MECHANISM OF THE INHIBITORY EFFECT OF ETHOXYMETHYLENE-ATP ON THE ACTIVITY OF HEPATIC ADENYLATE CYCLASE

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1. Introduction

In the previous paper [1] it has been demonstrated that the 2′,3′*O*-ethoxymethylene derivatives of adenosine, AMP, ADP and ATP possess strong inhibitory effects on the activity of hepatic adenylate cyclase. Ethoxymethylene-ATP (ATP-EM) was the most potent inhibitor from these compounds which contain the blocked *cis*-diol grouping on the ribose. This paper describes the mechanism of the inhibitory effect of ATP-EM on the activity of hepatic adenylate cyclase.

2. Materials and methods

2′,3′*O*-Ethoxymethylene-ATP (ATP-EM) and 2′,3′-*O*-Isopropylidene-ATP (ATP-IPI) were synthesized as described previously [1]. Guanylylmidodiphosphate (Gpp(NH)p) was a product of Böhringer, glucagon was supplied by Eli Lilly, neutral aluminium oxide for chromatographic adsorption analysis according to Brockmann (act. II) was a product of Reanal, Hungary, and [α-^{32}P] ATP, trisodium salt (3–10 Ci/mmol) was supplied by the Radiochemical Centre, Amersham. Other chemicals used were from the sources described earlier [2].

The enzyme preparation for adenylate cyclase activity determination was made by a mild homogenization of 0.5 g rat liver in 5 ml ice-cold 75 mM Tris–HCl buffer, pH 7.8, with 26 mM $MgSO_4$ and 1 mM EDTA and subsequent centrifugation of the sample for 2 min at 900 × *g*; the sediment fraction was suspended in 5–10 ml of the same homogenization medium and it was considered as a crude plasma membranes fraction. A 20 μl fraction, corresponding to 40–80 μg protein [3], was used for adenylate cyclase assay. In experiments with preincubation, 1 ml plasma membrane fraction was incubated with additions for 10 min at 37°C. Then the samples were cooled, centrifuged at 0–4°C for 5 min at 900 × *g*, resuspended in 1 ml of the above-described homogenization medium, and the whole procedure repeated three times. Volumes (20 μl) of this washed plasma membranes fraction from samples after preincubation were again used for adenylate cyclase assay.

The activity of adenylate cyclase was determined according to Ramachandran [4] with modifications described earlier [2]. The final composition of the incubation mixture was: 0.1 mM ATP, 30 mM Tris–HCl, pH 7.8, 2 mM cyclic AMP, 10.4 mM Mg^{2+}, 1 mM K^+, 0.4 mM EDTA, 5 mM phosphoenolpyruvate, phosphoenol pyruvate kinase 40 μg/ml and myokinase 20 μg/ml. The ^{32}P radioactivity was measured by Cerenkov's radiation [5]. The results are presented in pico- or nano moles of cyclic AMP produced by 1 mg enzyme protein/10 min incubation. Given values are mean ± SE of two or three experiments.

3. Results

The inhibitory effect of ATP-EM was tested on the basal activity of hepatic adenylate cyclase and the activity of the same enzyme stimulated by glucagon or Gpp(NH)p or sodium fluoride. The results in table1 show that percentage inhibition of adenylate cyclase activity by increasing concentrations of ATP-EM (0.03–1.0 mM) was the same regardless to the enzyme activity and to the type of the stimulatory agent used.

Figure 1 shows that the nonproductive substrate analogue ATP-EM which cannot be converted to cyclic AMP by adenylate cyclase competitively inhibits the interaction of ATP with the enzyme stimulated by glucagon. In the presence of 10.4 mM Mg^{2+} and 0.4 mM EDTA, linear double reciprocal plots were obtained at pH 7.8. The kinetic constants are summarized in the inserted table. The inhibitory effects of ATP-EM are purely competitive at 32 μM and 100 μM concentrations while at 320 μM concentration the effects appear to be of noncompetitive type of inhibition as it is seen from the drop of $V_{max.}$ The K_i for competitive antagonism is 25–30 μM; thus K_i is only 1/4–1/3 of K_m for ATP. These data indicate that the affinity of the active site for ATP-EM is greater than that for the natural substrate.

The aim of the following experiments was to determine whether ATP-EM inhibits the activation process of adenylate cyclase or whether this drug can antagonize the activity of the enzyme in the already active state. Following the pretreatment with a combination of Gpp(NH)p and glucagon, and extensive washing of hepatic membranes, the enzyme displayed 5–7-times higher activity than the enzyme preincubated in the absence of these stimulatory drugs. The latter enzyme could be activated by the addition of glucagon or Gpp(NH)p or sodium fluoride to the assay system. However, this stimulated activity did not reach the values of activity of the enzyme preincubated in the presence of Gpp(NH)p plus glucagon. The enzyme in a persistent active state [6] could not be further activated by the addition of glucagon or Gpp(NH)p or sodium fluoride to the assay system. On the contrary the addition of sodium fluoride partially reduced the enzyme activity (table 2).

Figure 2 compares the inhibitory effect of ATP-EM with that of another nonproductive substrate ATP-IPI on cyclic AMP production by hepatic adenylate cyclase preparations in a persistent active state elicited by the combined actions of Gpp(NH)p and glucagon. The inhibitory effect of ATP-EM was stronger than the effect of ATP-IPI.

The action of ATP-EM on the activation process of the combination of Gpp(NH)p and glucagon during the time of enzyme preincubation was tested in order to determine whether the inhibititory effects of this nonproductive substrate are confined to the binding site of ATP at the catalytic unit of adenylate cyclase or whether this drug also acts on the nucleotide regulatory site [7]. ATP-EM added to the adenylate cyclase

Table 1
Inhibitory effect of ATP-EM on basal activity of hepatic adenylate cyclase and activity stimulated by glucagon or Gpp(NH)p or sodium fluoride

ATP-EM (μM)	Adenylate cyclase activity (%)			
	None[a]	Glucagon[b] (1 μM)	Gpp(NH)p[c] (0.1 mM)	NaF[d] (10 mM)
None	100	100	100	100
32	61 ± 6	59 ± 6	60 ± 1	59 ± 1
100	35 ± 2	37 ± 1	34 ± 2	35 ± 1
320	13 ± 4	9 ± 1	13 ± 0	13 ± 2
1000	4 ± 2	2 ± 0	4 ± 1	3 ± 0

[a–d]pmol cyclic AMP formed/mg protein/10 min
[a] 61 ± 11
[b]300 ± 42
[c]334 ± 2
[d]432 ± 11

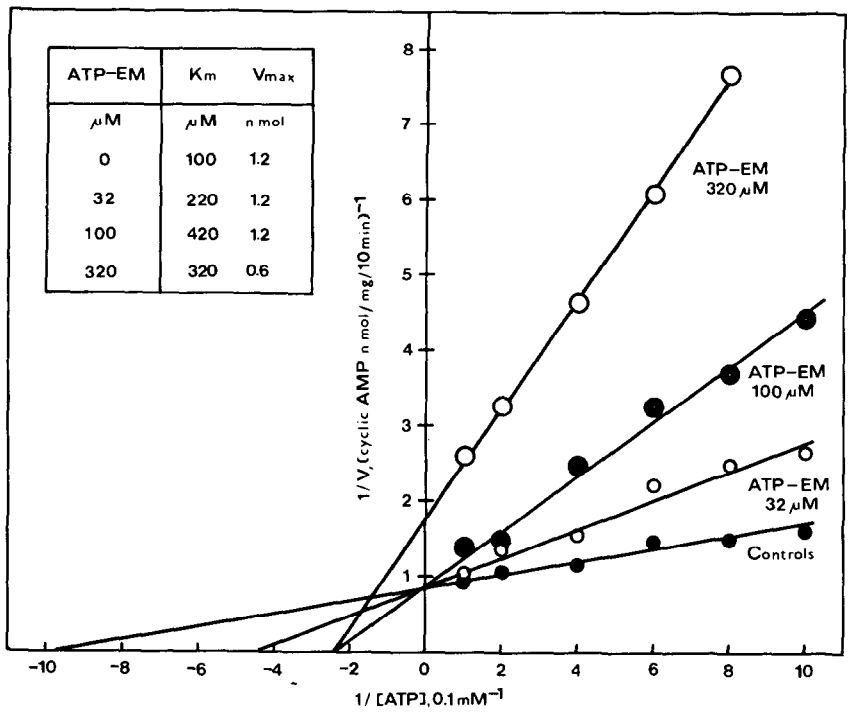

Fig.1. Double reciprocal plots of activity versus substrate (ATP) for hepatic adenylate cyclase stimulated by 1 μM glucagon; inhibitor ATP-EM was used in concentrations 32 μM, 100 μM and 320 μM. K_m and V_{max} are given in the inserted table.

preparations during the preincubation without Gpp(NH)p did not lead to the activation of the enzyme (results not shown). The results in table 2 show that the production of the persistent active state due to a combined actions of Gpp(NH)p and glucagon was not reduced by the addition of ATP-EM during the time of preincubation. These results indicate that the inhibitory effects of ATP-EM on adenylate cyclase are not connected with its action on the nucleotide regulatory site of the enzyme.

4. Discussion

The strong inhibitory effect of ATP-EM and other adenine derivatives with blocked *cis*-diol grouping on

Table 2
The effect of ATP-EM on the activation of hepatic adenylate cyclase by combined actions of Gpp(NH)p and glucagon during the preincubation of the enzyme

Adenylate cyclase assay additions	Preincubation additions		
	None	Gpp(NH)p (0.1 mM) + Glucagon (1 μM)	Gpp(NH)p (0.1 mM) + Glucagon (1 μM) + ATP-EM (1 mM)
Basal[a]	77 ± 16	412 ± 12	502 ± 12
Glucagon (1 μM)[b]	165 ± 18	397 ± 26	458 ± 10
Gpp(NH)p (0.1 mM)[c]	247 ± 3	413 ± 5	515 ± 25
NaF (10 mM)[d]	163 ± 6	349 ± 3	398 ± 10

[a–d]pmol cyclic AMP formed/mg protein/10 min

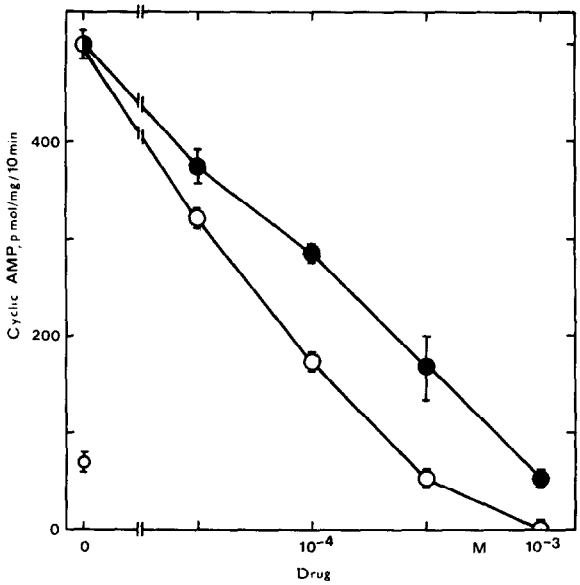

Fig.2. The inhibitory effect of ATP-EM and ATP-IPI on the persistent active state of hepatic adenylate cyclase due to a combined effect of 1 μM glucagon and 0.1 mM Gpp(NH)p during 10 min preincubation (•); preincubation without additions (♀); inhibitory drugs ATP-EM (□) and ATP-IPI (•) were added to the adenylate cyclase assay system.

the ribose on the activity of hepatic adenylate cyclase was described in the previous paper [1]. This inhibitory effect can be demonstrated both on the basal adenylate cyclase activity and on the enzyme activity stimulated by glucagon or Gpp(NH)p or sodium fluoride. These results indicate that the site of action of these inhibitory drugs is distal to the site of action of the stimulation agent, i.e, most probably at the binding site for ATP on the catalytic unit of adenylate cyclase. Direct evidence for this hypothesis is presented in this paper.

Quantitative analysis of the inhibitory effects of ATP-EM on basal activity of hepatic adenylate cyclase as well as on enzyme activity stimulated by glucagon or Gpp(NH)p or sodium fluoride revealed the equal percentage-inhibition of all these states of adenylate cyclase activity (table 1). Further indication for the action of ATP-EM at the active site of the catalytic unit of adenylate cyclase stems from the kinetic studies with glucagon-stimulated hepatic adenylate cyclase (fig.1). It is as evident from the data of these experiments, presented as double reciprocal plots of activity versus substrate, that ATP-EM in 32 μM and 100 μM concentrations revealed pure competitive antagonistic effects with the action of ATP, while at 320 μM concentrations the type of inhibition was noncompetitive. The affinity of active site for ATP-EM was 3–4 times greater than that for ATP alone.

In the second part of this study we investigated whether ATP-EM possesses, in addition to the competitive inhibitory action on the catalytic site, any effect on the nucleotide regulatory site of the enzyme studied. It is known that ATP, in addition to its effects as substrate, interacts in high concentrations also with the nucleotide regulatory site [7], which preferentially reacts with GTP and its analogues [8]. The absence of any stimulatory (data not shown) or inhibitory effects of ATP-EM on the production of the persistently active state, due to enzyme preincubation with Gpp(NH)p plus glucagon (table 2), may be considered as crucial evidence indicating that the strong inhibitory effects of ATP-EM on adenylate cyclase are not connected with its action on the nucleotide regulatory site.

In summary, it may be concluded that ATP-EM, and probably also other adenosine derivatives with a blocked *cis*-diol grouping on the ribose, like ATP-IPI, possesses a strong competitive inhibitory effect, with respect to ATP, on the catalytic site of adenylate cyclase.

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