition          | Calcium, 1.27 mM (present, +) (absent, −) | Cyclic GMP concentration | Discharge of β-glucuronidase |
|--------------------------------|-------------------------------------------|---------------------------|------------------------------|
|                                | pmol/10⁶ cells                           |                           | μg phenolphthalein/18 h/5 × 10⁶ cells |
| 5 × 10⁶ Neutrophils (N)        | +                                        | 0.72 ± 0.12               | 4.3 ± 0.2                   |
|                                | −                                        | 1.07 ± 0.19               | 3.9 ± 0.1                   |
| N + serum-treated zymosan (Z) | +                                        | 9.81 ± 2.10†              | 14.6 ± 1.2†                 |
|                                | −                                        | 1.34 ± 0.25               | 4.8 ± 0.4                   |
| N + RA serum-treated Z         | +                                        | 16.4 ± 1.92†              | 21.2 ± 1.8§                 |
|                                | −                                        | 2.53 ± 0.43               | 5.7 ± 0.4                   |
| N + RA serum-treated Z + acetylcholine, 10⁻⁶ M | +                                      | 53.5 ± 7.24§              | 49.5 ± 3.7§                 |
|                                | −                                        | 3.10 ± 0.37               | 6.3 ± 0.8                   |
| N + agg IgG                   | +                                        | 9.42 ± 1.28§              | 12.2 ± 0.9§                 |
|                                | −                                        | 1.87 ± 0.33               | 4.4 ± 0.3                   |
| N + agg IgG + acetylcholine, 10⁻⁶ M | +                                      | 28.1 ± 2.20§              | 38.9 ± 4.0§                 |
|                                | −                                        | 3.02 ± 0.31               | 6.0 ± 0.7                   |
| N + RA serum-treated Z + EGTA, 10⁻³ M | +                                      | 3.61 ± 0.47               | 7.4 ± 0.9                   |
| N + agg IgG + EGTA 10⁻³ M     | +                                        | 4.04 ± 0.33               | 6.2 ± 0.8                   |
| N + agg IgG + EGTA, 10⁻³ M +  | +                                        | 4.47 ± 0.31               | 8.5 ± 0.6                   |
| acetylcholine, 10⁻⁶ M         |                                           |                           |                             |

* Data represent the Mean ± SEM of four–six separate determinations.
† Neutrophils were preincubated with acetylcholine and/or EGTA at 37°C for 10 min before addition of immunologic reactants, and then further incubated at 37°C for 5 min.
§ Significantly different (P < 0.01) from corresponding values in the absence of calcium.
significantly by eliminating calcium from the extracellular medium (Table IV). However, in the absence of extracellular calcium the marked elevation of cyclic GMP levels as well as the concomitant discharge of β-glucuronidase, that were associated with contact of neutrophils with both immunologic reactants and calcium, failed to occur (Table IV). Further, in the absence of calcium acetylcholine failed to provoke further enzyme release or further elevation of cyclic GMP levels. Similar results to those reported above were obtained when a calcium chelating agent, EGTA, was added to the incubation systems containing calcium (Table IV).

**DISCUSSION**

The experiments described in this report were conducted in order to ascertain the roles of cyclic GMP and calcium in mediating or signaling the immunologic discharge of lysosomal enzymes from human neutrophils. The reasons for initiating these studies derived from previous findings in this laboratory that cyclic GMP and certain cholinergic agents, that are known to elevate the levels of cyclic GMP in various tissues (5-9), enhanced the discharge of lysosomal enzymes from neutrophils during cell contact with particulate agg IgG treated with RA serum (2). Lysosomal enzyme discharge required the presence of extracellular calcium (3). Cyclic AMP and catecholamines, on the other hand, inhibited enzyme release from leukocytes and purified neutrophils (1, 3). In addition to the above studies with intact cells opposing influences of cyclic GMP (and cholinergic agents) and cyclic AMP (and catecholamines) on the osmotic release of enzyme protein from isolated lysosome fractions were observed (10, 14). These data support the original hypothesis proposed by Goldberg et al. (7) that many cellular functions are affected in opposite directions, and thus regulated, by intracellular cyclic GMP and cyclic AMP. Moreover, cellular concentrations of both cyclic nucleotides, and thus cellular functions, can be influenced by autonomic neurohormones (6, 8). Evidence for the existence of such a bidirectionally regulated biologic system in neutrophils was reported recently (4, 15). For example, acetylcholine enhanced both the immunologic discharge of lysosomal enzymes and the accumulation of neutrophil cyclic GMP whereas epinephrine inhibited enzyme discharge and promoted the accumulation of cyclic AMP. Our attention was then focused primarily on the triggering or signaling mechanism, namely cyclic GMP, by which immunologic stimuli provoke neutrophils to discharge their lysosomal contents into the extracellular environment.

The time course analysis of nucleotide levels in neutrophils indicates that cyclic GMP levels are elevated at 1 min and peak at 5-10 min after cell contact with zymosan particles. A good correlation was obtained between the capacities of immunologic reactants to provoke β-glucuronidase release and to elevate neutrophil cyclic GMP levels without altering cyclic AMP levels. For example, serum or RA serum-treated zymosan particles, agg IgG, or RA serum-treated...
agg IgG, and zymosan-treated serum each triggered both β-glucuronidase extrusion from and elevation of cyclic GMP levels in human neutrophils. Further, untreated zymosan, unaltered IgG, and serum failed to provoke either enzyme release or cyclic GMP accumulation. These findings illustrate that cellular accumulation of cyclic GMP is associated with the immunologic discharge of β-glucuronidase from neutrophils and suggest that cellular cyclic GMP plays a role in mediating lysosomal enzyme secretion.

A time-course analysis of the effects of acetylcholine and epinephrine on the immunologic discharge of β-glucuronidase from neutrophils revealed that the enhancement and inhibition of enzyme release by acetylcholine and epinephrine, respectively, were apparent 2 min after, and lasted for at least 30 min after, contact of cells with RA serum-treated zymosan particles. Similar results, employing only one or two time intervals of incubation, were reported previously (4). The purpose of the time course experiments was to relate both times of appearance and durations of effects of autonomic neurohormones on enzyme discharge to those on cyclic nucleotide accumulation. A close correlation was obtained between the time course of cyclic GMP accumulation and that of β-glucuronidase discharge during contact of neutrophils with RA serum-treated zymosan particles. Acetylcholine enhanced the magnitude of both neutrophil responses without altering the time course relationship. Simultaneous increases in β-glucuronidase extrusion and cyclic GMP accumulation by acetylcholine were observed during contact of neutrophils with each of the immunologic reactants tested. Acetylcholine stimulated neither enzyme release nor cyclic GMP accumulation in the absence of a suitable immunologic reactant. Acetylcholine did not affect cyclic AMP concentrations in neutrophils at any time when cyclic GMP levels were elevated. Thus, once again a close association was found between cyclic GMP levels and lysosomal enzyme discharge, this time under the enhancing influence of acetylcholine.

Viability of neutrophils was maintained under the experimental conditions employed as indicated by the failure of the various immunologic and pharmacologic stimuli either to provoke the discharge of cytoplasmic lactate dehydrogenase from cells or to bring about cellular uptake of eosin Y.

Inhibition of the immunologic discharge of β-glucuronidase from neutrophils by epinephrine was associated, in every instance, with a concomitant accumulation of cyclic AMP. Epinephrine inhibited the secretion of β-glucuronidase that was provoked by several different immune reactants, and these inhibitory effects were accompanied by marked elevations of the neutrophil levels of cyclic AMP. Moreover, the time course of accumulation of cyclic AMP in neutrophils during cell contact with RA serum-treated zymosan particles and epinephrine resembled closely that of inhibition of β-glucuronidase discharge. In the absence of a suitable immunologic stimulus (absence of lysosomal enzyme release) epinephrine failed to elevate cyclic AMP levels. Further, epinephrine
did not alter the time-course of accumulation of cyclic GMP in neutrophils during inhibition of immunologic enzyme discharge and the accompanying rise in cyclic AMP levels. Thus, a close association was found between cyclic AMP levels and inhibition of the immunologic secretion of lysosomal enzymes from human neutrophils by epinephrine.

Several reports have illustrated the requirement of extracellular calcium for the selective discharge of lysosomal enzymes from granulocytes (3, 15, 16). The data in the present study reveal that extracellular calcium is required for the immunologic discharge of β-glucuronidase from purified human neutrophils regardless of the type of immunologic stimulus employed. To illustrate, phagocytosable zymosan particles, agg IgG, and zymosan-treated serum all require extracellular calcium in order to provoke neutrophils to secrete β-glucuronidase. Moreover, the capacities of each of these immune reactants to elevate the levels of cyclic GMP in neutrophils were abolished in the absence of extracellular calcium. Extracellular calcium was required also for the pharmacologic (acetylcholine) enhancement of immunologic release of lysosomal enzyme from, and the accompanying enhanced accumulation of cyclic GMP in, neutrophils. The consistent requirement of calcium for both enzyme release and cyclic GMP accumulation provides additional evidence in support of the hypothesis that cyclic GMP is involved closely in mediating the selective discharge of lysosomal enzymes from granulocytes. The precise sequence of events and mechanisms by which calcium and cyclic GMP trigger lysosomal enzyme discharge are not known. However, preliminary studies in this laboratory indicate that immune reactants stimulate the uptake of extracellular calcium (45Ca) into, or binding of calcium to, neutrophils at 37°C at times (1-5 min) when intracellular cyclic GMP levels are elevated and β-glucuronidase is discharged. Therefore, intracellular calcium may be involved in promoting the accumulation of cyclic GMP. Indeed, calcium was reported to stimulate the accumulation of cyclic GMP in vitro in lung (17) and heart (18) tissue.

The experimental evidence suggests that cyclic GMP and calcium are involved in mediating, whereas cyclic AMP appears to signal the inhibition of, the immunologic discharge of lysosomal enzymes from human neutrophils. Thus, the presence in human neutrophils of the biochemical machinery to accumulate cyclic GMP and/or cyclic AMP, in response to various extracellular chemical stimuli, provides the granulocytes with the capacity to control the extrusion of their lysosome granule contents. A consistent association of cyclic GMP accumulation and β-glucuronidase discharge in neutrophils is observed regardless of the type of extracellular immunologic stimulus. Further, these cell functions, both of which require extracellular calcium, are accentuated by acetylcholine. On the other hand, cyclic AMP accumulation is associated closely with inhibition of β-glucuronidase release and both events are elicited by epinephrine. Indeed, direct opposing influences of cyclic GMP and cyclic AMP (dibutyryl
analog) on the release of $\beta$-glucuronidase from neutrophils during cell contact with each of the immunologic reactants were observed. The opposing effects of cyclic GMP and cyclic AMP on neutrophil function represent a clear demonstration of the "Yin Yang" concept of bioregulatory mechanisms as proposed originally by Goldberg et al. (7), whereby the two nucleotides elicit direct oppositional actions on cellular processes. Parasympathetic and sympathetic neurohormones, by virtue of their capacities to influence nucleotide levels in neutrophils, are also capable of regulating the release of lysosomal enzymes. In view of the experimental evidence obtained thus far, we propose that cyclic GMP accumulation, in the presence of extracellular calcium, mediates or signals the immunologic secretion of lysosomal granule contents from human neutrophils and that the potential for cyclic AMP accumulation as well enables the granulocytes to exercise a bidirectional control of lysosomal enzyme discharge.

SUMMARY

The purpose of this investigation was to elucidate the relationship of cyclic GMP and calcium to the immunologic discharge of lysosomal enzymes from purified human neutrophils. Contact of neutrophils with a variety of immunologic stimuli, including zymosan particles treated with either normal or rheumatoid arthritic (RA) serum, heat-aggregated (agg) IgG, particulate and immobilized agg IgG each treated with RA serum, and zymosan-treated serum, provoked the discharge of $\beta$-glucuronidase, but not cytoplasmic lactate dehydrogenase, and stimulated the accumulation of cyclic GMP. Both enzyme release and elevation of cyclic GMP levels required the presence of extracellular calcium as neither cellular event proceeded in its absence. Cholinergic enhancement of the immunologic secretion of $\beta$-glucuronidase from neutrophils by acetylcholine was associated with a concomitant accumulation of cyclic GMP. These actions of acetylcholine on neutrophils did not proceed in the absence of extracellular calcium. Whereas the concentrations of cyclic GMP in neutrophils were elevated by both immune reactants and a combination of the latter and acetylcholine, cyclic AMP levels remained unaltered. Thus, cyclic GMP, but not cyclic AMP, was associated with the immunologic and pharmacologic discharge of lysosomal enzymes from neutrophils. Contrariwise, cyclic AMP, but not cyclic GMP, was associated with inhibition of lysosomal enzyme release. For example, dibutyryl cyclic AMP and epinephrine inhibited the release of $\beta$-glucuronidase from neutrophils that was elicited by each of the immune reactants tested. Moreover, cyclic AMP levels in the cells were elevated markedly in every instance that enzyme discharge was inhibited by epinephrine. Epinephrine did not alter the neutrophil concentrations of cyclic GMP at times when those of cyclic AMP were elevated. The data in this report constitute partial evidence that the immunologic discharge of lysosomal enzymes from human neutrophils is mediated or signaled by intracellular cyclic GMP and that calcium is linked to this stimulation of enzyme secretion.
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