(giving 67 percent absorption at λ_{max}) and for the cones 0.384 (giving 59 percent absorption at λ_{max}). These densities are probably too high, since the spectral sensitivities of the photoreceptors are narrower than pigment absorption curves based upon these peak densities.

5. The electroretinogram was measured by placing a cotton wick or silver–silver chloride electrode into the vitreous of the eyecup; the moist cotton on which the eyecup rested served as the reference electrode. For evidence that the dark-adaptation of the photoreceptors is similar to that of the b-wave, see J. E. Dowling and H. Ripps, *J. Gen. Physiol.* **56**, 491 (1970), and **60**, 698 (1972).
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12. Experiments on mudpuppy horizontal cells (*11*) have demonstrated that dark-adapted cone receptors show complete temporal summation of absorbed photons for flash durations up to 500 msec (that is, the product of the duration of stimulation and the intensity of light necessary to produce a criterion response is a constant for durations as long as 500 msec). Rod responses decay more slowly than cone responses, and so rods probably sum photons for durations at least as long as cones. The flashes of light used to measure rod and cone absolute sensitivity (as in Fig. 1D) were less than 200 msec in duration and so were almost certainly within the summation times of both receptors.
13. Both stimulators were calibrated for absolute light intensity by placing a United Detector Technology PIN-5 diode in the position normally occupied by the eyecup. The output of the diode in the unbiased mode measured against a calibrated Kipp and Zonen compensated thermopile was 2.80 ± 0.47 watts/amp at 550 nm (13 measurements). A calibrated Eppley thermopile gave a second value within the standard deviation of the first. Lamps in both stimulators were driven by regulated power supplies, and lamp outputs did not vary from day to day by more than ± 10 percent. Variations of intensity across the field of stimulation were no greater than ± 5 percent.
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20. V_{max} ranged from 3.5 to 21 mv for rods and 5 to 14.5 mv for cones. For the photoreceptors shown in Fig. 1, A and D, V_{max} was 5.6 mv (open squares) and 13 mv (filled squares) for the two rods and 12.5 mv (open and filled circles) for the two cones. V_{max} for the cones was not actually measured but was estimated from the shapes of the intensity-response curves for all of the wavelengths where the sensitivity was determined. The largest responses actually measured for the two cones were 11.1 mv (open circles) and 11.8 mv (filled circles). Hence it is unlikely that V_{max} was overestimated. It is possible that V_{max} was underestimated; but if V_{max} was underestimated, σ would also have been underestimated, and the difference between the sensitivities of rods and cones would be even greater than that given in Table 1.
21. Table 1 includes only determinations of σ made from recordings of receptor potentials. The determinations of σ for skate and rat rods and for primate cones were made by extracellular recording; those for mudpuppy rods and mudpuppy and turtle cones were made by intracellular recording. Since the isolated extracellular receptor response has been shown to be proportional to the intracellular receptor potential (*16*), the values of σ determined extracellularly can be compared to those determined intracellularly. However, the values of V_{max} measured extracellularly cannot be compared to those measured intracellularly. Hence Table 1 can be used to compare only the sensitivities of these various rods and cones and not their gains.
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23. Preliminary results of these experiments were reported at the meeting of the Association for Research in Vision and Ophthalmology, Sarasota, Florida, 24–28 April 1972. Supported in part by an NIH traineeship to G.L.F. (P10-6606) and an NIH research grant to J.E.D. (EY-00824). This research was begun at the Wilmer Institute, Johns Hopkins University School of Medicine, Baltimore. We thank Paul K. Brown, Daniel G. Green, John E. Lisman, and William R. Wooten for helpful advice and for critical readings of the manuscript, Ralph Nelson and Jung Ming Wu for assistance in designing and building electronic equipment, and P. S. Sheppard for preparing the illustrations. This report is part of a dissertation submitted by G.L.F. in partial fulfillment of the requirements for a Ph.D. degree from the Biophysics Department, Johns Hopkins University.
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23 March 1973

Enzyme Release from Polymorphonuclear Leukocyte Lysosomes: Regulation by Autonomic Drugs and Cyclic Nucleotides

Abstract. *Osmotic release of β-glucuronidase from polymorphonuclear leukocyte lysosomes is inhibited by catecholamines and adenosine 3′,5′-monophosphate, and accelerated by cholinergic agents and guanosine 3′,5′-monophosphate. These actions are specific for the sympathetic and parasympathetic neurotransmitters and for the two cyclic nucleotides, as phenylephrine, tyramine, choline, adenosine 5′-monophosphate and guanosine 5′-monophosphate do not modify lysosomal enzyme release.*

That norepinephrine and epinephrine inhibit the release of enzymes from liver lysosomes in vitro (*1*) may be an indication that the sympathetic nervous system participates in modulating lysosome membrane integrity and that the action of the catecholamines is mediated by adenosine 3′,5′-monophosphate (cyclic AMP), since phosphodiesterase inhibitors enhance and β-adrenergic receptor antagonists block the action on lysosomes. The significance of these re-

Table 1. Inhibition by sympathomimetic and acceleration by parasympathomimetic amines of release of β-glucuronidase from polymorphonuclear leukocyte lysosomes. Data represent the mean ± S.E.M. from three separate experiments. Release of enzyme from a granule suspension incubated without compound for 0 and 60 minutes, respectively, yielded extinction values (540 nm) of 0.170 to 0.215 and 0.470 to 0.540.

Agent*	Release of β-glucuronidase (percent of control) at concentrations of the agent:			
	$10^{-4}M$	$10^{-5}M$	$10^{-6}M$	$10^{-7}M$
Norepinephrine	27 ± 2.4	42 ± 5.2	53 ± 5.5	64 ± 5.1
Epinephrine	14 ± 1.2	25 ± 2.7	42 ± 5.0	58 ± 4.1
Phenylephrine	99 ± 7.3	102 ± 6.6	98 ± 5.7	100 ± 4.8
Tyramine	100 ± 3.9	98 ± 6.1	99 ± 5.3	101 ± 4.5
Acetylcholine	191 ± 14	157 ± 9.2	124 ± 7.2	110 ± 4.7
Acetyl-β-methylcholine	172 ± 12	144 ± 6.8	121 ± 6.0	104 ± 5.5
Choline	99 ± 3.9	102 ± 5.1	98 ± 5.0	99 ± 4.3

* The forms of the agents tested: *l*-norepinephrine bitartrate, *l*-epinephrine bitartrate, *l*-phenylephrine hydrochloride, tyramine hydrochloride, acetylcholine chloride, and acetyl-β-methylcholine chloride.

Table 2. Inhibition by adenosine 3′,5′-monophosphate and acceleration by guanosine 3′,5′-monophosphate of release of β-glucuronidase from polymorphonuclear leukocyte lysosomes. The data represent the mean ± S.E.M. from three separate experiments. Release of enzyme from a granule suspension incubated without compound for 0 and 60 minutes, respectively, yielded extinction values (540 nm) of 0.160 to 0.185 and 0.485 to 0.500.

Agent	Release of β-glucuronidase (percent of control) at the molar concentrations of the agent:				
	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}
Cyclic AMP	84 ± 5.8	70 ± 5.2	52 ± 4.9	46 ± 4.8	88 ± 5.6
AMP	76 ± 6.1	92 ± 7.1	101 ± 7.3	100 ± 6.8	102 ± 7.7
Cyclic GMP	78 ± 7.4	128 ± 9.0	189 ± 12	172 ± 11	120 ± 6.2
GMP	89 ± 6.7	100 ± 8.4	99 ± 7.0	101 ± 6.4	100 ± 5.3

sults derives from the knowledge that lysosomal enzymes are important mediators of the inflammatory process (*2*) and that inhibition of enzyme release from lysosomes is one possible mechanism by which antirheumatic drugs elicit therapeutic effects (*3, 4*).

Epinephrine and other catecholamines may have a role in the regulation or control of the inflammatory process. Epinephrine evokes an anti-inflammatory action in various animal models of acute (*5*) and chronic (*6*) inflammation. Although the mechanism of therapeutic action of epinephrine in these models of inflammation is not clearly understood, the β-adrenergic receptor appears to be involved since antagonists of β-receptors attenuate the anti-inflammatory action of the catecholamine.

We report here that the neurotransmitter of the sympathetic nervous system, norepinephrine, inhibits—whereas the neurotransmitter of the parasympathetic nervous system, acetylcholine, accelerates—the release of enzymes from polymorphonuclear leukocyte (PMN) lysosomes in vitro. Similarly, epinephrine, a sympathomimetic agent, inhibits—whereas acetyl-β-methylcholine, a parasympathomimetic agent, enhances—lysosomal enzyme release. Further, opposing actions on the release of lysosomal enzymes is observed with two different naturally occurring cyclic nucleotides. Cyclic AMP and guanosine 3′,5′-monophosphate (cyclic GMP) inhibit and accelerate, respectively, the release of enzymes from lysosomes. Thus, autonomic agents and cyclic nucleotides elicit oppositional actions on lysosomal enzyme release.

Guinea pig PMN's were isolated, and the granule fraction from these cells was prepared as described (*4*). After resuspension of the lysosome granule fraction in cold 0.45*M* sucrose, 0.1-ml portions were added to 2 ml of 0.12*M* sucrose–0.04*M* tris · acetate, *p*H 7.4, with or without compound. All compounds tested were soluble in aqueous buffer and solutions were prepared immediately prior to use. Care was taken to avoid decomposition and reduce discoloration of catecholamines by working quickly with freshly prepared solutions maintained at 0° to 4°C. Samples were incubated at 37°C for 60 minutes, with agitation, and then centrifuged (*4*). β-Glucuronidase (*3*) (the substrate is phenolphthalein glucuronide) was the lysosomal marker enzyme. None of the agents tested altered directly the activity of the marker enzyme released.

The characteristic property of latency of lysosomal enzymes was demonstrated for granule-bound β-glucuronidase in guinea pig PMN (*4*). Norepinephrine and epinephrine, sympathomimetic catecholamines that stimulate β-adrenergic receptors, inhibit the release of β-glucuronidase from PMN lysosomes (Table 1). Phenylephrine and tyramine, sympathomimetic amines that do not possess a catechol moiety and therefore do not interact directly with β-receptors, do not affect lysosomal enzyme release. Significant effects ($P < .05$) are observed down to $10^{-8}M$ catecholamine. These data indicate that interaction with β-receptors might account for the inhibitory actions of catecholamines on lysosomal enzyme release. Similar data and conclusions regarding the effects of catecholamines on rat liver lysosomes have been reported (*1*).

In most physiologic systems acetylcholine, a parasympathomimetic amine, elicits actions that are opposite to those of norepinephrine and epinephrine. Acetylcholine and acetyl-β-methylcholine accelerate release of β-glucuronidase from PMN lysosomes (Table 1), an effect opposite to that exhibited by the catecholamines. Significant effects ($P < .05$) are observed down to $10^{-7}M$ acetylcholine and $10^{-6}M$ acetyl-β-methylcholine.

Cyclic AMP inhibits whereas cyclic GMP accelerates the release of β-glucuronidase (Table 2). Cyclic AMP elicits a maximum effect at $10^{-7}M$ and appreciably less effect at higher or lower concentrations. Cyclic GMP elicits somewhat of a biphasic action. Inhibition of enzyme release is obtained at $10^{-4}M$, but enhancement of release is evident at $10^{-5}M$ to $10^{-8}M$. Thus, within the concentration range of $10^{-5}M$ to $10^{-8}M$, opposing actions on lysosomal enzyme release are exhibited by cyclic AMP and cyclic GMP. These actions are specific for the respective cyclic nucleotides as neither AMP nor GMP, at $10^{-5}M$ to $10^{-8}M$, alter enzyme release appreciably (Table 2).

Cyclic AMP mediates numerous physiologic actions of endogenous catecholamines (*7*). Among these are inhibition of histamine release from sensitized human leukocytes (*8*) and inhibition of platelet aggregation (*9*). Recent studies have suggested that the inhibitory action of catecholamines on the release of enzymes from rat liver lysosomes is mediated by cyclic AMP (*1*). The specificity of the inhibitory action of catecholamines and cyclic AMP on the release of enzymes from PMN lysosomes is consistent with the view that cyclic AMP mediates the effect of the catecholamines. Direct evidence for such a mechanism of action for epinephrine was obtained when adenylate cyclase activity, stimulatable by epinephrine, was detected in the lysosome fractions employed in the present study (*10*).

The mechanism by which parasympathomimetic agents accelerate lysosomal enzyme release is not clearly understood. Low concentrations of cyclic GMP accelerate the release of enzymes from lysosomes. In view of the findings that acetylcholine can stimulate synthesis and elevate tissue levels of cyclic GMP, and that certain physiologic effects of this cholinergic agent might be mediated by cyclic GMP (*11*), it is not unreasonable to consider that acetylcholine and acetyl-β-methylcholine accelerate lysosomal enzyme release via a mechanism involving cyclic GMP. The specificity of the enhancing action of cholinergic agents and cyclic GMP (choline and GMP are without effect) on enzyme release supports this view. Consistent with this hypothesis are the findings (*12*) that acetylcholine and cyclic GMP accelerate the release of histamine from sensitized human lung fragments in vitro.

Inhibition of release of lysosomal

enzymes from phagocytosing leukocytes by cyclic AMP and agents such as prostaglandins that elevate tissue levels of cyclic AMP has been reported (*13*). This action might be explained, at least partially, by our findings that catecholamines and cyclic AMP strengthen the integrity of lysosomes. The apparent alteration of physical properties of the lysosome membrane, perhaps induced by cyclic AMP, might influence certain intracellular events, such as the peripheral migration of lysosomes and subsequent fusion with heterophagic vacuoles, that lead ultimately to the extracellular release of lysosomal contents. Moreover, this action of the catecholamines might be responsible for their anti-inflammatory effects. If this hypothesis is valid, then our finding that cholinergic agents elicit an opposing action to that of catecholamines on lysosomes suggests that the autonomic nervous system might play an important role in regulating or controlling the inflammatory process by virtue of the capacity of neurohormones to modulate the structural integrity of lysosomes.

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Complement-Induced Platelet Protein Alterations

Abstract. *Polypeptides of high molecular weight are deleted from the sedimentable fraction of platelets subjected, while intact, to complement action. These polypeptides, distinct from the previously described thrombin-sensitive protein of this fraction, also diminish after platelets are exposed to thrombin. They may be of importance in the molecular events underlying complement-triggered changes in platelet function.*

The capacity of complement to generate platelet coagulant activity has been demonstrated (*1*). It is likely that failure of complement-platelet interaction is responsible for the coagulation abnormality in the blood of rabbits congenitally deficient in the sixth component of complement (C6) (*1, 2*). Activation of complement can trigger aggregation and release of vasoactive amines (*3*) and has been implicated in the precipitation of intravascular coagulation (*4*). We now report complement-induced alterations in high-molecular-weight polypeptides residing in the sedimentable portion of human and rabbit platelets. Similar alterations can be induced by thrombin. These changes may reflect molecular events underlying complement-triggered changes in platelet function.

Citrate was added to human and rabbit blood to prevent coagulation. Platelets were washed and isolated by modifications of methods already described (*5*). The cellular constituents of the blood were separated from plasma by centrifugation and washed twice in Tyrode buffer containing 2 percent bovine serum albumin; calcium was omitted, and 0.03*M* adenosine was added to prevent aggregation. These procedures were carried out at room temperature. The cells were then resuspended in the buffer, and the erythrocytes and leukocytes were removed by centrifugation at 800*g* for 2 minutes. Platelet preparations contained less than three erythrocytes or leukocytes per 10,000 platelets. Platelets were gently sedimented by centrifugation at 800*g* for 5 minutes and resuspended in plasma, serum, or buffer to a concentration ranging from 200,000 to 700,000 per cubic millimeter.

Antiserum to human platelets was produced in C6-deficient rabbits. Disrupted platelets were subjected to molecular sieve chromatography in a column of Sepharose 2B (2.5 by 34 cm), and the void volume fraction was used for immunization. Citrated plasma was prepared from the blood of the immunized animals and heated to 56°C for 1 hour; the heat precipitable material was removed by centrifugation.

Inulin was prepared as described (*6*). Human thrombin (1000 U.S.P. unit/mg) was provided by D. L. Aronson and prepared as described (*7*). Citrated human or rabbit plasma was freed of platelets by centrifugation at 51,000*g* (average) for 30 minutes in a swinging bucket rotor. Rabbit serum was prepared by allowing blood to clot in a glass for 2 hours at 37°C and was twice absorbed with tribasic calcium phosphate (10 mg/ml) to remove residual prothrombin.

The effect of complement on human platelets was determined in the following manner. Washed platelets were resuspended in normal human plasma, normal rabbit plasma, or normal rabbit serum. As a control, platelets were suspended in C6-deficient plasma or C6-deficient serum. Complement was then activated by the addition of 1/20 volume of undiluted heated antiserum to platelets, and the mixture was incubated for 30 minutes at 37°C. The mixture was then sonicated at setting number 1 on a Bronson sonifer with a microtip for two 15-second periods. After separation by centrifugation at 51,000*g* (average) for 30 minutes, the sedimentable fraction was then solubilized and reduced in a mixture of 6 percent sodium dodecyl sulfate (SDS), 0.04*M* dithiothreitol, 0.1*M* EDTA, 0.1*M* tris, *p*H 8.0; this reaction mixture was kept for 30 minutes at 37°C and then for 18 hours at room temperature. It was then alkylated with 0.33*M* iodoacetamide (15 minutes at 37°C). Samples (250 μg) of this material—measured as protein (*8*) with purified C3 as a standard—were then subjected to electrophoresis at 5 Ma for 12 hours in columns (6 by 200 mm) of 5 percent polyacrylamide gel containing 1 percent SDS. The gels were stained with Coomassie blue.

The changes induced in rabbit platelets by complement were determined in a similar manner, except that colloidal inulin (1 mg/ml) was used to activate complement. The effect of thrombin on human platelets was assessed by adding 10 unit/ml to a suspension of platelets in Tyrode albumin buffer (without calcium) and incubating at