Evidence for involvement of cyclic nucleotides in intrinsic factor secretion by isolated rabbit gastric mucosa.

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Research Article

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Evidence for Involvement of Cyclic Nucleotides in

Intrinsic Factor Secretion by

Isolated Rabbit Gastric Mucosa

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A B S T R A C T   Secretion of intrinsic factor (IF) has previously been demonstrated in isolated rabbit fundic mucosa maintained in organ culture. We have now investigated the possibility that cyclic nucleotides may play a role in IF secretion. A phosphodiesterase inhibitor, 3-isobutyl methylxanthine (IBMX), stimulated IF secretion nearly fourfold while increasing tissue levels of both cyclic AMP (cAMP) and cyclic GMP (cGMP). Peak IF secretion in response to IBMX was not reached until after tissue cAMP levels were maximal. Dibutyryl cAMP and 8-Br-cAMP increased secretion by the same order of magnitude as did IBMX, whereas corresponding analogues of cGMP had no such effect. Histamine increased secretion of IF. In the presence of 40 μM IBMX, histamine elevated tissue levels of cAMP, but not of cGMP, and the stimulating effect of 10 μM histamine on IF secretion was potentiated. An H_2 receptor antagonist, cimetidine, blocked the increases in IF secretion and tissue cAMP levels due to histamine, and the increase in IF secretion due to IBMX. These observations are consistent with a role for cAMP in the secretion of IF by isolated gastric mucosa.

INTRODUCTION

Since Kimberg's comprehensive review (1) appeared in 1974, the results of numerous investigations have argued for (2–7) and against (8, 9) an important role for cyclic nucleotides in mammalian gastric secretion. These investigations have been concerned with hydrochloric acid secretion (6, 8) and with adenylate cyclase activity and levels of adenosine 3',5'-cyclic AMP (cAMP)[1] in gastric mucosa (2–5, 7–9). We are unaware of studies on the possible relationships between cyclic nucleotides and gastric secretion of macromolecules.

Mucosal organ culture has shown promise as a means of investigating gastrointestinal mucosal function in vitro (10). Reports from this laboratory have described organ culture of gastric mucosa (11), *de novo* production, and steady-state secretion of pepsinogen and intrinsic factor (IF) by biopsies of rabbit fundic mucosa maintained in organ culture for 24 h (11, 12), and blockade of histamine-stimulated secretion of IF by cimetidine (13). In the rabbit, as in man, IF appears to be secreted by the parietal cell (14). We have now used organ culture of rabbit gastric mucosa to examine whether cyclic nucleotides play a role in IF secretion.

METHODS

Organ culture technique. The mucosal organ culture technique (15), adapted for rabbit gastric fundus, has been described in detail (11). White New Zealand rabbits weighing 2–5 kg were fasted overnight. Stomachs of ether-anesthetized animals were rapidly excised, the mucosa was washed with cold 0.15 M NaCl, and fundic mucosal biopsies were obtained with a suction biopsy tube. Biopsies were mounted on steel grids, mucosal surfaces up, and placed in contact with culture medium in organ culture dishes. For cyclic nucleotide determinations two biopsies were mounted on each grid. The culture medium was Trowell's medium T8 plus 10% fetal calf serum, 100 U/ml of penicillin, and 100 μg/ml of streptomycin.

Biopsies were cultured for 3 h over this control medium for stabilization, and then transferred to fresh control medium or experimental medium for periods that never exceeded 4.5 h. Experimental and control biopsies from the same rabbit were compared in each experiment.

[1] *Abbreviations used in this paper*: cAMP and cGMP, cyclic AMP and GMP; ^{57}Co-CNCbl, ^{57}Co-labeled cyanocobalamin; dbcAMP and dbcGMP, $N^6,O^{2'}$-dibutyryl cyclic AMP and GMP; IBMX, 3-isobutyl-1-methylxanthine; IF, intrinsic factor.

In vitro effects of humoral agents on cyclic GMP (cGMP) levels are often very early and short-lived (16–18). To measure levels of cyclic nucleotides at very short incubation periods (1–2 min), we modified the culture technique. After the 3-h stabilization, grids were transferred to medium continuously equilibrated, before and after transfer, with 95% O_2–5% CO_2 at 37°C. For precise timing only two dishes were handled at a time.

IF assay. IF secretion was determined as described (13), using ^{57}Co-labeled cyanocobalamin (^{57}Co-CNCbl). After an excess of ^{57}Co-CNCbl of known specific activity was added to the sample, it was exhaustively dialyzed against 0.15 M NaCl. After culture, 92–96% of the total ^{57}Co-CNCbl-binding capacity of the medium was blocked by anti-IF antibody in serum from a single pernicious anemia patient. Results were expressed as picograms cyanocobalamin bound per milligram biopsy protein.

In each experiment the amount of ^{57}Co-CNCbl bound by fresh medium was determined and subtracted from values for media over which biopsies were cultured. Protein present in the culture medium never bound more than half the amount of ^{57}Co-CNCbl bound by media obtained after incubation with control tissues. Binding by fresh medium was not inhibited by anti-IF antibody.

Assay for cyclic nucleotides. After incubation biopsies were immediately frozen in liquid nitrogen and homogenized within 1 h in 2 ml of trichloroacetic acid (6%). Homogenates were centrifuged at 27,750 g for 20 min at 4°C. Pellets were digested in 1 N NaOH for 30 min at 80°C, and assayed for protein content (19).

cAMP and cGMP in the supernate were separated by standard methods (20). The supernate was applied to a column of dry neutral aluminum oxide (0.5 × 1 cm), which was then washed with 10 ml water. The cyclic nucleotides were eluted with 7.5 ml of 200 mM ammonium formate, pH 9, onto a Dowex-1-formate column, 0.5 × 2.0 cm (generated from Dowex-1-chloride and washed with 20 ml of distilled water). The column was washed with another 10 ml of water. cAMP was eluted with 10 ml of 1 N formic acid and cGMP with 10 ml of 4.5 N formic acid. Eluates were dried and reconstituted in 0.05 M sodium acetate buffer, pH 6.2.

cAMP was assayed by the method of Steiner (21), and cGMP by the acetylation method, exactly as described by Harper and Brooker (22). To measure cGMP it was necessary to pool four to six biopsies. In the relevant concentration ranges, there was virtually no cross-reactivity between the two nucleotides.

cAMP values were corrected for recovery of [^{3}H]cAMP added to homogenates before centrifugation (mean recovery, 71±2%). Mean recovery of [^{3}H]cGMP in representative experiments was 59±2%; cGMP values cited below are not corrected. In other experiments, recoveries of nonlabeled nucleotides added to homogenates agreed with labeled recoveries to within 15%.

Statistical analyses. Results have been expressed as means±1 SE. Differences between means for three or more animals were tested for significance by Student's t test for unpaired data (23); all P values reported here refer to such comparisons.

Materials. The suction biopsy tube was manufactured by Quinton Instruments (Seattle, Wash.). Organ culture dishes were from Falcon Labware Div., Becton, Dickinson & Co. (Oxnard, Calif.). Trowell's Medium T8 and fetal calf serum were obtained from Grand Island Biological Co. (Grand Island, N. Y.).

3-isobutyl-1-methylxanthine (IBMX) was from Aldrich Chemical Co., Inc. (Milwaukee, Wis.). Cimetidine was kindly donated by Smith Kline & French Laboratories, Pharmaceutical Div. of SmithKline Corp. (Philadelphia, Pa.). $N^6,O^{2'}$-dibutyryl cAMP (dbcAMP) and $N^2,O^{2'}$-dibutyryl cGMP (dbcGMP), and also the 8-Br derivatives of the two cyclic nucleotides (8-Br-cAMP and 8-Br-cGMP), all as the sodium salts, histamine dihydrochloride, and all other reagents were obtained from Sigma Chemical Co. (St. Louis, Mo.). Dowex-1 (AG 1-X8, Cl$^-$, 200–400 mesh) was from Bio-Rad Laboratories (Richmond, Calif.). Neutral aluminum oxide was from J. T. Baker Chemical Co. (Phillipsburg, N. J.).

[^{3}H]cAMP and [^{3}H]cGMP were purchased from New England Nuclear (Boston, Mass.). ^{57}Co-B$_{12}$, 66.7 ng/μCi sp act, was from Radiochemical Centre (Amersham, England). Radioimmunoassay materials were obtained from Schwarz/Mann Div., Becton, Dickinson & Co. (Orangeburg, N. Y.) for the cAMP assay and from Collaborative Research Inc. (Waltham, Mass.) for the cGMP assay.

RESULTS

Effects of IBMX. In seven experiments we cultured biopsies for 4.5 h in the presence of various concentrations of IBMX and measured IF secretion. At IBMX concentrations of 40 μM or above, secretion was invariably greater than control values (Fig. 1). IBMX at 0.2 mM increased secretion four- to fivefold.

We then cultured biopsies from a total of six animals for various intervals with 40 μM or 0.2 mM IBMX and measured tissue levels of cyclic nucleotides. In the presence of IBMX, levels of both cyclic nucleotides were uniformly increased. At 0.2 mM IBMX, the increase was two- to threefold; in a representative experiment, the mean tissue cAMP concentration increased in 30 min from 7.1±0.6 pmol/mg protein to 20.3±1.8 pmol/mg protein, while the concentration of cGMP increased in the same interval from 0.07±0.001 pmol/mg protein to 0.27±0.07 pmol/mg protein.

Next, we examined the temporal relation between tissue cyclic nucleotide levels and secretion rates in response to IBMX (0.2 mM). Hourly IF secretion rates were calculated as differences in mean amounts of IF accumulated in the medium at successive hours. The mean tissue cAMP level rose steeply during the first
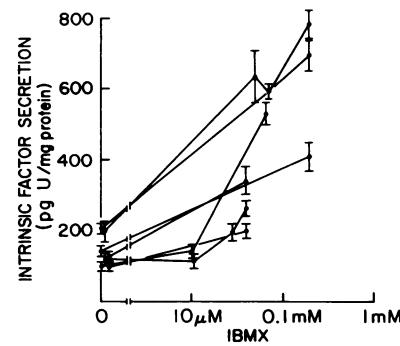

FIGURE 1 Effect of IBMX on IF secretion. Biopsies were cultured for 4.5 h over medium with or without IBMX. Each curve represents the data from an individual animal. Each point is the mean±1 SE for 6–12 biopsies at a given IBMX concentration.

30 min (Fig. 2) and reached a maximum of 25.6±2.0 pmol/mg protein within 1 h. Changes in cGMP levels paralleled those of cAMP (data not shown). By contrast, the peak rate of IF secretion, 272±28 pg U/mg protein per h, was not attained until the 2nd h.

Effects of cyclic nucleotide analogues. We tested the effects of cAMP and cGMP and some of their derivatives on IF secretion. Neither cAMP nor cGMP at a concentration of 1 mM increased secretion. In contrast, dbcAMP at 50 μM and above uniformly elevated secretion (Fig. 3). These effects were not due to butyric acid contamination, since 1 and 10 mM butyric acid had no effect on secretion. 8-Br-cAMP at concentrations of 0.1 and 1 mM increased secretion from 122±20 to 374±40 and 775±124 pg U/mg protein, respectively. Neither dbcGMP (5 mM) nor 8-Br-cGMP (1 mM) had any effect on secretion.

We measured the effect on tissue cGMP levels of dbcAMP, which can act as a phosphodiesterase inhibitor (24). In the presence of 0.5 mM dbcAMP, which stimulated secretion, tissue cGMP levels increased from 0.06±0.005 pmol/mg protein to 0.16±0.025 pmol/mg protein.

Effects of histamine. Because histamine stimulates IF secretion (13) and elevates adenylate cyclase activity (25, 26) in rabbit gastric mucosa, we examined the

FIGURE 3   Effects of dbcAMP on IF secretion. Biopsies were cultured for 4.5 h over media containing various concentrations of dbcAMP. Each curve represents the data from an individual animal. Values are means±1 SE for approximately six biopsies.

effects of histamine on cyclic nucleotide levels. In a single preliminary experiment where biopsies were cultured for 30 min over media containing a high concentration (10 mM) of histamine, no increase was detected in mean tissue levels of either cyclic nucleotide. We therefore examined the effects of histamine in the presence of IBMX. In each of four separate experiments, histamine at concentrations of 10 μM or greater uniformly increased mean tissue cAMP levels in the presence of 40 μM IBMX (Fig. 4). Under the same conditions, 10 mM histamine increased tissue cAMP levels from 13.9±0.7 to 27.5±3.0 pmol/mg protein ($n = 3$; $P < 0.01$) in 15 min of incubation; by contrast, in two experiments there was no indication of an increase in cGMP levels due to histamine at 1, 2, or 15 min.

We determined the effects of 40 μM IBMX on histamine-stimulated secretion of IF. It had previously been established that 0.1 mM histamine alone increased secretion over control levels by 158±44 pg U/mg protein (five animals; six to eight biopsies per experimental group in each animal). Histamine at 10 μM caused a much smaller increase, only 34±13 pg U/mg protein (three animals, three to five biopsies per group in each animal). In contrast, in the presence of 40 μM IBMX, 10 μM histamine caused a mean increase in secretion, over and above that produced by IBMX alone, of 523±129 pg U/mg protein in four animals (five biopsies per group in each animal). This increase was significantly greater than the mean increase not only for 10 μM histamine alone ($P < 0.025$), but also for 0.1 mM histamine alone ($P < 0.025$).

Effects of cimetidine. We measured effects of histamine on IF secretion and tissue cAMP levels in the presence and absence of an H_2 receptor antagonist, cimetidine. IBMX (40 μM) was added when cAMP
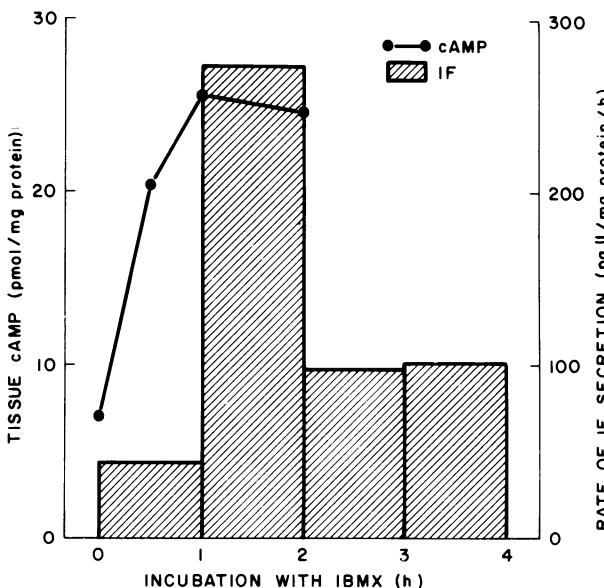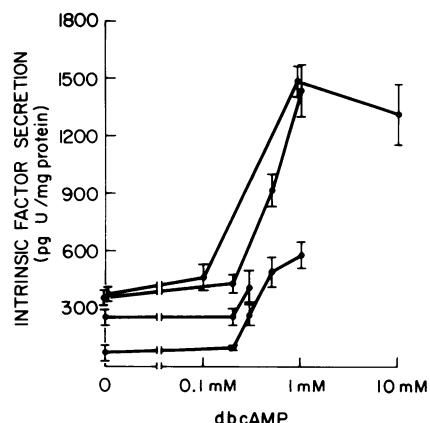
FIGURE 2   Tissue cAMP levels and IF secretion rates as functions of incubation time with IBMX. Biopsies were cultured for various periods of time over medium containing IBMX (0.2 mM), and cAMP concentration or IF accumulation was then determined in tissue or medium, respectively. Bars represent mean hourly rates of IF secretion (differences in amounts accumulated in successive hours) by 7–16 biopsies from one to two animals. Each closed circle represents a mean tissue concentration of cAMP from 5 to 14 determinations in one to two animals.
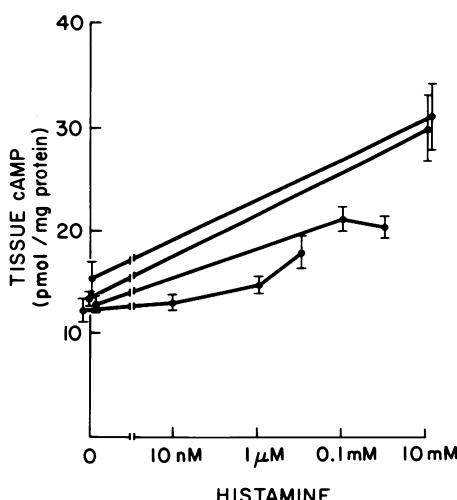
FIGURE 4   Effect of histamine on tissue cyclic AMP concentrations. Biopsies were cultured for 4.5 h over media containing 40 μM IBMX and various concentrations of histamine. Each curve represents the data from an individual animal. Values are means±1 SE for approximately six biopsies.

levels were to be determined. 2 mM cimetidine abolished the stimulating effect of 0.1 mM histamine on IF secretion (Table I) and reversed the elevation of tissue cAMP concentrations by 0.1 mM histamine in the presence of IBMX.

We next determined whether cimetidine would alter the effects of IBMX on IF secretion or tissue cAMP. The increase in secretion caused by IBMX (0.2 mM) was completely prevented when cimetidine (2 mM) was added to the medium (Table II). In the presence of cimetidine, IBMX still elicited a marked rise in mean tissue cAMP levels.

TABLE I
Effects of Cimetidine on Histamine-Stimulated Elevations of IF Secretion and Tissue cAMP Levels

	IF secretion	Tissue cAMP
	pgU/mg protein	pmol/mg protein
Control	353±49 (5)	16.7±2.0 (5)
Histamine (0.1 mM)	511±39 (5)	26.0±3.0 (3)
Histamine + cimetidine (2 mM)	350±22 (3)	14.5±1.9 (3)

IF secretion and tissue cAMP were measured under different conditions, as follows: For experiments with IF secretion, biopsies were cultured for 4.5 h in the absence of IBMX. For experiments in which tissue cAMP was determined, biopsies were cultured for 30 min in the presence of IBMX (40 μM). The number of experiments is shown in parentheses. For each experiment, mean values were obtained from 5 to 12 biopsies taken from a single animal. The results shown in the table represent the means±1 SE of these means.

TABLE II
Effects of Cimetidine on IBMX-Stimulated Elevations of IF Secretion and Tissue cAMP Levels

	IF secretion	Tissue cAMP
	pgU/mg protein	pmol/mg protein
Control	189±38 (6)	8.6±1.3 (4)
IBMX (0.2 mM)	597±58 (6)	25.1±3.7 (4)
IBMX + cimetidine (2 mM)	188±89 (3)	20.2±1.9 (3)

For measurement of IF secretion and tissue cAMP, biopsies were cultured for 4.5 h and 30 min, respectively. The number of experiments is shown in parentheses. Means for 5–12 biopsies from a single animal were obtained in each experiment. The values shown represent the means±1 SE of these means.

We determined the effect of cimetidine on tissue cAMP levels in a total of six animals. In each experiment the mean cAMP concentration, based on 5–12 biopsies, was lower in the presence than in the absence of cimetidine, regardless of whether histamine was present. In the first three experiments (Table I) cimetidine, even in the presence of 0.1 mM histamine, reduced cAMP levels below control values by 3.7±1.2 pmol/mg protein. In the other three experiments (Table II), the mean tissue cAMP level was lower by 6.3±3.0 pmol/mg protein in the presence of both cimetidine and IBMX than in the presence of IBMX alone.

DISCUSSION

This investigation provides several observations that are consistent with the involvement of cAMP in the secretion of intrinsic factor (IF) by isolated gastric mucosa. First, the phosphodiesterase inhibitor IBMX not only elevated mean tissue levels of both cAMP and cGMP in cultured mucosal biopsies from the fundus of rabbit stomach, but also markedly stimulated IF secretion by these biopsies. In addition, peak mean tissue levels of both cyclic nucleotides developed in IBMX-treated biopsies before maximal rates of IF secretion were observed.

Although IBMX increased the mean levels of both cAMP and cGMP in cultured biopsies, only analogues of cAMP stimulated IF secretion. Addition of either dbcAMP or 8-Br-cAMP to the culture medium increased IF secretion to approximately the same extent as did IBMX. In contrast, analogues of cGMP in concentrations as high as 1 mM had no detectable effect on IF secretion by cultured biopsies. Thus, the analogue experiments suggest that cAMP, rather than cGMP, might be involved in the stimulatory effect of IBMX on IF secretion.

Further evidence that cAMP may be involved in IF secretion is provided by our experiments with hista-

mine and cimetidine. The present results confirm previous observations that histamine stimulates IF secretion in rabbit gastric mucosa (13). In separate studies (27, 28), we have also confirmed previous reports (25, 26) that histamine stimulates adenylate cyclase activity in this tissue. In addition, the present observations show that histamine in the presence of IBMX elevates mean levels of cAMP, but apparently not those of cGMP, in rabbit fundic mucosal biopsies. Furthermore, at a concentration of IBMX which by itself submaximally increases mean cAMP levels in these biopsies, histamine clearly potentiated the stimulation of IF secretion by IBMX. Finally, 2 mM cimetidine not only blocked the stimulating action of histamine on both IF secretion and mean cAMP levels, but also completely blocked the stimulation of IF secretion by IBMX. All these observations with histamine and cimetidine are consistent with the possibility that cAMP may play a role in the stimulation of IF secretion by histamine.

The fact that dbcAMP elevated mucosal concentrations of cGMP in separate experiments raises the possibility that cGMP might mediate the stimulation of IF secretion by dbcAMP in rabbit gastric mucosa. This possibility must be viewed in the light of the failure of cGMP analogues to stimulate IF secretion to any obvious degree. Moreover, it is improbable that cGMP acts as a mediator of the effects of histamine in our system, because we did not observe any effect of histamine on tissue levels of cGMP, either in the presence or in the absence of IBMX, and since histamine (H_2) is not known to stimulate guanylate cyclase. These considerations make it appear unlikely that cGMP is involved in the stimulation of IF secretion by IBMX, dbcAMP, or histamine in our preparation. It is possible, however, that dbcAMP, in addition to its direct action as a cAMP analogue and its elevation of cGMP concentrations, may also raise cAMP levels by inhibiting cAMP phosphodiesterase, and influence IF secretion in this manner.

We have previously reported (13) that cimetidine blocks "basal" IF secretion in our preparation. The simplest interpretation of this finding is that basal IF secretion depends on the presence of endogenous histamine (or another H_2 agonist) in the tissue. Similarly, the present observation that cimetidine totally blocks the increase in IF secretion induced by IBMX (Table II) strongly suggests that the stimulating effect of IBMX on IF secretion in our experiments is dependent on the presence of endogenous histamine. This interpretation is completely consistent with the results of our potentiation experiments, in which the effect of histamine on IF secretion was greatly enhanced by IBMX. The importance of endogenous histamine is supported by recent studies (29) indicating that although cimetidine inhibits the responses of parietal

cells in the intact canine gastric mucosa to stimulation not only by histamine but also by gastrin and carbamylcholine, this agent inhibits only responses to histamine in isolated parietal cells obtained from the same mucosa. Also conceivable is the possibility that cimetidine, in addition to its action as an H_2 blocker, acts at a site distal to the locus of action of IBMX, but we know of no evidence to support this possibility.

Two aspects of our observations concerning the effects of IBMX and cimetidine on mucosal cAMP levels (Table II) merit further comment. If IBMX raises cAMP levels primarily by inhibiting phosphodiesterase, this action of IBMX requires the presence of one or more active adenylate cyclases. Moreover, if the stimulation of IF secretion by IBMX implies the presence of endogenous histamine in the tissue, as we have reasoned in the preceding paragraph, and if histamine stimulates IF secretion by stimulating adenylate cyclase and raising cAMP levels, then a portion of the IBMX-stimulated rise in cAMP levels should also reflect the presence of such endogenous histamine, and therefore cimetidine should block this part of the increase.

The present data suggest that cimetidine may in fact inhibit part of the rise in cAMP levels induced by IBMX. As has been noted above, in each of six independent experiments mean tissue cAMP levels were substantially lower in the presence of cimetidine and IBMX (in three cases, even with histamine present) than in the presence of IBMX alone. Since the standard errors of the means in such experiments are usually considerable, however, it is apparent that a large number of experiments would be required to demonstrate a statistically significant reduction of cAMP levels by cimetidine.

In any case, we would not anticipate that cimetidine would completely inhibit the IBMX-stimulated increase in mucosal cAMP levels, since the parietal cells, which are clearly responsive to histamine, constitute no more than half the fundic mucosal cell mass in those mammalian species where cell distributions have been investigated (30). Of the remaining cell types, most have not been shown to be responsive to histamine and presumably would not be affected by cimetidine, on the basis of our present knowledge. IBMX would be expected to permit increased accumulation of cAMP in any of these cells that contained active adenylate cyclase-phosphodiesterase systems not dependent on histamine. Moreover, it is possible that part of the adenylate cyclase of parietal cells may also be active in the intact mucosa in the presence of cimetidine.

ACKNOWLEDGMENTS

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