MOLECULAR PHARMACOLOGY, 6, 24–30 (1970)

Analogues of Adenosine 3′,5′-Cyclic Phosphate as Activators of Phosphorylase *b* Kinase and as Substrates for Cyclic 3′,5′-Nucleotide Phosphodiesterase

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(Received July 18, 1969)

SUMMARY

The ability of several analogues of adenosine cyclic 3′,5′-phosphate to activate phosphorylase *b* kinase was investigated. Tubercidin 3′,5′-cyclic phosphate was slightly more active than cyclic 3′,5′-AMP. Compounds that involve structural alteration of the phosphate moiety, such as adenosine 3′,5′-cyclic phosphorothioate and two isomeric cyclic phosphonate compounds, were either extremely weak activators or inactive. Adenine 9-β-D-xylofuranosyl 3′,5′-cyclic phosphate and N^6,2′-*O*-dibutyryl cyclic 3′,5′-AMP, structures which involve modification of the sugar moiety, were also relatively inactive. The studies point to a remarkable specificity, especially with regard to the intactness of the phosphate moiety.

Of the compounds tested, only cyclic 3′,5′-AMP and tubercidin 3′,5′-cyclic phosphate were effective substrates for cyclic 3′,5′-nucleotide phosphodiesterase. A marked requirement for the unmodified phosphate group is apparent for interaction with this enzyme, whereas there is less specificity with regard to the base moiety.

INTRODUCTION

Adenosine 3′,5′-cyclic phosphate is recognized as an important intracellular messenger and mediator of hormone action (1, 2). At least some of its actions can be explained through a regulatory action on a number of enzymes (1, 3). One of the more thoroughly studied enzymes in this regard is phosphorylase *b* kinase, the enzyme that catalyzes the conversion of phosphorylase *b* to phosphorylase *a*. Krebs *et al.* (4) have shown that the ATP-dependent activation of this enzyme *in vitro* is facilitated by cyclic 3′,5′-AMP at concentrations of the order of 10^{-8} M. This reaction is utilized as a precise and sensitive assay of the cyclic nucleotide in biological materials (5, 6). Clarification of the actions of cyclic 3′,5′-AMP has been assisted by the availability of several analogues, particularly those differing in the base moiety (7, 8), and certain *N*-and *O*-substituted derivatives such as N^6,2′-*O*-dibutyryl cyclic 3′,5′-AMP (9, 10). Recently several analogues which involve modification of the phosphate moiety have become available, namely, adenosine 3′,5′-cyclic phosphorothioate and two isomeric cyclic phosphonate derivatives. Another compound, adenine xylofuranosyl 3′,5′-cyclic phosphate, involves modification of the sugar moiety. The present report is concerned with the ability of these compounds and certain other analogues to facilitate the ATP-dependent activation of phosphorylase *b* kinase. Their ability to act

This work was supported by grants from the British Columbia Heart Foundation and the Medical Research Council of Canada.

as substrates or inhibitors of cyclic 3′,5′-nucleotide phosphodiesterase is also examined.

MATERIALS AND METHODS

Tubercidin 3′,5′-cyclic phosphate (11) and tubercidin 5′-phosphate were obtained from Dr. Arthur R. Hanze, the Upjohn Company, Kalamazoo, Michigan. Adenosine 3′,5′-cyclic phosphorothioate was provided by Dr. F. Eckstein, Max-Planck Institut für experimentelle Medizin, Göttingen, Germany. Adenine 9-β-D-xylofuranosyl 3′,5′-cyclic phosphate was obtained through the courtesy of Dr. Harry B. Wood, Jr., Cancer Chemotherapy National Service Center, Bethesda, Maryland. The cyclic phosphonate compounds, the 3′-cyclic ester of 9-[5′-deoxy-5′ - dihydroxyphosphinylmethyl - β - D - ribofuranosyl]adenine (compound I), and the 5′-cyclic ester of 9-[3′-deoxy-3′-dihydroxyphosphinylmethyl - β - D - ribofuranosyl] - adenine (compound II),[1] as well as adenosine 5′-deoxy-5′-methylenephosphonic acid, were gifts from Dr. J. G. Moffatt, Syntex Institute of Molecular Biology, Palo Alto, California. The structural similarities between these compounds and cyclic 3′,5′-AMP are apparent from Fig. 1. Other cyclic nucleotides and tubercidin (7-deazaadenosine) were obtained from commercial sources.

Paper chromatography was effected by the descending technique on Whatman No. 1 or 3MM paper. The solvent systems used were: 2-propanol-ammonia-water (7:1:2 by volume) (solvent I) and 1 M ammonium acetate–95% ethanol (15:35 by volume) (solvent II).

Phosphorylase *b* kinase was prepared (30 S fraction) from rabbit skeletal muscle by the procedure of Krebs *et al.* (4). Cyclic 3′,5′-nucleotide phosphodiesterase was prepared from rabbit brain by a modification of the procedure used previously (12). The cerebral cortices from two rabbits were homogenized in 10 volumes of 0.25 M sucrose in a Potter-Elvehjem homogenizer. The

[1] Compounds I and II will be referred to in the text as the 5′-methylene cyclic phosphonate and the 3′-methylene cyclic phosphonate, respectively.

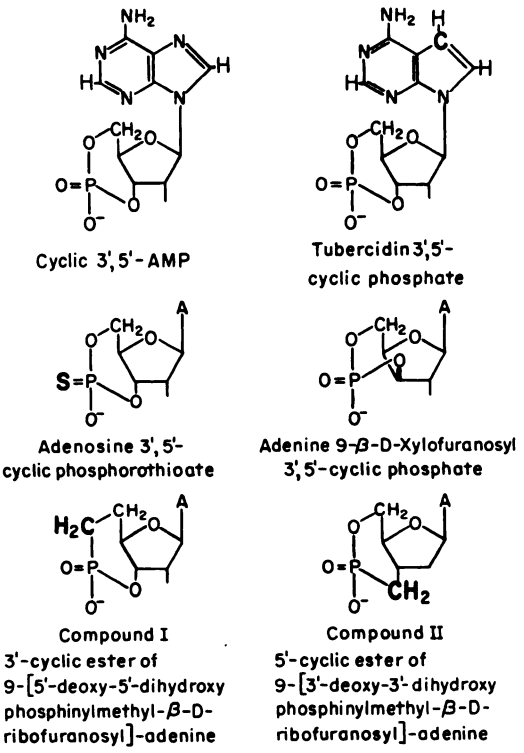

FIG. 1. *Analogues of cyclic 3′,5′-AMP*
The structural differences from cyclic 3′,5′-AMP are shown in bold lettering.

homogenate was adjusted to pH 7.0 and centrifuged for 1 hr at 38,000 × *g*. The supernatant solution was set aside, and the residue was homogenized in 2 volumes of sucrose containing 1% sodium deoxycholate. After 1 hr at 4°, the preparation was centrifuged as before. The combined supernatant fluids were brought to 30% saturation with ammonium sulfate at pH 7.0. After centrifugation at 38,000 × *g* for 20 min, the precipitate was suspended in 15 ml of 1 mM imidazole containing 1 mM $MgSO_4$, pH 7.0. The opalescent solution was centrifuged at 100,000 × *g* for 90 min, and the recovered supernatant fluid was dialyzed against 3 liters of the above buffer. The protein content was 5.6 mg/ml. The preparation catalyzed the hydrolysis of 0.087 μmole of cyclic 3′,5′-AMP per milligram per minute in the test tube assay described below.

Activation of phosphorylase kinase. Meas-

urements of the activation of phosphorylase kinase were conducted essentially as described by Posner *et al.* (5) for the assay of cyclic 3′,5′-AMP, except that the activation reaction was carried out at 4° for 30 min (13). Solutions of the compounds to be analyzed were made up in 25 mM potassium phosphate, pH 7.5, and 0.1-ml aliquots were added to the activation assay. Each compound was tested at 6–10 concentrations in the range over which it was active, and each assay was conducted coincidentally with cyclic 3′,5′-AMP concentrations ranging from 1 to 20 × 10^{-8} M. A control tube contained ATP and Mg^{++} but no cyclic nucleotide. In this way the activation above that produced by ATP alone was readily determined. Following 30 min of incubation at 4°, the reaction was terminated by diluting a 0.1-ml aliquot of each tube 15- or 30-fold with ice-cold 15 mM neutral cysteine. A 0.2-ml aliquot of this dilute solution was then assayed for phosphorylase kinase at pH 6.8 according to Posner *et al.* (5), except that the substrate was 10,000 Cori units of phosphorylase (contained in 0.1 ml).

Cyclic 3′,5′-nucleotide phosphodiesterase measurements. Each cyclic nucleotide (1.5 μmoles) was added to tubes containing Tris-HCl, pH 7.5 (50 μmoles), magnesium acetate (0.2 μmole), and water, the final volume being 0.2 ml. Brain extract (11.2–564 μg of protein) was added, and the tubes were incubated at 30°. Tubes containing the cyclic nucleotide and buffer, but no enzyme, served as controls. At the appropriate interval (20 min for cyclic 3′,5′-AMP and tubercidin 3′,5′-cyclic phosphate, 120 min in all other cases) the reaction was stopped by the addition of glacial acetic acid (20 μl), and the tubes were placed in ice. An aliquot (25 μl) of each was subjected to paper chromatography in solvent I. When dibutyryl cyclic 3′,5′-AMP was tested, the chromatograms were developed in solvent II. Unreacted substrate spots were cut out and eluted with water to a final volume of 1.5 ml. The absorbance of the eluates was determined at 258 mμ, and the quantity of substrate hydrolyzed was calculated using a molecular extinction for each compound equivalent to that of cyclic 3′,5′-AMP, i.e., 14,650 M^{-1} cm^{-1} at 258 mμ and pH 7.0 (7). In some cases, diesterase activity was measured by a spectrophotometric method (12) in which hydrolysis is coupled to the deamination of the nucleotide product with adenylic deaminase.

RESULTS

Activation of phosphorylase kinase. A typical activation of phosphorylase kinase by

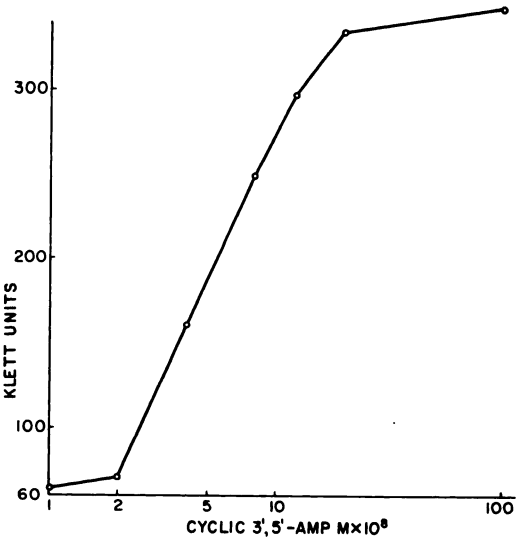

FIG. 2. *Activation of phosphorylase b kinase by cyclic 3′,5′-AMP*

The activation reaction was carried out as follows. To tubes in an ice-water bath were added 0.1 ml of 0.125 M Tris–0.125 M β-glycerophosphate buffer, pH 6.8; 0.1 ml of a solution of 18 mM ATP and 60 mM magnesium acetate (pH 7.0); 0.1 ml of cyclic 3′,5′-AMP (or other analogue) in 25 mM potassium phosphate, pH 7.5, at the appropriate concentration; and 0.1 ml of H_2O. A control tube contained the ATP-magnesium acetate solution, but no cyclic nucleotide. The reaction was started by the addition of 0.1 ml of phosphorylase *b* kinase solution [30 S fraction, 25,000 units/ml (13)]. The tubes were incubated for 30 min at 4°. A 0.1-ml aliquot of each tube was diluted with 2.9 ml of cold, neutral 15 mM cysteine (which effectively terminated the activation), and a 0.2-ml aliquot of this dilute solution was assayed immediately for phosphorylase kinase at pH 6.8 by the procedure of Posner *et al.* (5), except that the substrate was 10,000 Cori units of phosphorylase (13). Inorganic phosphate released was measured in a Klett colorimeter, and the readings are plotted against the concentration of cyclic 3′,5′-AMP in the activation reaction.

cyclic 3′,5′-AMP is shown in Fig. 2. From 10 such determinations, the mean concentration for half-maximal activation was found to be 7.3×10^{-8} M. This is in agreement with the apparent K_m for the cyclic nucleotide (7×10^{-8} M) recently established by DeLange *et al.* (14). The effectiveness of the various cyclic nucleotides in activating the enzyme is shown in Table 1. Tubercidin 3′,5′-cyclic phosphate proved to be slightly more active than cyclic 3′,5′-AMP. The other compounds tested were strikingly less effective; concentrations for half-maximal activation ranged from 6×10^{-6} M for compound I (the 5′-methylene cyclic phosphonate) to 2×10^{-5} M for adenosine 3′,5′-cyclic phosphorothioate. Adenine xylofuranosyl 3′,5′-cyclic phosphate produced no activation at concentrations as high as 6×10^{-4} M, and compound II (the 3′-methylene cyclic phosphonate) was inactive at concentrations as high as 4×10^{-4} M. Larger amounts were not available for testing, but if these latter two compounds possess any activity, it would be less than 0.01 % of that of cyclic 3′,5′-AMP.

Because the latter compounds failed to activate the enzyme, they were tested as possible inhibitors of cyclic 3′,5′-AMP. When present in the activation reaction at 1×10^{-4} M, each caused no inhibition of the activation produced by 12×10^{-8} M cyclic 3′,5′-AMP.

Hydrolysis by cyclic 3′,5′-nucleotide diesterase. The ability of the several analogues to act as substrate for the diesterase is shown in Table 2. Tubercidin 3′,5′-cyclic phosphate was hydrolyzed almost 3 times more rapidly than cyclic 3′,5′-AMP. Of the remaining compounds, only compound I (the 5′-methylene cyclic phosphonate) and adenine xylofuranosyl 3′,5′-cyclic phosphate were hydrolyzed at measurable rates. The product of hydrolysis of tubercidin 3′,5′-cyclic phosphate migrated identically with authentic tubercidin 5′-phosphate on chromatography in solvent I and on electrophoresis at pH 7.5 (3000 V). A second, minor ultraviolet-absorbing spot was also evident, which migrated with authentic tubercidin on chromatography and, like authentic tubercidin, failed to migrate on high-

TABLE 1

Effect of cyclic 3′,5′-AMP analogues on the activation of phosphorylase kinase

Conditions are described in the text. Except for cyclic 3′,5′-AMP, each value is the mean of two separate determinations. The value for cyclic 3′,5′-AMP was arbitrarily taken as 100. The relative activity of each compound is expressed as a percentage of that value.

Compound	Concentration for half-maximal activation	Relative activity
	M × 10^8	%
Cyclic 3′,5′-AMP	7.3	100
Tubercidin 3′,5′-cyclic phosphate	5.5	133
Adenosine 3′,5′-cyclic phosphorothioate	2000	0.36
Adenine xylofuranosyl 3′,5′-cyclic phosphate	ND[a]	<0.01
Compound I	600	1.22
Compound II	ND[b]	<0.01
N^6,2′-*O*-Dibutyryl cyclic 3′,5′-AMP	700	1.04
Cyclic 3′,5′-UMP	850	0.83
Cyclic 3′,5′-CMP	750	0.97

[a] No activation was detectable at 6×10^{-4} M.

[b] No activation was detectable at 4×10^{-4} M (see the text).

TABLE 2

Action of cyclic 3′,5′-nucleotide phosphodiesterase on analogues

Except for cyclic 3′,5′-AMP and tubercidin 3′,5′-cyclic phosphate, all compounds were incubated with the diesterase (564 μg of protein) for 120 min at 30° (see the text).

Compound	Velocity	Relative rate
	μmoles/mg/20 min	%
Cyclic 3′,5′-AMP	1.7	100
Tubercidin 3′,5′-cyclic phosphate	5.0	295
Adenosine 3′,5′-cyclic phosphorothioate	0	0
Adenine xylofuranosyl 3′,5′-cyclic phosphate	0.066	3.9
Compound I	0.071	4.2
Compound II	0	0
N^6,2′-*O*-Dibutyryl cyclic 3′,5′-AMP	0	0
Cyclic 3′,5′-CMP	0	0

voltage electrophoresis at pH 7.5. This suggests that the primary product, tubercidin 5′-phosphate, was dephosphorylated presumably by a phosphatase or 5′-nucleotidase present in the brain preparation. The optical cyclic nucleotide phosphodiesterase assay (12) coupled to adenylic deaminase could not be used for tubercidin 3′,5′-cyclic phosphate, because the product, tubercidin 5′-phosphate, was not deaminated by the latter enzyme. The product of the enzymatic hydrolysis of compound I (the 5′-methylene cyclic phosphonate) was identified as the 6′-phosphonate, i.e., adenosine 5′-deoxy-5′-methylenephosphonic acid, by virtue of cochromatography with the authentic compound in solvent I and by identical mobility on electrophoresis at pH 7.5. Moreover, the product underwent deamination with adenylic deaminase to the corresponding inosine derivative, as shown by the ultraviolet absorption spectrum. In separate experiments, it was determined that authentic adenosine 5′-deoxy-5′-methylenephosphonic acid was deaminated by adenylic deaminase at 26% of the rate of 5′-AMP. In fact, the hydrolysis of the 5′-methylene cyclic phosphonate by the diesterase could readily be followed spectrophotometrically by coupling with adenylic deaminase and following the decrease in absorbance at 265 mμ. The hydrolysis of adenine xylofuranosyl 3′,5′-cyclic phosphate, surprisingly, could not be followed in the spectrophotometric assay, because the product (presumably the 5′-phosphate) was not a substrate for adenylic deaminase.

Because adenosine 3′,5′-cyclic phosphorothioate and compound II (the 3′-methylene cyclic phosphonate) were not attacked by the diesterase, the possibility was considered that they might act to inhibit the enzyme. When tested in the spectrophotometric assay (cyclic 3′,5′-AMP concentration, 1.35×10^{-4} M), the cyclic phosphorothioate produced no inhibition at 2×10^{-4} M. This would indicate that this compound is neither a substrate nor an inhibitor of the enzyme. Similar observations have been made by Bär and Eckstein.[2] Compound II (the 3′-methylene cyclic phosphonate) was tested as an inhibitor of the diesterase in the spectrophotometric assay, using a cyclic 3′,5′-AMP concentration of 1×10^{-4} M. Under these conditions the 3′-methylene cyclic phosphonate produced no measurable inhibition at 1×10^{-4} M, but when present at 2×10^{-4} M (the maximum that could be added in the optical assay) it caused 20% inhibition. In another experiment, the compound was tested for inhibition in the standard test tube assay at a concentration of 7.5 mM (equivalent to that of cyclic 3′,5′-AMP). Since both cyclic compounds migrated identically in solvents I and II, unreacted substrate could not be used for the determination of reaction rates. Instead the product (5′-AMP) was eluted and determined optically. Under these conditions the 3′-methylene cyclic phosphonate produced 30% inhibition. It might be concluded that this compound is a weak inhibitor of the diesterase.

[2] H. P. Bär and F. Eckstein, personal communications.

DISCUSSION

We have shown previously (15), from studies on heart phosphorylase kinase, that a variety of ribonucleoside and deoxyribonucleoside 3′,5′-cyclic phosphates other than cyclic 3′,5′-AMP were unable to activate the enzyme except at concentrations above 1×10^{-5} M. These and the present data point to a striking specificity of the activation reaction for cyclic 3′,5′-AMP, and to the probability that the base, the sugar moiety, and the phosphate diester group all influence binding. The activity of tubercidin 3′,5′-cyclic phosphate indicates that the N^7 position of the ring probably does not participate in binding. That the integrity of the ribofuranosyl moiety must remain unaltered is apparent from the fact that the deoxyadenosine 3′,5′-cyclic phosphate (15) and adenine xylofuranosyl 3′,5′-cyclic phosphate are inactive. The latter compound, in particular, is an extremely close analogue of cyclic 3′,5′-AMP. However, the 3′-hydroxyl above the plane of the ring allows for the formation of an unstrained phosphate diester linkage, in contrast to the highly strained grouping in cyclic 3′,5′-AMP. Displacement of the phosphate moiety above the plane of the ribofuranosyl ring could greatly affect binding. The very low activity of the

5′-methylene cyclic phosphonate and the complete lack of activity of the 3′-methylene cyclic phosphonate and of adenosine 3′,5′-cyclic phosphorothioate, as well as the failure of the latter two compounds to act as inhibitors of the activation, indicate a striking selectivity for the unmodified phosphate group.

DeLange *et al.* (14) have reported that although cyclic 3′,5′-AMP markedly activated phosphorylase *b* kinase, no binding of the cyclic nucleotide to the enzyme could be demonstrated. More recently Walsh, Perkins, and Krebs (16) have described a protein kinase that catalyzes the ATP-dependent phosphorylation and activation of phosphorylase kinase. This protein kinase has an absolute requirement for cyclic 3′,5′-AMP. Walsh *et al.* suggested that activation of phosphorylase kinase by the cyclic nucleotide is due to the presence of protein kinase. The protein kinase also catalyzed the cyclic 3′,5′-AMP-dependent phosphorylation of casein and protamine, so that a more direct method of examining the specificity of binding may now be available. By the use of this enzyme and analogues of cyclic 3′,5′-AMP, it will undoubtedly be possible to gain a precise understanding of the mechanism whereby cyclic 3′,5′-AMP activates phosphorylase kinase.

The present data provide some further information on the specificity of the cyclic 3′,5′-nucleotide phosphodiesterase. Previous studies (8, 13, 17) have revealed that, with the exception of cytidine 3′,5′-cyclic phosphate, various ribonucleoside and deoxyribonucleoside 3′,5′-cyclic phosphates are hydrolyzed at significant rates. This would indicate a low degree of specificity with regard to the base moiety. The rapid rate of hydrolysis of tubercidin 3′,5′-cyclic phosphate is in accord with this. The present studies indicate a much more striking requirement for an unmodified phosphate group. Thus adenosine 3′,5′-cyclic phosphorothioate and the 3′-methylene cyclic phosphonate analogue are neither substrates nor effective inhibitors, which would indicate a very low degree of binding. In addition, the 5′-methylene cyclic phosphonate is a poor substrate, as is adenine xylofuranosyl 3′,5′-cyclic phosphate. Although in the latter compound the phosphate group is intact, its planar position is altered.

The low activity of N^6,2′-*O*-dibutyryl cyclic 3′,5′-AMP in the kinase activation assay is of interest, since the compound is widely used in intact cellular preparations and is known to activate phosphorylase in dog liver slices (10), to increase blood glucose (18), and to promote lipolysis in rat epididymal fat pads (6). Posternak, Sutherland, and Henion (18) have reported that an esterase present in dog liver effectively removes the acyl group in the 2′-*O* position. It does not seem entirely clear whether this compound itself is active in intact preparations or whether it is converted to cyclic 3′,5′-AMP or to the N^6-acyl derivative. The compound is not hydrolyzed by the brain diesterase or by the enzyme from heart (18). The brain preparation used here is still relatively crude, but no alteration of the dibutyryl derivative was noted. It would be of interest to know whether this compound is catabolized differently in various tissues.

ACKNOWLEDGMENTS

We wish to thank the various individuals named in the text for providing samples of the compounds tested. We are grateful to Drs. H. P. Bär and J. G. Moffatt for several helpful discussions, and to Dr. F. Eckstein for providing us information on adenosine 3′,5′-cyclic phosphorothioate.

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