Biochemical Pharmacology, Vol. 22, pp. 221–228. Pergamon Press, 1973. Printed in Great Britain.

EFFECT OF CYCLIC NUCLEOTIDES ON ACTIVITY OF CYCLIC 3′,5′-ADENOSINE MONOPHOSPHATE PHOSPHODIESTERASE*

DON N. HARRIS, MARK CHASIN, MARIE B. PHILLIPS, HAROLD GOLDENBERG, SYLVIA SAMANIEGO and SIDNEY M. HESS

Department of Biochemical Pharmacology, Squibb Institute for Medical Research, New Brunswick, N.J. 08903, U.S.A.

(*Received* 10 *May* 1972; *accepted* 28 *July* 1972)

Abstract—A series of cyclic 2′,3′-nucleotides, cyclic 3′,5′-nucleotides and derivatives of cyclic 3′,5′-adenosine monophosphate (cyclic AMP) with a substituent at the C-8 position were investigated as inhibitors of partially purified cyclic AMP phosphodiesterases (PDE) of cat heart and rat brain. The assays were carried out at a substrate concentration (0·06 μM) where the contribution to the total enzyme activity by phosphodiesterases with K_m values for cyclic AMP above 100 μM was insignificant; consequently the activity measured was that of low K_m enzymes.

Cyclic 3′,5′-guanosine monophosphate (cyclic GMP) and cyclic 3′,5′-inosine monophosphate (cyclic IMP) were shown to be the most potent inhibitors of PDE of cat heart (I_{50} = 1 and 2 μM, respectively). Of the cyclic AMP derivatives tested that have a substituent at the C-8 position, 8-bromo cyclic AMP was the most potent inhibitor; next most potent were the derivatives with a sulfur atom, whereas derivatives with oxygen- or nitrogen-containing substituents were the least potent inhibitors of PDE of cat heart or rat brain. Most of the cyclic nucleotides that were tested were more potent inhibitors of the PDE of cat heart than that of PDE of rat brain.

The kinetic properties of PDE of cat heart were also investigated in the presence of cyclic GMP, cyclic IMP and 8-bromocyclic AMP. All three compounds were found to be competitive inhibitors, with apparent K_i values of 0·51, 2·3 and 20 μM respectively. The possible pharmacologic role of cyclic nucleotides is discussed.

THE ROLE of cyclic 3′,5′-adenosine monophosphate (cyclic AMP) as the "second messenger" for an increasing number of hormonal actions has been summarized in several review articles.[1–4] Intracellular concentrations of cyclic AMP can be increased by the stimulation of adenylate cyclase or by the inhibition of cyclic AMP phosphodiesterase (PDE). Another naturally occurring cyclic nucleotide, cyclic 3′,5′-guanosine monophosphate (cyclic GMP), is normally found in the fluids and tissues of the body. The extracellular levels of cyclic GMP are increased by administration of parathyroid hormone, calcium ion, and alpha adrenergic agents, whereas acetylcholine causes an increase in cyclic GMP levels in isolated perfused hearts of rats.[3] It has been suggested that cyclic GMP at physiological levels may indirectly alter intracellular concentrations of cyclic AMP, by virtue of its ability to regulate the hydrolysis of cyclic AMP by PDE.[5–11]

* A preliminary report of these studies has been previously communicated: D. N. Harris, M. B. Phillips and H. J. Goldenberg, *Fedn Proc.* **30**, 219 (1971).

Derivatives of cyclic AMP such as adenosine 3′,5′-cyclic phosphorothioate,[12,13] the 3′-methylene cyclic phosphonate,[13] and 8-thio-, 8-methylthio- and 8-benzylthio cyclic AMP,[14] have been tested as inhibitors of PDE, but only the latter two compounds were found to be more potent than theophylline against the enzymes examined.

Recent studies using preparations of bovine heart,[7] rat brain,[15–18] cat heart,[18] frog bladder and rat kidney,[19] rabbit skeletal muscle,[20] and rat adipose tissue[21] have demonstrated the presence of two PDE activities with widely different K_m values for cyclic AMP. Thompson and Appleman,[16,17] moreover, have separated the PDE activity of various rat tissues into a high molecular weight fraction with a high affinity for cyclic GMP and a low affinity for cyclic AMP and a low molecular weight fraction with a high affinity specifically for cyclic AMP. Their studies indicate that the high molecular weight form of the phosphodiesterase is actually a cyclic GMP-PDE.

In the present study, cyclic 2′,3′-nucleotides, cyclic 3′,5′-nucleotides other than cyclic AMP, and derivatives of cyclic AMP with a substituent at the C-8 position were investigated to determine if they could influence the rate of hydrolysis of cyclic AMP at low substrate concentration (0·05 μM). Partially purified preparations of PDE from cat heart and rat brain were compared for possible differences in the specificity of these enzymes to inhibition by cyclic nucleotides.

METHODS

Materials. The 8-^{3}H-cyclic AMP (12·7 or 16·3 Ci/m-mole) was purchased from Schwarz/Mann. The cyclic AMP derivatives with a substituent at the C-8 position were supplied by Dr. Roland K. Robins of the ICN Nucleic Acid Research Institute. The liquid-scintillation solvent consisted of 8 g of 2,5-diphenyloxazole, 0·6 g of 1,4-*bis*[2-(2-methyl-5-phenyloxazolyl)]-benzene, 150 g of naphthalene, 10 ml of 2-ethoxyethanol, 20 ml of ethylene glycol and 1,4-dioxane to make 1000 ml.* Snake venom (*Ophiophagus hannah*) was obtained from Sigma Chemical Company. The anion-exchange resin (AG 1-X2, minus 400 mesh, chloride form) was obtained from Bio-Rad Laboratories and prepared as described by Brooker *et al.*[15] All other chemicals used were of reagent grade, obtained from commercial sources.

Preparation of phosphodiesterase. A mongrel cat (1–2 kg) or 10–12 male Sprague–Dawley rats (150–200 g) were sacrificed by cervical dislocation and decapitation respectively. The cat heart and rat brains were removed and immediately placed on cracked ice. After the auricular tissue of the heart was removed and discarded, the following steps were carried out at 0–4°. The organs were minced and homogenized in 5–10 vol. of 0·05 M imidazole buffer (pH 7·5), that also contained 5 mM dithiothreitol (Cleland's reagent) in the case of the heart preparations. The homogenates were immediately centrifuged for 15–20 min at 39,000 *g*. The supernatant fractions were adjusted to 50 per cent saturation with ammonium sulfate, the pH was adjusted to 7·5 with 1 N NaOH, and the mixture was allowed to stand for 1 hr. The solutions were centrifuged as before, and the precipitates were taken up in a minimum volume of the imidazole buffer and dialyzed against 20 vol. of the buffer overnight. Using this procedure there was approximately a 5-fold purification of each enzyme preparation. Protein concentrations were from 5 to 7 mg/ml for cat heart PDE by the micro-modification[22] of the biuret method,[23] and from 15 to 20 mg/ml for rat brain PDE as

* The liquid-scintillation solution was prepared according to the directions given in the Packard Instrument Catalog, page 22, 1962.

determined by the procedure of Lowry *et al.*[24] Both enzyme preparations had a sp. act. of about 0·25 nmole of cyclic AMP hydrolyzed/mg of protein/min. They were stored at 0–4° until used. In the case of the rat brain enzyme, enzymatic activity was stable for up to 12 months. The cat heart enzyme was less stable, however, and was freshly prepared each month. The yield of enzyme activity was about 20 and 50 per cent of that appearing in the crude homogenates of cat hearts and rat brains respectively.

Phosphodiesterase assay. The hydrolysis of cyclic AMP at concentrations near physiological levels was measured by the radiometric assay described by Brooker *et al.*,[15] and Thompson and Appleman,[16] modified so that the incubation mixture contained potential inhibitors of phosphodiesterase activity. The assay is based on the conversion of ^{3}H-cyclic AMP by phosphodiesterase to ^{3}H-5′-AMP, which is further hydrolyzed to ^{3}H-adenosine by the nucleotidase of snake venom. The reaction was stopped by the addition of an anion-exchange resin which binds and quenches all charged nucleotides and leaves ^{3}H-adenosine as the only labeled compound to be counted. Incubations were carried out for 10 min at 37° in a plastic liquid-scintillation counting vial containing the following components: 50 μl of an aqueous solution of inhibitor; 50 μl of the substrate solution used by Brooker *et al.*[15] containing 0·17 μM ^{3}H-cyclic AMP; and 50 μl of PDE solution ($\simeq$1 μg protein) containing human serum albumin (1 mg/ml), and an excess of snake venom nucleotidase (1 mg/ml). Full enzyme activity was determined by substituting 50 μl of 60 mM Tris-HCl buffer, pH 8·0, for the 50 μl of an aqueous solution of inhibitor. Incubation blanks contained all ingredients of the incubation mixture except 50 μl of the PDE solution. For kinetic experiments, the amount of substrate used was as indicated in the figures. All assays were performed between 20 and 30 per cent of the total enzymatic reaction, where the reaction rate was a linear function of enzyme protein concentration and time. Inhibitory potency was determined by comparing the micromolar concentration (I_{50}) of compounds that caused a 50 per cent inhibition of the enzymatic activity.

RESULTS

The results in Table 1 show the influence of a variety of cyclic nucleotides on the activities of partially purified phosphodiesterases prepared from cat heart and rat brain. The enzyme from cat heart was more sensitive to these compounds than was PDE from rat brain. The most potent inhibitors, the cyclic 3′,5′-purine nucleotides, were 200–1000 times more potent than were the corresponding cyclic 2′,3′-purine nucleotides. The cyclic 3′,5′-pyrimidine nucleotides exhibited only borderline inhibition or were inactive.

It has been demonstrated that similar preparations of cyclic nucleotide phosphodiesterase from different tissues have two K_m values for cyclic AMP.[7,15–21] Two different K_m values for cyclic AMP were also demonstrated for our preparations of PDE from cat heart (2 and 1000 μM) and rat brain (4 and 120 μM).[18] The contribution to the total activity by the high-K_m enzyme is insignificant using our assay conditions, since the preparations of PDE were assayed at a cyclic AMP concentration of 0·06 μM. The relative potencies of the compounds reported here, therefore, only refer to inhibition of the low-K_m enzyme.

Kinetic experiments with the PDE from cat heart were carried out to determine the type of inhibition that occurred during the hydrolysis of cyclic AMP in the presence of

TABLE 1. INHIBITION OF CYCLIC 3′,5′-ADENOSINE MONOPHOSPHATE PHOSPHODIESTERASE ACTIVITY BY CYCLIC NUCLEOTIDES*

Compound	I_{50}† (μM)	
	Cat heart PDE	Rat brain PDE
Cyclic 3′,5′-GMP	1	700
Cyclic 3′,5′-IMP	2	940
Cyclic 3′,5′-UMP	1600	Inactive‡
Cyclic 3′,5′-CMP	Inactive	Inactive
Cyclic 3′,5′-TMP	2000	Inactive
Cyclic 2′,3′-GMP	210	Inactive
Cyclic 2′,3′-AMP	600	1700
Cyclic 2′,3′-IMP	2000	NT§
Cyclic 2′,3 -UMP	Inactive	Inactive
Cyclic 2′,3′-CMP	NT	Inactive

* Assays were performed as described in Methods.
† Concentration causing a 50 per cent inhibition of enzymatic activity.
‡ Inactive = $I_{50} > 10^{-2}$ M.
§ NT = not tested.

cyclic GMP. These data were analyzed by the method of Dixon,[25] and are shown in Fig. 1. The inhibition was of the competitive type, with an apparent inhibition constant (K_i) of 0·53 μM. The kinetics of the inhibition of cat heart PDE by cyclic 3′,5′-inosine monophosphate (cyclic IMP) were also studied by the above procedure. These data are not shown, but the inhibition was also competitive, with an apparent K_i value of 2·3 μM.

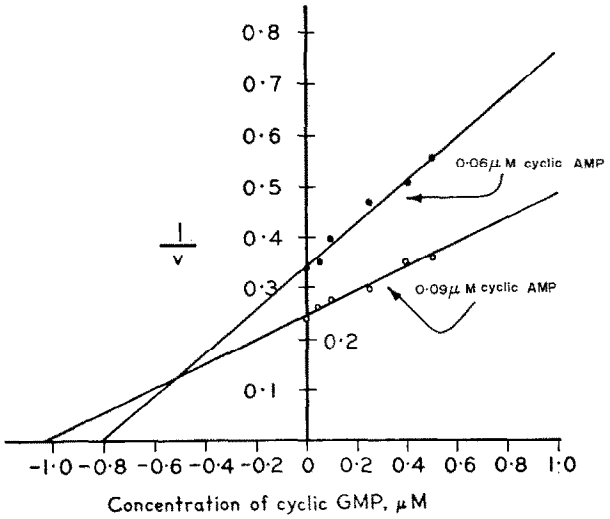

FIG. 1. Dixon[25] analysis of the inhibition of cat heart cyclic AMP phosphodiesterase by cyclic GMP. Each point is the mean of three determinations. The curves represent the best fit, as determined by least-squares regression analysis, using the #. Curfit program supplied by Tymeshare Corp. of Englewood Cliffs, N.J. The unit of velocity is picomoles per microgram of protein per 10 min.

The effects that derivatives of cyclic AMP with a substituent at the C-8 position had on the enzymatic activities of PDE of cat heart and rat brain are shown in Table 2. N^6,2′-*O*-dibutyryl cyclic AMP (dibutyryl cyclic AMP) was included in this study as an example of a frequently used derivative of cyclic AMP. As with the unsubstituted cyclic nucleotides, PDE from cat heart was more sensitive to inhibition by these compounds than was PDE of rat brain. The only exception was the 8-dimethylamino derivative of cyclic AMP, which was a slightly more potent inhibitor of PDE of rat brain.

TABLE 2. INHIBITION OF CYCLIC 3′,5′-ADENOSINE MONOPHOSPHATE PHOSPHODIESTERASE ACTIVITY BY 8-SUBSTITUTED DERIVATIVES OF CYCLIC 3′,5′-ADENOSINE MONOPHOSPHATE*

Cyclic AMP derivatives	I_{50}† (μM)	
	Cat heart PDE	Rat brain PDE
8-Thio cyclic AMP	330	540
8-Methylthio cyclic AMP	39	125
8-Ethylthio cyclic AMP	41	52
8-(2-Hydroxyethyl)thio cyclic AMP	44	200
8-Benzylthio cyclic AMP	24	40
8-Amino cyclic AMP	23	400
8-Azido cyclic AMP	90	150
8-Methylamino cyclic AMP	160	700
8-Dimethylamino cyclic AMP	3300	2300
8-(2-Hydroxyethyl)amino cyclic AMP	270	4400
8-Hydroxy cyclic AMP	Inactive‡	Inactive
8-Methoxy cyclic AMP	130	600
8-Bromo cyclic AMP	16	67
N^6,2′-*O*-dibutyryl cyclic AMP	100	650

* Assays were performed as described in Methods. The 8-substituted derivatives of cyclic AMP were dissolved in 0·01 N NaOH.

† Concentration causing a 50 per cent inhibition of enzymatic activity.

‡ Inactive = $I_{50} > 10^{-2}$ M.

As a group, the most potent inhibitors were those that were substituted at the C-8 position with a sulfur-containing substituent. With the exception of the 8-thio derivative, all of the I_{50} values were below 50 μM for PDE of cat heart. These compounds were only one-half to one-fourth as potent as inhibitors of PDE of rat brain. In general, the potency of these compounds as inhibitors of both enzymes increased as the side-chain size increased. All compounds of this group, except 8-thio cyclic AMP, were more potent inhibitors of both preparations of PDE than was dibutyryl cyclic AMP.

Derivatives of cyclic AMP with a nitrogen-containing substituent at the C-8 position were somewhat less potent inhibitors of the enzymes from both sources. The exception was 8-amino cyclic AMP, which was a potent inhibitor (I_{50} = 23 μM) of PDE of cat heart. In contrast to the 8-sulfur-substituted nucleotides, the extent of the inhibition decreased as the side-chain size of the 8-nitrogen-substituted nucleotides increased. No definite trend could be ascertained for cyclic AMP derivatives with oxygen-containing groups at C-8, since only two representatives of this class were studied.

One of the most potent inhibitors of the 8-substituted cyclic nucleotides was 8-bromo-cyclic AMP, with I_{50} values of 16 and 67 μM for PDE of cat heart and rat brain respectively. Figure 2 shows the inhibition of PDE of cat heart as a function of the concentration of 8-bromo cyclic AMP, as analyzed by the Dixon method.[25] The inhibition exhibited by this compound was competitive, with an apparent K_i value of 20 μM.

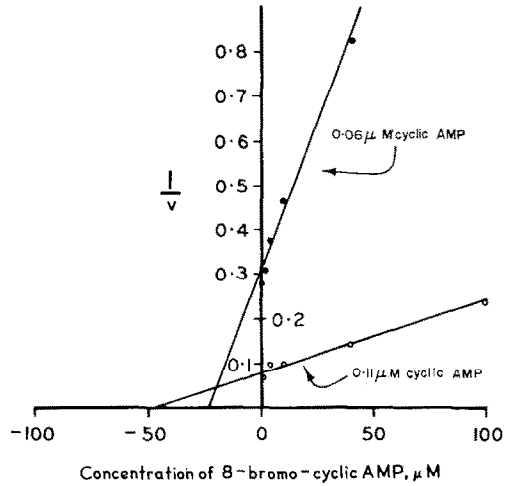

FIG. 2. Dixon[25] analysis of the inhibition of cat heart cyclic AMP phosphodiesterase by 8-bromo-cyclic AMP. Each point is the average of two determinations. The curves represent the best fit, as determined by least-squares regression analysis, as described in Fig. 1. The unit of velocity is picomoles per microgram of protein per 10 min.

DISCUSSION

It has been suggested that cyclic GMP, the only cyclic nucleotide other than cyclic AMP known to occur naturally, may be involved in regulating the intracellular levels of cyclic AMP. Goren and Rosen,[9] utilizing a high concentration of cyclic AMP (1000 μM) as substrate, reported that cyclic GMP was a potent competitive inhibitor of PDE isolated from the supernatant of homogenates of bovine heart ("soluble" PDE). Working with substrate concentrations closer to physiological levels (1 μM), Beavo *et al.*[7,10] showed that cyclic GMP (2 μM) inhibited "soluble" PDE of bovine heart, but had no effect on similar preparations of rat heart and brain. The latter workers also found that this concentration of cyclic GMP stimulated the PDE activity of particulate fractions of rat heart and brain. We have found that cyclic GMP was a potent inhibitor of the "soluble" PDE of cat heart (I_{50} = 1 μM) and a weak inhibitor of this enzyme in rat brain (I_{50} = 700 μM).

Our studies have also shown that cyclic IMP was a potent inhibitor of PDE of cat heart, and that other commercially available cyclic nucleotides were much less potent inhibitors or were inactive. These results agree with those of Rosen[5] who used PDE of frog erythrocyte in her studies. We have also shown that members of a new series of cyclic AMP derivatives with substituents at the C-8 position inhibited PDE isolated from rat brain and cat heart.

If cyclic GMP plays a physiological role in controlling the intracellular levels of cyclic AMP by inhibiting PDE, we suggest that compounds such as the 8-substituted derivatives of cyclic AMP reported here may play a similar role pharmacologically. A prime prerequisite for pharmacologic activity is the ability of such compounds to penetrate the cell membrane. This penetration has been demonstrated by 8-bromo cyclic AMP and by derivatives of cyclic AMP with a sulfur-containing group at the C-8 position. These inhibitors of PDE from both cat heart and rat brain stimulated lipolysis in fat cells and steroidogenesis in adrenal cells,[26] and relaxed tracheal and vascular smooth muscle.[27] A second criterion is that the agent reach the appropriate receptor in an active form. Evidence to support the view that these compounds are stable may be derived from the studies of Muneyama *et al.*,[14] who reported that these 8-substituted derivatives of cyclic AMP, with the exception of 8-amino cyclic AMP, were not hydrolyzed appreciably by PDE of pig brain. Whereas this is good evidence to support the second criterion, degradation by other biochemical reactions may not be ruled out. Another desirable feature of these compounds is their apparent enzyme specificity. We have demonstrated that cyclic nucleotides are, generally, more potent inhibitors of PDE of cat heart than of PDE of rat brain; for example, cyclic GMP and cyclic IMP were approximately 700 and 500 times, respectively, more potent as inhibitors of PDE of cat heart than of PDE of rat brain.

In addition to the properties mentioned above, Muneyama *et al.*[14] have shown that these 8-substituted derivatives of cyclic AMP mimic endogenous cyclic AMP by activating cyclic AMP-dependent protein kinase. Activation of protein kinase may be the primary mechanism of action of compounds of this type, but it is likely that this activity may be enhanced by endogenous cyclic AMP that is protected from hydrolysis by those cyclic nucleotides that are also inhibitors of PDE. The combination of these two activities should be particularly significant under conditions where endogenous cyclic AMP levels are elevated by hormonal stimulation.

REFERENCES

1. C. G. Smith, *A. Rep. med. Chem.* **2,** 286 (1966).
2. G. A. Robison, R. W. Butcher and E. W. Sutherland, *A. Rev. Biochem.* **37,** 149 (1968).
3. E. W. Sutherland, *J. Am. med. Ass.* **214,** 1281 (1970).
4. G. W. Liddle and J. G. Hardman, *New Engl. J. Med.* **285,** 560 (1971).
5. O. M. Rosen, *Archs Biochem. Biophys.* **137,** 435 (1970).
6. O. M. Rosen, *Archs Biochem. Biophys.* **139,** 447 (1970).
7. J. A. Beavo, J. G. Hardman and E. W. Sutherland, *J. biol. Chem.* **245,** 5649 (1970).
8. F. Murad, V. Manganiello and M. Vaughan, *J. biol. Chem.* **245,** 3352 (1970).
9. E. N. Goren and O. M. Rosen, *Archs Biochem. Biophys.* **142,** 720 (1971).
10. J. A. Beavo, J. G. Hardman and E. W. Sutherland, *J. biol. Chem.* **246,** 3841 (1971).
11. D. J. Franks and J. P. MacManus, *Biochem. biophys. Res. Commun.* **42,** 844 (1971).
12. F. Eckstein and H.-P. Bar, *Biochim. biophys. Acta* **191,** 316 (1969).
13. G. I. Drummond and C. A. Powell, *Molec. Pharmac.* **6,** 24 (1970).
14. K. Muneyama, R. J. Bauer, D. A. Shuman, R. K. Robins and L. N. Simon, *Biochemistry, N.Y.* **10,** 2390 (1971).
15. G. Brooker, L. J. Thomas, Jr. and M. M. Appleman, *Biochemistry, N.Y.* **7,** 4177 (1968).
16. W. J. Thompson and M. M. Appleman, *Biochemistry, N.Y.* **10,** 311 (1971).
17. W. J. Thompson and M. M. Appleman, *J. biol. Chem.* **246,** 3145 (1971).
18. M. Chasin, D. N. Harris, M. B. Phillips and S. M. Hess, *Biochem. Pharmac.*, **21,** 2443 (1972).
19. S. Jard and M. Bernard, *Biochem. biophys. Res. Commun.* **41,** 781 (1970).
20. Y.-C. Huang and R. Kemp, *Biochemistry, N.Y.* **10,** 2278 (1971).
21. E. G. Loten and J. G. T. Sneyd, *Biochem. J.* **120,** 187 (1970).
22. S. Chaykin, *Biochemistry Laboratory Techniques*, p. 17. John Wiley, New York (1966).

23. A. G. GORNALL, C. G. BANDAWILL and M. M. DAVID, *J. biol. Chem.* **177,** 751 (1949).
24. O. H. LOWRY, N. J. ROSEBROUGH, A. L. FARR and R. J. RANDALL, *J. biol. Chem.* **193,** 265 (1951).
25. M. DIXON, *Biochem. J.* **55,** 170 (1953).
26. C. A. FREE, M. CHASIN, V. PAIK and S. M. HESS, *Biochemistry, N.Y.* **10,** 3785 (1971).
27. B. RUBIN, E. H. O'KEEFE, M. H. WAUGH, D. A. DEMAIO and D. G. KOTLER, *Proc. Soc. exp. Biol. Med.* **137,** 1244 (1971).