ated histamine release at an early stage after exposure to antigen, a stage that is not dependent on divalent cations.

Unlike the other drugs listed, histamine is an endogenous compound which may be present at high concentration in the extracellular fluid of an inflammatory reaction, of which it may actually be a chemical mediator. It is possible, however, that histamine plays an additional role: by inhibiting release of additional histamine from leukocytes newly arrived at the scene of an allergic reaction, histamine may act as a negative modulator of the intensity of inflammation. If such a concept proves to be correct, cyclic AMP may act in leukocytes as an inhibitor of processes which are ordinarily necessary for host defenses, but which may become so intense that they endanger the host.

It is interesting that most effects so far attributed to cyclic AMP in either leukocytes or platelets are inhibitory: antigenic release (presumably from mast cells) of histamine from primate lung (*3*) or of slow-reacting substance of anaphylaxis into rat peritoneum (*11*), neutrophil candidacidal activity (*5*), lymphocyte transformation (*12*), platelet aggregation (*13*), and phagocytic release of lysosomal enzymes (*14*) are all thought to be inhibited by intracellular cyclic AMP. It would be of interest to test the ability of histamine to interfere with these processes.

HENRY R. BOURNE
KENNETH L. MELMON
Departments of Medicine and *Pharmacology, University of California School of Medicine, San Francisco 94122*
LAWRENCE M. LICHTENSTEIN
Department of Medicine, Johns Hopkins University School of Medicine at the Good Samaritan Hospital, Baltimore, Maryland 21212

References and Notes

1. L. M. Lichtenstein and A. G. Osler, *J. Exp. Med.* **120**, 507 (1964).
2. L. M. Lichtenstein, P. S. Norman, A. G. Osler, *J. Clin. Invest.* **45**, 1126 (1966); L. M. Lichtenstein, D. A. Levy, K. Ishizaka, *Immunology* **19**, 831 (1970).
3. L. M. Lichtenstein and S. Margolis, *Science* **160**, 903 (1968); E. S. K. Assem and H. O. Schild, *Nature (Lond.)* **224**, 1028 (1969); T. Ishizaka, K. Ishizaka, R. P. Orange, K. F. Austen, *Fed. Proc.* **29**, 575 (1970).
4. H. Shimizu, J. W. Daly, C. R. Creveling, *J. Neurochem.* **16**, 1609 (1969).
5. H. R. Bourne, R. I. Lehrer, M. J. Cline, K. L. Melmon, *J. Clin. Invest.*, in press.
6. In the absence of theophylline the effects of histamine on ^{3}H-nucleotide accumulation were small and inconsistent. Theophylline by itself produced no change in ^{3}H-nucleotide accumulation. Similar results were reported in brain tissue (*4*).
7. G. Krishna, B. Weiss, B. B. Brodie, *J. Pharmacol. Exp. Ther.* **163**, 379 (1968).
8. L. M. Lichtenstein, *Int. Arch. Allergy Appl. Immunol.*, in press.
9. R. E. Scott, *Blood* **35**, 914 (1970); H. R. Bourne and K. L. Melmon, *J. Pharmacol. Exp. Ther.*, in press.
10. E. W. Sutherland, G. A. Robison, R. W. Butcher, *Circulation* **37**, 279 (1968).
11. W. J. Koopman, R. P. Orange, K. F. Austen, *J. Immunol.* **105**, 1096 (1970).
12. J. W. Smith, A. L. Steiner, C. W. Parker, *J. Clin. Invest.* **50**, 442 (1971).
13. N. R. Marquis, R. L. Vigdahl, P. A. Tavormina, *Biochem. Biophys. Res. Commun.* **36**, 965 (1969).
14. C. D. May, B. B. Levine, G. Weissman, *Fed. Proc.* **37**, 394 (1968).
15. We thank Sally Dion, Ann Solootka, and Jeanne Harrison for skillful technical assistance. This work was supported by PHS grants HE 09964, 6M01791, and AI 07290, and by a Research Career Development Award from the National Institute of Allergy and Infectious Diseases to L.M.L.

21 May 1971

Cyclic 3′,5′-Nucleotide Phosphodiesterase: Cytochemical Localization in Cerebral Cortex

Abstract. *The distribution of cyclic 3′,5′-nucleotide phosphodiesterase activity in rat cerebral cortex was determined by cytochemical methods. The detectable phosphodiesterase activity was localized in postsynaptic (dendritic) nerve endings, most of it immediately adjacent to the synaptic membrane. Most of the postsynaptic nerve endings showed phosphodiesterase activity.*

An increasing amount of evidence suggests that adenosine 3′,5′-monophosphate (cyclic AMP) may be involved in the regulation of metabolism and function in nervous tissue (*1*). Certain of the data suggest that cyclic AMP may be intimately associated with the molecular events underlying the process of synaptic transmission. For instance, evidence has been presented that exogenous cyclic AMP can mimic the inhibitory effect of norepinephrine on cerebellar Purkinje cells (*2*). Moreover, brief periods of synaptic activity in autonomic ganglia caused a severalfold increase in the amount of cyclic AMP in the ganglia, which occurred largely, if not entirely, in postsynaptic cells (*3*). This increase in cyclic AMP may modify the excitability of certain postsynaptic cells by the following chain of events (*3, 4*): (i) activation of a protein kinase by cyclic AMP, (ii) phosphorylation of the synaptic membrane by the kinase, (iii) change of ionic permeability of the membrane, (iv) change of membrane potential, and (v) change of excitability. This scheme provides a plausible molecular mechanism for prolonged modulation of synaptic transmission either by facilitation or by inhibition.

An important factor limiting the magnitude and duration of action of cyclic AMP in tissues is the rate of enzymatic destruction of the nucleotide by cyclic 3′,5′-nucleotide phosphodiesterase. Of all mammalian tissues studied, the brain has the highest phosphodiesterase activity (*5*). A knowledge of the precise location of phosphodiesterase activity within the cells should be valuable in clarifying the function of cyclic AMP in nervous tissue. We have, therefore, undertaken a cytochemical study of the localization of phosphodiesterase in brain tissue. In our initial investigations, the parietal lobe of the rat cerebral cortex has been used.

Cyclic 3′,5′-nucleotide phosphodiesterase hydrolyzes cyclic AMP to 5′-AMP. The basis of our cytochemical procedure was to convert this 5′-AMP to adenosine and inorganic phosphate with an excess of 5′-nucleotidase, and to precipitate the newly formed inorganic phosphate with lead ions, thus forming an electron-opaque product at or near the site of phosphodiesterase activity.

Adult male albino rats (160 to 180 g) were anesthetized by intraperitoneal injection of 3.1 percent chloral hydrate (1 ml per 100 g of body weight) and then perfused through the left ventricle with 400 ml of 2 percent glutaraldehyde in 0.05*M* cacodylate-nitrate buffer (*p*H 7.4) containing 0.25*M* dextrose. The cerebral hemispheres were removed and washed overnight in the same buffer. The hemispheres were then cut into strips 2 to 3 mm thick, from which 50-μm slices of tissue were prepared in a Smith-Farquhar tissue chopper. The slices were first incubated for 30 minutes at room temperature in a solution containing 60 m*M* tris maleate buffer (*p*H 7.4), 2 m*M* $MgCl_2$, and 0.25*M* sucrose (TMS buffer) containing 5′-nucleotidase (*6*). The slices were next incubated for 30 minutes at 37°C in TMS buffer containing 2 m*M* lead nitrate, 5′-nucleotidase (3 mg/ml), and 3 m*M* cyclic AMP, either in the ab-

sence or in the presence of 50 m*M* theophylline, an inhibitor of phosphodiesterase. After incubation the slices were washed briefly in TMS buffer, fixed in 2 percent osmium tetroxide in 0.05*M* cacodylate-nitrate buffer (*p*H 7.4) containing 0.25*M* sucrose, dehydrated in ethanol, and embedded in Epon 812. Thin sections were cut on a Porter-Blum MT1 microtome, lightly stained with uranyl acetate, and examined in a Hitachi 11B electron microscope.

When sections were incubated in medium containing 3 m*M* cyclic AMP, the reaction product was localized almost exclusively in postsynaptic nerve endings (Fig. 1, A and B). Most of this reaction product occurred in the immediate vicinity of the synaptic membrane. It was possible to distinguish between pre- and postsynaptic membranes only when the plane of section was perpendicular to the synaptic membranes; in such cases it was clear that the reaction product was located on or adjacent to the area of thickening of the postsynaptic membrane (arrow plus asterisk in Fig. 1A). That minor portion of the reaction product not located in the immediate vicinity of the synaptic membrane was usually observed in close association with smooth endoplasmic reticulum and possibly with microtubules. The reaction product was found within about 85 percent of the identifiable postsynaptic nerve endings examined. Little or no reaction product was observed in association with axonal structures. Sections incubated in medium containing 3 m*M* cyclic AMP and 50 m*M* theophylline showed little or no reaction product (Fig. 1C), indicating inhibition of cytochemically demonstrable phosphodiesterase activity.

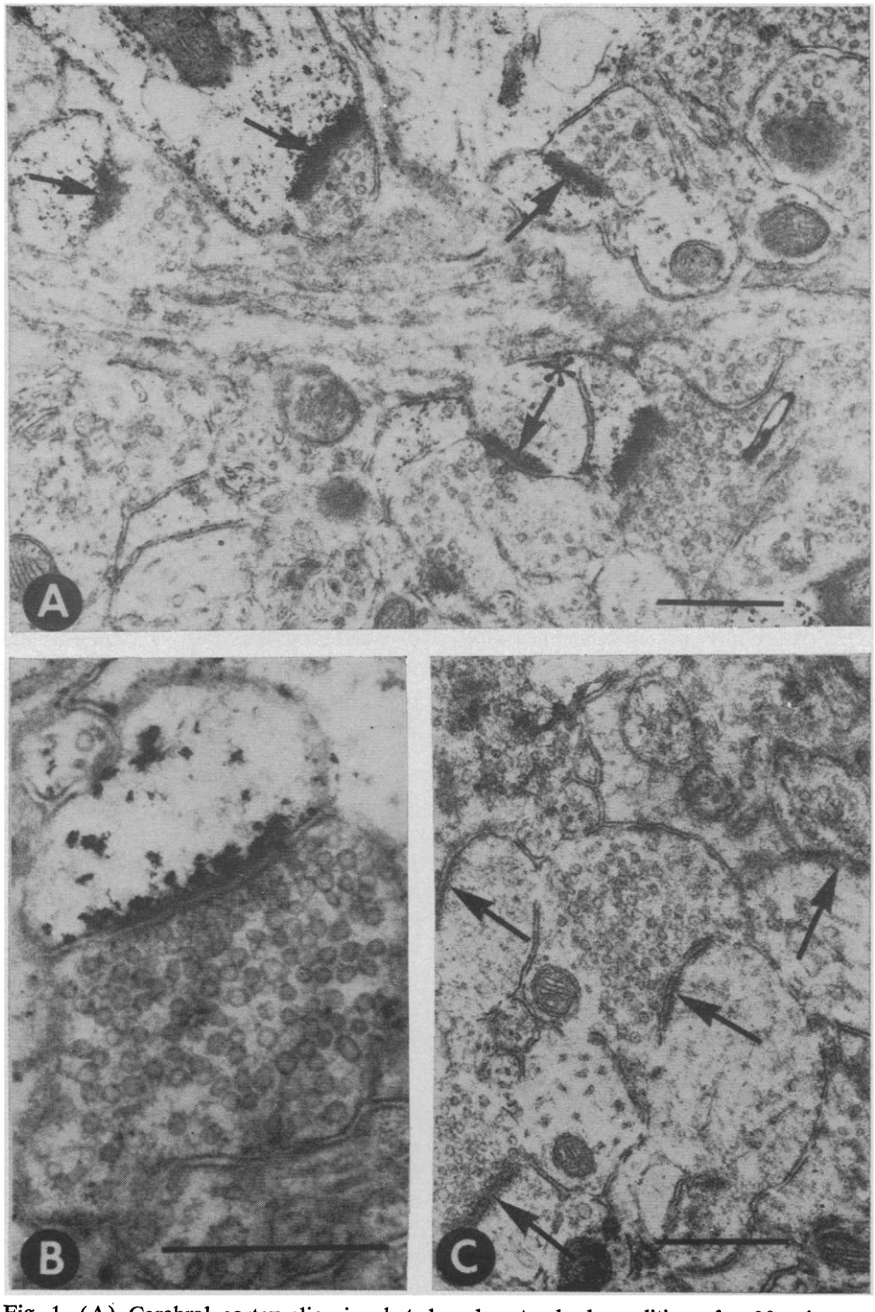

Fig. 1. (A) Cerebral cortex slice incubated under standard conditions for 30 minutes at 37°C (× 38,000). Note that almost all of the reaction product is localized postsynaptically, most of it immediately adjacent to the postsynaptic membrane (arrows). For explanation of asterisk, see text. (B) Similar to (A), but higher magnification (× 62,000). (C) Similar to (A), except that 50 m*M* theophylline was included in the incubation mixture in order to inhibit phosphodiesterase activity (× 38,000). Note absence of reaction product. Arrows indicate typical density of the postsynaptic membrane produced by staining with osmium tetroxide and uranylacetate. Scale in each picture is 0.5 μm.

A variety of control experiments were performed in order to check the validity of the cytochemical observations. In some experiments slices were first incubated in TMS buffer in the absence of 5′-nucleotidase for 30 minutes at room temperature and then incubated, still in the absence of 5′-nucleotidase, for 30 minutes at 37°C in TMS buffer to which 2 m*M* lead nitrate and either 3 m*M* 5′-AMP or 3 m*M* cyclic AMP had been added. Such slices showed no reaction product, indicating that endogenous 5′-nucleotidase activity did not contribute to the observed localization of reaction product. (Presumably, any endogenous 5′-nucleotidase did not survive the experimental procedures used. In contrast, liver slices taken from some of the animals used for the brain studies, when incubated in the identical medium with 5′-AMP as substrate in the absence of added 5′-nucleotidase, showed a typical reaction product associated with bile canalicular membranes.) When 5′-AMP and 5′-nucleotidase were both present in the incubation medium, a heavy precipitate formed in the solution, as would be expected from the formation of lead phosphate. When sections were first incubated with 5′-nucleotidase and then incubated with 5′-AMP in the

absence of exogenous 5′-nucleotidase, deposition of reaction product occurred throughout the sections over both pre- and postsynaptic nerve endings, indicating that the exogenous 5′-nucleotidase present during the first incubation period had penetrated the entire section and did not bind specifically to material in the area of the postsynaptic membrane. These results also exclude, as an explanation for the observed distribution of reaction product, selective binding of lead ions, 5′-AMP, or of inorganic phosphate to synaptic structures. The various control experiments thus support the conclusion that the postsynaptic localization of reaction product observed under standard conditions (Fig. 1, A and B) represents the actual site of phosphodiesterase activity surviving the experimental procedure (*7*).

Breckenridge and Johnston (*8*), using a quantitative micromethod for measuring cyclic nucleotide phosphodiesterase activity, found that the enzyme activities in different regions of the rabbit central nervous system varied greatly, although correlation with other biochemical parameters or with cellular density was not observed. A histochemical attempt to localize phosphodiesterase activity in a light microscopic study (*9*), in unfixed tissue sections incubated for up to 3 hours, did not yield direct information about the subcellular site of enzyme activity; the study has been received with reservation (*8*) because of procedural limitations in the technique. Moreover, there were major discrepancies between the results obtained with the histochemical procedure (*9*) and those obtained by the quantitative micromethod (*8*).

Cytochemical methods provide a unique approach to determining the location of enzymes, particularly in heterogeneous tissue. The conclusion that phosphodiesterase activity is concentrated in the postsynaptic nerve endings in the immediate vicinity of the synaptic membrane is consistent with other, albeit much less precise, evidence concerning the location of the enzyme. Subcellular fractionation studies of the distribution of phosphodiesterase activity in rat brain cortex indicate that the enzyme is present in relatively high concentrations in those subcellular fractions containing nerve endings (*10*, *11*).

The same subcellular fractions also contain high concentrations of adenyl cyclase (*10*), the enzyme which catalyzes the synthesis of cyclic AMP, as well as high concentrations of cyclic AMP–dependent protein kinase (*12*) which is postulated to mediate the actions of cyclic AMP in nervous and other tissues (*13*). Unfortunately, the techniques of subcellular fractionation used to date do not permit precise localization of these enzymes in cerebral cortex because both pre- and postsynaptic elements occur in subcellular fractions containing nerve endings. Denervation studies have also provided evidence consistent with our results. Breckenridge and Johnston (*8*), studying the superior cervical ganglion, demonstrated that phosphodiesterase activity in these tissues was not altered significantly by denervation. These results indicate that the bulk of phosphodiesterase activity is not located in the prejunctional nerve fibers in those tissues.

In view of our results, it is important to consider the possible physiological significance of the phosphodiesterase located at the postsynaptic membrane. The postsynaptic membrane is the site at which neural transmitter substances alter permeability and thereby initiate or inhibit the generation of propagated responses in postsynaptic neurons. It is plausible that the effects of neural transmitter substances on amounts of cyclic AMP, observed during synaptic activity (*3*), are also brought about through an action on postsynaptic membranes, in this case by regulating the activity of adenyl cyclase present in these membranes. If, as seems possible (*2–4*), cyclic AMP mediates the physiological actions of certain neural transmitter substances on neurons, then the termination of the action of the neurotransmitters would require removal not only of the neurotransmitter itself, but also of the cyclic AMP formed in response to the action of the neurotransmitter. The presence of phosphodiesterase in the immediate vicinity of the postsynaptic membrane, demonstrated in our study, would fulfill such a function; its occurrence there provides further evidence for a role of cyclic AMP in the physiology of synaptic transmission.

NOEL T. FLORENDO
RUSSELL J. BARRNETT
PAUL GREENGARD
Departments of Anatomy and
Pharmacology, Yale University
School of Medicine,
New Haven, Connecticut 06510

References and Notes

1. P. Greengard and E. Costa, Eds. *Role of Cyclic AMP in Cell Function* (Raven, New York, 1970).
2. B. J. Hoffer, G. R. Siggins, F. E. Bloom, in *ibid.*, p. 349; G. R. Siggins, A. P. Oliver, B. J. Hoffer, F. E. Bloom, *Science* **171**, 192 (1971).
3. D. A. McAfee, M. Schorderet, P. Greengard, *Pharmacologist* **12**, 290 (1970); *Science* **171**, 1156 (1971).
4. P. Greengard and J. F. Kuo, in *Role of Cylic AMP in Cell Function*, P. Greengard and E. Costa, Eds. (Raven, New York, 1970), p. 287.
5. R. W. Butcher and E. W. Sutherland, *J. Biol. Chem.* **237**, 1244 (1962).
6. Since in the method 5′-nucleotidase is used as an auxiliary enzyme, tissue slices were first incubated with high concentrations of 5′-nucleotidase (5 mg of lyophilized *Crotalus atrox* venom per milliliter, containing no detectable phosphodiesterase activity) to ensure the presence of an excess amount of this enzyme throughout the tissue slices during the incubation period. (5′-Nucleotidase purified from *Crotalus atrox* venom appeared to be strongly inhibited by lead ions.)
7. Studies were performed, under conditions of incubation similar to those used for cytochemical localization, to determine whether phosphodiesterase activity could be demonstrated biochemically after fixation in glutaraldehyde, extensive washing, and the presence of lead ions in the incubation medium. Cerebral hemispheres were either chopped into blocks measuring less than 1 mm^3, or they were homogenized. Phosphodiesterase activity was determined by the procedure of Butcher and Sutherland (*5*), except that a minor modification of the method of O. H. Lowry and J. A. Lopez [*J. Biol. Chem.* **162**, 421 (1946)] was used for measuring inorganic phosphate. The procedure of glutaraldehyde fixation plus washing (for anywhere from 3 to 16 hours) caused a loss of about two-thirds of the enzyme activity in slices or homogenates. The presence of 2 m*M* lead nitrate did not cause a further decrease in activity in slices, in contrast to the inhibition obtained with homogenates. Enzyme activity of fixed slices was inhibited about 80 percent by 50 m*M* theophylline; presumably, the residual activity was too diffuse to cause identifiable reaction product. Studies in several laboratories have indicated that about two-thirds of the phosphodiesterase activity in homogenates of cerebral cortex is recovered in the particulate fraction and about one-third in the soluble fraction after differential centrifugation. It seems likely that, in our experiments, any soluble phosphodiesterase activity was removed by the extensive washing procedure used. It seems reasonable to suppose that presynaptic terminals as well as cells other than neurons might contain phosphodiesterase activity. The absence of any demonstrable activity in these cells may be due either to a low natural amount of the enzyme or to extensive inactivation by the experimental procedure used.
8. B. McL. Breckenridge and R. E. Johnston, *J. Histochem. Cytochem.* **17**, 505 (1969).
9. T. R. Shanta, W. D. Woods, M. B. Waitzman, G. H. Bourne, *Histochemie* **7**, 177 (1966).
10. E. De Robertis, G. Rodriguez De Lores Arnaiz, M. Alberici, R. W. Butcher, E. W. Sutherland, *J. Biol. Chem.* **242**, 3487 (1967).
11. W. Y. Cheung and L. Salganicoff, *Nature* **214**, 90 (1967).
12. H. Maeno, E. M. Johnson, P. Greengard, *J. Biol. Chem.* **246**, 134 (1971).
13. J. F. Kuo and P. Greengard, *Proc. Nat. Acad. Sci. U.S.* **64**, 1349 (1969); *J. Biol. Chem.* **244**, 3417 (1969); *ibid.* **245**, 2493 (1970).
14. Supported by Public Health Service grants 5-T01-CA-05055, 5-R01-AM-03688, NS 08440, and MH 17387 and National Science Foundation grant GB 27510.

6 May 1971