Hydrolysis of Cyclic Guanosine and Adenosine 3',5'-Monophosphates by Rat and Bovine Tissues*

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J. A. Beavo,‡ Joel G. Hardman, and Earl W. Sutherland§

From the Department of Physiology, School of Medicine, Vanderbilt University, Nashville, Tennessee 37203

SUMMARY

An assay for measuring 3',5'-cyclic nucleotide phosphodiesterase activity over a wide range of substrate concentrations has been described. The ratio of cyclic adenosine 3',5'-monophosphate (cyclic AMP) to cyclic guanosine 3',5'-monophosphate (cyclic GMP) hydrolysis varied several fold among crude subcellular fractions from bovine and rat heart when measured at micromolar but not millimolar substrate. In rat brain, liver or skeletal muscle, this ratio was virtually the same in crude subcellular fractions as in the homogenate when determined at either micromolar or millimolar substrate.

A partially purified phosphodiesterase from the bovine heart supernatant had apparent $K_m$ values of 1 to 3 $\mu M$ for cyclic GMP and 25 to 45 $\mu M$ for cyclic AMP. Cyclic AMP and cyclic GMP interfered with the hydrolysis of each other in a manner predictable on the basis of their apparent $K_m$ values, suggesting that this bovine heart preparation contained only a single phosphodiesterase. However, in a crude liver supernatant cyclic GMP stimulated rather than inhibited cyclic AMP hydrolysis. A crude particulate fraction from bovine heart had two apparent $K_m$ values for cyclic AMP of about 0.8 and 25 $\mu M$. Most, if not all, of the cyclic GMP phosphodiesterase activity in this fraction had an apparent $K_m$ of about 20 $\mu M$.

Data obtained using the partially purified bovine heart enzyme indicate that both cyclic AMP and cyclic GMP can serve as substrates for a single enzyme. However, differences in the ratios of hydrolysis of the two nucleotides among subcellular fractions and kinetic data suggest the existence of more than one 3',5'-cyclic nucleotide phosphodiesterase within a tissue. Whether these phosphodiesterase activities have relative substrate preferences or whether one or more might have specific substrate requirements has not been established.

Cyclic adenosine 3',5'-monophosphate has been shown to be the intracellular agent which mediates the action of a number of hormones. The literature concerning the formation and functions of cyclic AMP has been reviewed several times (2-6). Cyclic guanosine 3',5'-monophosphate has been identified in urine and in a number of tissues (7-12). The enzyme which forms cyclic GMP from guanosine triphosphate has been investigated (10, 13, 14), but no specific function of this cyclic nucleotide has yet been established.

The intracellular concentrations of cyclic AMP and cyclic GMP are determined in part by the activities of one or more nucleoside 3',5'-monophosphate phosphodiesterases which catalyze the hydrolysis of the 3'-phosphate bond yielding the nucleoside 5'-monophosphate. Studies of cyclic nucleotide phosphodiesterase activity from several tissues have been conducted (15-20). We wanted to determine whether or not more than one cyclic nucleotide phosphodiesterase was present within a tissue and if so, whether they had relative or absolute substrate specificities. One of the approaches we used was to examine the hydrolysis of cyclic AMP and cyclic GMP by crude subcellular fractions from bovine heart and rat liver, brain, heart, and skeletal muscle. In addition, we determined some of the kinetic features of cyclic AMP and cyclic GMP hydrolysis by a partially purified soluble preparation (16) and a crude particulate fraction from bovine heart.

Butcher and Sutherland (16) described a convenient assay for determining phosphodiesterase activity which is applicable to cyclic nucleotides at concentrations greater than 10 $\mu M$. In order to study the hydrolysis of cyclic AMP and cyclic GMP with substrate concentrations closer to physiological levels, we used an assay based on the conversion of tritium-labeled substrate to labeled product. Analogous assays have been used by others (21-26).

EXPERIMENTAL PROCEDURE

Assay Procedures—The reaction sequence involved in this assay is similar in principle to that described by Butcher and Sutherland (16) except that tritiated substrate is used.

1 The abbreviations used are: cyclic AMP, cyclic adenosine 3',5'-monophosphate; cyclic GMP, cyclic guanosine 3',5'-monophosphate.
Hydrolysis of Cyclic GMP and Cyclic AMP

Remaining cyclic nucleotide is separated from the dephosphorylated tritiated products by anion exchange chromatography, and the amounts of both the final product and remaining substrate are determined.

Several concentrations of cyclic nucleotide containing about 500,000 dpm of tritiated nucleotide were incubated at 30° with a phosphodiesterase preparation for an appropriate time interval, usually 15 min, in a medium containing 10 pmoles of Tris-Cl, pH 7.5, and 0.5 μmole of MgCl₂ in a final volume of 0.25 ml. In kinetic studies, initial rates were estimated from incubations at time intervals or at several dilutions of the enzyme. In other studies, a suitable dilution of the phosphodiesterase preparation was chosen to produce a 15 to 30% conversion of substrate to product. Dilutions of the purified bovine heart enzyme were made in an aqueous solution of bovine serum albumin (1 mg per ml). Dilutions of the other enzyme preparations were made with the homogenization medium. The reaction was started by addition of the enzyme preparation and stopped by heating in a water bath at 95-98° for 75 sec, after which excess Crotalus atrox venom (0.05 to 0.10 mg) was added and the mixture incubated for 10 min at 30°. In some experiments, the reaction mixture was again heated at 95-98° for 60 sec, but this step was found to be unnecessary if the samples were fractionated immediately. Since boiling raised the blank, the second heat step was usually omitted. Control (blank) values were obtained by replacing the enzyme with water or with a previously boiled enzyme preparation. No differences were noted between the two controls.

After incubation with venom, the reaction mixture was applied to a column (4.5 × 0.5 cm) of Dowex-2 resin which had been washed to pH 7.5 with 3 bed volumes of 0.04 M Tris-Cl buffer. Pilot experiments showed that no cyclic nucleotide was eluted in the Tris wash, but that more than 95% of it was contained in the following 35 ml of HCl. When the cyclic nucleotides were completely hydrolyzed by excess phosphodiesterase and venom, more than 95% of the radioactivity was eluted in this Tris fraction. Aliquots of both the Tris and acid fractions were counted in a liquid scintillation spectrometer and the results corrected for quenching by the use of internal standards.

Preparation of Subcellular Fractions—Fresh bovine hearts, obtained at a local slaughter house and kept on ice, were used within 2 hours after being removed from the animals. Rat tissues, obtained from female Sprague-Dawley rats weighing 120 to 200 g, were removed immediately after stunning the animal by a blow to the head. The tissues were freed of any large pieces of connective tissue and finely minced with scissors. They were then homogenized in 9 volumes of 0.04 M Tris-Cl, pH 7.5-0.25 M sucrose medium by six passes of a motor-driven pestle in a loose fitting glass-Teflon homogenizer. The homogenate was centrifuged at 1,000 × g for 6 min at 0°, the supernatant decanted, and the precipitate washed 3 times by resuspending it in the original homogenate volume of fresh medium. The 1,000 × g supernatant fraction was then centrifuged at 15,000 × g for 15 min at 0° and this precipitate washed twice as described for the 1,000 × g precipitate. The supernatant fraction from the 15,000 × g centrifugation was centrifuged at 70,000 × g for 1 hour at 0° and the supernatant fraction decanted. All precipitates were resuspended in Tris-sucrose medium at the volume of the original homogenate.

Materials—Tritiated and nonlabeled cyclic AMP were purchased from Schwarz BioResearch, and cyclic GMP, prepared by Dr. T. Posternak, was custom labeled by Schwarz BioResearch. All the cyclic nucleotides were purified on Dowex-50 cation exchange resin columns (13). Crotalus atrox venom was purchased from the Ross Allen Reptile Institute, Silver Springs, Florida. Dowex-2 (Bio-Rad, AG2 X8, 100 to 200 mesh, chloride form) was obtained from Bio-Rad, Richmond, California, and prepared for use essentially as described previously.
RESULTS

Validation of Assay One possible metabolite of cyclic GMP in crude enzyme preparations, xanthosine, was found in pilot studies not to be separated from cyclic GMP by the Dowex-2 fractionation system. Therefore, with each tissue the results obtained by Dowex-2 fractionation were compared with those obtained by another assay or fractionation procedure since transformation to xanthosine or another such metabolite might have occurred in the crude preparations. At the higher substrate concentrations, the results were compared to those obtained by the method of Butcher and Sutherland (16). At lower substrate levels the results were compared to those obtained by isolation and assay of the remaining substrate using Dowex-50 cation exchange resin chromatography columns (13). At all substrate concentrations used, the results obtained by the Dowex-2 fractionation agreed well with the values obtained by the other methods.

Distribution among Subcellular Fractions—Phosphodiesterase activity in all fractions was measured at both millimolar and micromolar substrate levels. The results obtained at the high substrate level probably reflect relative V_max values while those obtained at the lower substrate level probably reflect both the relative V_max and K_m values. The results from subcellular distribution studies carried out using four tissues of the rat are shown in Figs. 1 through 4.

In brain the ratio of cyclic AMP to cyclic GMP hydrolysis did not vary appreciably among any of the fractions at either substrate level (Fig. 1). Cyclic GMP was hydrolyzed significantly faster than cyclic AMP at all substrate levels (p < 0.05 or less). In the 70,000 × g particles, cyclic AMP was hydrolyzed slightly faster (p < 0.05).

Graphs and figures are not transcribed here.
and Wieland (20) in that most of the total phosphodiesterase activity was contained in the soluble fraction, but they differ in that no apparent specificity for cyclic GMP was noted in the 1,000 x g particulate fraction as these workers have reported.

In the rat skeletal muscle, no large differences in the cyclic AMP to cyclic GMP hydrolysis ratios were noted among the fractions at either substrate level (Fig. 3). However, this tissue was different from others examined in that, at micromolar substrate, cyclic AMP was hydrolyzed considerably faster than cyclic GMP even in the supernatant fraction.

In the rat heart there was a considerable difference between the supernatant and 1000 x g particulate fractions in the ratio of cyclic AMP to cyclic GMP hydrolysis as measured at millimolar substrate (Fig. 4). At the low substrate level, cyclic GMP was hydrolyzed as fast as cyclic AMP by the supernatant, while cyclic AMP was hydrolyzed faster by the particles. Cyclic AMP was hydrolyzed slightly faster than cyclic GMP in the homogenate and supernatant when measured at millimolar substrate. In this tissue most of the activity was found in the supernatant and 1000 x g particles.

The hydrolysis of cyclic AMP and cyclic GMP by crude subcellular fractions from bovine heart is shown in Fig. 5. As in the rat heart there were large differences in the apparent substrate specificity among various fractions when measured at

![Graph](image-url)

**FIG. 4.** Distribution of cyclic nucleotide phosphodiesterase activity among crude subcellular fractions from rat heart. Conditions and procedures were the same as described in the legend to Fig. 1. Values are the average of four experiments ± S.E. At 1 mM substrate, cyclic AMP was hydrolyzed significantly faster than cyclic GMP in the homogenate (p < 0.005, paired analysis) and in the supernatant (p < 0.05). At 1 μM substrate cyclic AMP was hydrolyzed faster than cyclic GMP by the 1000 x g particles (p < 0.05). No significant difference was noted in the other fractions.

![Graph](image-url)

**FIG. 5.** Distribution of cyclic nucleotide phosphodiesterase activity among crude subcellular fractions from bovine heart. Conditions and procedures were the same as described in the legend to Fig. 1. Values are given as the means of four experiments ± S.E. The value for cyclic AMP hydrolysis at the 1 μM substrate level was statistically significantly different from that of cyclic GMP hydrolysis in the supernatant fraction (p < 0.005, paired analysis). Likewise, the values for the hydrolysis of the two nucleotides were significantly different in the 1000 x g particulate fraction (p < 0.005).

![Graph](image-url)

**FIG. 6.** Hofstee plots of the rates of hydrolysis of cyclic AMP and cyclic GMP by a partially purified phosphodiesterase preparation from bovine heart. Rates were estimated from values obtained by the measurement of radioactive product isolated on anion exchange chromatography columns as described under "Experimental Procedure." The slope is the negative value for the apparent K_m and the intercept on the ordinate is the value for the apparent V_max of the preparation. The K_m for cyclic AMP was about 39 μM and the K_m for cyclic GMP was about 2 μM. The unit of velocity is μmoles per mg of protein per 15 min. Substrate concentrations ranged from 0.35 to 30 μM for cyclic GMP and 0.35 to 1000 μM for cyclic AMP.
FIG. 7. Hofstee plot of the rate of cyclic AMP hydrolysis by a particulate fraction (1000 × g, 6 min) from bovine heart. The unit of velocity is µmoles per 15 min per g of wet weight from which the fraction was derived. Substrate concentrations ranged from 0.2 to 100 µM.

FIG. 8. Hofstee plot of the rate of cyclic GMP hydrolysis by a particulate fraction (1000 × g, 6 min) from bovine heart. The unit of velocity is µmoles per 15 min per g of wet weight from which the fraction was derived. Substrate concentrations ranged from 0.1 to 40 µM.

Kinetic Parameters for Cyclic AMP and Cyclic GMP Hydrolysis by Particulate and Soluble Bovine Heart Preparations*—To explore further the possibility that the phosphodiesterase activities in the bovine heart particles and supernatant were functionally different, we measured some of the kinetic parameters for both the cyclic nucleotides in the crude particulate fraction and in a partially purified soluble preparation (16). In the partially purified preparation from the supernatant, cyclic GMP was hydrolyzed nearly 5 times faster than cyclic AMP at a 1 µM substrate level. However, at a 1 mM level, cyclic AMP was hydrolyzed about 40% faster. With this partially purified preparation, the apparent Kₘ and Vₘₐₓ values for cyclic AMP and cyclic GMP were consistent with those hydrolysis ratios at both substrate levels. Fig. 6 shows a Hofstee (25) plot for cyclic AMP and cyclic GMP hydrolysis by the purified bovine heart enzyme. The apparent Kₘ for cyclic AMP was 25 to 45 µM, which is similar to previously reported values (16, 26). The apparent Kₘ for cyclic GMP was 1 to 3 µM and the apparent Vₘₐₓ was about 40% lower than that for cyclic AMP. If the enzyme dilutions were made without albumin (1 mg per ml), the Hofstee plots obtained were curvilinear. A similar effect of albumin has been reported by Cheung (27) for a bovine brain phosphodiesterase preparation.

Kinetic studies of the hydrolysis of cyclic AMP by the 1000 × g particulate fraction from bovine heart indicated that it contained two apparent phosphodiesterase activities (Fig. 7). One had an apparent Kₘ of about 25 µM, and the other had an apparent Kₘ of about 0.8 µM. Similar studies with cyclic GMP as substrate indicated that most if not all of the activity was associated with a phosphodiesterase having a Kₘ in the range of 20 µM although there may have been a small amount of activity which had a lower apparent Kₘ (Fig. 8).
of more than one cyclic nucleotide phosphodiesterase activity, it seemed likely that the partially purified preparation contained a single enzyme which hydrolyzed both cyclic nucleotides. In order to test this possibility we measured the hydrolysis of tritiated substrate in the presence of varying amounts of the other nonlabeled substrate. The working hypothesis was that if both nucleotides were substrates for the same enzyme, then each should interfere with the hydrolysis of the other in a manner predictable on the basis of their apparent $K_m$ values. As shown in Fig. 9, cyclic AMP hydrolysis (A) was more effectively inhibited by cyclic GMP than was cyclic GMP hydrolysis (B) inhibited by cyclic AMP. The inhibitory effect of either cyclic nucleotide on the hydrolysis of the other could be decreased by addition of more of the substrate being measured.\(^3\) For an inhibitor used as an alternate substrate by the same enzyme, one would predict that the apparent $K_m$ would equal the apparent $K_i$. The apparent $K_i$ for cyclic AMP was found to be about 40 $\mu$M (Fig. 10), virtually the same as the apparent $K_m$ for cyclic AMP as measured in the absence of cyclic GMP.

Cyclic GMP also inhibited cyclic AMP hydrolysis by a crude supernatant preparation from the bovine heart (Table I). However, when a crude supernatant from rat liver was used, over a wide range of concentrations cyclic GMP actually stimulated cyclic AMP hydrolysis rather than inhibiting it. Inhibition did not occur until the concentration of cyclic GMP was greater than 50 $\mu$M.

Identification of Product—The following criteria indicate that 5'-GMP was the only product formed from cyclic GMP by the partially purified bovine heart phosphodiesterase (16). First, when cyclic GMP was incubated with an excess of the phosphodiesterase, descending paper chromatography of the reaction mixture gave only one spot corresponding to 5'-GMP. The solvent system was 2 volumes of 1 m ammonium acetate (pH 7.5) and 5 volumes of ethyl alcohol (29). Second, treatment of the above reaction mixture with excess Crotalus atrox venom released a stoichiometric amount of inorganic phosphate. Sutherland and Rall (15) have previously reported similar data for cyclic AMP hydrolysis by a similar bovine heart preparation as did Drummond and Perrott-Yee (17) by a partially purified phosphodiesterase preparation from rabbit brain.

**DISCUSSION**

Studies using the partially purified preparation from bovine heart indicate that only a single cyclic nucleotide phosphodiesterase was present. In particular, the apparent competitive inhibitory effect of each cyclic nucleotide on the hydrolysis of the other, while not ruling out the existence of more than one phosphodiesterase, does suggest that both nucleotides were hydrolyzed by the same enzyme in this preparation. This is further indicated by the finding that the apparent $K_i$ for cyclic AMP as an inhibitor of cyclic GMP hydrolysis was virtually the same as the apparent $K_m$ for cyclic AMP hydrolysis.

On the other hand, there were pronounced differences in the kinetic behavior of phosphodiesterase activities in the 1000 $\times$ g particulate and the supernatant fractions of both rat and bovine heart. This is most easily explained by considering that more than one phosphodiesterase was present in the heart homogenates. It is possible that these kinetic differences resulted from the loss of a modifier of phosphodiesterase activity during preparation of the subcellular fractions. However, no inhibitory or synergistic effects were detected when the various fractions were added together. It is also possible that kinetic differences could be caused by preferential binding of one of the nucleotides to some component of the incubation medium or that one cyclic nucleotide, but not the other, was partially excluded from the particulate phosphodiesterase activity. However, neither of the latter two possibilities would have been likely to give a lower apparent $K_m$ for cyclic AMP hydrolysis in the particles than in the soluble fraction. The probability that the kinetic differences between the fractions were due to different enzymes with different kinetic parameters and not to permeability differences is further strengthened by the observations that treatment of the 1000 $\times$ g particulate fraction with Triton, sonic oscillation, or repeated freezing and thawing caused no increase in the cyclic GMP to cyclic AMP hydrolysis ratio.
With a crude enzyme preparation as used in this study, nonlinearity of a Hofstee plot may be indicative of multiple enzymes. However, several other alternatives should be considered. First, all of the assays available at the present time which are applicable to measurement of phosphodiesterase activity at low substrate concentrations require that most enzyme preparations be diluted to varying degrees in order to obtain reliable measurements. As a result it is difficult to rule out the possibility that the enzyme activity was changed by dilution. For instance, it was found in this study that dilution of the partially purified preparation without albumin caused an apparent lowering of the activity. Another possibility is that the enzyme might have multiple substrate binding sites and that the binding of substrate to one of the sites might affect the binding to another. Such a phenomenon in which the binding of the first ligand decreases the affinity for the next ligand has been termed negative cooperativity by Conway and Koshland (30) and evoked by them to explain anomalies in DPN binding to glycerol-3-phosphate dehydrogenase. Analogous binding properties have also been postulated by Teipel and Koshland (31) to explain several types of apparently anomalous kinetic results involving other ligands and enzymes. Such an effect also could result in nonlinear Hofstee plots. For these reasons conclusive proof of the existence of multiple cyclic nucleotide phosphodiesterases awaits further purification of the various enzyme fractions which have been noted and more detailed studies of their physical and kinetic properties.

The fact that one cyclic nucleotide can alter the rate of hydrolysis of the other raises the possibility that the intracellular concentration of one cyclic nucleotide may affect the level of the other by influencing its rate of degradation. However, since in at least one tissue, rat liver, cyclic GMP stimulates the hydrolysis of cyclic AMP, the influence of one cyclic nucleotide on the metabolism of the other may be considerably more complex than simple substrate competition in some tissues.

Finally, the results presented in this paper illustrate the importance of measuring cyclic nucleotide phosphodiesterase activity at subsaturating as well as at saturating substrate concentrations. For instance, the large differences in ratios of cyclic AMP to cyclic GMP hydrolysis among the bovine and rat heart subcellular fractions were not seen at saturating substrate concentrations. Alterations in phosphodiesterase activity involving differences in the apparent $K_m$, as are often encountered with modifiers of enzyme activity, could be overlooked when the activity is measured only at saturating substrate levels.

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