EVIDENCE FOR AND SOME PROPERTIES OF A 3', 5'- CYCLIC AMP PHOSPHODIESTERASE INHIBITOR IN POTATO

Makoto Shimoyama*, Masami Kawai#, Yoshinori Tanigawa, and Iwao Ueda
Department of Medical Chemistry, Osaka Medical College
Takatsuki, Osaka, Japan

Masako Sakamoto and Kazunari Hagiwara
Shitennoji Women's Jounior College, Habikino, Osaka

Yoshiaki Yamashita and Eiichi Sakakibara
General Research Center of Funai Pharmaceutical Co., Ltd., Hirakata, Osaka

Received February 23, 1972

SUMMARY

Dialysis of potato 105,000 x g supernatant resulted in a dramatic increase in 3', 5'- cyclic AMP phosphodiesterase activity, due to the removal of some unknown inhibitor of small molecular size, through this process. The inhibitor obtained by gel filtration was resistant to boiling at neutral, acidic, or alkaline pH and also to charcoal treatment. Kinetic analysis showed that inhibition was dependent on inhibitor concentration in the reaction mixture and that it was noncompetitive.

INTRODUCTION

The diesterase which converts 3', 5'- cyclic AMP to 5'- AMP plays an important role in the regulation of the intracellular level of 3', 5'- cyclic AMP in mammalian tissue and bacteria (1, 2).

During our study on the adenyl cyclase - phosphodiesterase system in various organs, we observed the presence of a potent low molecular inhibitor of 3', 5'- cyclic AMP phosphodiesterase in potato. The present report treats of some characteristics of this inhibitor.

MATERIALS AND METHODS

*All correspondence should be addressed to this author.

#Permanent address: General Research Center of Funai Pharmaceutical Co., Ltd., Hirakata, Osaka.

Potato (Salanum tuberosum), weighing 200 - 250 g was used. ^{3}H - 3', 5'- cyclic AMP was obtained from New England Nuclear. Snake venom (Naja naja) was purchased from Sigma Chemical Company. All the other chemicals were of reagent grade or the best commercially available.

Enzyme assay

The assay for phosphodiesterase activity was carried out by the two stage isotopic procedure described by Thompson and Appleman (3). The first stage reaction mixture of 0.5 ml contained 20 μmoles acetate buffer, pH 4.5*, 2.5 μmoles $MgCl_2$, 2.5 mg of bovine serum albumin, 50 nmoles ^{3}H - 3', 5'- cyclic AMP (12,000 cpm), and an appropriate concentration of enzyme. The reaction was initiated by the addition of cyclic AMP. After incubation for 20 min at 37°, the tubes containing the reaction mixture were transferred to a boiling water bath for 2 min to terminate the reaction. The reaction mixture was then further incubated with sufficient snake venom plus 50 μmoles Tris - Cl buffer, pH 7.5, for 30 min at 37°. The reaction was stopped by the additions of 0.8 ml of a 1:1 slurry Bio - Rad resin, AG 1 x 2, 200 - 400 mesh. The amount of radioactivity of the ^{3}H - adenosine left in the supernatant after centrifugation in a clinical centrifuge was measured by means of a liquid scintillation spectrometer. All assays were carried out at 20 % or less total reaction to be in the linear portion of the enzyme assay. One unit of enzyme activity was defined as the amount cleavage nmole of 3', 5'- cyclic AMP per hour. In some cases, the analysis of ^{3}H - 5'- AMP formed during first stage incubation was performed by paper chromatography as described in the legend to Table II. Protein was determined by the method of Lowry et al. (4).

Phosphodiesterase and inhibitor preparations

About 50 g of peeled potato was ground for 5 min in a Waring blender with 25 ml of 0.01 M Tris - Cl buffer, pH 7.5, containing 5 mM $MgCl_2$, pressed through cheesecloth; then the extract was centrifuged at 105,000 x g for 60

*We have found that the optimum pH of potato phosphodiesterase is 4.5 (report in preparation).

min and the supernatant solution was dialyzed overnight at 4° against a large volume of the same buffer. The precipitate was removed by centrifugation and the supernatant solution was empolyed as enzyme. The gel filtration was also carried out for the separation of phosphodiesterase and an inhibitor. 4 ml of 105,000 x g supernatant was applied to an Agarose A - 1.5 m column (1.5 x 90 cm), which was eluted with the same buffer. The standard elution pattern of the enzyme and an inhibitor was illustrated in Fig. 1. The flow rates for the separation did not exceed 6 ml / hour; the fraction volumes were normaly about 3 ml, and the V_o was 45 ml.

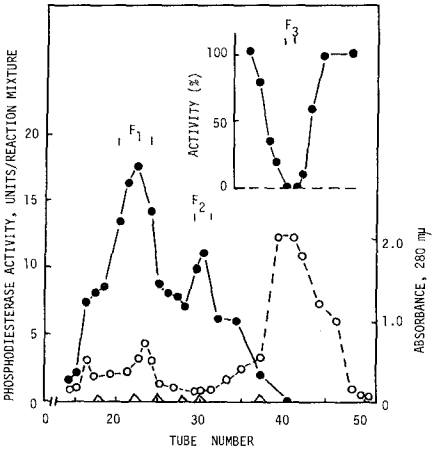

Fig. 1. 8 % Agarose gel filtration of a 105,000 x g supernatant. 4 ml of the supernatant was applied to a column as described in the text. 0.15 ml of the eluate was used as enzyme. o----o, absorbance at 280 mμ ; ●——●, enzyme actvity ;△——△, enzyme activity in the presence of 0.15 ml F_3 in the reaction mixture (see inset). Inset, per cent inhibition by eluate ; the assay of the dialyzed enzyme activity was carried out as described in the text except for the addition of 0.25 ml of each fraction. The reaction mixture involved 11.3 units of the dialyzed enzyme. Enzyme activity in the absence of eluate was settled at 100 %.

RESULTS AND DISCUSSION

During the course of enzyme preparation, we observed that dialysis dramatically increased enzyme activity. As shown in Table I, the dialyzed enzyme showed an activity 56 times higher, in terms of units per mg protein, than the non - dialyzed enzyme. Furthermore, a mixture of equal volumes of both enzyme

preparations showed the same activity as the non - dialyzed enzyme (data not shown). These results prompted further investigation by means of gel filtration.

Fig. 1 shows that two distinct fractions presenting phosphodiesterase activity have been isolated as significant levels from an Agarose A - 1.5 m column; these have been designated as F_1 and F_2, respectively. If the increase in enzyme activity shown in Table I had been caused by the dialytic removal of a certain inhibitor of small molecular size, this inhibitor should be eluted by gel filtration. To establish this possibility, the dialyzed enzyme activity was assayed in the presence of an aliquot of the eluate from tubes No. 38 to 44. As shown in the inset in Fig. 1, complete inhibition was seen in the fractions in tubes No. 40 and 41 (designated as F_3). Similar results were obtained when appropriate fractions, including F_1 and F_2, were employed

Table I

Effect of dialysis on enzyme activity

Fraction	Activity	Protein	Yield	Specific activity
	units	mg	%	units/mg protein
Before dialysis	26.1	18.2	100	1.44
After dialysis	788.9	9.7	321.4	81.5

4 ml of 105,000 x g supernatant solution was dialyzed overnight at 4° against 2 l of 0.01 M Tris-Cl buffer, pH 7.5, containing 5 mM $MgCl_2$. The assay of phosphodiesterase activity was carried out as described in the text. The reaction mixture contains 0.1 ml enzyme.

as enzyme. To make certain that this inhibition was not due to the influence of F_3 on the conversion of 5'- AMP to adenosine by the snake venom added to the 2nd stage reaction mixture, the following experiments were carried out (Table II): in Experiment A, the F_1 preparation had no F_3 effect on enzyme activity when it was added to the 2nd stage reaction mixture, although complete

Table II

Effect of F_3 on phosphodiesterase activity

Addition	Activity
	units
Experiment A, F_1 enzyme (0.08 mg protein)	
1. None	8.2 (2.9)
2. F_3 (1st stage reaction mixture)	Nil
3. F_3 (2nd stage reaction mixture)	8.5
Experiment B, F_2 enzyme (0.02 mg protein)	
1. None	3.8
2. F_3 (1st stage reaction mixture)	0.5
Experiment C, rat brain enzyme (0.08 mg protein)	
1. None	54.3 (17.4)
2. F_3 (1st stage reaction mixture)	55.2

Assays were carried out by the usual two stage procedure except in Experiment B. The reaction mixtures contained the indicated amounts of enzyme. Tubes 2 and 3 contained 0.15 ml of F_3 in the 1st and 2nd stage reaction mixtures of the standard assay system. In Experiment B, paper chromatography was carried out for separation of ^{3}H-5'-AMP from the substrate following 1st stage incubation. In this case, the radioactivity of the substrate used was 5 times higher than usual. A mixture of ammonium sulfate-1 M sodium acetate-isopropanol, 80:18:2 (5) was employed as solvent for paper chromatography. The radioactive spots corresponding to the ultraviolet absorption of 5'-AMP on paper were measured by means of a liquid scintillation spectrometer. The Rf values for 3',5'-cyclic AMP and 5'-AMP were 0.1 and 0.34, respectively. In Experiment C, the supernatant solution of rat brain homogenized with 9 volumes of cold 0.25 M sucrose following centrifugation at 105,000 x g for 60 min was employed as enzyme. The assay conditions were the same as that in Experiment A except that the 1st stage incubation time was 10 min and 50 μmoles Tris-Cl buffer, pH 8.5, were used instead of acetate buffer. Activity in the absence of snake venom is shown in parentheses.

inhibition of enzyme activity had been observed on its addition to the 1st stage reaction mixture; and, furthermore, in Experiment B, the formation of 5'- AMP by the F_2 preparation was strongly inhibited in the presence of F_3. In contrast to the potato enzyme, rat brain enzyme was not inhibited by F_3 (Experiment C). If snake venom was omitted from the 2nd stage reaction mixture, significantly less adenosine was produced in the F_1 and the brain preparations

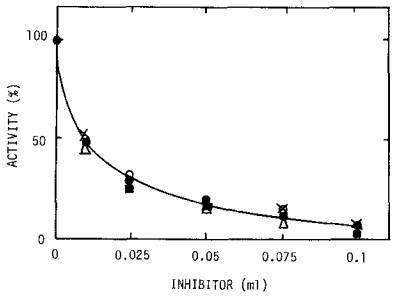

Fig. 2. Effect of inhibitor concentration on phosphodiesterase activity and stability of the inhibitor in F_3. As inhibitor source, F_3 was used after treatment as follows : F_3 was adjusted to the desired pH with 2 N HCl or 2 N NaOH, and was then heated in a boiling water bath for 5 min, cooled, and adjusted to pH 7.5. In some cases, 50 mg of charcoal was added to 1 ml of F_3 and the mixture was stirred for 5 min. These preparations were centrifuged at 8,000 x g for 15 min, and the supernatant solution was then adjusted to twice the original volume with water and used as inhibitor. 8.5 units of F_1 preparation was employed as enzyme. The other assay conditions are described in the text. The absence of inhibitor value was settled as 100 %. ●—●, F_3 (diluted to double volume with water); o—o, F_3, pH 7.5, boiled; x—x, F_3, pH 2.0, boiled; ■—■, F_3, pH 12.0, boiled; ▲—▲, charcoal treated.

(Experiments A and C). These results indicate that F_3 does not to inhibit the action of snake venom during the assay system of phosphodiesterase activity.

The stability of the inhibitor in F_3, together with the effect of the inhibitor concentration on phosphodiesterase activity is shown in Fig. 2. The relation between inhibition degree and inhibitor concentration was observed, and such exposures as boiling at neutral, acidic, or alkaline pH and as subjection to charcoal treatment had no effect on inhibitory potency.

Lineweaver - Burk plots of 1/v versus 1/[S] in both inhibitor absence and presence are shown in Fig. 3. The nature of F_3 inhibition seems simple as the Lineweaver - Burk plots give straight lines and the reaction is noncompetitive.

The data given here indicate that 3', 5'- cyclic AMP levels in potato would be influenced by this inhibitor. Of interest is the evidence for the existence of a phosphodiesterase inhibitor in potato in contrast to the evidence for an enzyme activator in mammals (6).

Investigation towards identification of this inhibitor in the potato is now in progress.

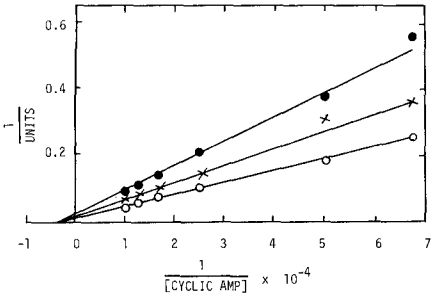

Fig. 3. Double reciprocal plots of the initial velocity of the phosphodiesterase versus cyclic AMP concentration at constant inhibitor level. Initial reaction velocity was determined by utilizing the standard assay system containing 19.3 units of F_1 preparation. The amounts of F_3 used were ; o——o, none; •—•, 0.015 ml; x——x, 0.006 ml, respectively.

ACKNOWLEDGEMENT

We wish to thank Miss T. Shimada for excellent her technical assistance.

REFERENCES

1. Robinson, G. A., Bucher, R. W., and Sutherland, E. W., Ann. Rew. Biochem., 37 : 149, 1968.
2. Sutherland, E. W. and Rall, T. W., Pharmacol. Rev., 12 : 265, 1960.
3. Thompson, W. J. and Appleman, M. M., Biochemistry, 10 : 311, 1971.
4. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J., J. Biol. Chem., 193 : 265, 1951.
5. Randerath, K., Biochem. Biophys. Rec. Comm., 6 : 452, 1961.
6. Cheung, W. Y., J. Biol. Chem., 10 : 2859, 1971.