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# Guanosine Cyclic 3′, 5′-Monophosphate and Guanylate Cyclase Activity in Guinea Pig Lung: Effects of Acetylcholine and Cholinesterase Inhibitors

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SUMMARY

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The effects of acetylcholine, other neurotransmitters, and hormones on the guanosine cyclic 3′,5′-monophosphate and adenosine cyclic 3′,5′-monophosphate contents of guinea pig lung were investigated. When lung slices were incubated in the presence of 0.1 mM physostigmine and 1 mM theophylline (which themselves had little effect), the addition of acetylcholine produced a prompt rise in cyclic GMP concentration, which reached a maximum (about 250% of basal) in 1–3 min and began to decline by 6 min, approaching control levels in 12 min. When measured at 2 min the effect of 0.1 μM acetylcholine was negligible, and that of 1 μM was essentially maximal. Acetylcholine also increased the cyclic AMP content of lung slices 2–3-fold in 2 min. The effects on both nucleotides were prevented by 0.5 mM atropine. Prostaglandins $E_1$, $E_2$, $A_2$ and $F_{2\alpha}$ (2 μM), 1 μM isoproterenol, and 0.1 mM serotonin had no effect on basal cyclic GMP concentration or on the increment produced by acetylcholine. The cyclic AMP content of lung slices was markedly increased by exposure (for 2 min) to prostaglandin $E_1$, isoproterenol, or epinephrine. The effect of epinephrine was prevented by propranolol and unaffected by phentolamine. Neither of the adrenergic blocking agents alone or in combination with epinephrine significantly affected cyclic GMP levels. Guanylate cyclase activity assayed in whole homogenates of lung was not demonstrably affected by acetylcholine or other cholinergic agents. It was significantly increased by 1 mM physostigmine (alone or in the presence of acetylcholine) but not by other inhibitors of cholinesterase (neostigmine or edrophonium). This effect of physostigmine was not prevented by atropine.

## INTRODUCTION

Although guanosine cyclic 3′,5′-monophosphate has been identified in all mammalian tissues examined, relatively little is known about the regulation of its formation and degradation. Acetylcholine can increase the cyclic GMP content of perfused rat heart (1) and of ventricular slices incubated *in vitro* (2). In slices of brain (2), small intestine (3), and thyroid (4) the cyclic GMP concentration also increases rapidly on exposure to acetylcholine or other muscarinic agents. It has further been suggested that *beta* adrenergic stimulation may decrease the effect of acetylcholine on tissue cyclic GMP (1–3). We have investigated the effects of

acetylcholine, other neurotransmitters, and hormones on the cyclic GMP and cyclic AMP contents of lung slices. Of the agents used, only acetylcholine increased cyclic GMP in lung slices. It was, however, without demonstrable effect on guanylate cyclase activity in homogenates of lung.

## METHODS

*Incubation of lung slices.* Female Hartley guinea pigs (approximately 750 g), which had been given free access to food and water, were anesthetized with sodium pentobarbital (60 mg, intraperitoneally). The lungs were excised through a ventral thoracotomy and placed in Krebs-Ringer-bicarbonate medium, pH 7.4, at 37°. Lung lobes, freed of extrapulmonary bronchi and blood vessels, were sliced with a Stadie-Riggs slicer. Slices from several lungs were collected in the same medium, through which 95% $O_2$–5% $CO_2$ was bubbled. Portions of 400–600 mg were placed in 25-ml Erlenmeyer flasks containing 3 ml of medium, usually with 1 mM theophylline and 0.1 mM physostigmine, and incubated for 15 min at 37° in an atmosphere of 95% $O_2$–5% $CO_2$. Other additions, in volumes of 30 μl, were then made, and after the indicated period the incubation was ended by addition of 5 ml of cold 10% trichloracetic acid followed by homogenization of tissue plus medium with a Brinkman Polytron. After centrifugation, the precipitate was dissolved in 1 N NaOH and the protein content was determined (5) using bovine serum albumin as a standard. A small amount of [$^3$H]cyclic GMP (less than 1 pmole) was added to each supernatant fraction for the purpose of calculating final recoveries.

*Purification and assay of cyclic GMP.* Supernatant fractions were acidified and extracted six times with water-saturated ether (10 ml) to remove the trichloracetic acid. They were then applied to 0.5 × 3 cm columns of AG1-X8 (formate, 200–400 mesh) and eluted as described previously (6). The 4 N formic acid eluates were lyophilized. The residue was dissolved in 150 μl of 50% ethanol and further purified with thin-layer chromatography on cellulose plates developed with ethanol–0.5 M ammonium acetate, 5:2 (v/v). Cyclic GMP was eluted with 4 ml of distilled water, lyophilized, and dissolved in 200 μl of distilled water. Recoveries of [$^3$H]cyclic GMP tracer ranged from 60 to 90%. Portions of some samples were incubated with cyclic 3′,5′-phosphodiesterase before assay. In all cases more than 90% of the assayable cyclic GMP was destroyed by this treatment. On the other hand, as shown in Table 1, samples of cyclic GMP purified by column chromatography alone were only partially hydrolyzed by the phosphodiesterase. These samples also inhibited the degradation of added authentic cyclic GMP, although they did not interfere with its assay. The apparent cyclic GMP content of the column-purified extracts was independent of the amount assayed (Table 1). For all experiments reported here, however, all samples were purified with thin-layer chromatography in order to be able to verify the essentially complete degradation of cyclic GMP with phosphodiesterase.

The cyclic GMP content of the purified samples was assayed by the competitive protein binding method of Murad *et al.* (6). After ammonium sulfate precipitation, the binding protein was further purified by chromatography on DEAE-cellulose (7). The assay was carried out in 10 x 70 mm glass tubes which contained, in a final volume of 100 μl, 40 μl of purified tissue extract or standard cyclic GMP, 40 μl of binding protein (30 mg/ml), and 20 μl of 0.25 M sodium acetate buffer, pH 4.0, containing 10 pmoles of [$^3$H]cyclic FMP. Samples were incubated at 4° for 2 hr, then diluted with 1 ml of cold 20 mM potassium phosphate buffer, pH 6.2, and applied to cellulose ester Millipore filters (HAWP-02500). The filters were washed with 10 ml of the same buffer before radioassay (6). Data presented are from representative experiments, each of which was repeated at least twice.

*Assay of guanylate cyclase.* Guinea pig lungs were placed in a cold (4°) medium containing 10 mM Tris-Cl (pH 7.4), 10 mM NaCl, 10 mM KCl, 5 μM EDTA, and 0.1 mM dithiothreitol. The tissue was minced with scissors and homogenized in 9 volumes of the same medium, using a ten Broeck homogenizer. The assay for guanylate cyclase activity was carried out in 10 x 70 mm glass tubes which contained, in a final volume of

TABLE 1

*Effect of lung extract on hydrolysis of cyclic GMP by phosphodiesterase*

Samples of lung extract purified through chromatography on AG1-X8 (formate, 200–400 mesh) and dissolved in water were assayed before and after treatment with phosphodiesterase. To some, 10 pmoles of authentic cyclic GMP were added before incubation with phosphodiesterase and/or assay.

| Sample assayed | | Apparent cyclic GMP | |
|---|---|---|---|
| Lung | Cyclic GMP | No phosphodiesterase | After phosphodiesterase |
| *μl* | *pmoles* | *pmoles* | *pmoles* |
| 20 | | 4.9 | 3.5 |
| 40 | | 10.2 | 7.3 |
| 20 | 10 | 15.5 | 11.5 |
| 40 | 10 | 19.8 | 14.8 |

100 μl, 40 μl of lung homogenate (approximately 350 μg of protein; range, 250–400), 5 μmoles of Tris-Cl (pH 8.0), 0.5 μmole of $MnCl_2$, 0.1 μmole of NADP, 0.1 μmole of cyclic GMP, and 0.1 μmole of GTP (approximately $10^6$ cpm of [$^3$H]GTP). After incubation at 37° for 10 min in a shaking water bath, 0.5 ml of a solution of 2 mM GTP and 2 mM cyclic GMP was added, and the mixture was placed in a boiling water bath for 5 min and then centrifuged at 4000 rpm for 10 min. The supernatant fraction was applied to a 0.5 x 3 cm column of neutral alumina previously washed with 5 mM Tris-Cl, pH 7.4. The material eluted with 15 ml of the same buffer was applied to a 0.5 x 3 cm column of AG1-X8 (formate, 200–400 mesh), followed by 15 ml of 2 N formic acid. Cyclic GMP was then eluted with 10 ml of 4 N formic acid. A portion of the latter was added to 15 ml of Aquasol (New England Nuclear) for radioassay, and another was used for determination of absorbance at 260 nm, from which recovery of cyclic GMP (70–85%) was calculated. The $^3$H-labeled product in the 4 N formic acid eluate was characterized by two-dimensional thin-layer chromatography, using cellulose plates developed first with ethanol–0.5 M ammonium acetate, 5:2 (v/v), and then, after drying, with distilled water. In all samples examined, the radioactivity migrated with authentic cyclic GMP.

*Purification and assay of cyclic AMP.* When cyclic AMP as well as cyclic GMP was to be measured, the trichloracetic acid supernatant from the homogenized lung slices was divided into two portions. One was used for determination of cyclic GMP as described above, and the other for assay of cyclic AMP by the method of Gilman (8) after purification as previously reported (9).

*Materials.* [$^3$H]Cyclic GMP (4.45 Ci/mmole) and [$^3$H]cyclic AMP (16.3 Ci/mmole) were purchased from Schwarz BioResearch and purified by thin-layer chromatography as described above. [$^3$H]GTP (5.28 Ci/mmole) was obtained from New England Nuclear. Cyclic GMP, cyclic AMP, GTP, cyclic 3′,5′-nucleotide phosphodiesterase, methacholine, bethanechol, and neutral alumina were purchased from Sigma. Acetylcholine, pilocarpine, serotonin, *l*-epinephrine, and NADP were purchased from Calbiochem; carbachol, from Aldrich Chemical Company; theophylline, from Nutritional Biochemicals; physostigmine sulfate, from Merck & Company; reserpine and phentolamine, from Ciba; *l*-isoproterenol *d* bitartrate, from Sterling Winthrop Research Institute; histamine, from Mann Research Laboratories; *dl*-propranolol, from Ayerst Laboratories, AG1-X8, from Bio-Rad; and cellulose F plates, from E. M. Reagents. Prostaglandins $E_1$, $E_2$, $A_2$, and $F_{2\alpha}$ were kindly donated by Dr. J. E. Pike of Upjohn. Edrophonium was the gift of Hoffmann–La Roche. Atropine sulfate was provided by Dr. Stephen Hajdu, and physostigmine salicylate and neostigmine, by Dr. Elwood Titus.

## RESULTS

*Cyclic GMP in lung slices.* Incubation of lung slices for 15 min with 1 mM theophylline produced a small increase in cyclic GMP concentration and also increased the amount of cyclic GMP accumulated during a 2-min exposure to acetylcholine (Table 2). Physostigmine, 0.1 mM, did not itself alter cyclic GMP concentrations significantly, but did augment the increase produced by a

TABLE 2

*Effects of theophylline, physostigmine, acetylcholine, and atropine on cyclic GMP in lung slices plus medium*

Lung slices were incubated for 15 min with or without 1 mM theophylline and 0.1 mM physostigmine. Additions were then made as indicated, and incubation was continued for 2 min. Cyclic GMP levels, in slices plus medium, are reported as the mean of values from duplicate incubations, which are given in parentheses.

| Additions | Theophylline (1 mM) | Physostigmine (0.1 mM) | Cyclic GMP |
|---|---|---|---|
| | | | *omples/mg protein* |
| Experiment 1 | | | |
| None | 0 | + | 0.8 (0.8, 0.8) |
| None | + | + | 1.6 (1.6, 1.6) |
| Acetylcholine, 10 μM | 0 | + | 3.3 (3.1, 3.5) |
| Acetylcholine, 10 μM | + | + | 5.3 (4.9, 5.7) |
| Experiment 2 | | | |
| None | + | 0 | 1.1 (1.1, 1.2) |
| None | + | + | 1.4 (1.3, 1.5) |
| Acetylcholine, 10 μM | + | 0 | 4.7 (4.0, 5.4) |
| Acetylcholine, 10 μM | + | + | 5.7 (6.0, 5.4) |
| Experiment 3 | | | |
| None | + | 0 | 1.6 (1.5, 1.7) |
| None | + | + | 1.5 (1.3, 1.7) |
| Acetylcholine, 10 μM | + | 0 | 3.8 (3.1, 4.5) |
| Acetylcholine, 10 μM | + | + | 9.0 |
| Atropine, 0.5 mM | + | + | 1.4 (1.4, 1.4) |
| Acetylcholine + atropine | + | + | 1.5 (1.3, 1.7) |
| Experiment 4 | | | |
| None | + | + | 1.5 (1.6, 1.4) |
| Acetylcholine, 10 μM | + | + | 5.3 |
| Atropine, 0.5 mM | + | + | 2.1 (2.0, 2.2) |
| Acetylcholine + atropine | + | + | 1.6 (1.9, 1.2) |

2-min exposure to acetylcholine. Both 1 mM theophylline and 0.1 mM physostigmine were included in the medium for the other experiments reported here. Under these conditions, in 16 experiments, the cyclic GMP content of slices plus medium after 15 min of incubation was 1.98 ± 0.23 pmoles/mg of protein (mean ± SE). Two minutes after the addition of acetylcholine (final concentration, 10 μM), this was increased 3.37 ± 0.46 pmoles/mg of protein (mean of the difference of paired samples ± SE). Atropine, 0.5 mM, had no effect on basal levels of cyclic GMP but completely prevented the effect of acetylcholine (Table 2).

As shown in Fig. 1, the effect of 10 μM acetylcholine was nearly maximal within 1-3 min and began to decline after 6 min, approaching zero by 12 min. In this experiment and in a few others the cyclic GMP content of incubations containing theophylline and physostigmine but no acetylcholine increased slightly with time. Usually, however, it was essentially constant. When measured at 2 min, the effect of 0.1 μM acetylcholine was negligible, and that of 1 μM was almost maximal (Fig. 2).

Prostaglandins $E_1$, $E_2$, $A_2$, and $F_{2\alpha}$ (2 μM), 1 μM isoproterenol, and 0.1 mM serotonin had no demonstrable effect on basal cyclic GMP concentration or on the increment produced by 10 μM acetylcholine (Table 3). With lung slices from guinea pigs given 1 mg of reserpine subcutaneously 24 hr prior to death to deplete endogenous catecholamine stores, the effects of acetylcholine (time course and dose response) were not significantly different from those observed with tissue from untreated animals (data not shown). Neither 10 μM propranolol nor 10 μM phentolamine significantly altered the cyclic GMP content of lung slices, whether

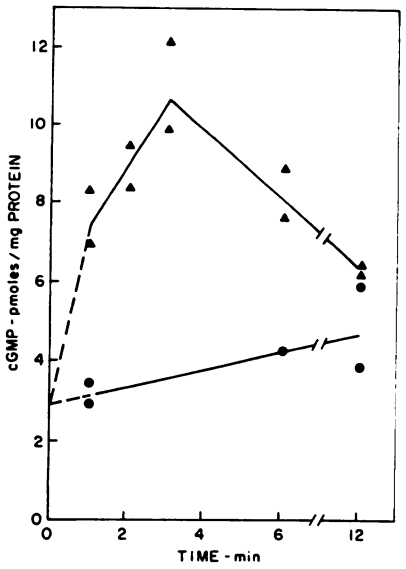


FIG. 1. *Effect of acetylcholine on cyclic GMP content of lung slices plus medium*

Lung slices were incubated with 1 mM theophylline and 0.1 mM physostigmine as described in Table 2 before addition of acetylcholine to a final concentration of 10 μM (▲) or diluent (●) at zero time. Data from individual incubations are shown.

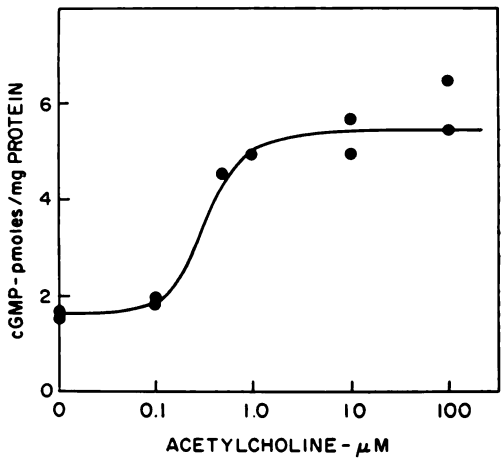


FIG. 2. *Effect of acetylcholine concentration on cyclic GMP in lung slices plus medium*

Incubations were performed as described in Table 2 in the presence of 1 mM theophylline and 0.1 mM physostigmine. Each point represents the cyclic GMP content of a single incubation.

added alone or in the presence of epinephrine; i.e., no evidence of an *alpha* or *beta* adrenergic effect on cyclic GMP levels was obtained (data not shown).

*Cyclic AMP in lung slices.* As shown in Table 3, incubation of lung slices with 10 μM acetylcholine for 2 min also increased the cyclic AMP content 2–3-fold. This effect was completely prevented by 0.5 mM atropine. $PGE_1$,[1] 2 μM, and 1 μM isoproterenol increased the cyclic AMP concentration to about the same extent, and their effects were observed whether or not acetylcholine was present. The effect of 2 μM $PGE_2$ was much smaller than that of $PGE_1$, while $PGA_2$ and $PGF_{2\alpha}$ at the same concentration were without effect. The effect of epinephrine on cyclic AMP was prevented by propranolol but not by phentolamine (data not shown).

*Guanylate cyclase.* Guanylate cyclase activity of homogenates of guinea pig lung

[1] The abbreviations used are: $PGE_1$, $PGE_2$, $PGA_2$, and $PGF_{2\alpha}$, prostaglandins $E_1$, $E_2$, $A_2$, and $F_{2\alpha}$.

TABLE 3

*Effects of various agents, singly or in combination with acetylcholine, on cyclic GMP and cyclic AMP levels in lung slices plus medium*

Incubations were carried out (with 1 mM theophylline and 0.1 mM physostigmine) as described in Table 2. Means of values from duplicate incubations are reported.

| Additions | Cyclic GMP | | Cyclic AMP | |
|---|---|---|---|---|
| | No acetylcholine | 10 μM acetylcholine | No acetylcholine | 10 μM acetylcholine |
| | *pmoles/mg protein* | | *pmoles/mg protein* | |
| Experiment 1 | | | | |
| None | 3.3 | 6.4 | 11 | 40 |
| $PGE_1$, 2 μM | 1.8 | 7.0 | 56 | 119 |
| $PGE_2$, 2 μM | 2.1 | 5.8 | 16 | 55 |
| Experiment 2 | | | | |
| None | 1.7 | 4.6 | 10 | 33 |
| $PGE_1$, 2 μM | 1.6 | 3.4 | 42 | 96 |
| $PGA_2$, 2 μM | 1.6 | 4.4 | 11 | 38 |
| $PGF_{2\alpha}$, 2 μM | 1.7 | 4.2 | 10 | 30 |
| Experiment 3 | | | | |
| None | 1.9 | 5.4 | 12 | 31 |
| Isoproterenol, 1 μM | 0.8 | 4.6 | 58 | 89 |
| Experiment 4 | | | | |
| None | 1.9 | 4.3 | 11 | 30 |
| Serotonin, 0.1 mM | 1.5 | 4.2 | 12 | 24 |

ranged from 41 to 102 pmoles/min/mg of protein (64.9 ± 5.0, $n$ = 16), values similar to those reported for rat lung (10). The accumulation of cyclic GMP was proportional to the amount of homogenate (between 50 and 400 μg of protein) and proceeded at a constant rate for at least 15 min. Acetylcholine, 0.1 mM, alone or in the presence of cholinesterase inhibitors, failed in a number of experiments to increase cyclic GMP accumulation. In the presence of 1, 10, or 100 μM $CaCl_2$ or 0.1, 1, or 100 μM $MgCl_2$, acetylcholine (0.1 nM, 0.1 μM, and 0.1 mM) was likewise without effect.

Physostigmine sulfate and physostigmine salicylate, 1 mM, consistently increased guanylate cyclase activity by about 50% whether or not acetylcholine was added (Table 4). This effect of physostigmine was not prevented by atropine (Fig. 3), nor was it altered by incubation of the lung homogenate at 37° for 15 min before assay to permit degradation of endogenous acetylcholine. Guanylate cyclase activity was unaffected by neostigmine sulfate, edrophonium bromide (Table 4), or 1 mM carbachol, methacholine, bethanechol, and pilocarpine (data not shown).

TABLE 4

*Guanylate cyclase activity in lung homogenates*

Guanylate cyclase activity was assayed in whole homogenates as described in METHODS. Data are the means of duplicate assays, with the individual values in parentheses.

| Additions | Acetylcholine (0.1 mM) | Cyclic GMP accumulated |
|---|---|---|
| | | *pmoles/min/mg protein* |
| None | 0 | 90 (88, 93) |
| | + | 86 (83, 90) |
| Physostigmine sulfate, 1 mM | 0 | 151 (148, 154) |
| | + | 154 (151, 156) |
| Physostigmine salicylate, 1 mM | 0 | 136 (122, 149) |
| | + | 136 (128, 143) |
| Neostigmine sulfate, 1 mM | 0 | 100 (89, 100) |
| | + | 98 (98, 99) |
| Edrophonium chloride, 1 mM | 0 | 92 (90, 93) |
| | + | 94 (87, 101) |

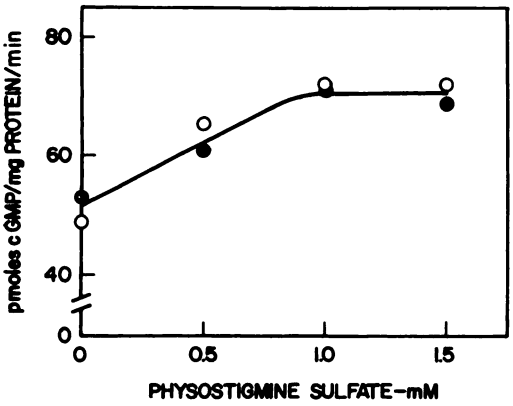


FIG. 3. *Effect of physostigmine sulfate on guanylate cyclase activity in lung homogenate*

Incubations were performed as described in METHODS in the presence of physostigmine sulfate alone (●) or with atropine in 5-fold greater concentration (○). When physostigmine was absent 7.5 mM atropine was used.

## DISCUSSION

Acetylcholine increased the cyclic GMP content of lung slices, as it does in slices of heart, brain, small intestine, and thyroid (2–4). The concentrations required to produce maximal effects (0.5–1.0 μM) and the rapidity of the response were similar in all tissues. In thyroid slices (4), however, the cyclic GMP concentration remained elevated for at least 20 min in the presence of acetylcholine (and physostigmine), whereas in lung and the other tissues the cyclic GMP concentration, after an initial rise, returned rather rapidly to basal levels. The lack of effect of *beta* adrenergic stimulation (isoproterenol or epinephrine plus phentolamine) on lung cyclic GMP concentration is in contrast to findings in the heart and small intestine, where isoproterenol inhibited the effects of acetycholine or bethanechol (1–3). In addition, treatment of guinea pigs with reserpine did not increase the cyclic GMP content of lung slices or the magnitude of the response to acetylcholine, as might have been observed if endogenous catecholamines had a significant depressant effect on cyclic GMP concentration. Isoproterenol and epinephrine did produce a marked increase in the cyclic AMP content of lung slices, which was prevented by the *beta* adrenergic blocking agent propranolol and was uninfluenced by phentolamine.

Prostaglandin $E_1$ also caused marked accumulation of cyclic AMP in the lung slices without demonstrably affecting cyclic GMP content.

It is, clearly, difficult to assign a specific site or function to the changes in cyclic nucleotide concentrations in a tissue as heterogeneous as the lung. Nevertheless, it seems probable that the increase in cyclic GMP concentration produced by acetylcholine occurs in cells innervated by the parasympathetic nervous system; i.e., cyclic GMP serves as a "second messenger" for acetylcholine. This is consistent with data from the perfused rat heart (1) and oxotremorine-treated mice (11, in which typically cholinergic physiological responses paralleled increases in cyclic GMP concentration. Although one cannot exclude the possibility that acetylcholine causes accumulation of cyclic GMP by interfering with its degradation, and despite our failure to demonstrate an effect of acetylcholine on guanylate cyclase, it seems likely that in the intact cell cyclic GMP formation is enhanced by stimulation of muscarininc receptors (3). The relatively specific effect of physostigmine on lung guanylate cyclase could, in fact, reflect interaction of this agent with receptors which, because of inappropriate conditions of preparation and/or assay, do not exhibit a functional response to acetylcholine.

In addition to its effect on cyclic GMP, acetylcholine produced a marked increase in the cyclic AMP content of lung slices. This effect of acetylcholine may be indirect, since effects on cyclic AMP have not been observed in other, more homogeneous tissues (1–3). The lung is a site for synthesis or storage of a number of agents that can increase cyclic AMP concentration, e.g., prostaglandins, norepinephrine, and histamine, and it seems likely that the rise in cyclic AMP concentrations produced by acetylcholine is secondary to the release of one of these substances in the lung slices. Alternatively, the accumulation of cyclic GMP might itself increase cyclic AMP concentration, as it apparently does in fat cells, kidney slices, and avian erythrocytes (12), presumably by inhibiting cyclic AMP phosphodiesterase (11, 13).

ACKNOWLEDGMENT

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NOTE ADDED IN PROOF:

We have recently found that bradykinin like acetylcholine increases the cyclic GMP and cyclic AMP content of lung slices and have presented evidence consistent with the view that the effects of both agents on cyclic AMP are secondary to the induced release of prostaglandins (Stoner, J., Manganiello, V. C., and Vaughan, M., Proc. Nat. Acad. Sci., U.S.A. (1973) in press).

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