# DEMONSTRATION OF AN "ACTIVATOR FACTOR" AND AN "INHIBITOR FACTOR" IN THE CYCLIC AMP PHOSPHODIESTERASE FROM OXYNTIC CELLS OF BULLFROG GASTRIC MUCOSA

Tushar K. RAY and John G. FORTE
*Department of Physiology-Anatomy, University of California, Berkeley, Calif. 94720, USA*

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## 1. Introduction

The involvement of cyclic AMP in the control of acid secretion by gastric mucosa has been suggested [1–3]. One experimental line of evidence has concerned the stimulatory action of methyl xanthines which are known to inhibit cyclic AMP-phosphodiesterase. This enzyme, which converts cyclic AMP to 5′-AMP, is the only known enzymatic regulator of cyclic AMP catabolism [4]. Thus the extent and duration of any cellular effect mediated by cyclic AMP will be controlled by the activity of the phosphodiesterase. The importance of this enzyme has been realized in recent years, and has been documented by a number of publications [5–12]. Cheung [6] and others [7, 8] have demonstrated a heat-stable, nondialysable "active factor" from brain and liver tissues. Such findings suggest that the activator might be playing a fundamental role in regulating the phosphodiesterase *in vivo*.

In the present paper we demonstrate a heat-stable dialysable "activator" and a heat-stable nondialysable "inhibitor" of the cyclic AMP-phosphodiesterase activity obtained from the oxyntic, or acid-secreting, cells of bullfrog gastric mucosa.

## 2. Materials and methods

The oxyntic cells from bullfrog gastric mucosa were collected by a slight modification of the method reported earlier [13]. Details of this procedure will be published elsewhere [14].

The oxyntic cells were homogenized in either 2.0 mM acetate buffer pH 6.8 or 0.25 M sucrose, adjusted to pH 7.3 with Tris. The phosphodiesterase was prepared by $(NH_4)_2SO_4$ fractionation of the 100,000 *g* supernatant. All operations were run at 0–4°. The supernatant was first saturated with $(NH_4)_2SO_4$ to 16% (w/v), centrifuged at 15,000 *g* for 10 min and the precipitate discarded. The supernatant fraction was then brought up to 34% saturation with $(NH_4)_2SO_4$. After centrifugation at 15,000 *g*, the pellet contained about 85–90% of the total cyclic AMP phosphodiesterase activity. This pellet was resuspended in 0.05 M Tris buffer, pH 8.0 containing 0.1% β-mercaptoethanol and stored between 0–4° for use as the enzyme source. For preparation of the dialysable "active factor" the pellet was dissolved in glass distilled water containing 0.1% β-mercaptoethanol and the pH adjusted to 8.0 by adding Tris.

The heat-stable, dialysable "active factor" was prepared in the following 2 ways: 1) The enzyme preparation (ca. 5 mg protein/ml) was heat inactivated at 85° for 5 min. Denatured protein was removed by centrifugation and the supernatant was dialyzed against glass distilled water overnight in the cold. The dialysate was lyophilized and dissolved in a volume equal to that of the original solution. 2) Without denaturation the enzyme solution was dialyzed overnight against cold glass distilled water containing 0.1% β-mercaptoethanol. The dialysate was lyophilized and redissolved in a volume of glass distilled water equal to that of the original solution. A portion was assayed after one month (aged dialysate).

The material retained in the dialysis bag after dialysis of the heat inactivated supernatant was the source of the heat-stable, nondialysable "inhibitor factor".

Table 1
Effect of $Mg^{2+}$, theophylline and the supernatant of the heat inactivated (85° for 5 min) enzyme solution on the dialysed and nondialysed cyclic AMP phosphodiesterase.

| Fraction | Activity (nmole/mg protein/10 min) |
|---|---|
| 1. Nondialysed enzyme (without $Mg^{2+}$) | 10.0 |
| 2. Nondialysed enzyme | 94.0 |
| 3. Nondialysed + 85°, 5 min supernatant | 90.0 |
| 4. Nondialysed + theophylline | 61.3 |
| 5. Dialysed enzyme (without $Mg^{2+}$) | 0.0 |
| 6. Dialysed enzyme | 35.0 |
| 7. Dialysed + 85°, 5 min supernatant | 80.0 |
| 8. Dialysed + supernatant + theophylline | 41.6 |
| 9. Dialysed + theophylline | 31.0 |
| 10. 85°, 5 min supernatant | 0.0 |

Each result represents average values from 3 different preparations. Details of the experimental and assay conditions are given in Materials and methods. Theophylline was used at a final concentration of 10 mM. For the dialysed enzyme preparation, the enzyme solution was dialysed at 4° overnight against 0.05 M Tris-Cl pH 8.0 containing 0.1% β-mercaptoethanol and 1.0 mM EDTA. 500–700 μg of the enzyme protein was used for each assay. 0.1 ml of the 85°, 5 min supernatant was used.

The activity of cyclic AMP phosphodiesterase was assayed using $^3$H-cyclic AMP (Schwarz Radiochemicals) as substrate. Advantage was taken of the fact that oxyntic cells contain virtually no 5′-nucleotidase activity [12]. The reaction mixture contained, in a total volume of 0.5 ml, 1.0 μmole cyclic AMP, 5.0 μmole Tris pH 9.0, 2.5 μmole $MgCl_2$ and necessary amounts of enzyme and other test substances. The reaction was carried out for 10 min at 37°, stopped in a boiling water bath for 3 min, centrifuged and then 0.1 ml of the supernatant was used for chromatographic assay. 5′-AMP formed in the reaction was separated by paper chromatography using isopropanol: $NH_3$ :$H_2O$ (7:2:1) solvent system. The 5′-AMP spots were detected under UV, cut out and then counted in "Aquasol" scintillation fluid (New England Nuclear).

## 3. Results and discussion

Table 1 compares the phosphodiesterase activity of the nondialyzed and dialyzed enzyme preparations and the effects of $Mg^{2+}$, heat inactivated supernatant and theophylline on the enzyme activity. Dialysis of the enzyme caused more than 50% reduction of the activity which can be almost fully restored by adding back the 85°, 5 min supernatant. Theophylline (10 mM) can only inhibit 30–50% of the total activity both from the nondialysed enzyme preparation as well as dialysed reconstituted enzyme preparation. It is noteworthy that theophylline has practically no effect on the activity of the dialysed enzyme preparation. This experiment shows that the 85°, 5 min supernatant contains some heat-stable factor(s) which can restore to a great extent the activity of the dialyzed enzyme. This also indicates that theophylline can only prevent the stimulation caused by the 85°, 5 min supernatant fraction but has no effect on the activity of the dialysed enzyme (basal activity).

Table 2
Effect of "inhibitor factor", "activator factor" and theophylline on phosphodiesterase activity of dialyzed enzyme preparation.

| Fractions | Activity (nmole/mg protein/10 min) |
|---|---|
| 1. Dialyzed enzyme | 41.0 |
| 2. Dialyzed enzyme + inhibitor | 40.8 |
| 3. Dialyzed enzyme + activator | 103.8 |
| 4. Dialyzed enzyme + inhibitor + activator | 85.2 |
| 5. 4 + theophylline | 65.6 |
| 6. 3 + theophylline | 82.0 |

Each result represents average values from 4 different preparations. Preparation of the "inhibitor" and "activator" fractions have been described in Materials and methods. 0.1 ml of the "activator" and "inhibitor" were used. 500–700 μg of enzyme protein was used for each assay.

Dialysis of the 85°, 5 min supernatant produced interesting fractions in both the dialysable and nondialysable portions of the material as shown in table 2. The dialysable material produced a marked stimulatory effect on phosphodiesterase activity (about 2–3 fold) and we refer to this fraction as "activator". On the other hand we refer to the nondialysable fraction as "inhibitor" since it was effective in abolishing the stimulation produced by the dialysable active factor. However, the "inhibitor" fraction had no effect when added to the dialysed enzyme alone. The combined effect of the dialysable and the non-

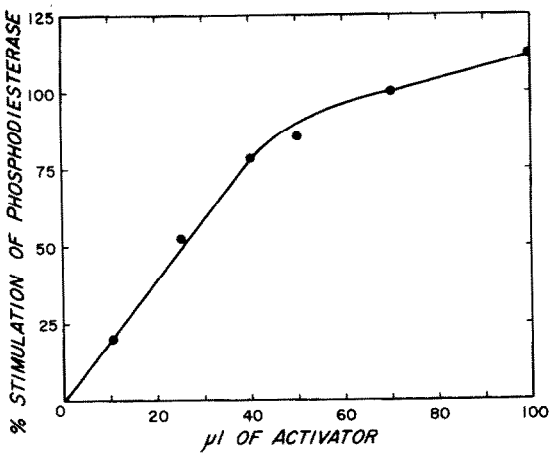


Fig. 1. Stimulation of cyclic AMP phosphodiesterase activity by different amounts of "active factor" prepared from the 85°, 5 min supernatant. The fraction collected by saturation with 34% $(NH_4)_2SO_4$ was dialyzed overnight and used as the enzyme source. The methods for preparation of dialyzed enzyme and activator are described in Materials and methods. 500 µg of enzyme protein was used for each assay.

dialysable factors is like that of the total 85°, 5 min supernatant. But in the absence of the "inhibitor factor", the stimulation caused by the dialysable "active factor" is somewhat more than that caused by the 85°, 5 min supernatant. These observations clearly demonstrate the presence of 2 distinct factors, one acting as an "inhibitor" and the other acting as an "activator" of the phosphodiesterase enzyme. In the absence of the "activator" and "inhibitor" factors, theophylline seems to be a relatively poor inhibitor of phosphodiesterase activity. Thus there appears to be a complex interplay between inhibitor, activator and theophylline in the regulation of phosphodiesterase activity.

Fig. 1 shows the dose-dependent activation of the phosphodiesterase by the "active factor" fraction from the heated supernatant. The nature of the curve indicates a stoichiometric relation between the two.

We have also obtained an active factor by dialysing the enzyme preparation without the heat inactivation process. The dialysable fraction from this procedure is also active in stimulating phosphodiesterase as shown in table 3. Apparent differences in activation

Table 3
Comparison of fresh and aged dialysates of the enzyme solution on phosphodiesterase activity.

| Fraction | Activity (nmole/mg protein/10 min) |
|---|---|
| 1. Dialyzed enzyme | 35.0 |
| 2. Dialyzed enzyme + fresh dialysate (active factor) | 86.6 |
| 3. Dialyzed enzyme + aged dialysate (aged active factor) | 35.6 |

Each result represents average values from 3 different preparations. Details of the preparation of "fresh" and "aged" factors are described in Materials and methods. 0.1 ml of fresh and aged preparations of the "active factor" was used. 500–700 µg of enzyme protein was used for each assay.

by fractions prepared by the 2 methods may be due to differences in yield (c.f. table 2 and 3). On standing at 0–4° for a period of 1 month, the "active factor" completely lost its activity.

The nature of the "active factor" and the cause for its deterioration with time are still not clear. However, we have performed some preliminary experiments directed toward characterization of the material. Pronase digestion for as long as 30 hr does not inactivate the "active factor", indicating that the material is not a polypeptide nor is its activity related to conjugation to some dialysable polypeptide.

Spectral analysis of the dialysable active material prepared by either of the 2 methods revealed absorption maxima at 207 and 244 with a shoulder at 282 nm. Interestingly, when the "active factor" prepared by the latter method was inactivated after long term storage the spectral properties were somewhat altered: the shoulder, initially at 282, shifted to 292 nm, while the other peaks remained unchanged. Furthermore, when the inactivated factor was chromatographed in the isopropanol: $NH_3$:$H_2O$ system a UV absorbable material was detected with an $R_f$ value of about 0.95. After eluting the spot in 0.05 M phosphate buffer pH 7.4 the UV spectra revealed a peak of 207 nm with a shoulder at 244 nm. Chromatography of the fresh, active material did not reveal such a spot.

Although the results presented in this paper do not elucidate the mechanism of action of the "inhibi-

tor" and "activator" factors on cyclic AMP phosphodiesterase activity, the possibility is suggested that these factors might be playing an important role in the regulation of the phosphodiesterase *in vivo* in bullfrog gastric mucosa. Consequent alterations in tissue level of cyclic AMP would have profound effects on secretory activity. Furthermore, it would be of interest to determine whether such regulating factors are more general for cyclic AMP phosphodiesterase activity in other systems.

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