Mechanisms of Lysosomal Enzyme Release from Human Leukocytes

II. EFFECTS OF cAMP AND cGMP, AUTONOMIC AGONISTS, AND AGENTS WHICH AFFECT MICROTUBULE FUNCTION

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A B S T R A C T Selective release of inflammatory materials from leukocyte lysosomes is reduced by compounds which increase cyclic 3',5'-adenosine monophosphate (cAMP) levels in suspensions of human leukocytes and is augmented by agents which increase cyclic 3',5'-guanosine monophosphate (cGMP) levels in these cell suspensions. Lysosomal enzymes are released in the absence of phagocytosis when cytochalasin B (5 μg/ml) converts polymorphonuclear leukocytes (PMN) to secretory cells: lysosomes merge directly with the plasma membrane upon encounter of PMN with zymosan, and cells selectively extrude substantial proportions of lysosomal, but not cytoplasmic enzymes. β-Adrenergic stimulation of human leukocytes produced a dose-related reduction in β-glucuronidase release (blocked by 10^{-6} M propranolol) whereas α-adrenergic stimulation (phenylephrine plus propranolol) was ineffective. In contrast, the cholinergic agonist carbamylcholine chloride enhanced enzyme secretion, an effect blocked by 10^{-6} M atropine. Incubation of cells with exogenous cAMP or with agents that increase endogenous cAMP levels (prostaglandin E_1, histamine, isoproterenol, and cholera enterotoxin) reduced extrusion of lysosomal enzymes; in contrast, exogenous cGMP and carbamylcholine chloride (which increases endogenous cGMP levels), increased β-glucuronidase release. Whereas colchicine (5 $\times$ 10^{-4} M), a drug which impairs microtubule integrity, reduced selective enzyme release, deuterium oxide, which favors microtubule assembly, enhanced selective release of lyosomal enzymes. The data suggest that granule movement and acid hydrolase release from leukocyte lysosomes requires intact microtubules and may be modulated by adrenergic and cholinergic agents which appear to provoke changes in concentrations of cyclic nucleotides.

INTRODUCTION

Phagocytic cells become the center of inflammatory lesions (1) due, in part, to release of substances previously sequestered within lysosomes. During uptake of particles such as zymosan and immune complexes, polymorphonuclear leukocytes of human blood (PMN)[1] remain viable and selectively extrude lysosomal, but not cytoplasmic, enzymes (2–5). Selective lysosomal enzyme release proceeds independently of particle ingestion when cytochalasin B (CB) converts PMN to "secretory" cells in which an intracellular event (fusion of lysosomes with phagosomes) becomes converted to an extracellular event (fusion of lysosomes with the plasma membrane). Under these conditions leukocytes release substantial proportions of lysosomal enzymes. Zymosan particles are not ingested but remain adherent to the cell surface (6–8).

Intracellular movement of lysosomes, and of secretory granules in a variety of cell types, appears to be

[1] Abbreviations used in this paper: carbachol, carbamylcholine chloride; CB, cytochalasin B; DMSO, dimethyl sulfoxide; D_2O, deuterium oxide; LDH, lactate dehydrogenase; PGE$_1$, prostaglandin E$_1$; PMN, polymorphonuclear leukocytes, SRS-A, slow-reacting substance of anaphylaxis.

regulated by an as yet unclear interaction between cyclic nucleotides, microtubules, and microfilaments (9). Although microtubules are in a dynamic state of assembly and disassembly, it is probably in their aggregated state that microtubules exert their influence on cell mechanics (10). Exogenous cyclic 3',5'-adenosine monophosphate (cAMP) and colchicine both favor disassembly of microtubules (11) and both reduce selective enzyme release (2, 3, 8, 12, 13). Exogenous cAMP, or compounds which increase its cellular concentration, also reduces antigen-induced histamine release from sensitized leukocytes (14, 15) and lung tissue (16, 17). In contrast, deuterium oxide (D₂O) favors formation of microtubules (18, 19) and augments histamine release from leukocytes (20, 21). Moreover, it has been reported that α-adrenergic agents (which decrease cAMP levels) and cholinergic agonists, enhance the immunologic release of histamine and slow-reacting substance of anaphylaxis (SRS-A) from human lung fragments (22). Cholinergic stimulation of heart and brain preparations produces increases in levels of cyclic guanosine 3',5'-monophosphate (cGMP) (23) and the introduction of 8-bromo-cGMP to sensitized lung tissue (22) was associated with enhancement of antigen-induced release of histamine and SRS-A. It therefore seemed possible that movement of lysosomes to phagosomes or the plasma membrane might also be controlled by autonomic agonists, cyclic nucleotides, and agents which influence assembly of microtubules.

Consequently, following the experimental protocol suggested by Kaliner, Orange, and Austen (22), CB-treated human leukocytes were incubated with a series of autonomic agonists before zymosan challenge. The results obtained in these studies indicate that, in CB-treated human leukocytes, release of acid hydrolases may be modulated by adrenergic and cholinergic agents as well as by cyclic nucleotides. It is further demonstrated that agents which increase cAMP levels in suspensions of CB-treated human PMN (isoproterenol, prostaglandin E₁ (PGE₁), histamine, cholera enterotoxin) reduce enzyme release from these cells whereas carbamylcholine chloride (carbachol), which raises cGMP levels, increases lysosomal enzyme release.

The role of microtubules in lysosomal enzyme release was also evaluated by means of D₂O and colchicine, following the protocol suggested by Gillespie and Lichtenstein (21). It was found that D₂O enhanced enzyme release whereas colchicine, a compound known to impair microtubule integrity (10) and reduce selective enzyme extrusion (2, 8, 12, 13) modified the D₂O effect.

METHODS

Medium. Cells were incubated in a medium consisting of (millimolars): NaCl, 138; KCl, 2.7; Na₂H PO₄, 8.1; KH₂PO₄, 1.5; CaCl, 0.6; MgCl, 1.0; pH 7.4. The osmo-larity of the buffer was within 5% of 300 mosmol by freezing point depression technique. D₂O replaced H₂O in the medium as required.

Human peripheral blood leukocytes were separated and suspended (4×10^6 cells/ml) as previously described (2, 3). Leukocyte suspensions used in the majority of studies contained 83±4% polymorphs; the remaining cells were identified as monocytes and lymphocytes. Hypo-osmolar lysis of erythrocytes, and subsequent differential centrifugation, removed the bulk of contaminating platelets and mononuclear cells. In other experiments purified preparations of human PMN were obtained by means of Hypaque/Ficoll gradients (24), allowing studies of cell suspensions which contained 98±1% polymorphs. Portions of cell suspensions (1.0 ml) were dispensed into 10×75-mm test tubes and incubated with CB (5 μg/ml × 5 min). Cells were then incubated with the test compounds. A dose of theophylline (5×10^{-4} M), insufficient to affect enzyme release by itself, was usually added when cells were incubated with cAMP, β-adrenergic agonists, and histamine. Metabisulfite, to a final concentration of 0.01%, was added in experiments with adrenergic agonists. Autologous serum (10% vol/vol) was then added and cells were exposed to zymosan. As detailed in previous studies (3), appropriate control experiments (a) indicated there was no preferential degradation of enzyme activity in resting or treated cells, (b) excluded the possibilities that particles and test compounds interfered with enzyme assays, or (c) that there was selective adsorption of enzymes to cells or particles after their release into the medium. At the end of experiments, tubes were centrifuged at 755 g at 4°C. The cell-free supernates were removed for enzyme determination.

Cytochemical localization of myeloperoxidase was carried out by a modification of the method of Graham and Karnovsky (25) in which the cells were fixed for 5 h at room temperature in 4% formaldehyde and 5% glutaraldehyde and washed overnight in 0.1 M cacodylate buffer (pH 7.2). They were then incubated at room temperature in a medium that contained 10% 3,3'-diaminobenzidine and 0.01% H₂O₂ in 0.5 M tris buffer (pH 7.6). The cells were rinsed briefly in distilled water and postfixed in 2% O₅O₄ in distilled water for 1 h. The fixed cells were washed twice in distilled water, en bloc, stained in 2% aqueous uranyl acetate for 15 min, and washed again in water. They were then dehydrated in ethanol and embedded in Spurr's low molecular weight epoxy. Thin sections were taken with a diamond knife on a Porter-Blum MT 2-B ultramicrotome (Ivan Sorvall, Inc., Newtown, Conn.) and viewed with a Zeiss EM 9s (Carl Zeiss, Inc., New York).

Viability of cells. The integrity of leukocytes in the pellets at the end of experiments was assayed by several techniques. (a) Exclusion of eosin Y: a cell pellet was diluted in 0.5% eosin Y in saline, mixed, and cells were counted in a hemocytometer. Nonviable cells lost their ability to exclude eosin Y and stained pink. In no instance did the proportion of nonviable cells exceed 3%. (b) Phagocytosis: the percent of untreated cells capable of phagocytosis (85%) when zymosan was added after a 5 h incubation period was no less than when cells were incubated immediately with zymosan, and CB-treated cells regained their capacity to the same extent in CB-free media. (c) Release of cytoplasmic lactate dehydrogenase (LDH) was used as an indicator of cell death, since rupture of cells by detergent (Triton X-100), sonication, or hypo-osmolar lysis (60 mosmol buffer) leads to release of total assayable LDH.

Enzyme assays. β-Glucuronidase was determined at 18 h of incubation with phenolphthalein glucuronic acid as substrate, as previously described (26). LDH was determined by the method of Wacker, Ulmer, and Vallee (27).

The platelet:leukocyte ratio of the cell suspensions was 5:1. Platelets, present even in 20-fold excess, contained negligible proportions of total β-glucuronidase activity (3). The contribution to total enzyme activity of leukocyte subpopulations was determined after separation of cells by means of Hypaque/Ficoll gradients (24).

cAMP determinations. At appropriate times, 0.1 ml 60% trichloroacetic acid (TCA) was added to purified preparations of PMN suspensions (10^7 cells). After sonication (Sonifier Cell Disruptor; Heat System-Ultrasonics, Inc., Plainview, N. Y.) particulate material was removed by centrifugation and TCA was extracted from the supernatant fluid by four washes with 5 vol of water-saturated ether. Duplicate 50-μl samples were assayed for cAMP by the protein-binding competition method of Gilman (28). The following modifications were employed: (*a*) Protein kinase was purified from frozen bovine heart muscle through the ammonium sulfate fractionation step and subsequent dialysis (29). (*b*) Each tube containing standard cAMP (Calbiochem, San Diego, Calif.), solutions also contained a volume of ether-extracted TCA solution equal to the volume of the unknown sample to minimize any possible effects of these substances on the assay. (*c*) Dextran-coated charcoal was used to separate free cAMP from protein kinase-bound cAMP according to the method of Mashiter, Mashiter, Hauger, and Field (30). Liquid scintillation counting of the supernate was performed in 10-ml samples of a 1:2 mixture of Triton X-100: Permafluortoluene solution (Packard Instrument Co., Inc., Downers Grove, Ill.

Although addition to assay tubes of lysosomal enzyme-rich supernates from zymosan stimulated PMN suspensions completely inhibited binding of cAMP, a finding in agreement with observations of a cAMP-binding inhibitor in human PMN lysosomes (31), there was no inhibition of cAMP in PMN preparations from which protein had been removed by TCA precipitation and centrifugation. Recoveries were 100±5%. Individual values reported for cAMP are the means of duplicate determinations from separate incubation tubes. Duplicate determinations varied by no more than 10%.

cGMP determinations. Hypaque/Ficoll-purified PMN suspensions were prepared (TCA addition, sonication, ether extraction) as outlined for cAMP determinations. Extracts were frozen and lyophilized overnight. The powder was redissolved in 0.05 M sodium phosphate buffer pH 6.2 and cGMP determined by radioimmunoassay as detailed by Steiner, Parker, and Kipnis (32), with minor modifications. Sodium phosphate buffer was substituted for sodium acetate since it has greater buffering capacity at pH 6.2. Separation of free and bound forms of cyclic nucleotide was achieved by addition of dextran-coated charcoal instead of by second antibody precipitation. Contents of the assay tubes were mixed with 1 ml of dextran-coated charcoal adsorption mixture (2.5% charcoal, 0.3% dextran, 0.25% heated human plasma protein fraction, 20 mM KH_2PO_4, pH 6.0). The mixture was allowed to stand at 4°C for 10 min before centrifugation (760 *g*) for 15 min. Radioactivity of the supernates was determined.

Radioimmunoassay of cGMP as developed by Steiner et al. (32) did not require purification of the nucleotide from TCA extracts of tissues or cells. TCA extracts of human

PMN, however, contained a factor(s) which interfered with the binding to antibody of cGMP and recovery of known amounts of cGMP added to TCA extracts of PMN suspensions averaged 70±10%. We are currently investigating a simplified purification procedure to eliminate the cGMP-binding inhibitor from extracts of PMN suspensions. Individual values reported for cGMP are the means of duplicate determinations and are not corrected for the decreased recovery. Duplicate determinations varied by no more than 5%.

MATERIALS

Prostaglandins were kindly furnished by Dr. John Pike, Upjohn Co., Kalamazoo, Mich.; theophylline, Mann Research Lab Inc., New York; cyclic nucleotides, adrenergic and cholinergic agents, histamine, and atropine, Sigma Chemical Co., St. Louis, Mo.; propranolol (Inderal) provided by Ayerst Laboratories, New York; zymosan: ICK Nutritional Biochemicals Div., Cleveland, Ohio; CB: ICI Research Laboratories, Alderley Park, Cheshire, England. CB was dissolved in 0.1% dimethyl sulfoxide (DMSO). This concentration of DMSO did not alter cell morphology or enzyme release (6). Cholera enterotoxin was provided by Dr. R. A. Finkelstein, The University of Texas Southwestern Medical School, Dallas, Tex., who prepared it according to published procedures (33, 34). There is evidence that cholera enterotoxin loses activity rapidly when dilute solutions are prepared in protein-free phosphate-buffered saline.[2] Portions of enterotoxin were therefore added to PMN suspensions (containing serum) within 30 min of their preparation. In previous studies, where cholera enterotoxin did not reduce lysosomal enzyme release from phagocytic cells (35), care was not taken to use the enterotoxin soon after dilution with phosphate-buffered saline. Indeed, dilute solutions were often stored frozen and used after thawing.

RESULTS

Time of preincubation with pharmacological agents. The time of preincubation with β-adrenergic agents was critical for reduction of enzyme release to be demonstrated. Preliminary experiments indicated that a lag period of 5 min after addition of agents was required for inhibition. Peak effects were observed when cells were incubated 5–20 min. Consequently, cells were preincubated 15 min with β-adrenergic agents in all experiments. Zymosan-induced release of β-glucuronidase from CB-treated cells was assayed at timed intervals after stimulation with carbachol. Enhancement of enzyme release was evident at 2 min, reached a peak effect within 5 min, and was minimal by 10 min. In experiments with carbachol and cGMP, 3 min proved to be the optimum time for preincubation (Fig. 1).

Distribution of enzyme activity. Suspensions of mixed leukocytes contained 83±4% polymorphonuclear cells. To exclude the possibility that mononuclear cells contributed significantly to β-glucuronidase release, cells were separated by means of Hypaque/Ficoll gradients

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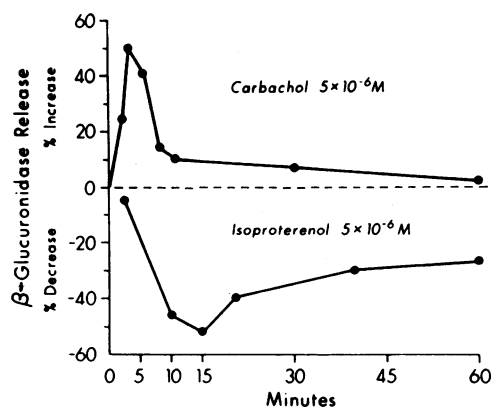
[2] Craig, J. 1973. Personal communication.

FIGURE 1 Lysosomal enzyme release from CB-treated human leukocytes: effect of incubation time with autonomic agonists before exposure of cells to zymosan. Leukocytes $(4 \times 10^6/ml)$ incubated at 37°C with 5×10^{-6} M carbachol or isoproterenol for times indicated, then exposed to zymosan (particle:cell ratio $= 30:1$) for 30 min. The percent change in β-glucuronidase release is compared with enzyme release from untreated control cells.

(24). The fraction enriched in mononuclear cells (lymphocytes and monocytes) proved to contain only 1.5% of the *total* β-glucuronidase activity and 9.2% of the total LDH activity of the lymphocyte/monocyte-contaminated leukocyte preparation. For example, the total β-glucuronidase (released by 0.2% Triton X-100) in the lymphocytes and monocytes normally present in a suspension of 4×10^6 leukocytes (83% PMN) was 0.34 μg phenolphthalein/h, whereas the total β-glucuronidase content of the leukocyte suspension was 22.2 μg phenolphthalein/h. (numbers are means; $n = 3$).

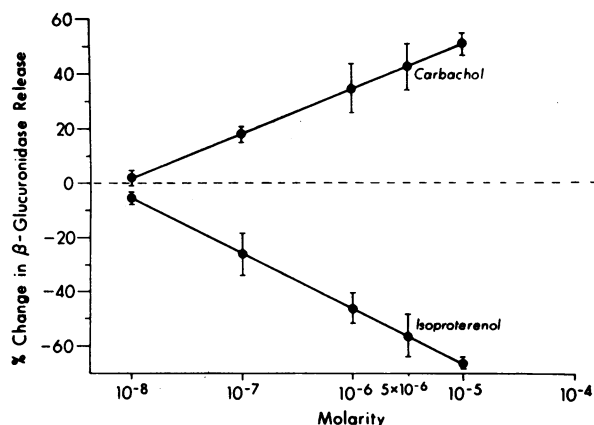FIGURE 2 Pharmacological regulation of lysosomal enzyme release from CB-treated human leukocytes. Leukocytes $(4 \times 10^6/ml)$ incubated 5 min with 5 μg/ml CB, then 15 min with isoproterenol (plus 5×10^{-4} M theophylline), or 3 min with carbachol, then exposed to zymosan (particle: cell ratio $= 30:1$) for 30 min. Control cells released $25.6 \pm 2.8\%$ of total β-glucuronidase (5.2 ± 0.7 μg/phenolphthalein/h/4×10^6 cells).

Effect on enzyme release of autonomic agonists. Cell suspensions from several ($n = 7$) subjects were exposed to the β-adrenergic agonist isoproterenol. Inhibition of enzyme release varied directly with the concentration of isoproterenol. In direct contrast, exposure to carbachol resulted in a dose-related enhancement of β-glucuronidase release (Fig. 2). In the absence of zymosan, carbachol did not enhance enzyme release. Epinephrine produced inhibition equivalent to that of isoproterenol (measured at 30 min).

In leukocytes from a minority of subjects ($n = 3$), enzyme release measured at 30 min appeared to be unaffected by autonomic agonists. These were studied in detail and kinetic analysis demonstrated that the *rate* of enzyme release was invariably modified: decrease in the presence of isoproterenol, enhancement with carbachol. Moreover, when the concentrations of agonists were raised by one log order, the expected results were again obtained. An experiment with leukocytes from such a subject is shown in Fig. 3. The rate of enzyme release was decreased by isoproterenol (Fig. 3A) and increased by carbachol (Fig. 3B), compared with control cells, during the first 15 min of zymosan exposure, despite the fact that cumulative enzyme release after 30 min of zymosan contact was similar in control and treated cells.

The effect of autonomic antagonists on enzyme release. The effects on enzyme release of isoproterenol and epinephrine were prevented when cells were incubated with β-adrenergic-blocking agent propranolol for 2 min before β-adrenergic stimulation, whereas introduction of the antimuscarinic agent, atropine sulfate, (2 min before addition of carbachol to CB-treated cells) prevented the cholinergic effect (Table I). Propranolol (10^{-6} M) and atropine (10^{-6} M) had no effect on enzyme release. Neither norepinephrine nor the relatively specific α-adrenergic agent phenylephrine (10^{-12}–10^{-3} M) had sig-
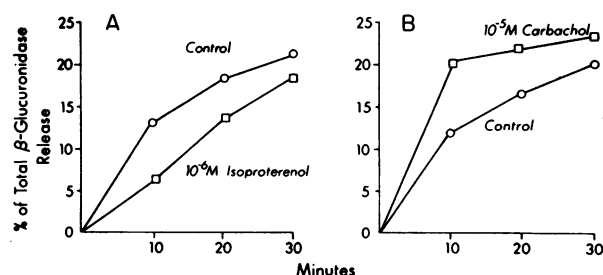

FIGURE 3 Kinetics of enzyme release from CB-treated human leukocytes: regulation by autonomic agonists. Leukocytes (4×10^6) incubated 5 min with 5 μg/ml CB then 15 min with isoproterenol (Fig. 3A), or 3 min with carbachol (Fig. 3B). Curves document reduction by isoproterenol and acceleration by carbachol of rate of β-glucuronidase release despite the similarity in cumulative release at 30 min in control and treated cells.

TABLE I

Pharmacological Regulation by Autonomic Agonists of Lysosomal Enzyme Release
*from Human Leukocytes**

| | Enzyme release | | |
| Compound (agonist ±antagonist) | β-Glucuronidase | | LDH percent total‡ |
	Percent total‡	Percent(±)change	
Resting	1.1±0.6	—	0.7±0.4
Stimulation (zymosan)	24.5±1.7	—	1.1±0.7
(β-Adrenergic)			
Isoproterenol	10.8±1.0	−56.5	1.4±0.7
Isoproterenol + propranolol (10⁻⁶ M)	24.0±1.9	0	1.7±1.0
Epinephrine (5 × 10⁻⁵ M)	14.7±0.9	−40.0	1.7±0.9
Epinephrine + propranolol (10⁻⁶ M)	22.3±1.1	0	1.8±0.7
(α-Adrenergic)			
Phenylephrine	20.8±1.4	−15.1	1.9±1.1
Phenylephrine + propranolol (10⁻⁶ M)	23.9±1.3	0	2.4±0.9
(Cholinergic)			
Carbachol	34.9±2.6	+42.6	2.1±0.7
Carbachol + atropine (10⁻⁶ M)	23.9±2.1	0	2.0±1.1
cGMP	35.6±3.0	+45.2	1.7±1.0

* All incubations at 37°C. Leukocytes (4 × 10⁶) pretreated with CB (5 μg/ml × 10 min). Agonists present at 5 × 10⁻⁶ M. Cells exposed to zymosan (particle:cell ratio:30:1) × 30 min. Values = mean ±SEM. n = 4.

‡ Expressed as percent of total (100%) activity released by 0.2% Triton X-100: 100% β-glucuronidase = 26.4±1.6 μg phenolphthalein/4 × 10⁶ leukocytes/h; 100% LDH = 1718±101 AU/4 × 10⁶/leukocytes.

nificant effects on lysosomal enzyme release. At the higher concentrations, phenylephrine displayed only its β-adrenergic effect. Addition of propranolol with the α-adrenergic agents did not significantly alter enzyme release.

Effect of exogenous cyclic nucleotides on enzyme release. Incubation of cells with cGMP, the presumed mediator of cholinergic stimulation (22), before challenge by zymosan, resulted in augmented β-glucuronidase release (Table I). In contrast, incubation of leukocytes with exogenous cAMP, with its dibutyryl analogue, or with compounds which augment endogenous cAMP concentrations in mixed leukocyte preparations, consistently reduced lysosomal enzyme release from CB-treated cells exposed to zymosan (Table II).

Studies with purified PMN. Experiments with purified (98±1%) preparations of PMN obtained from Hypaque/Ficoll gradients indicated that these cells were capable of selective enzyme extrusion; this could be reduced by addition to cells of agents known to increase endogenous cAMP (Table II). Whereas reduction of zymosan induced enzyme release by dibutyryl cAMP and PGE₁ was comparable in PMN and mixed leukocyte preparations, the effects of histamine, isoproterenol, and

cholera enterotoxin were less marked in PMN than in mixed leukocyte suspensions.

Measurements of cAMP and cGMP in PMN. It has been demonstrated (35, 36) that isoproterenol and PGE₁

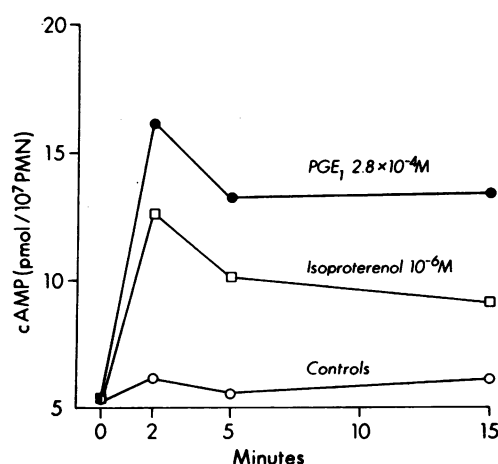FIGURE 4 Stimulation of cAMP in suspensions of Hypaque/Ficoll-purified human PMN. PMN (10⁷) incubated at 37°C with 5 × 10⁻⁴ M theophylline and with or without (controls) test compounds. Each point represents the mean of three experiments.

TABLE II

TABLE II

*Pharmacological Regulation of Lysosomal Enzyme Release from Human Leukocytes**

	Mixed leukocyte suspensions			Purified PMN preparations (Hypaque/Ficoll)		
	β-Glucuronidase		LDH Percent total‡	β-Glucuronidase		LDH, Percent total‡
Compound	Percent total*	Percent change		Percent total‡	Percent change	
Resting	2.9±1.1	—	2.8±0.8	3.6	—	4.0
Stimulation (zymosan)	23.4±2.0	—	3.7±1.0	26.2	—	2.0
Theophylline, 5 × 10⁻⁴ M	24.1±1.2	0	2.4±1.1	25.4	0	1.6
cAMP, 10⁻³ M*	16.6±1.4	−28.7	1.9±0.6	ND	ND	ND
dibutyryl cAMP, 10⁻³ M*	14.1±2.1	−39.6	2.3±1.0	16.0	−38.8	5.5
PGE₁, 2.8 × 10⁻⁴ M§	13.4±1.1	−43.7	2.6±1.2	14.0	−46.7	3.4
Histamine, 10⁻⁴ M*	18.8±1.6	−39.4	2.8±1.0	18.5	−29.4	2.6
Isoproterenol, 10⁻⁶ M	12.6±1.9	−46.2	1.7±0.9	19.0	−27.4	2.4
Cholera enterotoxin, 10 ng‖	7.7±2.0	−67.1	2.9±1.3	14.5	−44.6	3.9

* All incubations at 37°C. Leukocytes (4 × 10⁶) pretreated with CB (5 μg/ml, 5 min), incubated with compounds 15 min then exposed to zymosan for 30 min. Theophylline (5 × 10⁻⁴ M) in all tubes.
‡ Expressed as percent of total activity (100%) released by 0.2% Triton X-100.
§ 30 min incubation.
‖ 60 min incubation.
ND = Not done; values = Mean ±SEM, *n* = 4; purified PMN preparations: mean of two experiments.

stimulate greater accumulations of cAMP in lymphocytes and mixed leukocytes than in PMN. We have confirmed these findings and have observed that preparations of purified PMN accumulated cAMP in response to isoproterenol and PGE₁ maximally within the first 5 min (Fig. 4). In addition, the rapid accumulation and/or release of cAMP which followed exposure of CB-treated

PMN to zymosan was enhanced and sustained when these cells were preincubated with PGE₁ isoproterenol, histamine, or cholera enterotoxin (Table III). These compounds simultaneously reduced zymosan-induced release of β-glucuronidase.

In contrast, PGF₂ₐ, which did not increase cAMP levels significantly, did not reduce zymosan-induced

TABLE III

*Pharmacological Regulation of Lysosomal Enzyme Release from Human PMN**

	n	Preincubation	cAMP		β-Glucuronidase release, percent inhibition
			Before zymosan	20 min after zymosan	
		min	*pmol/10⁷ cells*		
Control (no drug)	5	—	5.4	6.8	—
PGE₁, 2.8 × 10⁻⁴ M	5	30	18.0	37.0	36.8
PGE₁, 2.8 × 10⁻⁶ M	2	30	11.1	16.3	19.5
PGF₂ₐ, 2.8 × 10⁻⁶ M	3	30	8.4	10.2	0
Cholera enterotoxin, 1 ng	2	60	11.7	19.9	26.8
Cholera enterotoxin, 10 ng	3	60	16.0	85.0	49.9
Cholera enterotoxin, 100 ng	1	60	32.8	ND	ND
Histamine, 10⁻⁴ M	3	15	12.9	26.1	30.0
Isoproterenol, 10⁻⁶ M	4	15	14.4	23.8	27.9
Theophylline, 5 × 10⁻⁴ M	5	30	6.1	8.9	0

* All incubations at 37°C. Hypaque/Ficoll-purified PMN (4 × 10⁶) pretreated with CB (5 μg/ml × 5 min), incubated with compounds, then exposed to zymosan for 20 min. Theophylline (5 × 10⁻⁴ M) present in all experiments.
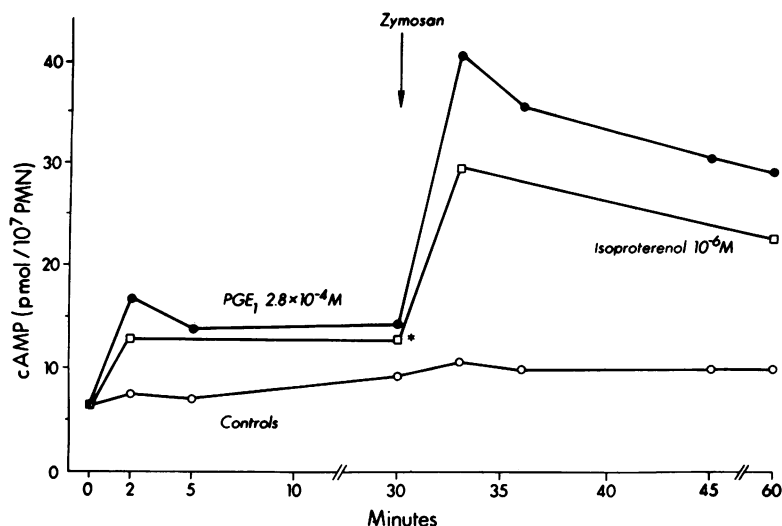Values are means for number of experiments noted.
ND = Not done.

FIGURE 5 Stimulation of cAMP in suspensions of Hypaque/Ficoll-purified human PMN. PMN (10^7) incubated at $37°C$ with 5 μg/ml CB plus 5×10^{-4} M theophylline and with or without (controls) PGE$_1$ or isoproterenol and then exposed to zymosan. Each point represents the mean of three experiments. (*) Cells were incubated 30 min with PGE$_1$ and 15 min with isoproterenol before their exposure to zymosan.

β-glucuronidase release. (Concentrations of PGF$_{2α}$ greater than 2.8×10^{-6} M, cause enzyme release by virture of cell damage [3]). The cAMP response of CB-treated human PMN to 2.8×10^{-4} M PGE$_1$ and 10^{-6} M isoproterenol is shown in Fig. 5.

The cGMP response of human PMN to carbachol also appeared to be maximal within the first 5 min of incubation. An experiment with two concentrations of carbachol is shown in Fig. 6. Carbachol enhanced and maintained cGMP concentration at significantly higher levels than in suspensions of untreated control cells. Carbachol (10^{-6} M) did not stimulate accumulation of cAMP.
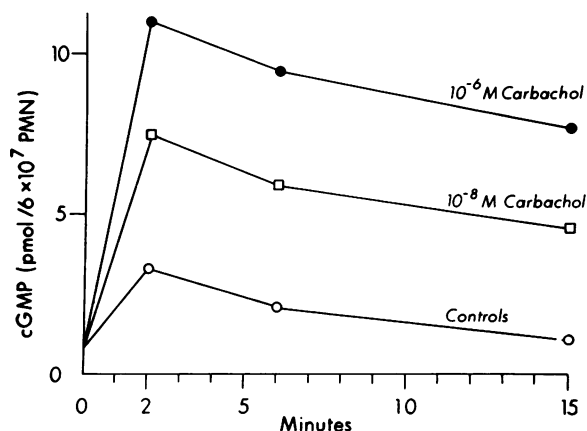
Effects of D$_2$O and colchicine on enzyme release. Cells were incubated with or without colchicine (5×10^{-4} M) for 60 min at $37°C$, washed, and resuspended in medium containing 0 or 50% D$_2$O. Cytochalasin B (5 μg/ml) and autologous serum (10% vol/vol) were added, cells were incubated for 10 min and then exposed to zymosan (particle:cell ratio 30:1) for 30 min at
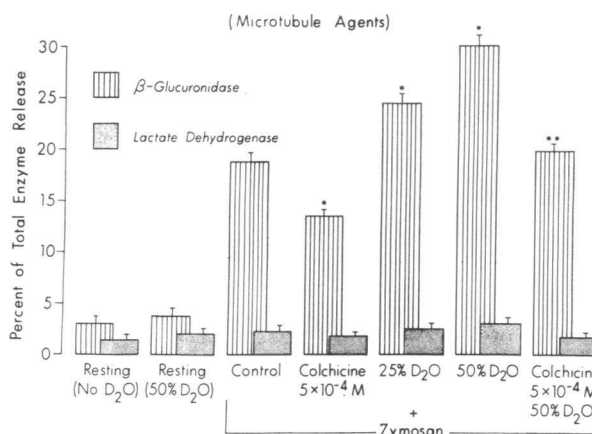
FIGURE 7 Lysosomal enzyme release from CB-treated cells. Human leukocytes (4×10^6) incubated 10 min with 5 μg/ml CB, incubated 60 min with or without colchicine in appropriate buffer (±D$_2$O), and exposed to zymosan 30 min. Total (100%) activity (released by 0.2% Triton X-100) for β-glucuronidase = 22.8 ± 2.1 μg phenolphthalein/h/4×10^6 cells. Total activity LDH = 462 ± 51 AU/4×10^6 cells. (*) Significant at $P < 0.01$ vs. control. (**) Significant at $P < 0.01$ vs. 50% D$_2$O.

FIGURE 6 Stimulation of cGMP in suspensions of human PMN. PMN (6×10^7) incubated at $37°C$ with and without (controls) carbachol (10^{-6} and 10^{-8} M) for times indicated.

Lysosomal Enzyme Release from Human Leukocytes   303

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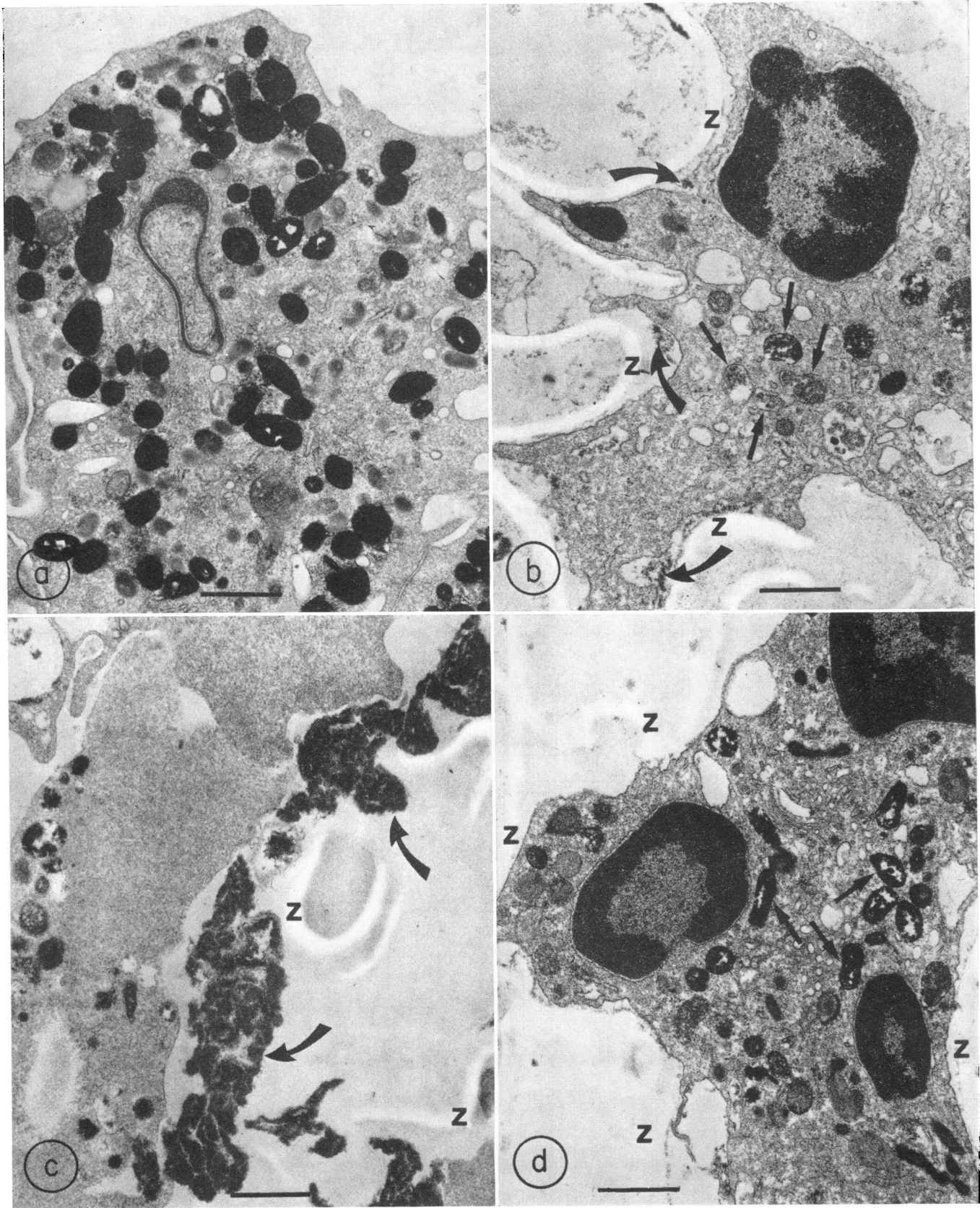
37°C. D_2O alone did not induce enzyme release in unstimulated cells. Colchicine, as previously reported (8, 12), reduced β-glucuronidase release, whereas D_2O enhanced release. In the presence of both D_2O and colchicine, release was intermediate between that induced by either agent alone (Fig. 7).

Morphology of enzyme secretion. Cells incubated with CB, then fixed and stained for endogenous peroxidase, showed many dense, peroxidase-positive, azurophilic granules as well as peroxidase negative specific granules scattered throughout the cytoplasm (Fig. 8a). Whereas large organelles such as the nucleus, centrioles, mitochondria, and granules could be seen, fine structures of the cytoplasm such as microtubules, microfilaments, or glycogen granules were not visualized by the cytochemical procedure used specifically to stain myeloperoxidase-containing azurophils (25).

When CB-treated cells were exposed to zymosan for 30 min and subsequently fixed and stained for peroxidase, degranulation to the plasma membrane could be demonstrated morphologically (Fig. 8b). Peroxidase-positive granules were neither so numerous nor as densely stained in the cell interior when compared with resting cells. Indeed, peroxidase-positive deposits could be seen at the cell-zymosan interface (Fig. 8b).

Cells incubated with carbachol (10^{-6} M) as well as with CB, before exposure to zymosan, underwent extensive degranulation. Large, dense masses of peroxidase-positive material were observed to lie between the cells and the adherent zymosan particles, whereas relatively few lysosomes remained intact in the cell cytoplasm. Furthermore, in cells treated with carbachol and CB, fewer lysosomal residua remained visible in the cell interior. Large areas of featureless cytoplasm appeared which were free of granules and vacuoles (Fig. 8c). Similar extensive degranulation was observed when CB-treated PMN were incubated with cGMP before their exposure to zymosan (not shown). In contrast, when PMN were incubated with PGE_1 (not shown), isoproterenol

TABLE IV

Controls of Lysosomal Enzyme Secretion from Human PMN

Control	Secretion↓	Secretion↑
(a) Autonomic agonist	β-Adrenergic	Cholinergic
(b) Agonist blocked by	Propranolol	Atropine
(c) Exogenous cyclic nucleotide	cAMP	cGMP
(d) Endogenous cyclic nucleotide accumulated	cAMP	cGMP
(e) Enzyme activated by cyclic nucleotide	Protein kinase	Unknown
(f) State of microtubules	Disassembled = colchicine, vinblastine effect	Assembled D_2O effect

(Fig. 8d), or cAMP (not shown) before exposure to zymosan, degranulation was retarded and the cells were intermediate in appearance between resting cells and untreated cells exposed to zymosan. This inhibition of degranulation has been quantitated and is presented in detail elsewhere (37).

DISCUSSION

It has been demonstrated (6-8, 38, 39) that the CB-treated leukocyte resembles a secretory cell in which the influence of various compounds on membrane fusion and enzyme extrusion may be measured directly. These cells, upon contact with a phagocytic stimulus (e.g., zymosan), selectively merge lysosomes with the plasma membrane as if the latter were a phagocytic vacuole. Selective secretion of lysosomal materials after this merger occurs in the absence of cell death and despite absence of particle ingestion. The results of experiments presented in this communication suggest that the secretion of lysosomal hydrolases from human PMN can be modified by agents which affect cyclic nucleotides and microtubules. Control of lysosomal enzyme release, summarized in Table IV, may be compared with the regulation of release of mediators of inflammation and secretion of exportable proteins from other cell types:

FIGURE 8 (a) A human peripheral blood neutrophil treated with CB (5 μg/ml) for 5 min then fixed and stained for endogenous peroxidase. Numerous black, peroxidase-positive azurophilic granules as well as many peroxidase-negative specific granules can be seen in the cytoplasm of this cell. Magnification × 14,500. Line equals 1 μm. (b) Another neutrophil, pretreated with CB and then exposed to zymosan (Z) for 30 min before fixing and staining for peroxidase. Only two intact azurophilic granules are visible in this cell although many lysosomal residua can be seen with some peroxidase-positive material persisting within them (arrows). Some peroxidase-positive material can also be seen at the cell-zymosan interface (curved arrows). Magnification × 14,500. Line equals 1 μm. (c) A neutrophil pretreated with CB, incubated (37°C) with 10^{-6} M carbachol for 3 min, and exposed to zymosan for 30 min. Masses of peroxidase-positive material are visible (arrows) between the cell surface and the zymosan (Z) with large expanses of featureless cytoplasm adjacent to these masses. Magnification × 14,500. Line equals 1 μm. (d) CB-treated neutrophil incubated (37°C) 15 min with 10^{-6} M isoproterenol and 5×10^4 M theophylline, then exposed to zymosan for 30 min. Although four zymosan particles are seen in contact with the cell, many peroxidase-positive azurophilic granules and peroxidase-negative specific granules, remain in the cytoplasm. Magnification × 14,500. Line equals 1 μm.

(a) β-Adrenergic catecholamines reduce selective extrusion of lysosomal enzymes from human PMN exposed to zymosan. Similarly, β-adrenergic stimulation reduces immunologically induced histamine release from sensitized basophils and lung tissue (14, 16, 17). In contrast, the cholinergic agonist carbachol enhances lysosomal enzyme release from leukocytes, augments histamine and SRS-A release from lung fragments (18, 22), evokes secretion of lysosomal acid hydrolases and catecholamines from the bovine adrenal gland (40), and induces gastric secretion (41) and mucin secretion from submaxillary gland (42).

(b) Appropriate blockade of neurohumoral receptors (by propranolol or atropine) inhibits actions of the autonomic agonists upon lysosomal enzyme extrusion from human leukocytes. Release of histamine and SRS-A from human lung fragments is similarly controlled (16, 17, 22).

(c) Human leukocytes possess specific and separate receptors for endogenous hormones including β-adrenergic catecholamines, histamine, and prostaglandins (43). Each of these agents stimulates accumulation of cAMP in suspensions of mixed human leukocytes (15, 44, 46). Our experiments demonstrate that each of these agents, as well as exogenous cAMP (and its dibutyryl analogue), added directly to leukocyte suspensions, increases cAMP levels and reduces selective extrusion of lysosomal enzymes from human leukocytes exposed to zymosan. Consequently, it is reasonable to suppose that it is accumulation of cAMP which causes inhibition of enzyme secretion. Similarly, exogenously added cAMP inhibits the release of lymphocytoxins (47), histamine, and SRS-A (16, 17). Although β-adrenergic agents, prostaglandin E_1, histamine, and cholera enterotoxin cause accumulation of cAMP in neutrophil suspensions, far greater increments of cAMP are found in mononuclear fractions (35, 36). Moreover, when neutrophils were separated from mixed suspensions, after incubation with isoproterenol, it was not possible to demonstrate increments in cAMP (36) within cells.

In the experiments we report, as well as in those of others (35), small but significant increments of cAMP were detected after stimulation by isoproterenol. In such experiments, the measured cyclic nucleotide represents the sum of intracellular cAMP and that released into the medium. Since cAMP leaks from stimulated cells (48), it is important not only to measure intracellular, but also extracellular cAMP.

Our experiments with purified preparations of PMN indicate that isoproterenol, PGE_1, histamine, and cholera enterotoxin act to increase cAMP and to inhibit release of lysosomal enzymes in the absence of mononuclear cells. However, reduction of enzyme release is usually more marked in suspensions of mixed leukocytes than in suspensions of purified PMN. This would suggest that mononuclear cells, perhaps by releasing their cAMP, may further reduce enzyme release from PMN. It will be necessary to define more precisely the nature and extent of intercellular cAMP fluxes in this system.

(d) After incubation with polystyrene latex beads for 5 min the cAMP content of human peripheral blood leukocytes is increased severalfold (49). This increase, however, has been held to be due to the accumulation of the cyclic nucleotide mainly in mononuclear cells (50). CB-treated human PMN exposed to zymosan respond with only modest increments in cAMP. However, when the cells are incubated with compounds which produce these modest increases in cAMP levels and are then exposed to zymosan, the cAMP burst is enhanced and sustained. The reason for this is not apparent. It is possible that adenyl cyclase stimulated by zymosan contact with the plasma membane acts synergistically with adenyl cyclase stimulated by the test compound to produce the striking elevation in cAMP. Additionally, or alternatively, the test compound may interfere with the mechanisms responsible for regulating the return of cAMP to basal levels after zymosan contact.

Exogenous cGMP enhances secretion of lysosomal hydrolases from human leukocytes, results which are concordant with studies in which the introduction of 8-bromo-cGMP to sensitized lung tissue was associated with enhancement of antigen-induced release of histamine and SRS-A. There is evidence, in other tissues, that cholinergic agents stimulate the accumulation of endogenous cGMP (22, 23). Similarly, the results of the studies presented here indicate that the cholinergic agonist carbachol increases the cGMP level in suspensions of human PMN and increases zymosan-induced release of lysosomal enzymes. This reciprocal relationship between cGMP and cAMP, whether exogenously added or endogenously accumulated after appropriate stimulation, has been observed in other biological systems. Thus, exogenous cAMP inhibits the uptake of uridine, leucine, and 2-deoxyglucose by cultured mouse fibroblasts, an effect counteracted by cGMP (51). In addition, exogenous cAMP as well as agents that elevate intracellular concentrations of cAMP, prevent the transformation of lymphocytes by phytohemagglutinin (52, 53), and prevent the cytotoxic actions of lymphocytes upon cells bearing alloantigens to which they are sensitized (47), whereas cholinergic agents enhance the cytotoxicity of lymphocytes (54). Indeed, mitogenic concentrations of phytohemagglutinin produced 10 to 50-fold increments in the concentration of lymphocyte cGMP whereas lymphocyte cAMP was not elevated (55).

(e) A unique protein kinase, active upon histone, has been isolated from purified human PMN (56). This enzyme phosphorylates a number of substrates when ex-

posed to cAMP and cyclic inosine 5'-monophosphate (cIMP) ($> 10^{-3}$ M) but not to cGMP. At present no enzyme has been isolated, the activity of which is uniquely susceptible to stimulation of cGMP.

(f) D_2O favors the assembly of microtubules (18, 19) and enhanced the selective extrusion of lysosomal enzymes from human leukocytes. D_2O also favors the secretion of histamine from appropriately sensitized cells and appears to do so by virtue of an effect upon microtubule assembly as evidenced by the fact that colchicine, in histamine-rich cells, seems to reverse the effect of D_2O (20, 21). Colchicine, which binds to tubulin subunits (57), leads to disassembly of microtubules and reduces selective enzyme release. Moreover, prior incubation of cells with colchicine reduced the potentiating effect of D_2O, suggesting that granule movement and hydrolase release both depend upon intact microtubules. Similarly, vinblastine, which causes the disassembly of microtubules by forming dense precipitates (58), also inhibits release of lysosomal enzyme (8). Louie and Dixon (59) have shown that phosphorylation of histone, by introducing surface-negative charges, promotes disassembly of histones: such experiments provide a precedent for disassembly of tubulin after protein kinase-mediated phosphorylation.

It is therefore reasonable to suspect that each of these agents acts at the same, final, site to prevent or enhance the secretion of previously stored lysosomal hydrolases to the outside. Our ultrastructural studies have demonstrated that appearance of lysosomal enzymes in the supernate appears to be due to the direct secretion of the contents of lysosomes from a granule that has merged with the plasma membrane as if it were a phagocytic vacuole. Consequently, one explanation for our findings is that the cyclic nucleotides, cAMP and cGMP, effect the state of assembly of microtubules in PMN and that their state of assembly regulates the flow of lysosomes and the secretion of enzymes to the outside. This, of course, need not be the only site at which these agents act. The reciprocal relationships between cAMP and cGMP can be demonstrated not only when these agents are added exogenously, but also when compounds which influence their endogenous accumulation are exhibited.

Only studies in which it is not unlikely that inactive cholera enterotoxin was employed (35) appear to contradict the hypothesis presented above. We have now shown that freshly prepared enterotoxin protected by serum protein from inactivation stimulates accumulation of cAMP and inhibits hydrolase release. However, the results presented in this paper have been obtained with CB-treated cells and cannot be unequivocally demonstrated in cells not treated with this agent. The explanation may lie in the fact that, in cells not treated with CB, lysosomes merge with phagocytic vacuoles in the interior of the cell and enzyme release to the periphery only occurs by the process of "regurgitation during feeding" (3). Thus, direct quantitation of fusion is not afforded by measurements of lysosomal hydrolases in the suspending medium. The bulk of these enter phagocytic vacuoles intracellularly and drug effects may indicate actions upon phagocytosis per se and not upon the fusion process. It is only in cells in which phagocytosis is completely inhibited by CB that it becomes possible to quantitatively monitor this intracellular event by measurements of the extracellular release of lysosomal hydolases. However, our previous studies (37) have indicated that intracellular degranulation (measured morphometrically) is inhibited by exogenous cAMP and agents which elevate endogenous cAMP. Moreover, preliminary studies indicate that carbachol, which stimulates endogenous cGMP causes enhanced intracellular degranulation in cells not treated with CB.

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