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ADENYLATE CYCLASE IN SILKWORM

EFFECTS OF ADENOSINE 3'-PHOSPHATE AND 2'-DEOXYADENOSINE 3'-PHOSPHATE ON THE ENZYME SYSTEM IN FAT BODY

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Summary

Adenosine 3'-phosphate and 2'-deoxyadenosine 3'-phosphate inhibit silkworm fat body adenylate cyclase. The inhibition has a rapid onset, and is dependent on the concentration of Mn^{2+} or Mg^{2+}. The concentrations of 2'-deoxy-3'-AMP required for 50% inhibition (K_i) are 13 μM with 2 mM Mn^{2+} and 32 μM with 10 mM Mg^{2+}. These K_i values are 7—30 times lower than that for 2'-deoxyadenosine. Stimulation of adenylate cyclase by NaF renders the activity more sensitive to the nucleotide inhibition, reducing the K_i value to 4 μM in the presence of Mn^{2+}. The inhibitory activity is specific for adenine 3'-nucleotide; K_i for 2'-AMP and 5'-AMP are ten times or more higher than that for 3'-AMP, and the other 3'-nucleotides including 8-bromo-3'-AMP, 3'-IMP and 3'-GMP have little or no inhibitory activity.

Introduction

The inhibition by adenosine of adenylate cyclase (ATP pyrophosphate-lyase (cyclizing), EC 4.6.1.1) in silkworm pupal fat body has been previously studied [1]. The adenosine inhibitory site of the adenylate cyclase had stringent requirement for the purine ring, and was characterized as similar to the 'P' site defined by Londos and Wolff [2]. During the course of inhibition study, the 3'-phosphorylated derivative of adenosine, 3'-AMP*, and 2'-deoxy-3'-AMP as

Abbreviations: 3'-AMP, adenosine 3'-phosphate; 2'-deoxy-3'-AMP, 2'-deoxyadenosine 3'-phosphate; 8-Br-3'-AMP, 8-bromoadenosine 3'-phosphate.

well, were found to be more potent inhibitors of silkworm adenylate cyclase than the original nucleoside. The deoxy 3′-nucleotide has been reported to inhibit adenylate cyclase of rat fat cell [3] and beef thyroid [4]. The deoxynucleotide was also isolated from toad erythrocytes as a natural inhibitor for rat fat cell adenylate cyclase [5]. This report describes the characterization of the 3′-nucleotide inhibition of silkworm fat body adenylate cyclase, including kinetics and specificity of the inhibitory action of the nucleotide.

Materials and Methods

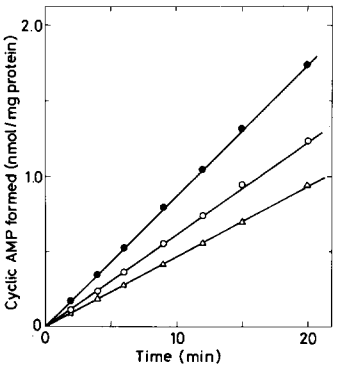
ATP, cyclic AMP, adenosine 3′,5′-diposphate, 2′-AMP and 3′-nucleotides were purchased from Sigma Chemicals Co. 8-Br-3′-AMP was a generous gift from Dr. M. Ikehara of Osaka University (Osaka, Japan). Labeled compound and other chemicals and reagents were as previously described [6]. Silkworms (*Bombyx mori*) were reared as previously described [7], and young male pupae were used throughout the experiments. Fat bodies were removed and homogenized as described [7]. The homogenate was centrifuged at $1000 \times g$ for 20 min and the resulting particulate fraction was resuspended in 0.05 M Tris-HCl, pH 7.2, and immediately used for the assay of adenylate cyclase activity. The assay medium contained 40 mM Tris-HCl, pH 7.2, 0.2 mM [^{3}H]ATP (50—60 cpm/pmol), 2 mM 1-methyl-3-isobutylxanthine, 10 mM creatine phosphate, 3 units of creatine phosphokinase, 0.08% bovine serum albumin, $MgCl_2$ or $MnCl_2$ as described and 100—200 μg protein of the particulate fraction in a total volume of 100 μl. Reactions were started with addition of the particulate fraction and carried out for 15 min at 30°C. The [^{3}H]cyclic AMP formed was isolated, and the radioactivity counted as previously described [6]. Values presented are means of duplicate incubations form representative experiments unless otherwise noted. Protein was determined by the method of Lowry et al. [8] with bovine serum albumin as standard.

Results

Inhibition of adenylate cyclase by 3′-AMP and 2′-deoxy-3′-AMP

Effects of the 3′-nucleotides on the time course of adenylate cyclase activity of silkworm fat body are shown in Fig. 1. The activity without added nucleotide was proportional to the incubation time at least for 20 min as was seen in previous experiments [1,6]. Addition of 3′-AMP or 2′-deoxy-3′-AMP at 10 μM caused a marked inhibition without any effect on the linearity of reaction, indicating no lag for the inhibitory effect. As 2′-deoxy-3′-AMP was more potent than 3′-AMP, the deoxynucleotide was preferred for subsequent experiments.

Fig. 2 shows the dose effect of 2′-deoxy-3′-AMP on basal and NaF-stimulated activities of fat body adenylate cyclase in the presence of 10 mM Mg^{2+} or 2 mM Mn^{2+}. Increasing concentrations of 2′-deoxy-3′-AMP over the range of 0.5 μM to 0.5 mM caused a typical dose-dependent inhibition of the activity. With either Mn^{2+} or Mg^{2+}, NaF stimulated the activity 2—3-fold and produced a shift in inhibitory potency of the nucleotide (Fig. 2); the fluoride lowered the concentration of 2′-deoxy-3′-AMP required for 50% inhibition (K_i)



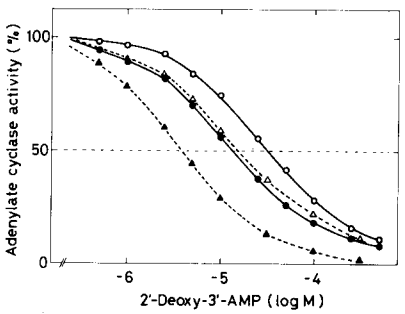


Fig. 1. Time course of inhibition of adenylate cyclase activity by 3'-nucleotides. Particulate fractions of silkworm fat body (150 μg protein/assay) were incubated for the indicated times with 2 mM Mn^{2+} in the absence (●———●) or presence of 10 μM 3'-AMP (○———○) or 10 μM 2'-deoxy-3'-AMP (△———△).

Fig. 2. Adenylate cyclase activity as a function of 2'-deoxy-3'-AMP concentration. Particulate fractions of fat body were incubated under the standard conditions with 2'-deoxy-3'-AMP of the indicated concentrations and either 2 mM Mn^{2+} (●, ▲) or 10 mM Mg^{2+} (○, △) in the absence (○———○, ●———●) or presence (△- - - - -△, ▲- - - - - -▲) of 5 mM NaF. Activity is expressed as the percent of activity in the absence of the inhibitor at each of the assay conditions. These activities were 53.1, 118, 101 and 243 pmol cyclic AMP per mg protein for Mg^{2+}, Mg^{2+} plus NaF, Mn^{2+} and Mn^{2+} plus NaF, respectively. Protein was 130 μg (●, ▲) and 97 μg (○, △) per assay.

from 13 to 4 μM with 2 mM Mn^{2+} and from 32 to 15 μM with 10 mM Mg^{2+}. Note that significant inhibition was seen even with nucleotide concentrations below 1 μM.

Effect of divalent cations on inhibition by 2'-deoxy-3'-AMP

Fat body adenylate cyclase activity with or without added 2'-deoxy-3'-AMP is shown as a function of free Mg^{2+} and Mn^{2+} concentrations in Figs. 3 and 4, respectively. The concentrations of free Mg^{2+} and Mn^{2+} were calculated by assuming stability constants for MgATP and MnATP as $1.66 \cdot 10^4$ and $6.03 \cdot 10^4$ M^{-1}, respectively [9]. On increasing metal concentrations, adenylate cyclase activity without added inhibitor increased and saturated at approx. 20 mM Mg^{2+} or 1 mM Mn^{2+} (Figs. 3A and 4A). The addition of 2'-deoxy-3'-AMP caused reduction of saturating concentration of metal ion, i.e., with 20 μM and 50 μM of the nucleotide, saturating concentrations of Mg^{2+} were reduced to 10 mM and 5 mM, respectively, and the higher Mg^{2+} concentrations appeared to be inhibitory. This was more evident with increasing concentrations of Mn^{2+} (Fig. 4A). In Figs. 3B and 4B, the percentage inhibition of adenylate cyclase activity by 2'-deoxy-3'-AMP is plotted as a function of free Mg^{2+} and Mn^{2+}, respectively. Both Mg^{2+} and Mn^{2+} increased the sensitivity of the enzyme to the nucleotide inhibition, Mn^{2+} was more potent than Mg^{2+}.

The Hill coefficients of adenylate cyclase activity with respect to Mg^{2+} and Mn^{2+} obtained by replots (not shown) of the data in Figs. 3A and 4A, respectively, were nearly 1 for both cations in the presence and absence of the inhibitor. The finding suggests that the cyclase exhibits no apparent cooperativity with respect to either Mg^{2+} or Mn^{2+}, and that the 3'-nucleotide does not affect this behavior.

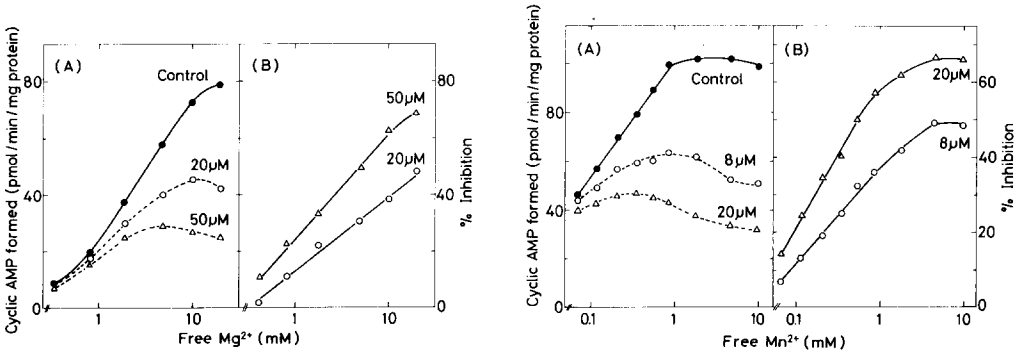


Fig. 3. A. Effect of Mg^{2+} on the activity of silkworm fat body adenylate cyclase. Particulate fractions of fat body (81 μg protein per assay) were incubated with various concentrations of Mg^{2+} and 0 (●———●), 20 (○- - - - -○) or 50 (△- - - - -△) μM of 2′-deoxy-3′-AMP under the standard conditions. B. Effect of Mg^{2+} on the extent of inhibition of adenylate cyclase by 2′-deoxy-3′-AMP. Percent inhibition of adenylate cyclase activity was calculated from the data in A. Concentrations of 2′-deoxy-3′-AMP are shown.

Fig. 4. A. Effect of Mn^{2+} on the activity of silkworm fat body adenylate cyclase. B. Effect of Mn^{2+} on the extent of inhibition of adenylate cyclase by 2′-deoxy-3′-AMP. The assay conditions and the expression of results are as described in the legend to Fig. 3, except Mg^{2+} was replaced by Mn^{2+}. Protein was 140 μg per assay. Concentrations of 2′-deoxy-3′-AMP are shown.

Kinetics of inhibition by 2′-deoxy-3′-AMP

The kinetics of inhibition of adenylate cyclase activity were investigated at various concentrations of MeATP and 2′-deoxy-3′-AMP with free Mn^{2+} or Mg^{2+} of a fixed concentration. Results of a typical experiment conducted with 2 mM free Mn^{2+} are shown in Fig. 5. The double reciprocal plots were linear, the common intercept, however, was not on the abscissa (Fig. 5A). Replots of the data according to Dixon [10] yielded a set of hyperbolae (Fig. 5B). These results indicate that inhibition by the 3′-nucleotide is of the mixed type, i.e., partially competitive and partially non-competitive. The results of experiments

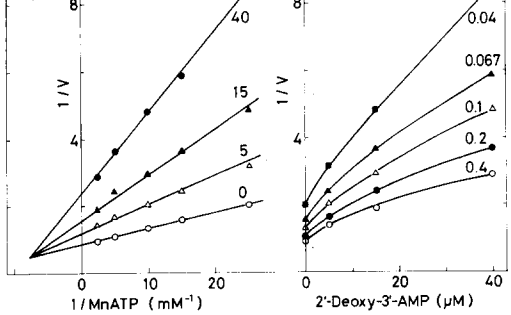


Fig. 5. Kinetics of inhibition of adenylate cyclase activity by 2′-deoxy-3′-AMP. Particulate fractions of silkworm fat body (115 μg protein per assay) were incubated under the standard conditions at the indicated concentrations of MnATP and 2′-deoxy-3′-AMP. The amounts of $MnCl_2$ added to the assay were adjusted so as to maintain a constant concentration of 2.0 mM free Mn^{2+}. A. Double-reciprocal plots. Concentrations (μM) of 2′-deoxy-3′-AMP are shown. Velocities (V) are expressed as pmol cyclic AMP formed per mg protein per min. B. Dixon plots of the data in A. Concentrations (mM) of MnATP are shown.

similarly conducted with 10 mM Mg^{2+} and MgATP were essentially the same as that found with Mn^{2+}. Similar mixed-type inhibition of adenylate cyclase has been previously found with inhibition by adenosine in various tissues [1,4,11—12].

Specificity of inhibition by nucleotides

To examine the specificity of inhibition of adenylate cyclase by nucleotide, effects of various adenine nucleotides and other 3′-nucleotides on adenylate cyclase were determined in the presence of 2 mM Mn^{2+} or 10 mM Mg^{2+} (Table I). Among the nucleotides tested, 2′-deoxy-3′-AMP was the most potent, and K_i value was found to be 13 μM with 2 mM Mn^{2+} and 32 μM with 10 mM Mg^{2+}. Adenosine 3′-phosphate was somewhat less potent than the deoxy-nucleotide. The nucleosides and 2′- and 5′-nucleotides were one order (or more) less potent than the corresponding 3′-nucleotide. Introduction of another phosphate group to the 5′-position of 3′-AMP greatly diminished the inhibitory potency. The order of potencies of adenine nucleotides was 2′-deoxy-3′-AMP > 3′-AMP >> 2′-AMP > adenosine 3′,5′-diphosphate > 2′-deoxy-5′-AMP > 5′-AMP >> cyclic AMP. As evident in the case of 8-Br-3′ AMP and 3′-IMP, modification of the purine ring virtually abolished the inhibitory potency, and the other purine and pyrimidine 3′-nucleotides were inactive up to 0.5 mM. Cyclic AMP was weakly active, at 1 mM it showed 5—10% inhibition, but no inhibition at 0.5 mM. For all of the nucleotides and nucleo-

TABLE I

EFFECTS OF VARIOUS NUCLEOTIDES ON ADENYLATE CYCLASE ACTIVITY

Particulate fractions of silkworm fat body were incubated under the standard conditions with either 2 mM Mn^{2+} or 10 mM Mg^{2+}. Values listed were the means of that obtained from two or three independent experiments, which were determined by concentration curves similar in form to that in Fig. 2. N.I. indicates that no inhibition was observed at 0.5 mM.

Compound	Concentration for 50% inhibition of activity (K_i)	
	2 mM Mn^{2+} (μM)	10 mM Mg^{2+} (μM)
3′-AMP	32	150
2′-Deoxy-3′-AMP	13	32
2′-AMP	270	1000
5′-AMP	800	1400
2′-Deoxy-5′-AMP	600	3000
8-Br-3′-AMP	>1000 *	>1000 **
Adenosine 3′,5′-diphosphate	320	>1000 ***
3′,5′-cyclic AMP	N.I.	N.I.
3′-IMP	N.I.	N.I.
3′-GMP	N.I.	N.I.
3′-CMP	N.I.	N.I.
Adenosine	180	2500
2′-Deoxyadenosine	95	1000

Inhibition at 1.0 mM:
* 33%.
** 18%.
*** 37%.

sides having inhibitory potency, Mn^{2+} was more potent than Mg^{2+} in increasing the inhibitory activity.

Discussion

The data in this report show that 3′-AMP and 2′-deoxy-3′-AMP are potent inhibitors for silkworm fat body adenylate cyclase. Minor modification in the purine ring of the nucleotide results in a loss of inhibitory potency, indicating that the nucleotide inhibitory site of adenylate cyclase has strict structural requirements in the purine ring. This inhibitory site also has selective preference for 3′-nucleotide; i.e., the 2′-nucleotide is 10-times less potent, the 5′-nucleotide and 3′,5′-cyclic nucleotide much less potent.

Silkworm fat body adenylate cyclase has an adenosine inhibitory site which has strict specificity for the purine ring [1]. The characteristics of the nucleotide inhibition of silkworm adenylate cyclase show some resemblance to that of the adenosine inhibition [1]: i.e., (1) stringent requirements for adenine ring, (2) greater inhibitory potency of the 2′-deoxy derivatives than the native compounds, (3) cation dependency of the inhibition, (4) kinetic properties of the inhibition and (5) greater sensitivity of the NaF-stimulated activity to the inhibition than the basal activity. The results suggest the possibility that both the 3′-nucleotide and adenosine may act on the same site of silkworm adenylate cyclase which was previously characterized as the adenosine inhibitory site [1], but the site originally had a preference for the 3′-nucleotide rather than the nucleoside. Recently, 2′-deoxy-3′-AMP has been described to inhibit beef thyroid adenylate cyclase [4]. The K_i value (70 μM) in the presence of Mn^{2+} is, however, similar to that for 2′-deoxyadenosine (95 μM), and no preference for 3′-nucleotide is observed.

Sahyoun et al. [3] have described the inhibition of rat fat cell adenylate cyclase by 2′-deoxy-3′-AMP, and they characterized in some detail the nature of the inhibition by the deoxynucleotide. The inhibition is specific for 2′-deoxy-3′-AMP, and the K_i value (10 μM) is similar to that found with silkworm adenylate cyclase. Some of their results, however, are incosistent with our findings with silkworm adenylate cyclase: (1) while the inhibition of rat adenylate cyclase is non-competitive with respect to metal ATP, the inhibition of the silkworm enzyme is of a mixed type; (2) in rat fat cell, the deoxynucleotide causes a change in the Hill coefficients with respect to Mg^{2+}-activation of adenylate cyclase from 0.9 in the absence of the inhibitor to 0.5 in its presence, in silkworm preparations, however, no such a change is induced and the Hill coefficients are nearly 1 regardless of the presence of the deoxynucleotide; (3) while stimulation of rat adenylate cyclase by NaF has no effect on the inhibition by 2′-deoxy-3′-AMP, stimulation of the silkworm enzyme by NaF renders the activity more sensitive to the nucleotide inhibition; (4) the inhibition of the rat enzyme is quite specific for 2′-deoxy-3′-AMP, and 3′-AMP is only a weak inhibitor as is 5′-AMP or 2′-deoxyadenosine. For silkworm adenylate cyclase, in contrast, 3′-AMP appears to be a considerably strong inhibitor as is 2′-deoxy-3′-AMP, a remarkable difference in the inhibitory potency being observed between 3′-AMP and 2′- or 5′-AMP. These apparent inconsistencies can be explained by the differences in the properties of

adenylate cyclase systems, although they need to be investigated for more detail.

Sahyoun et al. [5] also isolated a naturally occurring inhibitor of adenylate cyclase from toad erythrocytes, and identified it as 2′-deoxy-3′-AMP. They found the inhibitor in various rat tissues as well. Together with their findings, the high sensitivity of silkworm adenylate cyclase to the 3′-nucleotide inhibition may support the idea that 2′-deoxy-3′-AMP or 3′-AMP could play an important role in the regulation of intracellular metabolism through the action on the adenylate cyclase system in the insect tissues, as suggested by Sahyoun et al. [3] for rat tissues. At the present time, however, it is uncertain whether the 3′-nucleotide is present in silkworm tissues at effective concentration, although our preliminary experiments show the presence of heat-stable, low molecular weight inhibitor of adenylate cyclase in silkworm fat body and hemolymph. The isolation and characterization of the inhibitor are currently under investigation.

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