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Bioregulation of Lysosomal Enzyme Secretion from Human Neutrophils: Roles of Guanosine 3′:5′-Monophosphate and Calcium in Stimulus-Secretion Coupling

(adenosine 3′:5′-monophosphate/acetylcholine/immune reactants/calcium translocation/ionophores)

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ABSTRACT **The biologic role of calcium and guanosine 3′:5′-monophosphate (cyclic GMP) in the immunologic secretion of lysosomal enzymes from human neutrophils was studied. Contact of neutrophils with zymosan-treated serum or the divalent cation ionophore A-23187, in the presence of extracellular calcium, resulted in β-glucuronidase (β-D-glucuronide glucuronosohydrolase, EC 3.2.1.31) secretion and a concomitant accumulation of cyclic GMP without any loss of cell viability. Acetylcholine (0.1 μM), in the presence of calcium, enhanced the immunologic stimulation of cyclic GMP accumulation and lysosomal enzyme discharge. A marked and rapid association of $^{45}CaCl_2$ with neutrophils occurred during cell surface contact with zymosan-treated serum, and this effect on calcium association was enhanced with 0.1 μM acetylcholine. The precise mechanism of the neutrophil–calcium interaction is presently not well understood. However, the finding that 0.5–1.0 μM A-23187 also provoked a rapid association of extracellular calcium with neutrophils suggests that calcium mobilization into the intracellular environment may account, at least in part, for this association between cells and calcium. The close temporal relationship between β-glucuronidase secretion, cyclic GMP accumulation, and calcium mobilization during cell contact with membrane active agents such as immune reactants, acetylcholine, and ionophores suggests that these three cellular events bear a cause and effect relationship. On the basis of our findings to date, we propose that the immunologic secretion of lysosomal contents from human neutrophils is signaled by intracellular cyclic GMP and that extracellular calcium, by gaining access to the intracellular processes responsible for cyclic GMP accumulation, serves as the link to stimulus-secretion coupling.**

Recent reports from this laboratory indicated that the immunologic secretion of lysosomal enzymes from human neutrophils can be modulated by autonomic neurohormones, prostaglandins, glucocorticosteroids, and cyclic nucleotides (1–6). Guanosine 3′:5′-monophosphate (cyclic GMP) and pharmacologic agents that elevated neutrophil levels of cyclic GMP (acetylcholine, prostaglandin $F_{2\alpha}$) stimulated, whereas adenosine 3′:5′-monophosphate (cyclic AMP) and agents that elevated cyclic AMP levels (epinephrine, prostaglandins E_1, A_1, A_2) inhibited lysosomal enzyme secretion during cell contact with phagocytizable and nonphagocytizable immune reactants. These experimental findings support the "Yin and Yang" concept of bioregulation of cell function as proposed originally by Goldberg *et al.* (7), whereby cyclic GMP and cyclic AMP elicit opposing actions in bidirectionally regulated biologic systems (8–10). In view of the requirement of extracellular calcium for lysosomal enzyme secretion from human neutrophils (11, 12), we sought to ascertain the role of calcium in this model of stimulus-secretion coupling.

Immunologic secretion of lysosomal contents from human neutrophils is probably mediated by intracellular cyclic GMP, as a variety of immune reactants provoked cyclic GMP accumulation and a concomitant discharge of lysosomal enzymes in the presence of calcium (12). The rapid accumulation of cyclic GMP may be associated, among other factors, with guanylate cyclase stimulation. Calcium has been reported to stimulate guanylate cyclase activity (13, 14), and to influence cyclic GMP formation (15, 16) in other tissues. Extracellular calcium may gain entry into neutrophils, in light of recent reports that calcium influx into mast cells is associated with allergic histamine discharge (17). In fact, increased radiocalcium uptake into other secretory cells accompanies secretion of packaged intracellular constituents (18), and this sequence of events has been termed stimulus-secretion coupling, whereby calcium serves as the link between these two processes (19). Therefore, experiments were designed to ascertain whether calcium interaction with human neutrophils triggers the immunologic secretion of lysosomal enzymes.

MATERIALS AND METHODS

Isolation of Human Neutrophils, Preparation of Zymosan-Treated Serum, and Determination of Cyclic GMP Concentration and β-Glucuronidase Activity. Human neutrophils were isolated and suspended (5×10^6 cells per ml) in Hanks' balanced salt solution, pH 7.4, containing 0.1%, w/v glucose (Hanks'–glucose) as described previously (3). Final cell suspensions consisted of 96–99% neutrophils; erythrocytes and platelets were absent. Zymosan-treated serum (ZTS) was prepared and utilized as described previously (3). Cyclic GMP concentrations (in entire incubation media containing cells), and β-glucuronidase (β-D-glucuronide glucuronosohydrolase, EC 3.2.1.31) and lactate dehydrogenase (L-lactate: NAD^+ oxidoreductase, EC 1.1.1.27) activities (discharged into the extracellular incubation medium) were determined as described (2).

Incubation Procedures and Analysis of the Association Between Extracellular Calcium and Human Neutrophils.

Abbreviations: cyclic GMP, guanosine 3′:5′-monophosphate; cyclic AMP, adenosine 3′:5′-monophosphate; Hanks'–glucose, Hanks' balanced salt solution (pH 7.4) containing 0.1% glucose; ZTS, zymosan-treated serum.

TABLE 1. *Requirement of calcium for β-glucuronidase secretion and cyclic GMP accumulation in human neutrophils*

Experimental condition*	Calcium, 1.27 mM (present, +) (absent, −)	Cyclic GMP concentration (pmol/10^6 cells)	β-Glucuronidase secretion (μg phenolphthalein/18 hr per 5×10^6 cells)
5×10^6 Neutrophils (N)	+	0.48 ± 0.14	3.7 ± 0.3
	−	0.64 ± 0.11	4.3 ± 0.2
N + zymosan-treated serum (ZTS)	+	31.7 ± 7.6†	21.4 ± 1.8†
	−	2.4 ± 0.5	5.2 ± 0.4
N + ZTS + 0.1 μM acetylcholine	+	52.2 ± 5.4†	39.7 ± 5.4†
	−	3.6 ± 0.6	5.1 ± 0.5
N + 0.5 μM A-23187	+	38.6 ± 4.0†	40.3 ± 6.7†
	−	2.4 ± 0.3	4.9 ± 0.6

* Neutrophils were preincubated alone or with acetylcholine at 37° for 10 min before the addition of A-23187 or ZTS, and were then further incubated at 37° for 5 min. Data represent the mean ± SEM of six determinations.

† Significantly different ($P < 0.01$, Student's *t*-test) from corresponding values in the absence of calcium.

Neutrophils were used 30 min after preparation of the final suspensions, which were maintained at 26–27°. In each experiment the reactions consisted of 1.0 ml of neutrophil suspension (5×10^6 cells) in a total volume of 1.0–1.2 ml. Cell suspensions were preincubated either alone or with acetylcholine at 37° for 10 min before the addition of other materials such as ZTS, serum, or A-23187 and were then further incubated at 37° for up to 10 min. In those experiments where $^{45}CaCl_2$ was utilized, 1.0 μCi of $^{45}CaCl_2$ (2.18 Ci/mmol) was added at zero time (commencement of the incubation periods). Incubations with $^{45}CaCl_2$ were terminated by rapid vacuum filtration through 0.45 μm pore Millipore cellulose ester filters. The cells adhered to the upper surface of the filters and were washed with five volumes of cold Hanks'–glucose. Repeated washings with additional cold medium failed to remove any additional radioactivity from the cells. Filters were vacuum dried, dissolved in 10 ml of Fluoralloy (Beckman) scintillation cocktail (32.2 g of Fluoralloy dissolved in 3 parts of toluene and 1 part of 2-methoxyethanol), and the radioactivity (β-energy emission) was determined with a Packard Tri-Carb liquid scintillation spectrometer. Based on the extracellular concentration of calcium in the incubation media (1.27 mM) and the amount of added $^{45}CaCl_2$ (0.46 nmol of 2.18 μCi/nmol), each nmol of $^{45}CaCl_2$ is equivalent to 2.76 μmol of unlabeled $CaCl_2$. The data signify the increase in association of extracellular calcium with neutrophils and are expressed as μmol of calcium per 5×10^6 cells. This increase in calcium association with cells, whether it is influx and/or adsorption, has been termed the calcium concentration in Table 1 and Figs. 1 and 2.

Compounds. Solutions of acetylcholine chloride (Sigma Chemical Co., St. Louis, Mo.) were prepared and used immediately. A-23187 (a gift from Eli Lilly and Co., Indianapolis, Ind.) was dissolved in dimethylsulfoxide. Final concentrations of this solvent in incubation media never exceeded 0.1%, and this small amount of dimethylsulfoxide did not influence β-glucuronidase secretion, cyclic GMP accumulation, or $^{45}CaCl_2$ interaction with neutrophils.

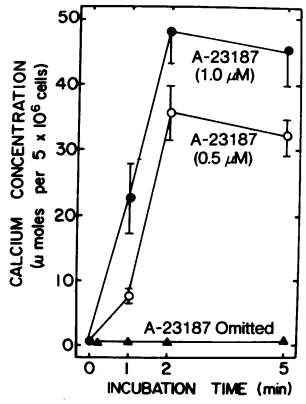

FIG. 1. Effect of A-23187 on the interaction between extracellular calcium and human neutrophils. Neutrophils (5×10^6) were preincubated alone at 37° for 10 min before the addition of A-23187 or buffer alone (A-23187 omitted) as indicated, and then further incubated at 37° for up to 5 min. $^{45}CaCl_2$ was added to the cell suspensions at zero time (0 min) and calcium concentration was determined according to the procedure outlined in the *text*. Data represent the mean ± SEM of four separate experiments. Each point for A-23187 (0.5 and 1.0 μM) at 1, 2, and 5 min is significantly different ($P < 0.001$, Student's *t*-test) from the corresponding point of A-23187 omitted.

RESULTS

Requirement of Calcium for β-Glucuronidase Secretion and Cyclic GMP Accumulation in Human Neutrophils. In the presence of extracellular calcium human neutrophils secreted β-glucuronidase during cell contact with particle-free, zymosan-treated serum (ZTS) (Table 1). Secretion was accompanied by a significant accumulation of cyclic GMP, and both cellular events required calcium. Acetylcholine enhanced the effects of ZTS on both enzyme secretion and cyclic GMP accumulation. In view of the knowledge that

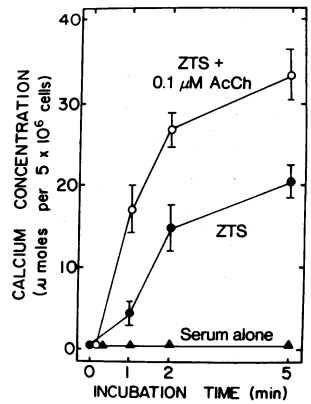

FIG. 2. Effect of zymosan-treated serum and acetylcholine on the interaction between extracellular calcium and human neutrophils. Neutrophils (5×10^6) were preincubated alone or with acetylcholine (AcCh) at 37° for 10 min before the addition of zymosan-treated serum (ZTS) or serum alone as indicated, and were then further incubated at 37° for up to 5 min. $^{45}CaCl_2$ was added to the cell suspensions at zero time (0 min) and calcium concentration was determined according to the procedure outlined in the text. Data represent the mean ± SEM of five separate experiments. Each point for ZTS + 0.1 μM AcCh and for ZTS at 1, 2, and 5 min is significantly different ($P < 0.01$, Student's *t*-test) from the corresponding time point at the other condition and from serum alone.

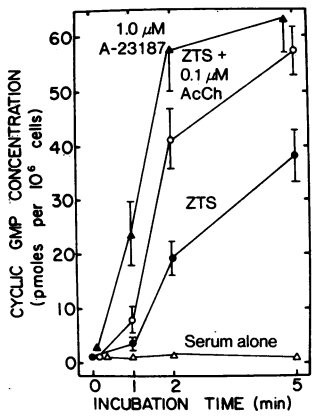

FIG. 3. Effect of zymosan-treated serum, acetylcholine, and A-23187 on the accumulation of cyclic GMP in human neutrophils. Neutrophils (5×10^6) were preincubated alone or with acetylcholine (AcCh) at 37° for 10 min before the addition of A-23187, zymosan-treated serum (ZTS), or serum alone as indicated, and were then further incubated at 37° for up to 5 min. Cyclic GMP concentration was determined as described previously (2). Data represent the mean ± SEM of six determinations. Each point for A-23187, ZTS + 0.1 μM AcCh, and ZTS at 1, 2, and 5 min is significantly different ($P < 0.05$, Student's t-test) from the corresponding point of serum alone. Further, each point for ZTS + 0.1 μM AcCh at 1, 2, and 5 min is significantly different ($P < 0.05$) from the corresponding point of ZTS.

calcium is required for allergic histamine release from mast cells and that certain ionophores provoke mast cell degranulation only in the presence of calcium, we tested the effect on neutrophils of A-23187, a divalent ionophore. A-23187 provoked β-glucuronidase secretion and cyclic GMP accumulation in the presence of extracellular calcium (Table 1).

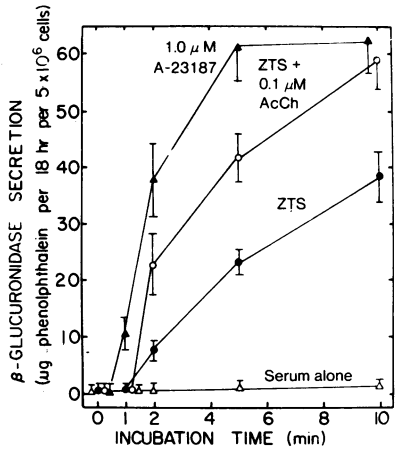

FIG. 4. Effect of zymosan-treated serum, acetylcholine, and A-23187 on the secretion of β-glucuronidase from human neutrophils. Neutrophils (5×10^6) were preincubated alone or with acetylcholine (AcCh) at 37° for 10 min before the addition of A-23187, zymosan-treated serum (ZTS), or serum alone as indicated, and were then further incubated at 37° for up to 10 min. Beta-glucuronidase activity was determined as described previously (2). Data represent the mean ± SEM of six determinations. Each point for A-23187, ZTS + 0.1 μM AcCh, and ZTS at 2, 5, and 10 min is significantly different ($P < 0.01$, Student's t-test) from the corresponding point of serum alone. At 1 min, only the point for A-23187 is significantly different ($P < 0.001$) from that for serum alone.

TABLE 2. *Effect of zymosan-treated serum, acetylcholine, and A-23187 on viability of human neutrophils*

Experimental condition*	Discharge of lactate dehydrogenase (Δ absorbancy at 366 nm/min per 5×10^6 cells)†	% of neutrophils with eosin Y uptake
5×10^6 Neutrophils (N)	0.0034 ± 0.0004	1.3 ± 0.2
N + zymosan-treated serum (ZTS)	0.0030 ± 0.0005	1.7 ± 0.4
N + ZTS + 0.1 μM acetylcholine	0.0035 ± 0.0004	1.1 ± 0.3
N + 0.5 μM A-23187	0.0028 ± 0.0003	1.8 ± 0.2
N + 1.0 μM A-23187	0.0038 ± 0.0005	1.6 ± 0.4

* Neutrophils were preincubated alone or with acetylcholine at 37° for 10 min before the addition of A-23187 or ZTS, and then further incubated at 37° for 10 min. Data represent the mean ± SEM of four separate experiments.

† Total cell enzyme activity, determined after 10 freeze-thaw cycles in dry ice–acetone mixture and running cool tap water, was 0.162 ± 0.012 Δ absorbancy (366 nm)/min per 5×10^6 neutrophils.

Strontium, but not magnesium, effectively substituted for calcium (80–90% as effective as calcium) in the incubation medium with regard to β-glucuronidase secretion provoked by ZTS and A-23187. The monovalent cationic requirements are not clear, as β-glucuronidase was discharged under conditions in which extracellular sodium had been replaced with an osmotically equivalent quantity of sucrose. The data illustrated in Table 2 indicate that ZTS, ZTS plus acetylcholine, and A-23187 did not impair cell viability (viable cells do not take up eosin Y or release the nonlysosomal enzyme lactate dehydrogenase).

The above data support further the view that extracellular calcium and cellular cyclic GMP are associated closely with the cellular events leading to lysosomal enzyme secretion (2, 3, 11, 12). Therefore, our attention was directed toward the interaction or association of extracellular calcium with human neutrophils.

Interaction of Extracellular Calcium with Human Neutrophils. Incubation of neutrophils in Hanks'–glucose containing $^{45}CaCl_2$ resulted in a time- and temperature-dependent association of radioactivity with the cells (Table 3). For example, at 4° the association between calcium and neutrophils was much greater than at 37°. Transfer of cells (entire incubation mixtures) from 4°, after allowing calcium to associate with cells for 10 min, to 37° resulted in a marked dissociation of radioactivity from the cells (Table 3). In contrast, transfer of incubation mixtures from 37° to 4° resulted in a gradual association of radioactivity with cells. At this time we do not know whether the association of calcium with neutrophils is due to influx and/or adsorption. The finding that A-23187 provoked calcium association with neutrophils at 37° (Fig. 1) suggests that calcium permeates these cells. In addition, ZTS and ZTS plus acetylcholine provoked a rapid association of extracellular calcium with neutrophils (Fig. 2). A soluble rather than particulate immune reactant was employed in order to avoid the complex interpretations associated with particle, and thus extracellular medium, internalization.

TABLE 3. *Interaction of extracellular calcium with human neutrophils at 37° and 4°*

Experimental condition*	Incubation time (min)			
	0	2	5	10
	Calcium concentration† (μmol per 5 × 10^6 cells)			
37°	0.21 ± 0.014	0.52 ± 0.046‡	0.38 ± 0.032‡	0.26 ± 0.020
4°	0.20 ± 0.011	1.87 ± 0.24‡	4.42 ± 0.66‡	7.15 ± 1.12‡
Transferred from 4° to 37°	6.88 ± 0.93	5.92 ± 0.57	4.72 ± 0.40‡	2.96 ± 0.31‡
Transferred from 37° to 4°	0.24 ± 0.019	1.60 ± 0.26‡	3.58 ± 0.54‡	6.22 ± 1.04‡

* Human neutrophils (5 × 10^6) were incubated in 1.0 ml of Hanks' balanced salt solution, pH 7.4 containing, 0.1% w/v glucose as indicated. In addition to the 1.27 mM $CaCl_2$ present in the Hanks' solution, tracer amounts of $^{45}CaCl_2$ (1.0 μCi; 0.46 nmol) were added to the cell suspensions at zero time (0 min). In the transfer experiments cells were incubated in the $^{45}CaCl_2$ medium for 10 min at either 4° or 37° and subsequently transferred to either 37° or 4° as indicated. Data represent the mean ± SEM of 4 separate experiments.

† Calcium concentration was determined according to the procedure outlined in the *text*.

‡ Significantly different ($P < 0.05$) from corresponding values at 0 min.

Effects of Immunologic and Pharmacologic Agents on Cyclic GMP Accumulation in and β-Glucuronidase Secretion from Human Neutrophils. Recent studies from this laboratory revealed that particulate immune reactants provoke cyclic GMP accumulation in, and a concomitant lysosomal enzyme discharge from, human neutrophils during particle ingestion (2, 3, 12). The data in Figs. 3 and 4 illustrate that a particle-free immune reactant (ZTS) also produced the same effects in the absence of particle ingestion. Further, 0.1 μM acetylcholine enhanced the effects of ZTS on both cyclic GMP accumulation (Fig. 3) and β-glucuronidase secretion (Fig. 4). Basal levels of cyclic AMP in neutrophils (3–9 pmol per 10^6 cells) did not change during cell contact with ZTS or ZTS plus acetylcholine.

The similar action of A-23187 and ZTS on calcium association with neutrophils prompted an analysis of the effect of the ionophore on the cellular levels of cyclic GMP. A-23187 provoked cyclic GMP accumulation (Fig. 3), which, once again, was accompanied by β-glucuronidase secretion (Fig. 4). In this regard we wish to emphasize the time course of changes in calcium association (Figs. 1 and 2), cyclic GMP accumulation (Fig. 3), and β-glucuronidase secretion (Fig. 4). Neutrophil calcium concentration and cyclic GMP accumulation increased within 1 min of addition of ZTS, ZTS plus acetylcholine, or A-23187 (Figs. 1, 2, and 3). Note that the greater the effect on calcium concentration by a given stimulus the greater was the effect on cyclic GMP accumulation (Fig. 3) and β-glucuronidase secretion (Fig. 4). Beta-glucuronidase discharge provoked by ZTS occurred by 2 min rather than within 1 min. A-23187 elicited a much greater effect on enzyme release at 2 min than at 1 min. Thus, cyclic GMP appears to accumulate before β-glucuronidase is secreted.

DISCUSSION

Experimental evidence obtained in this laboratory suggests that cyclic GMP and calcium are involved in mediating, whereas cyclic AMP signals the inhibition of, the immunologic secretion of lysosomal enzymes from human neutrophils (2, 3, 12, 20). Extracellular calcium is required for both cyclic GMP accumulation in and lysosomal enzyme discharge from neutrophils (11, 12). The data in the present report illustrate that contact of human neutrophils with a soluble immune reactant (ZTS) or a divalent cation ionophore (A-23187) provokes three cellular events: (*1*) increased association (influx and/or adsorption) between extracellular calcium and neutrophils, (*2*) accumulation of cyclic GMP, and (*3*) lysosomal enzyme secretion. A detailed analysis of the time course of appearance of these three cellular events suggests that they bear a cause and effect relationship to one another. For example, membrane active agents may promote mobilization of calcium into the cell, whereupon the calcium may cause the accumulation of cyclic GMP which, in turn, signals the secretion of lysosomal contents. Extracellular calcium is required for, and may be taken up by mast cells during, the allergic discharge of mast granule contents (17, 21). Similarly, earlier studies indicated that rabbit polymorphonuclear leukocytes concentrate extracellular calcium under certain experimental conditions (22). Results of the present studies suggest that calcium mobilization into neutrophils is a consequence of cell surface contact with agents that provoke lysosomal enzyme secretion. Accumulation of calcium within the neutrophil might be facilitated by the presence, at 6 to 30 times the concentration in the extracellular environment, of potential calcium-binding molecules such as phosphate, nucleotides, amino acids, and reducing sugars (23). Calcium plays a definite but not well understood role in stimulus-secretion coupling in various secretory cells (18). Calcium may serve as the link between the stimulus (immune reactant) and secretion in neutrophils by activating intracellular huanylate cyclase. Guanylate cyclase activation by calcium gas been reported (13, 14). This concept is supported further by the findings that A-23187 promoted calcium association with, cyclic GMP accumulation in, and β-glucuronidase secretion from human neutrophils. Thus, immune reactants that trigger lysosomal enzyme secretion may operate essentially as specific divalent cation ionophores.

The cell model depicted in Fig. 5 represents our current working hypothesis on the bioregulation of lysosomal enzyme secretion from human neutrophils. Cyclic GMP and cyclic AMP are visualized as second messengers which control dephosphorylating and phosphorylating reactions, respectively, involving one or more intracellular organelles. Some, but not all, of the essential enzymatic machinery postulated for neutrophils has been identified. For example, hormone-responsive adenylate cyclase (24), a cyclic AMP-dependent protein kinase (25), and soluble guanylate cyclase (preliminary observations in our laboratory) have been identified. Several aspects of this cell model have yet to be worked out, and these

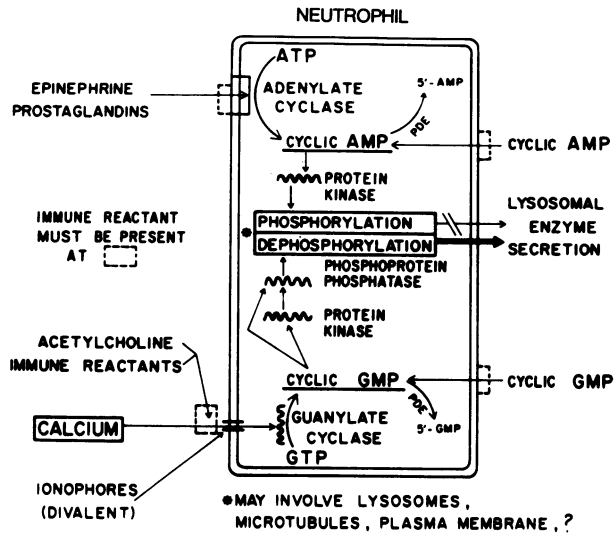

FIG. 5. Postulated cellular model depicting the bioregulation of lysosomal enzyme secretion from human neutrophils. Abbreviations: ATP, adenosine 5'-triphosphate; GTP, guanosine 5'-triphosphate; 5'-AMP, adenosine 5'-monophosphate; 5'-GMP, guanosine 5'-monophosphate; PDE, phosphodiesterase.

include the subcellular distribution of calcium after influx, stimulation of guanylate cyclase by calcium, the existence of a cyclic GMP-dependent phosphoprotein phosphatase (and possibly a cyclic GMP-dependent protein kinase), cyclic AMP-stimulatable phosphorylation, and cyclic GMP-stimulatable dephosphorylation of common intracellular organelles and/or the plasma membrane.

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1. Ignarro, L. J. (1973) *Nature New Biol.* **245**, 151–154.
2. Ignarro, L. J. & George, W. J. (1974) *Proc. Nat. Acad. Sci. USA* **71**, 2027–2031.
3. Ignarro, L. J., Lint, T. F. & George, W. J. (1974) *J. Exp. Med.* **139**, 1395–1414.
4. Ignarro, L. J. (1974) *J. Immunol.* **113**, 298–308.
5. Ignarro, L. J. (1974) *Agents and Actions*, in press.
6. Ignarro, L. J. (1975) in *Lysosomes in Biology and Pathology*, eds. Dingle, J. T. & Dean, R. (North-Holland, Amsterdam), Vol. 4, in press.
7. Goldberg, N. D., Haddox, M. K., Hartle, D. K. & Hadden, J. W. (1973) in *Proceedings of the Fifth International Congress of Pharmacology* (Karger, Basle), Vol. 5, pp. 146–169.
8. Goldberg, N. D., O'Dea, R. F. & Haddox, M. K. (1973) in *Advances in Cyclic Nucleotide Research*, eds. Greengard, P. & Robison, G. A. (Raven Press, New York), Vol. 3, pp. 155–223.
9. Hadden, J. W., Hadden, E. M., Haddox, M. K. & Goldberg, N. D. (1972) *Proc. Nat. Acad. Sci. USA* **69**, 3024–3027.
10. Estensen, R. D., Hill, H. R., Quie, P. G., Hogan, N. & Goldberg, N. D. (1973) *Nature* **245**, 458–460.
11. Ignarro, L. J. (1974) *Arthritis Rheum.* **17**, 25–36.
12. Ignarro, L. J. & George, W. J. (1974) *J. Exp. Med.* **140**, 225–238.
13. Hardman, J. G., Beavo, J. A., Gray, J. P., Chrisman, T. D., Patterson, W. D. & Sutherland, E. W. (1971) *Ann. N.Y. Acad. Sci.* **185**, 27–35.
14. White, L. E., George, W. J. & Ignarro, L. J. (1973) *Pharmacologist* **15**, 157.
15. Schultz, G., Hardman, J. G., Schultz, K., Baird, C. E. & Sutherland, E. W. (1973) *Proc. Nat. Acad. Sci. USA* **70**, 3889–3893.
16. Ferrendelli, F. A., Kinscherf, D. A. & Chang, M. M. (1973) *Mol. Pharmacol.* **9**, 445–454.
17. Foreman, J. C., Mongar, J. L. & Gomperts, B. D. (1973) *Nature* **245**, 249–251.
18. Rubin, R. P. (1970) *Pharmacol. Rev.* **22**, 389–428.
19. Douglas, W. W. & Rubin, R. P. (1963) *J. Physiol. (London)* **167**, 288–310.
20. Ignarro, L. J., Paddock, R. J. & George, W. J. (1974) *Science* **183**, 855–857.
21. Cochrane, D. E. & Douglas, W. W. (1974) *Proc. Nat. Acad. Sci. USA* **71**, 408–412.
22. Woodin, A. M. & Wieneke, A. A. (1963) *Biochem. J.* **87**, 487–495.
23. Woodin, A. M. (1968) in *The Biological Basis of Medicine*, ed. Bittar, E. E. (Academic Press, New York), Vol. 2, pp. 373–396.
24. Bourne, H. R. & Melmon, K. L. (1971) *J. Pharmacol. Exp. Ther.* **178**, 1–7.
25. Tsung, P. K., Hermina, N. & Weissmann, G. (1972) *Biochem. Biophys. Res. Commun.* **49**, 1657–1662.