ACYLPEPTIDES, THE INHIBITORS OF CYCLIC ADENOSINE 3′,5′-MONOPHOSPHATE PHOSPHODIESTERASE

III. INHIBITION OF CYCLIC AMP PHOSPHODIESTERASE

KUNIAKI HOSONO and HIDEO SUZUKI

Fermentation Research Institute, Agency of Industrial Science & Technology
Yatabe machi, Ibaraki, Japan

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Acylpeptides, APD-I, -II and -III, were inhibitors of cyclic adenosine 3′,5′-monophosphate (cAMP) phosphodiesterase, and their inhibition types were non-competitive. The inhibitory activity of APD-II was the most potent among them. Opening of the lactone linkage reduced the inhibitory activity to about half. The activity almost disappeared when an inhibitor or a derivative with opened lactone linkage was methylated with diazomethane. The activity was, however, restored by the addition of metal ions such as Ca^{2+}, Mn^{2+}, Fe^{2+}, and Co^{2+}. This suggests that the inhibition may be caused by a chelating action of the free carboxyl groups of glutamic acid and aspartic acid in the peptide.

As reported in previous papers[1~3], three kinds of cAMP phosphodiesterase inhibitors, APD-I, -II and -III, were isolated from the culture filtrate of *Bacillus subtilis* C-756. They were acylpeptides with differing β-hydroxy fatty acid residues, though the amino acid sequence of the heptapeptide and the location of lactone linkage were the same. Papaverine, methylxanthines such as caffeine and theophylline, reticulol[4], PDE-I and PDE-II[5] have been reported as inhibitors of cAMP phosphodiesterase. However, APD-I, -II and -III are acylpeptides and have no structural resemblance to the substrate.

This paper reports the relation between inhibitory activity and structure of the inhibitors.

Materials and Methods

Microorganism

Bacillus subtilis C-756 isolated from soil[6] was used.

Production, Isolation and Purification

The inhibitors, APD-I, -II and -III, were prepared as described in a previous paper[2].

Assay for cAMP Phosphodiesterase and Inhibitory Activity

The reaction mixture (total 1.0 ml) consisted of 40 mM tris-HCl (pH 7.5), 2 mM $MgSO_4 \cdot 7H_2O$, 0.2 mM cAMP, cAMP phosphodiesterase (*ca.* 140 μg-protein), alkaline phosphatase (*ca.* 70 μg-protein) and an inhibitor. The enzyme reaction was started by addition of cAMP phosphodiesterase and, following incubation at 38°C for 20 minutes, was stopped by addition of 5% trichloroacetic acid. The amount of inorganic phosphorus liberated from cAMP was colorimetrically measured by the ascorbic acid method[7]. Namely, to this mixture was added 1.0 ml of coloring reagent consisting of 1 volume of 6 N H_2SO_4, 2 volumes of H_2O, 1 volume of 2.5% ammonium molybdate and 1 volume of 10% ascorbic acid and left to incubate at 38°C for 90 minutes. The liberated phosphorus was measured by the absorbance at 820 nm. The activity of cAMP phosphodiesterase is proportional to the amount of liberated phosphorus under this condition.

The percent inhibition was calculated by the formula $(A-B)/A \times 100$, where in A is the phosphorus amount without an inhibitor and B is the amount with an inhibitor.

Derivation of the Inhibitor

To open the lactone linkage, the inhibitor was dissolved in 0.5 N NaOH and was allowed to stand for 1 hour at room temperature. The derivative was extracted with ethyl acetate at pH 2 and concentrated under reduced pressure.

To obtain the methylated derivatives, the inhibitor and the opened derivative were methylated with diazomethane.

Effect of Metal Ions

A 2 mM metal solution, namely, one of $CaCl_2 \cdot 2H_2O$, $MnCl_2 \cdot 4H_2O$, $FeSO_4 \cdot 7H_2O$, $FeCl_3 \cdot 6H_2O$, $CoCl_2 \cdot 6H_2O$, $NiCl_2 \cdot 6H_2O$, $CuSO_4 \cdot 5H_2O$ or $ZnSO_4 \cdot 7H_2O$, was added to the reaction mixture instead of 2 mM $MgSO_4 \cdot 7H_2O$, and the effect of the metal ion was examined.

To investigate the restoration of inhibitory activity which occurred with the addition of one of Mg^{2+}, Ca^{2+}, Mn^{2+}, Fe^{3+}, Co^{2+} or Ni^{2+}, a 5 mM metal solution was added to the enzyme system which had been adjusted to about 50% inhibition by the inhibitor or the opened derivative.

Chemicals

Alkaline phosphatase (Grade II) and cAMP phosphodiesterase [EC 3. 1. 4. 17] were obtained from Boehringer Mannheim GmbH, and ethylenediaminetetraacetic acid (EDTA) and ethylene glycol-bis(2-aminoethylether)-*N,N,N',N'*-tetraacetic acid (EGTA) from Dojindo Laboratories (Kumamoto, Japan). All the other chemicals were obtained from commercial sources.

Results and Discussion

Calculating from constitutive fatty acids and amino acids, the molecular weights of APD-I, -II and -III were 1,008, 1,022 and 1,036, respectively. As theophylline and papaverine are well-known inhibitors of cAMP phosphodiesterase, the concentrations of APD-I, -II and -III required for 50% inhibition (IC_{50}) in our assay system were compared with those of theophylline and papaverine·HCl. The results are shown in Table 1. The inhibitory activity of our inhibitors was slightly superior to that of papaverine, and the activity of APD-II was the most potent. Besides theophylline and papaverine, reticulol, PDE-I and PDE-II have been reported as inhibitors. Our inhibitors had potent inhibitory activities comparable to those of the reported inhibitors, because the concentrations required for 50% inhibition by reticulol, PDE-I and PDE-II have been shown to be almost equal to that of papaverine.

Table 1. IC_{50} for cAMP phosphodiesterase.

Inhibitor	IC_{50}
APD-I	8.5×10^{-5} M
APD-II	4.5×10^{-5} M
APD-III	8.1×10^{-5} M
Theophylline	2.8×10^{-3} M
Papaverine	1.1×10^{-4} M

IC_{50}: Inhibitor concentration in the reaction mixture for 50% inhibition. Substrate: 200 μM cAMP. Enzyme: 140 μg-protein cAMP phosphodiesterase.

In the assay system, the activity of cAMP phosphodiesterase was determined by measuring the amount of inorganic phosphorus liberated from cAMP (cAMP→adenosine 5'-monophosphate→adenosine+Pi). To convert adenosine 5'-monophosphate into adenosine and inorganic phosphorus, alkaline phosphatase was also used as an enzyme but the inhibitor did not affect the activity of alkaline phosphatase.

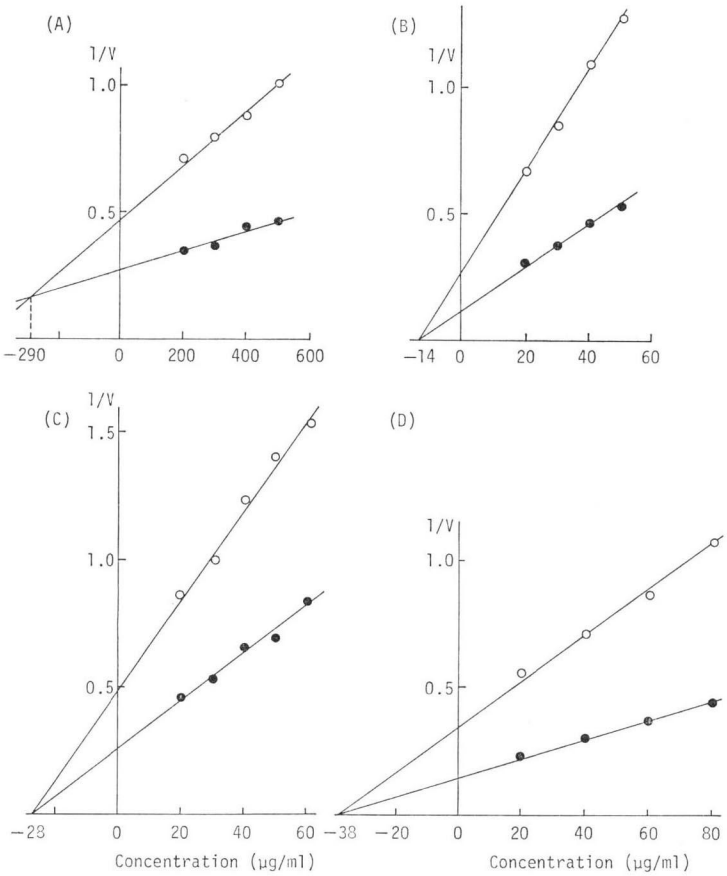
The inhibitor was an acylpeptide and seemed to have no structural resemblance with the substrate, cAMP. To clarify the relation between structure and inhibitory activity, the type of inhibition was examined. The results of theophylline, papaverine, APD-II and -III are shown by the DIXON plots (1/V to inhibitor concentration) in Fig. 1 (A~D). Theophylline is one of methylxanthines and its inhibition was competitive against cAMP but the inhibition by papaverine was non-competitive. The

Fig. 1. Inhibition of cAMP phosphodiesterase by theophylline, papaverine, APD-II and APD-III (DIXON plots).

The reaction mixture was as shown in the Materials and Methods. (A): Theophylline, (B): Papaverine, (C): APD-II, (D): APD-III. Enzyme: 140 μg-protein cAMP phosphodiesterase.

V: μg-liberated phosphorus/minute/mg protein.

Substrate concentration (cAMP): 100 μM (○), 200 μM (●).



Ki values of theophylline and papaverine were 1.6×10^{-3} M and 3.7×10^{-5} M, respectively. Under the same condition, the inhibitions by APD-II and -III were both non-competitive, and their *Ki* values were 2.7×10^{-5} M and 3.7×10^{-5} M, respectively.

The inhibitor molecule has a lactone linkage between carboxyl group of *C*-terminal leucine and β-hydroxyl group of fatty acid moiety, and free carboxyl groups of glutamic acid and aspartic acid. So it was presumed that the inhibition might be caused by a lactone linkage and/or free carboxyl groups. To clarify the role of the lactone linkage, the activity of the inhibitor was examined after it had been treated with alkali to open the linkage. The activity of derivatives with an opened lactone linkage was half or less than the parents. The presence of a lactone linkage was therefore not absolutely necessary to inhibit the reaction of cAMP phosphodiesterase. The concentrations required for 50% inhibition (IC_{50}) are shown in Table 2, compared with theophylline and papaverine.

To explain the role of free carboxyl groups, the inhibitor and the derivative with an opened lactone linkages were esterified to methyl esters with diazomethane. When the free carboxyl group was changed

Table 2. Effect of lactone linkage on the inhibition of cAMP phosphodiesterase.

Material	IC_{50} (μg/ml)
APD-II	43
APD-II opened	132
APD-III	78
APD-III opened	125
Theophylline	580
Papaverine	48

Conditions were the same as those of Table 1.

Table 3. Effect of free carboxyl group on the inhibition of cAMP phosphodiesterase.

Material	IC_{50} (μg/ml)
APD-II	43
APD-II methylated	>1,000
APD-II opened and methylated	>1,000
APD-III	78
APD-III methylated	>1,000
APD-III opened and methylated	>1,000

Conditions were the same as those of Table 1.

to ester, the activity almost disappeared, as shown in Table 3. These results show that the presence of free carboxyl groups is indispensable to inhibition of the enzyme.

It is well known that a free carboxyl group acts as a chelating agent to bind a metal ion. The inhibitor has two carboxyl groups per molecule and may pull out metal ions required for enzyme reaction. To examine the effect of metal ions, ethylenediaminetetraacetic acid (EDTA) and ethylene glycol-bis-(2-aminoethylether)-*N,N,N',N'*-tetraacetic acid (EGTA) were added to this system and their actions examined. The concentrations required for inhibition differed greatly, but these chelators inhibited the activity of cAMP phosphodiesterase as shown in Fig. 2. These results seem to show that some metal ions are necessary to enzyme reaction. Therefore one of eight metal ions, Ca^{2+}, Mn^{2+}, Fe^{2+}, Fe^{3+}, Co^{2+}, Ni^{2+}, Cu^{2+} and Zn^{2+}, which were thought to be related to the enzyme reaction, was added instead of magnesium. Though metal ions of Fe^{2+}, Cu^{2+} and Zn^{2+} inhibited the activity of cAMP phosphodiesterase, the other metal ions showed almost the same effect as Mg^{2+}. The activity of cAMP phosphodiesterase was little affected by omission of 2 mM $MgSO_4 \cdot 7H_2O$ from the reaction mixture. These results are shown in Table 4. Furthermore to investigate the chelating action, one of six metals, namely Mg^{2+}, Ca^{2+}, Mn^{2+}, Fe^{3+}, Co^{2+} and Ni^{2+}, was added to the enzyme system which had been adjusted to about 50% inhibition by the inhibitor or the opened derivative. Addition of Ca^{2+}, Mn^{2+}, Fe^{3+} and Co^{2+} restored the inhibitory activity, and the metals of Mn^{2+}, Fe^{3+} and Co^{2+} increased the phosphorus liberated from cAMP. The results are shown in Table 5.

Fig. 2. Inhibition of cAMP phosphodiesterase by EDTA and EGTA.

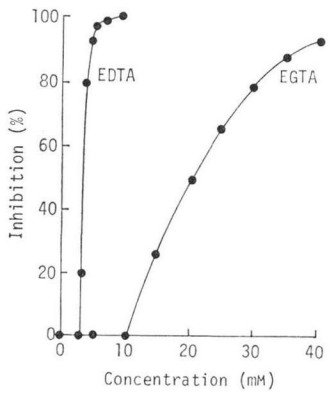


Table 4. Effect of metal ions on the inhibitory activity.

Metal ion	Relative activity of cAMP phosphodiesterase
None	96
Mg^{2+}	100
Ca^{2+}	89
Mn^{2+}	93
Fe^{2+}	46
Fe^{3+}	99
Co^{2+}	101
Ni^{2+}	85
Cu^{2+}	6
Zn^{2+}	5

Mg^{2+} ion contained in the reaction mixture was omitted or replaced by the other metal ions, and the activity was examined. The activity of cAMP phosphodiesterase in the reaction mixture with 2 mM $MgSO_4 \cdot 7H_2O$ was represented as 100.

Table 5. Restoration of the inhibitory activity with addition of metal ions.

Metal ion	Inhibitor			
	APD-II (40 μg/ml)	APD-II opened (120 μg/ml)	APD-III (80 μg/ml)	APD-III opened (120 μg/ml)
None	45	48	49	39
Mg^{2+}	58	55	62	49
Ca^{2+}	87	97	73	83
Mn^{2+}	106	101	101	99
Fe^{3+}	101	103	104	100
Co^{2+}	102	105	108	101
Ni^{2+}	83	57	78	67

The activity of cAMP phosphodiesterase without inhibitors was represented as 100. Metal ions were added to the enzyme system which had been adjusted to about 50% inhibition by addition of inhibitors.

The enzyme cAMP phosphodiesterase is widely distributed in animal tissues and exhibits various properties. However, this enzyme has been generally reported to require magnesium[8]. For full activity, one cAMP phosphodiesterase from rat brain requires Mn^{2+} and Mg^{2+} [9] and another from rat brain requires Ca^{2+} with the presence of calmodulin (calcium dependent modulator protein)[10]. So the active form of this enzyme seems to be a metal-enzyme complex. The commercial enzyme preparations contain slight amounts of $MgSO_4$ and $ZnCl_2$ as stabilizers. Though this must be taken into consideration, the difference in activity between the inhibitor and the opened derivative, and in restoration of inhibitory activity with addition of metal ions seems to derive from the difference in stability constants of metal-inhibitor complexes. Namely the inhibitor pulls out metal ions from a metal-enzyme complex, the active form of this enzyme, and the enzyme action is inhibited. Furthermore the inhibitor is presumed to be combined more tightly with Mn^{2+}, Fe^{3+} and Co^{2+} than with Mg^{2+}, Ca^{2+} and Ni^{2+}.

Finally, it was shown that free carboxyl groups of glutamic acid and aspartic acid were indispensable to the inhibition of cAMP phosphodiesterase.

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