Effects of 8-Substituted Adenosine 3′,5′-Monophosphate Derivatives on High K_m Phosphodiesterase Activity†

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ABSTRACT: Most of twenty-one 8-substituted adenosine 3′,5′-monophosphate derivatives were found to inhibit competitively the hydrolysis of adenosine 3′,5′-monophosphate by partially purified high K_m (Michaelis–Menten constant) phosphodiesterase from hog brain cortex, which had one active site at high concentration of adenosine 3′,5′-monophosphate (0.3 to 4.0 mM). The K_i value for the 8-substituted alkylaminoadenosine 3′,5′-monophosphate derivative was found to decrease with increasing unbranched carbon chain of the substituent, and a minimum value was obtained in the case of 8-octylaminoadenosine 3′,5′-monophosphate. The K_i value, however, increased gradually as the substituent of derivative became longer than that of 8-octylaminoadenosine 3′,5′-monophosphate. The similar phenomenon was observed in the 8-substituted alkylthioadenosine 3′,5′-monophosphate. The standard affinity for adenosine 3′,5′-monophosphate of the high K_m phosphodiesterase was 5.0 kcal/mol, which was calculated from K_m. The standard affinity for 8-hexylthioadenosine 3′,5′-monophosphate, which inhibited most strongly the enzyme activity, was 7.2 kcal/mol. The difference (2.2 kcal/mol) between the standard affinity for adenosine 3′,5′-monophosphate and that for 8-hexylthioadenosine 3′,5′-monophosphate seems to be based on the partial affinity for the substituent (hexylthio group) of the active site on the enzyme or its neighborhood.

A characteristic similar interrelation between substituent length of derivatives and their inhibitory effect on the enzyme activity was observed similarly in two different series of derivatives, 8-substituted alkylaminoadenosine 3′,5′-monophosphate and alkylthioadenosine 3′,5′-monophosphate. The results may indicate the characteristic structure at the active site of the enzyme or its neighborhood.

The intracellular levels of adenosine 3′,5′-monophosphate seem to be controlled by the difference in activities between adenylate cyclase and cyclic AMP[1] phosphodiesterase. Thompson and Appleman have demonstrated that most mammalian tissue contains at least two kinds of phosphodiesterase (1971a,b); one is low in molecular weight with low K_m for cyclic AMP and exists in the particulate fraction, and the other has a high molecular weight with high K_m and exists in the soluble fraction. Beavo et al. (1971) and Jard and Bernard (1970) have also reported similar facts.

This high K_m phosphodiesterase also hydrolyzes another cyclic nucleotide, guanosine 3′,5′-monophosphate. At a high concentration of either of these cyclic nucleotides, the hydrolysis of the other is inhibited noncompetitively but, at low concentration of cyclic GMP or cyclic IMP, the enzyme activity for the hydrolysis of cyclic AMP is enhanced (Beavo et al., 1971; Thompson and Appleman, 1971a,b). The high K_m phosphodiesterase obtained from supernatant fraction seems to be an allosteric enzyme consisting of heterogeneous subunits.

Cultured 3T3 fibroblast cells have been reported to contain both low K_m and high K_m phosphodiesterases (Heidrick and Ryan, 1971; Manganiello and Vanghan, 1972). But SV_{40}-3T3 cells (Armiento and Pastan, 1972) and human tumor (Perkins et al., 1971) have been shown to contain only low K_m phosphodiesterase. However, the function of high K_m phosphodiesterase in regulating intracellular levels of cyclic nucleotide is unknown.

We have, therefore, tried to elucidate the role of high K_m phosphodiesterase in regulating intracellular concentrations of cyclic nucleotides. In this paper, we describe some properties of high K_m phosphodiesterase partially purified from hog brain cortex and report the effects of cyclic AMP derivatives on the enzyme activity, specifically, in the cyclic AMP hydrolysis.

Materials and Methods

Preparation of Cyclic AMP. Cyclic AMP was prepared from the fermentation broth of a mutant derived from *Brevibacterium liquefaciens* (ATCC, 14929). The fermentation broth was centrifuged and the supernatant was applied to the column packed with Amberlite 900 (Cl^- type). Cyclic AMP was eluted as free type with 0.02 N HCl from the column and crystallized at pH 2.0. For the purpose of fur-

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[1] Abbreviations used are: cyclic AMP, adenosine 3′,5′-monophosphate; cyclic GMP, guanosine 3′,5′-monophosphate; cyclic IMP, inosine 3′,5′-monophosphate; 8-Ami-cAMP, 8-aminoadenosine 3′,5′-monophosphate; 8-Met-A-cAMP, 8-methylaminoadenosine 3′,5′-monophosphate; 8-Eth-A-cAMP, 8-ethylaminoadenosine 3′,5′-monophosphate; 8-Pro-A-cAMP, 8-propylaminoadenosine 3′,5′-monophosphate; 8-But-A-cAMP, 8-butylaminoadenosine 3′,5′-monophosphate; 8-Hex-A-cAMP, 8-hexylaminoadenosine 3′,5′-monophosphate; 8-Oct-A-cAMP, 8-octylaminoadenosine 3′,5′-monophosphate; 8-Dec-A-cAMP, 8-decylaminoadenosine 3′,5′-monophosphate; 8-Und-A-cAMP, 8-undecylaminoadenosine 3′,5′-monophosphate; 8-Dod-A-cAMP, 8-dodecylaminoadenosine 3′,5′-monophosphate; 8-Thi-cAMP, 8-thioadenosine 3′,5′-monophosphate; 8-Met-S-cAMP, 8-methylthioadenosine 3′,5′-monophosphate; 8-Eth-S-cAMP, 8-ethylthioadenosine 3′,5′-monophosphate; 8-Pro-S-cAMP, 8-propylthioadenosine 3′,5′-monophosphate; 8-But-S-cAMP, 8-butylthioadenosine 3′,5′-monophosphate; 8-Pen-S-cAMP, 8-pentylthioadenosine 3′,5′-monophosphate; 8-Hex-S-cAMP, 8-hexylthioadenosine 3′,5′-monophosphate; 8-Hep-S-cAMP, 8-heptylthioadenosine 3′,5′-monophosphate; 8-Oct-S-cAMP, 8-octylthioadenosine 3′,5′-monophosphate; 8-Dec-S-cAMP, 8-decylthioadenosine 3′,5′-monophosphate; 8-Dod-S-cAMP, 8-dodecylthioadenosine 3′,5′-monophosphate; 8-Br-cAMP, 8-bromoadenosine 3′,5′-monophosphate; K_m, Michaelis–Menten constant.

ther purification, this cyclic AMP crystallite was dissolved into cold alkaline solution (0.1 N NaOH) and then recrystallized at pH 2.0.

Organic Synthesis of 8-Substituted Cyclic AMP Derivatives. The solvent system used for paper chromatography was: (A) 1-butanol–acetate–water (4:1:5); (B) ethanol–0.5 N ammonium acetate (5:2). Ultraviolet spectra were determined using a Hitachi Perkin-Elmer Model 124 and 139.

The following derivatives were synthesized in the same manner as Muneyama's method (1971): 8-bromoadenosine 3′,5′-monophosphate; 8-aminoadenosine 3′,5′-monophosphate; 8-methylaminoadenosine 3′,5′-monophosphate; 8-ethylaminoadenosine 3′,5′-monophosphate; 8-thioadenosine 3′,5′-monophosphate; 8-methylthioadenosine 3′,5′-monophosphate; and 8-ethylthioadenosine 3′,5′-monophosphate. Muneyama's method with minor modification was also used for the synthesis of new 8-substituted cyclic AMP derivatives.

8-Propylaminoadenosine 3′,5′-Monophosphate. To a mixed solution of *n*-propylamine (25 ml, 0.31 mol) and ethylene glycol monomethyl ether (25 ml) was added 820 mg of 8-Br-cAMP (2 mmol). After the reaction mixture was refluxed at 100 °C for 5 h, the mixture was filtered and evaporated to dryness at below 40 °C. Fifteen milliliters of 1 N ammonia–water was added to the dried matter, this mixture was washed with 100 ml of ethyl acetate twice to remove the residual amine, and the water phase was dripped into 200 ml of cold acetone and adjusted to pH 2.5 with 5 N HCl in an ice–water bath. The obtained 8-Pro-A-cAMP (730 mg, 18.8 mmol) was further recrystallized. The crude crystallite was dissolved into 50 ml of cold 0.25 N NaOH, and then the solution was titrated with 5 N HCl to pH 2.5 slowly and kept in a cold room with stirring overnight to obtain the crystallite of 8-Pro-A-cAMP. The recrystallization for all derivatives was repeated until each derivative indicated one spot in paper chromatographies and electrophoresis. Finally, 535 mg of crystallite 8-Pro-A-cAMP was obtained in a 69% yield: λ_{max} (pH 2.0) 277 nm (ϵ 13 350), λ_{max} (pH 13.0) 277 nm; R_f (solvent A) 0.44, R_f (Solvent B) 0.55. The melting point was 226 °C. Anal. Calcd for $C_{13}H_{19}N_6O_6P_1 \cdot H_2O$: C, 38.61; H, 5.20; N, 20.79; O, 27.72. Found: C, 38.6; H, 5.1; N, 20.0; O, 28.2.

8-Butylaminoadenosine 3′,5′-Monophosphate. 8-But-A-cAMP (583 mg, 1.45 mmol) was synthesized in a 72% yield from 820 mg of 8-Br-cAMP (2 mmol) and 25 ml of *n*-butylamine (258 mmol) in the same manner as in the synthesis of 8-Pro-A-cAMP: λ_{max} (pH 2.0) 277 nm (ϵ 12 670), λ_{max} (pH 13.0) 277 nm; R_f (solvent A) 0.50, R_f (solvent B) 0.63. The melting point was 233 °C. Anal. Calcd for $C_{14}H_{21}N_6O_6P_1 \cdot 2H_2O$: C, 38.53; H, 5.73; N, 19.27; O, 29.36. Found: C, 38.1; H, 5.5; N, 19.8; O, 30.2.

8-Hexylaminoadenosine 3′,5′-Monophosphate. 8-Hex-A-cAMP (635 mg, 1.48 mmol) was synthesized in a 74% yield from 820 mg of 8-Br-cAMP (2 mmol) and 25 ml of *n*-hexylamine (188 mmol) in the same manner as in the synthesis of 8-Pro-A-cAMP: λ_{max} (pH 2.0) 277 nm (ϵ 13620), λ_{max} (pH 13.0) 277 nm; R_f (solvent A) 0.64, R_f (solvent B) 0.72. The melting point was 224 °C. Anal. Calcd for $C_{16}H_{25}N_6O_6P_1 \cdot H_2O$: C, 43.05; H, 6.05; N, 18.83; O, 25.11. Found: C, 43.0; H, 6.1; N, 19.3; O, 24.8.

8-Octylaminoadenosine 3′,5′-Monophosphate. 8-Oct-A-cAMP (650 mg, 1.43 mmol) was synthesized in a 71% yield from 820 mg of 8-Br-cAMP (2 mmol) and 25 ml of *n*-octylamine (151 mmol) in the same manner as in the synthesis of 8-Pro-A-cAMP: λ_{max} (pH 2.0) 277 nm (ϵ 13 040), λ_{max} (pH 13.0) 277 nm; R_f (solvent A) 0.65, R_f (solvent B) 0.78. The melting point was 223 °C. Anal. Calcd for $C_{18}H_{29}N_6O_6P_1$: C, 47.37; H, 6.36; N, 18.42; O, 21.11. Found: C, 46.6; H, 6.4; N, 18.2; O, 20.9.

8-Decylaminoadenosine 3′,5′-Monophosphate. *n*-Decylamine (3.14 g, 20 mmol), ten times in excess of amount of 8-Br-cAMP, was dissolved into 20 ml of methyl glycol monomethyl ether containing 820 mg of 8-Br-cAMP (2 mmol), and the mixed solution was refluxed for 2 h. The reaction mixture was cooled and then mixed with 200 ml of ethyl ether and 200 ml of 1 N ammonia–water and shaken vigorously to remove the residual excess amine. The lower phase was adjusted to pH 1.5 with 5 N HCl and then evaporated to 15 ml. The fluid was added to 100 ml of cold methyl alcohol and stirred in a cold room to crystallize the product. The crystallite (440 mg) was obtained in a 46% yield. This low yield was based on the loss of the compound in the course of evaporation: λ_{max} (pH 2.0) 277 nm (ϵ 10 760), λ_{max} (pH 13.0) 277 nm; R_f (solvent A) 0.72, R_f (solvent B) 0.82. The melting point was 226 °C. Anal. Calcd for $C_{20}H_{33}N_6O_6P_1 \cdot H_2O$: C, 47.81; H, 6.97; N, 16.73; O, 22.31. Found: C, 47.4; H, 6.9; N, 16.9; O, 22.3.

8-Undecylaminoadenosine 3′,5′-Monophosphate. 8-Und-A-cAMP (785 mg, 1.58 mmol) was synthesized in a 79% yield from 820 mg of 8-Br-cAMP (2 mmol) and 3.4 g of *n*-undecylamine (20 mmol) in the same manner as in the synthesis of 8-Dec-A-cAMP: λ_{max} (pH 2.0) 277 nm (ϵ 10 820), λ_{max} (pH 13.0) 277 nm; R_f (solvent A) 0.75, R_f (solvent B) 0.83. The melting point was 227 °C. Anal. Calcd for $C_{21}H_{35}N_6O_6P_1 \cdot H_2O$: C, 48.84; H, 7.17; N, 16.28; O, 21.71. Found: C, 48.8; H, 7.2; N, 16.1; O, 21.3.

8-Dodecylaminoadenosine 3′,5′-Monophosphate. 8-Dod-A-cAMP (750 mg, 1.46 mmol) was synthesized in a 73% yield from 820 mg of 8-Br-cAMP (2 mmol) and 3.7 g of *n*-laurylamine (20 mmol) in the same manner as in the synthesis of 8-Dec-A-cAMP: λ_{max} (pH 2.0) 277 nm (ϵ 11 180), λ_{max} (pH 13.0) 277 nm; R_f (solvent A) 0.77, R_f (solvent B) 0.85. The melting point was 234 °C. Anal. Calcd for $C_{22}H_{37}N_6O_6P_1 \cdot 2\frac{1}{2}H_2O$: C, 47.40; H, 7.54; N, 15.08; O, 24.41. Found: C, 47.8; H, 7.2; N, 15.5; O, 24.6.

8-Propylthioadenosine 3′,5′-Monophosphate. 8-Br-cAMP (2.46 g, 6 mmol) was refluxed in 50 ml of ethanol containing 2.18 ml of *n*-propanethiol (23 mmol) and sodium methoxide (18 mmol) for 1 h. After 1 h of reaction, the reaction mixture was cooled and then evaporated to dryness at temperatures below 40 °C. The residue was dissolved in 60 ml of cold water and the fluid was adjusted to pH 6.0. One hundred milliliters of ethyl ether was added to the pH 6.0 solution and shaken vigorously and the lower phase was adjusted to pH 2.0 slowly and kept in a cold room to crystallize. Finally, 1.93 g of 8-Pro-S-cAMP (4.79 mmol) was obtained in a 80% yield: λ_{max} (pH 2.0) 284 nm (ϵ 19 300), λ_{max} (pH 13.0) 282 nm; R_f (solvent A) 0.48, R_f (solvent B) 0.64. The melting point was 225 °C. Anal. Calcd for $C_{13}H_{18}N_5O_6P_1S_1$: C, 38.71; H, 4.47; N, 17.37; O, 23.82. Found: C, 38.7; H, 4.6; N, 16.9; O, 23.5.

8-Butylthioadenosine 3′,5′-Monophosphate. 8-But-S-cAMP (1.79 g, 4.29 mmol) was synthesized in a 72% yield from 2.46 g of 8-Br-cAMP (6 mmol) and 2.2 ml of *n*-butanethiol (19.6 mmol) in the same manner as in the synthesis of 8-Pro-S-cAMP: λ_{max} (pH 2.0) 284 nm (ϵ 20 090), λ_{max} (pH 13.0) 282 nm; R_f (solvent A) 0.53, R_f (solvent B) 0.73. The melting point was 224 °C. Anal. Calcd for $C_{14}H_{20}N_5O_6P_1S_1$: C, 40.29; H, 4.80; N, 16.79; O, 23.02. Found: C, 39.2; H, 4.7; N, 16.3; O, 23.4.

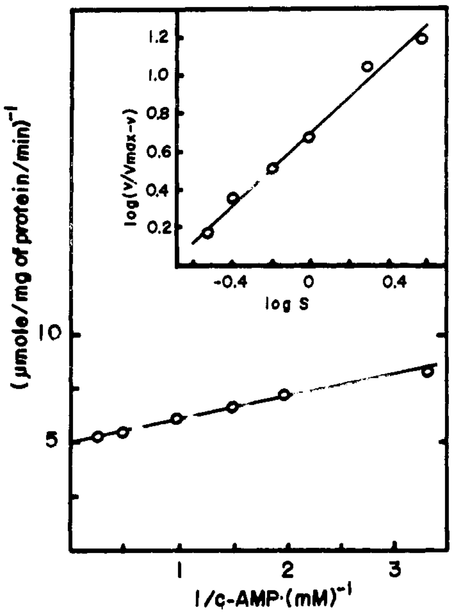

FIGURE 1: Kinetic analysis at pH 8.0 of cyclic AMP hydrolysis by DEAE fraction from hog brain cortex. The reaction was performed in 1 ml of reaction mixture for 15 min at 30 °C, which contained 40 mM Tris·HCl (pH 8.0), 5 mM $MgCl_2$, 0.3 to 4.0 mM cyclic AMP, and the enzyme preparation (82 μg of protein per tube). Hill plot was obtained from the same data as the double reciprocal plot.

8-Pentylthioadenosine 3′,5′-Monophosphate. 8-Pen-S-cAMP (2.22 g, 5.15 mmol) was synthesized in a 85% yield from 2.46 g of 8-Br-cAMP (6 mmol) and 2.2 ml of *n*-pentanethiol (16.9 mmol) in the same manner as in the synthesis of 8-Pro-S-cAMP: λ_{max} (pH 2.0) 284 nm (ϵ 19 310), λ_{max} (pH 13.0) 282 nm; R_f (solvent A) 0.60, R_f (solvent B) 0.77. The melting point was 226 °C. Anal. Calcd for $C_{15}H_{22}N_5O_6P_1S_1$: C, 41.76; H, 5.10; N, 16.24; O, 22.27. Found: C, 41.5; H, 5.0; N, 16.2; O, 22.3.

8-Hexylthioadenosine 3′,5′-Monophosphate. 8-Hex-S-cAMP (2.16 g, 4.92 mmoles) was synthesized in a 82% yield from 2.46 g of 8-Br-cAMP (6 mmol) and 2.2 ml of *n*-hexanethiol (13 mmol) in the same manner as in the synthesis of 8-Pro-S-cAMP: λ_{max} (pH 2.0) 285 nm (ϵ 20 330), λ_{max} (pH 13.0) 282 nm; R_f (solvent A) 0.66, R_f (solvent B) 0.83. The melting point was 228 °C. Anal. Calcd for $C_{16}H_{24}N_5O_6P_1S_1$: C, 43.14; H, 5.39; N, 15.73; O, 21.57. Found: C, 42.9; H, 5.2; N, 15.6; O, 21.0.

8-Heptylthioadenosine 3′,5′-Monophosphate. 8-Hep-S-cAMP (2.26 g, 4.9 mmol) was synthesized in a 82% yield from 2.46 g of 8-Br-cAMP (6 mmol) and 2.2 ml of *n*-heptanethiol (13.3 mmol) in the same manner as in the synthesis of 8-Pro-S-cAMP: λ_{max} (pH 2.0) 285 nm (ϵ 20 330), λ_{max} (pH 13.0) 282 nm; R_f (solvent A) 0.66, R_f (solvent B) 0.83. The melting point was 228 °C. Anal. Calcd for $C_{17}H_{26}N_5O_6P_1S_1$: C, 44.44; H, 5.66; N, 15.25; O, 20.91. Found: C, 43.9; H, 5.2; N, 15.6; O, 20.6.

8-Octylthioadenosine 3′,5′-Monophosphate. 8-Oct-S-cAMP (2.32 g, 4.95 mmol) was synthesized in a 83% yield from 2.46 g of 8-Br-cAMP (6 mmol) and 2.2 ml of *n*-octanethiol (12.1 mmol) in the same manner as in the synthesis of 8-Pro-S-cAMP: λ_{max} (pH 2.0) 285 nm (ϵ 18 260), λ_{max} (pH 13.0) 282 nm; R_f (solvent A) 0.67, R_f (solvent B) 0.85. The melting point was 223 °C. Anal. Calcd for $C_{18}H_{28}N_5O_6P_1S_1 \cdot H_2O$: C, 43.54; H, 6.11; N, 14.26; O, 22.81. Found: C, 43.2; H, 6.1; N, 14.1; O, 22.9.

8-Decylthioadenosine 3′,5′-Monophosphate. 8-Dec-S-cAMP (1.91 g, 3.81 mmol) was synthesized in a 64% yield from 2.46 g of 8-Br-cAMP (6 mmol) and 2.2 ml of *n*-decanethiol (10.1 mmol) in the same manner as in the synthesis of 8-Pro-S-cAMP: λ_{max} (pH 2.0) 285 nm (ϵ 17 790), λ_{max} (pH 13.0) 282 nm; R_f (solvent A) 0.68, R_f (solvent B) 0.86. The melting point was 227 °C. Anal. Calcd for $C_{20}H_{32}N_5O_6P_1S_1$: C, 47.90; H, 6.39; N, 13.97; O, 19.16. Found: C, 47.4; H, 6.2; N, 14.0; O, 20.5.

8-Dodecylthioadenosine 3′,5′-Monophosphate. 8-Dod-S-cAMP (2.62 g, 4.95 mmol) was synthesized in a 83% yield from 2.46 g of 8-Br-cAMP (6 mmol) and 2.2 ml of *n*-dodecanethiol (8.7 mmol) in the same manner as in the synthesis of 8-Pro-S-cAMP: λ_{max} (pH 2.0) 285 nm (ϵ 17 830), λ_{max} (pH 13.0) 282 nm; R_f (solvent A) 0.75, R_f (solvent B) 0.88. The melting point was 228 °C. Anal. Calcd for $C_{22}H_{36}N_5O_6P_1S_1$: C, 49.91; H, 6.81; N, 13.23; O, 18.15. Found: C, 49.7; H, 6.8; N, 13.3; O, 19.1.

Preparation of High K_m Phosphodiesterase. The high K_m phosphodiesterase (modulator-deficient form) was prepared by the method of Teo et al. (1973).

A fresh hog brain cortex was homogenized in four volumes of 0.1 M Tris·HCl buffer (pH 7.5) containing 1 mM $MgCl_2$ and 1 mM imidazole, and the homogenate was centrifuged at 12 000*g* for 30 min. The supernatant was taken to 55% saturation with $(NH_4)_2SO_4$ solution and centrifuged at 12 000*g*. The precipitate was suspended in 0.02 M Tris·HCl buffer (pH 7.5) containing 0.08 M NaCl, 1 mM $MgCl_2$, and 1 mM imidazole and then dialyzed against the same buffer. The dialyzed fluid was applied to a DEAE-cellulose column (3.7 × 18 cm), and an active fraction was obtained by eluting with the buffer containing 0.22 M NaCl. After the dialysis, the active fraction was used as enzyme preparation.

Phosphodiesterase Assay. The enzyme activity was measured by the procedure of Butcher and Sutherland (1962), employing 5′-nucleotidase. The reaction was initiated by adding phosphodiesterase preparation to the mixture of 1.0 ml containing 40 μmol of Tris·HCl (pH 8.0), 5 μmol of $MgCl_2$, and 1.0 μmol of cyclic AMP, conducted for 15 min at 30 °C, and terminated by boiling for 2 min. To liberate inorganic phosphate from 5′-AMP formed in the above reaction, the reaction mixtures were kept for another 30 min at 30 °C after 5′-nucleotidase (150 μg of protein per tube, 1.4 units) was added. The 5′-nucleotidase was provided by Mr. Kameyama, which was originated in bull sperm and purified by protamine treatment, $(NH_4)_2SO_4$ fractionation, and DEAE-cellulose column chromatography. One unit of the enzyme is defined as the amount converting 1 μmol of 5′-AMP to adenosine and inorganic phosphate per minute at 30 °C. The resulting inorganic phosphate was determined by the method of Takahashi (1955).

Determination of inorganic phosphate was performed under a strong acid condition, but no detectable amounts of inorganic phosphate were released from cyclic AMP and cyclic AMP derivatives.

The measurement of protein was carried out by the method of Lowry et al., using bovine serum albumin as standard (1951). One milligram of the enzyme protein was shown to hydrolyze 0.150 μmol of cyclic AMP per minute under the standard condition (1 mM cyclic AMP).

Results

Some Properties of the Phosphodiesterase Preparation. The enzyme preparation exhibited normal Michaelis-Menten kinetics in a high concentration range of cyclic AMP, 0.3 to 4 0 mM (Figure 1). K_m of this enzyme for cyclic

AMP was 0.20 mM. The Hill plot was almost straight and the binding constant for cyclic AMP was nearly 1. The above facts seem to indicate that the activity of the preparation is homogeneous at such high concentrations of cyclic AMP as shown in Figure 1 and the enzyme has a single binding site for cyclic AMP. Therefore, the mode of action of the enzyme was determined at unphysiologically high levels of cyclic AMP, 0.3 to 4.0 mM. In these experiments, the enzyme preparation was confirmed to contain no 5′-nucleotidase.

The optimum pH of the enzyme was examined at a high concentration of cyclic AMP (1 mM). The reaction was carried out under the standard condition described in the text. In Tris·HCl buffer, the enzyme preparation exhibited the highest activity in the range of pH 7.5 to 8.8. In glycine-NaCl-HCl buffer, the enzyme was fully active in the range of pH 8.5 to 9.0. These results indicate that the optimum pH of the high K_m phosphodiesterase from hog brain cortex is in the range of 7.5 to 9.0.

The effect of magnesium on the enzyme activity was examined. Although a relatively high enzyme activity was observed without addition of $MgCl_2$, much more high enzyme activity was obtained when $MgCl_2$ was added newly to the reaction mixture and the optimum concentration of $MgCl_2$ was found to be one mM. When EDTA was added to the reaction mixture without an additive Mg^{2+}, the enzyme activity was lost. The effect of EDTA was partially neutralized by the addition of excess magnesium ion: when 10 mM $MgCl_2$ was added to the reaction mixture containing 1 mM EDTA, the enzyme activity was restored to a level of approximately one-half of full activity. Therefore, magnesium ion was proved to be essential for the high K_m phosphodiesterase activity.

The Mode of Action of 8-Substituted Cyclic AMP Derivatives on the Phosphodiesterase Activity. The inhibitory effect of 8-Met-A-cAMP, 8-Met-S-cAMP, and 8-Eth-S-cAMP on the phosphodiesterase isolated from pig brain cortex has been evaluated already by Muneyama et al., but it is still obscure as to whether either the inhibitory mode of such derivatives is competitive or noncompetitive (1971).

Therefore, an effect of 8-substituted cyclic AMP derivatives on the phosphodiesterase activity was determined and the results depicted by Lineweaver–Burk plot are shown in Figure 2. The methods of this experiment are described in the figure legend. All of the 8-substituted cyclic AMP derivatives, except 8-Dod-A-cAMP and 8-Dod-S-cAMP, were found to inhibit competitively the cyclic AMP hydrolysis by the enzyme preparation. The mode of action of these derivatives is shown in Figure 2. Only 8-Dod-A-cAMP and 8-Dod-S-cAMP noncompetitively inhibited it. All of the derivatives, except 8-Ami-cAMP, were not hydrolyzed to their corresponding 5′-nucleotides by our phosphodiesterase preparation under the standard condition using 1 mM for each derivative as substrate. The rate of 8-Ami-cAMP hydrolysis was 57% of that of cyclic AMP hydrolysis.

On the other hand, the effects of free substituents, alkylamines, and alkylthiols were determined. Almost all of these compounds had no effect on the phosphodiesterase activity, except that decylamine, decanethiol, and dodecanethiol inhibited it noncompetitively.

The above results suggest that almost all of these derivatives bind to the same site as cyclic AMP, and they competitively inhibit the enzyme reaction at high concentrations of substrate. The noncompetitive inhibition observed in the case of 8-Dod-A-cAMP and 8-Dod-S-cAMP may result

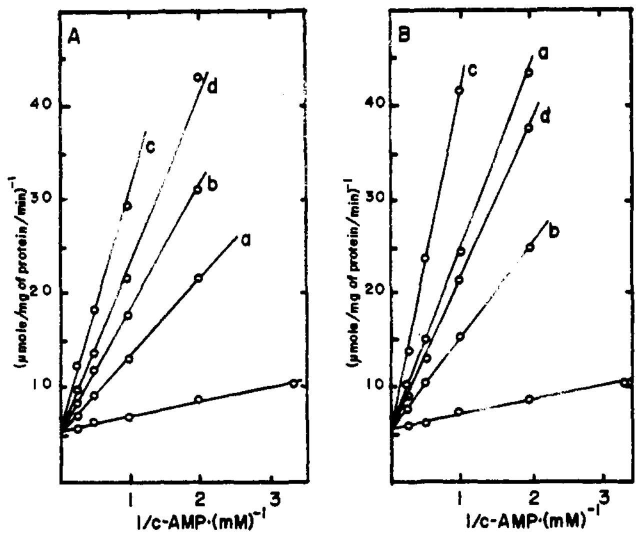

FIGURE 2: Effect of 8-substituted cyclic AMP derivatives on high K_m phosphodiesterase activity in DEAE fraction from hog brain cortex. The reaction was performed for 15 min at 30 °C in the same reaction mixture as in Figure 1, but containing 0.5 or 1.0 mM derivative as inhibitor. (A) Effects of 8-alkylamino cyclic AMP derivatives on high K_m phosphodiesterase activity: (a) with 1.0 mM 8-Met-A-cAMP; (b) with 1.0 mM 8-Eth-A-cAMP; (c) with 1.0 mM 8-Hex-A-cAMP; (d) with 0.5 mM 8-Dec-A-cAMP. (B) Effects of 8-alkylthio cyclic AMP derivatives: (a) with 0.5 mM 8-Eth-S-cAMP; (b) with 0.5 mM 8-Pro-S-cAMP; (c) with 0.5 mM 8-Hex-S-cAMP; (d) with 0.5 mM 8-Dec-S-cAMP.

from the interaction between their substituents and other than the active site on the enzyme.

In this experiment, we found that the inhibitory ability of 8-substituted cyclic AMP derivatives varied in a characteristic way according to the elongation of the carbon chain of the substituents. Therefore, to clarify this fact in more detail, K_i values for the derivatives were determined.

Relation between the K_i Value and the Length of the Substituent of Derivative. K_i values were determined from Lineweaver–Burk and Dixon plots for competitive inhibitors and just from Dixon plot for noncompetitive inhibitors. The reaction was carried out under the standard conditions: 40 mM Tris·HCl (pH 8.0), 5 mM $MgCl_2$, 0.3 to 4.0 mM cyclic AMP, 0.1 to 1.0 mM derivative, and the enzyme preparation (82 μg of protein per tube). The results are shown in Table I. To obtain the Lineweaver–Burk plot, the reaction was carried out in the mixtures containing 0.3 to 4.0 mM cyclic AMP as substrate and 1.0 mM cyclic AMP derivative as inhibitor. The Dixon plot was obtained using the reaction mixtures containing 0.5 mM cyclic AMP and varing concentrations of derivative (0.1 to 1.0 mM). In this case, K_m for cyclic AMP was 0.24 mM and V_{max} was 0.182 μmol per mg of protein per minute.

The K_i value for 8-alkylamino cyclic AMP derivatives was found to decrease as the carbon chain of substituents in derivatives was extended, and a minimum value was obtained in the case of 8-Oct-A-cAMP. The K_i value, thereafter, increased as the carbon chain was further extended and the K_i value for 8-Dod-A-cAMP became higher than the K_m value.

Phenomena quite similar to those described above were also observed in 8-alkylthio cyclic AMP derivatives. The inhibitory effect of the derivative became stronger with increasing the carbon chain of substituent, and 8-Hep-S-cAMP exhibited a maximum inhibitory activity. The inhibitory ability decreased with further increasing the carbon chain.

Table I: Standard Affinity of 8-Substituted Cyclic AMP Derivatives for Phosphodiesterase.

Substituent	K_m or K_i (mM)	Standard Affinity (kcal/mol)	Partial Affinity[a] (kcal/mol)
-H(cAMP)	0.24	5.00	
$-NH_2$	0.85	4.238	−0.76
$-NHCH_3$	0.21	5.08	0.84
$-NHCH_2CH_3$	0.12	5.41	0.33
$-NH(CH_2)_2CH_3$	0.23	5.02	−0.39
$-NH(CH_2)_3CH_3$	0.12	5.41	0.39
$-NH(CH_2)_5CH_3$	0.058	5.85	½(0.44)
$-NH(CH_2)_7CH_3$	0.0093	6.94	½(1.09)
$-NH(CH_2)_9CH_3$	0.082	5.64	½(−1.30)
$-NH(CH_2)_{10}CH_3$	0.17	5.20	−0.44
$-NH(CH_2)_{11}CH_3$	0.66	4.39	−0.81
-H(cAMP)	0.24	5.00	
-SH	0.81	4.27	−0.73
$-SCH_3$	0.079	5.66	1.39
$-SCH_2CH_3$	0.037	6.12	0.46
$-S(CH_2)_2CH_3$	0.078	5.67	−0.45
$-S(CH_2)_3CH_3$	0.074	5.70	0.03
$-S(CH_2)_4CH_3$	0.040	6.07	0.37
$-S(CH_2)_5CH_3$	0.019	6.52	0.45
$-S(CH_2)_6CH_3$	0.0095	6.93	0.49
$-S(CH_2)_7CH_3$	0.016	6.62	−0.31
$-S(CH_2)_9CH_3$	0.046	5.99	½(−0.63)
$-S(CH_2)_{11}CH_3$	0.14	5.32	½(−0.67)

[a] By one methylene group.

A considerable similarity in relation between substituent length in derivatives and their K_i values was confirmed in two different series of derivative.

When the K_i values described in Table I were plotted on a semilogarithm graph as a function of the carbon number of substituent in cyclic AMP derivatives, two minimum points were found in the case of 8-alkylamino cyclic AMP derivatives as shown obviously in Figure 3A: one was 8-Eth-A-cAMP (carbon number, 2) and the other was 8-Oct-A-cAMP (carbon number, 8). As the substituent in 8-Eth-A-cAMP obtained one more methylene group to form 8-Pro-A-cAMP, the K_i value increased. But the K_i value from 8-Pro-A-cAMP to 8-Oct-A-cAMP decreases almost linearly. As the length of the substituent became longer than that of 8-Oct-A-cAMP, however, on the contrary, the values increased linearly. The curve obtained from the 8-alkylthio cyclic AMP derivative was also found to be similar to that from the 8-alkylamino cyclic AMP derivative, as shown in Figure 3B. The minimum points were observed in both 8-Eth-S-cAMP and 8-Hep-S-cAMP.

The basic character of the interaction between the enzyme and the inhibitor is clearly indicated by these markedly similar and highly characteristic curves in two different series of cyclic AMP derivatives.

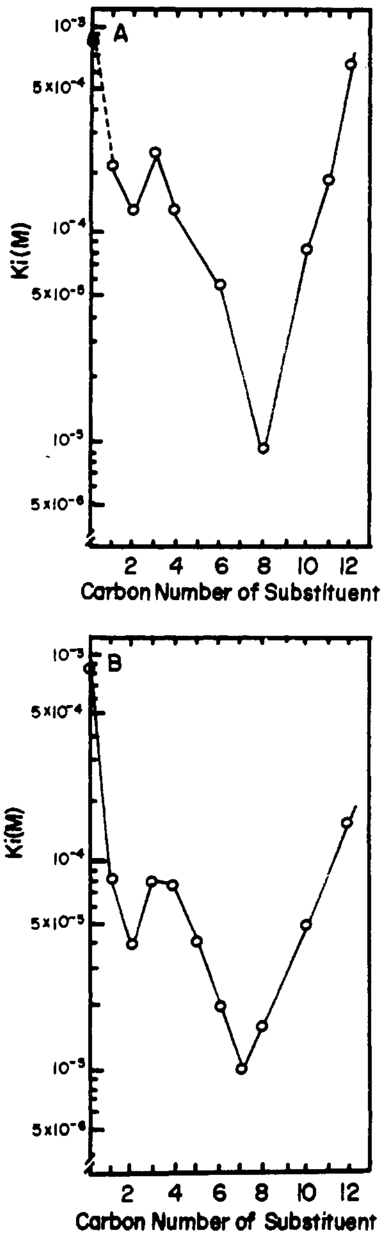

FIGURE 3: Relation between the logarithm of the K_i value and the length of the derivative's substituent. The K_i value for each cyclic AMP derivative was calculated from Lineweaver–Burk and Dixon plots and is shown in Table I. The reaction was carried out under conditions similar to those in Figure 2. The carbon number corresponds to the methylene (methyl) number of the substituent. A carbon number of zero indicates 8-Ami-cAMP (A) and 8-Thi-cAMP (B). (A) 8-Alkylamino cyclic AMP derivative series. (B) 8-Alkylthio cyclic AMP derivative series.

Discussion

High K_m phosphodiesterase from hog brain cortex was confirmed to be similar to that from brain of other mammals in such properties as optimum pH, magnesium requirement, and K_m for cyclic AMP. The enzyme was indicated to have one active site under the condition that cyclic AMP was used in the high concentration range from 0.3 to 4.0 mM.

The function of high K_m phosphodiesterase in regulating intracellular levels of cyclic nucleotide remains quite uncertain, even though the enzyme has been found to have the following properties. (1) The hydrolysis of cyclic nucleotides by the high K_m phosphodiesterase is stimulated by a protein-like modulator in the presence of magnesium and calcium ion (Cheung, 1967a,b, 1969; Kakiuchi, et al., 1970, 1973). (2) The hydrolysis of cyclic AMP is stimulated by low concentration of cyclic GMP or cyclic IMP (Beavo et al., 1970, 1971). (3) The high concentration of one cyclic nucleotide has been shown to interfere with the hydrolysis of the other (Beavo et al., 1970, 1971; Russell, et al., 1973).

Eighteen among the 8-substituted cyclic AMP derivatives described in this paper were found to inhibit competitively the hydrolysis of cyclic AMP by the high K_m phosphodiesterase, except that 8-Ami-cAMP, 8-Dod-A-cAMP, and 8-Dod-S-cAMP inhibited noncompetitively the cyclic AMP hydrolysis. To elucidate the inhibitory effect of cyclic AMP derivative on the enzyme activity, the standard affinity for cyclic AMP or cyclic AMP derivatives was calculated from the K_m or K_i values.

Generally, a standard affinity for a substrate or inhibitor of enzyme is provided by the following equation:

$$-\Delta G = -RT \ln K_m \text{ (or } K_i)$$

It is assumed that K_m is equal to K_s (dissociation constant). An alteration in affinity (partial affinity) is provided by

$$\Delta(-\Delta G) = (-\Delta G)(n + 1) - (-\Delta G)n$$

in this case, n is carbon number of substituent. In our experiment, the partial affinity should be based on the affinity for one methylene (or methyl) group of the substituent.

In the case of 8-alkylthio cyclic AMP derivatives, the partial affinity energy for each methylene group from the second to the seventh except both the third and fourth is almost close to one hydrogen bond energy, about 0.5 kcal/mol. 8-Hep-S-cAMP, formed by the addition of a methylene to 8-Hex-S-cAMP, is presumed to fit tightly to the active site on the enzyme and/or the adjacent site. Therefore, the partial affinity value for the seventh methylene group (0.49 kcal/mol) might be higher than that for other methylene groups. In the case of 8-alkylamino cyclic AMP derivatives, the partial affinity value for the eighth might be also interpreted in the same manner as described above.

The partial affinity value for the first methyl group is quite high, 1.38 kcal/mol, compared with the other methylene groups in the 8-alkylthio cyclic AMP derivative. This observation can be explained as follows. The hydrothio group at the eighth position of 8-Thi-cAMP may repel the active site or its adjacent site and thereby may reduce the affinity for the derivative. But the repulsion force of the hydrothio group is masked by the addition of the first methyl group to the hydrothio group, and thereby the first methyl group may appear to obtain such high affinity energy. Even in the case of 8-alkylamino cyclic AMP derivative, such high partial affinity energy for the first methyl group can be also confirmed in the same manner as described above.

The third methylene group is also found to reduce the affinity for cyclic AMP derivative and its partial affinity is negative value: −0.45 kcal/mol for the alkylthio derivative series and −0.39 kcal/mol for the alkylamino derivative series. These negative values are too small to indicate a strong electrostatic repulsion or a steric hindrance.

With increasing carbon chain of the substituent, the affinity for the derivative increases progressively, and 8-Hep-S-cAMP and 8-Oct-A-cAMP exhibit maxima in affinity energy. Any further lengthening, however, results in a reduction in the affinity for the derivatives.

The facts shown in Figure 3 and Table I are understood to be based on the characteristic structure at the active site or the adjacent site on high K_m phosphodiesterase.

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References

Armiento, M., and Pastan, I. (1972), *Proc. Natl. Acad. Sci. U.S.A. 69,* 459.

Beavo, J. A., Hardman, J. G., and Sutherland, E. W. (1970), *J. Biol. Chem. 245,* 5649.

Beavo, J. A., Hardman, J. G., and Sutherland, E. W. (1971), *J. Biol. Chem. 246,* 3841.

Butcher, R. W., and Sutherland, E. W. (1962), *J. Biol. Chem. 237,* 1244.

Cheung, W. Y. (1967a), *Biochim. Biophys. Res. Commun. 29,* 478.

Cheung, W. Y. (1967b), *Biochemistry 6,* 1079.

Cheung, W. Y. (1969), *Biochim. Biophys. Acta 191,* 303.

Heidrick, M. L., and Ryan, W. L. (1971), *Cancer Res. 31,* 1313.

Jard, S., and Bernard, M. (1970), *Biochem. Biophys. Res. Commun. 41,* 781.

Kakiuchi, S., and Yamazaki, R. (1970), *Biochem. Biophys. Res. Commun. 41,* 1104.

Kakiuchi, S., Yamazaki, R., Teshima, Y., and Uenishi, K. (1973), *Proc. Natl. Acad. Sci. U.S.A. 70,* 3526.

Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951), *J. Biol. Chem. 192,* 265.

Manganiello, V., and Vanghan, M. (1972), *Proc. Natl. Acad. Sci. U.S.A. 69,* 269.

Muneyama, K., Bauer, R. J., Shuman, D. A., Robins, R. K., and Simon, L. N. (1971), *Biochemistry 10,* 2390.

Perkins, J. P., MacIntyre, E. H., Riley, W. D., and Clard, R. B. (1971), *Life Sci. 10,* 1069.

Russell, T. R., Terasaki, W. L., and Appleman, M. M. (1973), *J. Biol. Chem. 248,* 1334.

Takahashi, T. (1955), *J. Biochem. (Tokyo) 26,* 690.

Teo, T. S., Wang, T. H., and Wang, J. H. (1973), *J. Biol. Chem. 248,* 585.

Thompson, W. J., and Appleman, M. M. (1971a), *Biochemistry 10,* 311.

Thompson, W. J., and Appleman, M. M. (1971b), *J. Biol. Chem. 246,* 3145.