Properties of a Phosphodiesterase with High Affinity for Adenosine 3′,5′-Cyclic Phosphate*

Yung-Chen Huang and Robert G. Kemp†

ABSTRACT: A phosphodiesterase with high affinity for adenosine 3′,5′-cyclic phosphate has been purified more than 300-fold from rabbit skeletal muscle. The enzyme displayed the following kinetic properties. (1) The enzyme had a Michaelis constant (K_m) of 4 μM at pH 7.0. The affinity decreased with increasing pH and at pH 9.0 the K_m was 26 μM. (2) The enzyme was also capable of hydrolyzing guanosine 3′,5′-cyclic phosphate but the K_m for this substrate was about 500 times higher than that observed with adenosine 3′,5′-cyclic phosphate. Competition between the two substrates suggested a common catalytic site. (3) Methylxanthines were capable of inhibiting the muscle phosphodiesterase but the enzyme was much less sensitive to inhibition than those phosphodiesterases described previously. Adenine and several derivatives of adenine were also found to be inhibitory. (4) Imidazole increased the maximal velocity of the muscle phosphodiesterase and shifted the pH optimum from 9.0 to about 8.0 to 8.5. Imidazole also decreased the affinity of the enzyme for adenosine 3′,5′-cyclic phosphate. Crude extracts of skeletal muscle were also studied at varying substrate concentrations. In addition to the low K_m adenosine 3′,5′-cyclic phosphate phosphodiesterase, kinetic plots suggested the presence of an enzyme activity with lower affinity for this nucleotide. An enzyme with a K_m of 15 μM for guanosine 3′,5′-cyclic phosphate was also detected in crude extracts.

The kinetic data are compared to previously published data obtained with phosphodiesterases having a relatively low affinity for adenosine 3′,5′-cyclic phosphate. The data are discussed in relation to the regulation of the concentration of adenosine 3′,5′-cyclic phosphate in mammalian tissues.

In recent years, the interest in the regulatory role of cAMP[1] has resulted in a number of studies of the kinetic properties of cyclic nucleotide phosphodiesterase. Earlier studies of the enzymes from bovine heart (Butcher and Sutherland, 1962), dog heart (Nair, 1966), and rat brain (Cheung, 1967) suggested a rather low affinity for cyclic AMP. More recent studies of bovine heart (Beavo *et al.*, 1970), rat brain (Brooker *et al.*, 1968), frog bladder and rat kidney (Jard and Bernard, 1970), and rate adipose tissue (Loten and Sneyd, 1970) have shown the presence of two phosphodiesterase activities distinguished by widely different affinities for cAMP. As suggested by Loten and Sneyd (1970), this type of behavior could be the result of two different catalytic activities or an example of a single enzyme displaying negative cooperativity (Levitzki and Koshland, 1969). We wish to report here the more than 300-fold purification of a phosphodiesterase from rabbit skeletal muscle. This enzyme displays a single low K_m for cAMP, and a very high K_m for cyclic 3′,5′-guanosine monophosphate.

Materials and Methods

Imidazole used in preparative work was grade I of Sigma Chemical Co., and that used in kinetic studies was grade III. *Crotalus atrox* venom was obtained from the Ross Allen Reptile Institute, Silver Springs, Fla. All nonradioactive nucleotides employed in this work were obtained from P-L Laboratories. [^{3}H]cAMP and [^{3}H]cGMP were obtained from New England Nuclear Corp. and were purified by thin-layer chromatography before use as follows. The material was streaked on precoated plates of silica gel F-254 (Brinkmann Instruments, Inc.) and developed with the solvent acetone–isopropyl alcohol–*n*-butyl alcohol–30 mM $(NH_4)HCO_3$ (2:2:3:2, v/v) (Jungas, 1966). cAMP and cGMP have R_F's of about 0.55 and 0.51, respectively, in this system and are well separated from their corresponding nucleosides and 5′- and 3′-nucleotides. The cyclic nucleotide, detected with ultraviolet light, was scraped off and stored on the silica powder at −20°. The nucleotide could be eluted from the silica with water.

The cyclic nucleotide phosphodiesterase was assayed at 30° by a modification of the procedure originally described by Appleman and Kemp (1966). The assay is based upon the production of [^{3}H]nucleoside from [^{3}H]cyclic nucleotide by the action of diesterase and snake venom nucleotidase. Reaction mixtures (1 ml) contained 50 mM Tris-HCl at the desired pH, 5 mM $MgCl_2$, 50,000 cpm of [^{3}H]cyclic nucleotide, and unlabeled cyclic nucleotide at the indicated concentration. In routine assays performed during purification, cAMP was added at a concentration of 0.1 mM. The reaction was started by the addition of 0.1 ml of the diesterase diluted to the desired concentration with buffer containing 50 mM Tris-HCl–2 mM EDTA–0.1 mM dithiothreitol, all adjusted to the pH used in that particular assay. The reaction was terminated by placing the sample in a boiling-water bath for exactly 1 min. The tubes were cooled, 0.2 ml of a solution of *C. atrox* venom (2 mg/ml) was added, and the reaction mixture was further incubated at 35°. After a 10-min incubation, the reaction mixture was put on a column of DEAE-Sephadex A-25 (0.5 × 7 cm) previously equilibrated with 0.05 M Tris-HCl buffer at pH 7.5. The column was washed with this same buffer and the initial effluent and wash were collected to combined volume of 5 ml. A 1-ml sample of this effluent was counted with a liquid scintillation spectrometer. The radioactivity in a blank (without diesterase) was subtracted from the sample radioactivity. The

* From the Department of Biochemistry, Medical College of Wisconsin, Milwaukee, Wisconsin 53233. *Received January 14, 1971.* Supported by a grant from the American Heart Association and U. S. Public Health Service Grant AM 11410.

† This work done under the tenure of an Established Investigatorship of the American Heart Association.

[1] Abbreviations used that are not listed in *Biochemistry 5*, 1445 (1966), are: cAMP, adenosine 3′,5′-cyclic monophosphate; cGMP, guanosine 3′,5′-cyclic monophosphate.

blank amounted to about 2% of the total radioactivity employed in the assay and was higher if unpurified tritiated cyclic nucleotide was used. Activity was determined by calculating the millimicromoles of cyclic nucleotide hydrolyzed, based on the specific radioactivity of the nucleotide in the original reaction mixture. One unit of enzyme activity represents that amount required to hydrolyze 1 mμmole of cAMP in 1 min at pH 7.5 in the presence of 0.1 mM cyclic nucleotide. The use of the substituted Sephadex as a means of separating the reaction products is an improvement over previous assays based on this principle. Approximately 98% of the [^{3}H]adenosine generated by the combined action of diesterase and the nucleotidase could be recovered in a total volume of 5 ml. In contrast, nucleosides appear to bind nonspecifically to Dowex-type resins and thus large volumes are required to elute the adenosine (DeLange *et al.*, 1968; Beavo *et al.*, 1970). This prolongs the procedure and necessitates the use of large amounts of radioactivity since only a small fraction of the total aqueous effluent can be counted. The Sephadex A-25 columns were prepared by adding the gel suspension to disposable Pasteur-type pipets containing a small glass wool plug. A week's supply of columns could be prepared at one time and then refrigerated in a plastic bag to retain the moisture. Before use, they were washed with a small amount of fresh buffer.

The assay for cAMP was linear for more than 1 hr as long as the substrate concentration remained high (Figure 1), indicating that under the conditions of the assay the enzyme remained stable. The assay is based upon measuring the fraction of total substrate hydrolyzed; thus, assays at different substrate concentrations were often carried out at widely different enzyme concentration in order that initial velocities might be measured conveniently. It was essential, therefore, to show that the assay was linear with enzyme concentration. The rate of cAMP hydrolysis was measured over a 35-fold range in enzyme concentration (0.4 to 14 μg per ml) and was observed to be linear with protein concentration.

Protein determinations were carried out by the procedure of Weichselbaum (1946) or by the method of Lowry *et al.* (1951). Bovine serum albumin was employed as a standard.

Results

Purification Procedure. cAMP phosphodiesterase was prepared from rabbit skeletal muscle by the following procedure. A female New Zealand white rabbit was killed with an overdose of Nembutal and bled by cutting the blood vessels in the neck. The muscle was passed through a meat grinder at 4° and homogenized in a Waring Blender for 30 sec in the presence of three volumes of a buffer containing 50 mM imidazole, 4 mM EDTA, and 0.1 mM dithiothreitol (pH 8.5). The homogenate was centrifuged for 40 min at 10,000*g* and 2° in a Lourdes centrifuge. The supernatant was passed through glass wool to remove lipid material and was adjusted to 0.60 saturation of $(NH_4)_2SO_4$ by the slow addition of a neutralized solution of saturated $(NH_4)_2SO_4$. The mixture was stirred for 30 min and the precipitate was collected by centrifugation at 10,000*g* for 40 min. It was dissolved in a volume of 10 mM Tris-HCl, 100 mM NaCl, 2 mM EDTA, and 0.1 mM dithiothreitol (pH 8.0) equal to one-tenth of the volume of the original extract. The yield was about 90% with a threefold increase in specific activity. The Tris-NaCl buffer will be subsequently referred to as buffer A. The enzyme solution was dialyzed against two changes of this same buffer overnight and then applied to a column of DEAE-Sephadex A-50 (5 × 35 cm) that had been equilibrated with buffer A. After the protein sample had entered the column bed, the column was washed with the buffer A until the OD of the effluent at 280 mμ dropped below 0.1. The enzyme was eluted with buffer A containing 0.8 M NaCl. Those fractions containing activity (50–60% yield) were pooled, dialyzed against buffer A, and again applied to column of DEAE-Sephadex A-50 (3.5 × 40 cm) equilibrated with buffer A. The enzyme was eluted with a linear gradient consisting of 1.5 l. of buffer A containing NaCl at a final concentration of 0.6 M flowing into buffer A (1.5 l.) in a mixing chamber. Fractions containing relatively high specific activity were pooled and concentrated by the addition of saturated $(NH_4)_2SO_4$ to a final saturation of 0.6. The overall yield was about 50% with a 40-fold increase in specific activity. The pellet was dissolved in a minimum amount of buffer A and the solution layered on a column of Sephadex G-200 (5 × 90 cm) equilibrated with buffer A containing 0.8 M NaCl. The enzyme eluted from the column usually had a specific activity in the order of 80 units/mg, representing a purification of about 160-fold over the crude extract. The yield from the crude extract was about 33%. Further purification usually involved repeating the steps on gradient elution from Sephadex A-50 and gel filtration on Sephadex G-200. Losses of activity in these steps were fairly large but specific activities were obtained that ranged from about 150 to 700 with overall yields of 4 to 10%. Kinetic analyses were carried out with enzyme preparations having specific activities greater than 200. Frozen rabbit muscle obtained from Pel-Freeze Biologicals (Rogers, Ark.) was also successfully employed for the preparation of the phosphodiesterase. Extracts of frozen muscle have somewhat higher activity than those from fresh animals, possibly because the frozen muscle has been taken only from young (8–12 weeks) animals. In some of the earlier preparations, an acid-precipitation step was used in place of the initial $(NH_4)_2SO_4$ fractionation. In this step, 1 N acetic acid is slowly added to the extract until the pH drops to 5.5. After stirring an additional 30 min, the precipitate was collected by centrifugation at 10,000*g* for 30 min. The yield in this step was lower than that obtained in the alternate ammonium sulfate precipitation although the purification was usually greater.

In 50 mM Tris-Cl buffer at pH 8.0 and at 4°, the purified enzyme lost about 50% of its activity in 1 month. Some stabilization was achieved in the presence of high concentrations of sodium chloride (0.1–0.5 M) but maximum stability was achieved in the presence of 0.05–0.2 M $MgCl_2$. Under these

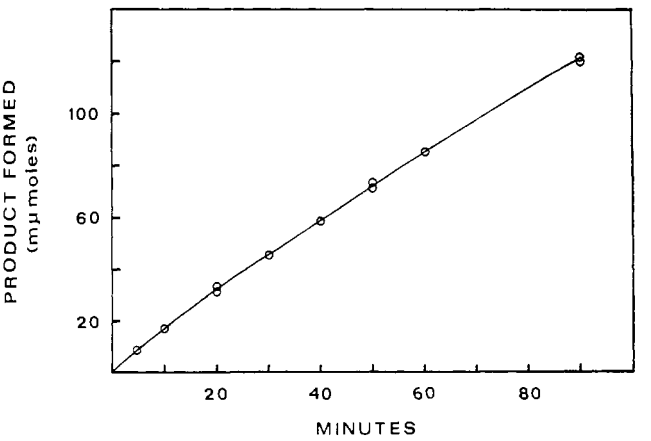

FIGURE 1: Progress curve of cAMP hydrolysis. Assays as described in Materials and Methods at pH 8.0 in the presence of 225 μM cAMP and 4.4 μg of purified phosphodiesterase. Each point represents an average of two determinations.

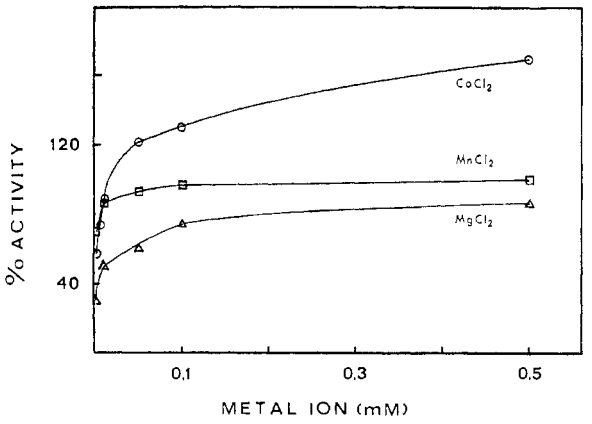

FIGURE 2: Effect of divalent metal ions on cAMP phosphodiesterase. Prior to assay, magnesium ion was removed from the purified enzyme by passing the preparation through a column of Sephadex G-25 previously equilibrated with 10 mM Tris-HCl at pH 7.5. Assays performed at pH 7.5 as described in Materials and Methods except that the indicated ions replaced $MgCl_2$. The cAMP concentration was 140 μM. For the second stage of assay, the snake venom concentration was doubled and $MgCl_2$ at 1 mM was added where not already present. The velocity is expressed as the per cent of that value obtained with 5 mM $MgCl_2$. Each point represents the average of three or more determinations.

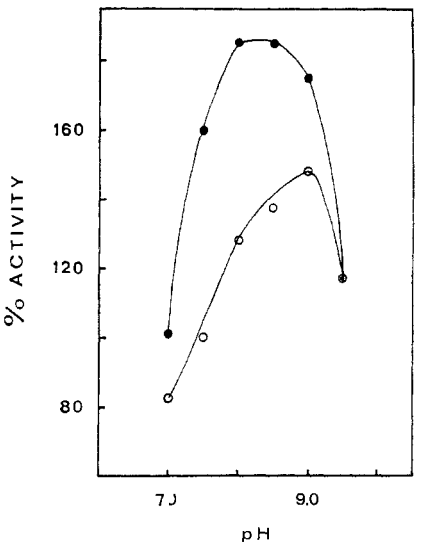

FIGURE 3: The pH and imidazole effect on cAMP phosphodiesterase. Reaction carried out with 100 μM cAMP 5 mM $MgCl_2$ in buffer consisting of 25 mM Tris and 25 mM *N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid at the indicated pH. The activity is expressed as the per cent of that activity given at pH 7.5 in the absence of imidazole. Each point represents the average of two determinations. Open circles, without imidazole; closed circles, plus 40 mM imidazole.

conditions, less than 5% of the initial activity was lost on storage for 30 days at 4°. The enzyme was therefore routinely stored in 10 mM Tris-Cl, 2 mM EDTA, 0.1 mM dithiothreitol, and 0.1 M $MgCl_2$ (pH 8.0).

Kinetics Metal Requirement. Purified muscle nucleotide phosphodiesterase that was passed through Sephadex G-25 to remove metal ions displayed approximately 20% of maximal activity in the absence of added metal ion. However, a low concentration of EDTA (0.1 mM) completely abolished activity. This would suggest that the enzyme has an absolute metal ion requirement and that the enzyme preparation contains metal ion that is not easily removed. Figure 2 shows the effect of low concentrations of magnesium, manganous, and cobalt ions on the activity of phosphodiesterase. Concentrations of cobalt ion in excess of 2 mM were inhibitory. The enzyme was either not activated or was inhibited by calcium, cupric, ferrous, zinc, and nickel ions when these ions were present at a concentration of 2 mM. Double-reciprocal plots of the metal ion activation data yielded straight lines indicating a single site or multiple sites of identical affinity. The data from these plots are given in Table I. The K_a for magnesium ion was not greatly different from the value of 13 μM reported by Cheung (1967) for a high K_m enzyme from brain. Cobalt ion gave the highest maximal activity and manganous ion was the most tightly bound of the three activating ions; but because magnesium ion is present in muscle in much higher concentration than the other two ions, magnesium ion was employed in all subsequent kinetic studies that are presented here.

pH. Figure 3 shows the variation of the activity of the phosphodiesterase *vs.* pH. The pH optimum was found to be about pH 9.0, similar to a number of other mammalian cyclic nucleotide phosphodiesterases (Butcher and Sutherland, 1962; Cheung, 1967). Butcher and Sutherland (1962) originally reported that imidazole stimulated beef heart diesterase and caused a shift in its pH optimum; but Cheung (1967) noted that although imidazole stimulated the rat brain enzyme, it did not shift the pH optimum. As indicated by Figure 3, the muscle enzyme gave an imidazole effect that was similar to that of the beef heart enzyme. It will be seen in the following section that imidazole also influence the affinity of the enzyme for cyclic AMP.

K_m. The muscle phosphodiesterase displayed a very high affinity for cAMP in the physiological pH range. The lower line in the double-reciprocal plot of Figure 4 represents the data obtained at pH 7.5 in the presence of varying concentrations of cAMP. The K_m calculated from these and similar data from other preparations is 6 μM. Table II gives these data as well as the K_m's and maximal velocities obtained at various hydrogen ion concentrations. At pH 7.0 the K_m was found to be 4 μM and increased with increasing pH to a value of 26 μM at pH 9.0. In comparison, values for K_m above 0.1 mM have been described for enzymes from beef heart (Butcher and Sutherland, 1962), dog heart (Nair, 1966), rat brain (Cheung, 1967), rat kidney (Dousa and Rychlik, 1970), and frog erythrocytes (Rosen, 1970). Crude enzyme preparations that display both high and low K_m values have been observed in ox heart (Beavo *et al.*, 1970), rat brain (Brooker *et al.*, 1968), and adipose tissue (Loten and Sneyd, 1970). With the purified rabbit muscle enzyme, assays were performed at concentra-

TABLE I: Kinetic Parameters for Metal Ion Effects on cAMP Phosphodiesterase.[a]

Addition	K_a (μM)	Rel V_{max}
$MgCl_2$	24	0.6
$MnCl_2$	1.2	0.7
$CoCl_2$	6	1.0

[a] The velocity obtained in the absence of added metal ion was subtracted from the velocities obtained in the presence of metal ions. These differences were plotted as reciprocals against the reciprocals of metal ion concentration and the values for K_a were obtained from the intercept on the abscissa.

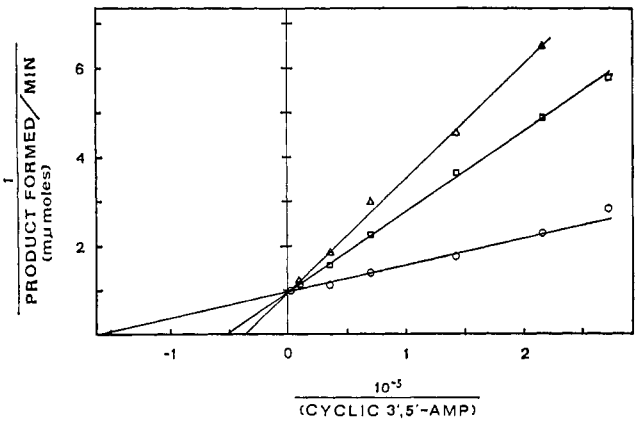

FIGURE 4: Competitive inhibition of cAMP phosphodiesterase by methylxanthines. Incubation carried out for 30 min at pH 7.5 as described in the Materials and Methods. The specific activity of the enzyme was 200 units/mg. (○) No additions; (△) plus 1 mM theophylline; (□) plus 1 mM caffeine.

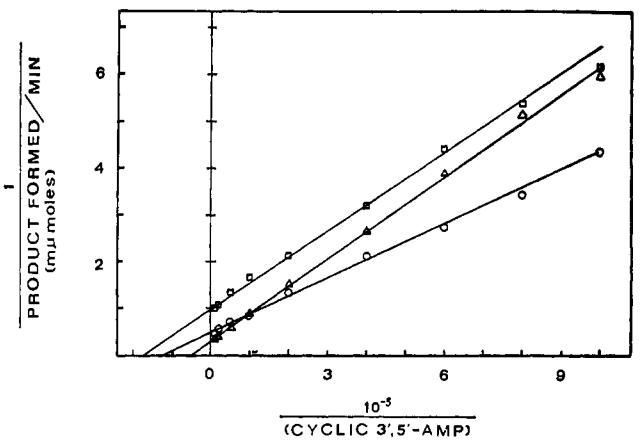

FIGURE 5: Effect of imidazole on substrate affinity. Assays were performed at pH 7.5 as described in the Materials and Methods. Each point represents the average of two determinations. (□) No additions; (○) plus 40 mM imidazole; (△) plus 120 mM imidazole.

tions ranging from 1 μM to 1.3 mM with no indication of a high K_m species. As will be shown, however, a lower affinity enzyme apparently is present in crude extracts of muscle.

Inhibitors and Activators. As observed with other mammalian phosphodiesterases, the methylxanthines were competitive inhibitors of muscle cyclic nucleotide phosphodiesterase. The double-reciprocal plot in Figure 4 describes the inhibitory action of caffeine and theophylline; the K_i's calculated from these data were 0.52 and 0.32 mM, respectively. Honda and Imamura (1968) reported a K_i for theophylline of 0.11 mM for the beef heart and the rabbit brain enzymes. It is obvious that at any given concentration of cAMP the muscle enzyme is much less sensitive than the heart enzyme to the inhibitory action of the methylxanthines. For example, at 0.4 mM cAMP, the heart enzyme was inhibited 70% by 1 mM theophylline (Butcher and Sutherland, 1962), while under the same conditions the rabbit muscle enzyme was inhibited less than 5%.

A number of other compounds have been tested for inhibitory action and these are given in Table III. In addition to the methylxanthines, adenine, adenosine, 6-dimethylaminopurine, and 6-hydroxypurine were inhibitory when present at a concentration of 1mM. Little or no inhibition was observed in the presence of 1 mM concentrations of guanosine, cytosine, hypoxanthine, uradine, uracil, and 6-aminouracil. In addition to purines and pyrimidines, a number of metabolic intermediates were also tested at 1 mM. No inhibition was observed with inorganic phosphate, adenosine mono-, di-, and triphosphate, citrate, phosphocreatine, and phosphoenolpyruvate. Cheung (1967), on the other hand, observed inhibition of rat brain phosphodiesterase by citrate and ATP; the concentrations employed, however, exceeded the concentration of magnesium ion and could have made the metal ion limiting by chelation. It is interesting that adenine and related compounds are effective inhibitors while the adenosine phosphates that are structurally even more similar to cAMP are not inhibitory.

The muscle phosphodiesterase was also inhibited by reagents that react with sulfhydryl groups. When the purified enzyme was incubated for 10 min at 30° in the presence of 10 μM concentrations of *p*-chloromercuribenzoate or 5,5′-dithiobis(2-nitrobenzoic acid), there was a loss of 82 and 55%, respectively, of the initial activity. Most of the activity was recovered following a brief incubation of the inhibited enzyme with dithiothreitol.

Imidazole, in addition to its influence on V_{max} and pH optimum, decreased the affinity of the phosphodiesterase for cAMP. Figure 5 shows the influence of imidazole on the kinetics of phosphodiesterase at pH 7.5. From these plots it was calculated that 40 mM imidazole increased V_{max} by a factor of 2.1 but K_m was also increased to 8 μM. In the presence of 120 mM imidazole, V_{max} was increased by a factor of 3.1 and the

TABLE II: Affinity of the Phosphodiesterase for cAMP as a Function of pH.[a]

pH	K_m (μM)	Rel V_{max}
7.0	4	0.6
7.5	6	1.0
8.0	10	1.2
9.0	26	2.2

[a] Data obtained from double-reciprocal plots of initial velocities from assays performed as described in the Materials and Methods. Concentrations of cAMP were varied from 1 μM to 1 mM. The enzyme employed in these assays had a specific activity of 200 units/mg.

TABLE III: Some Inhibitors of cAMP Phosphodiesterase.[a]

Addition (1 mM)	Act. (%)
None	100
Theophylline	45
Theobromine	72
Caffeine	62
Adenine	79
Adenosine	75
6-Dimethylaminopurine	51
6-Hydroxypurine	86

[a] Assay carried out for 30 min at pH 7.5 and a cAMP concentration of 5 μM and other conditions described in Materials and Methods. Values represent the average of duplicate assays.

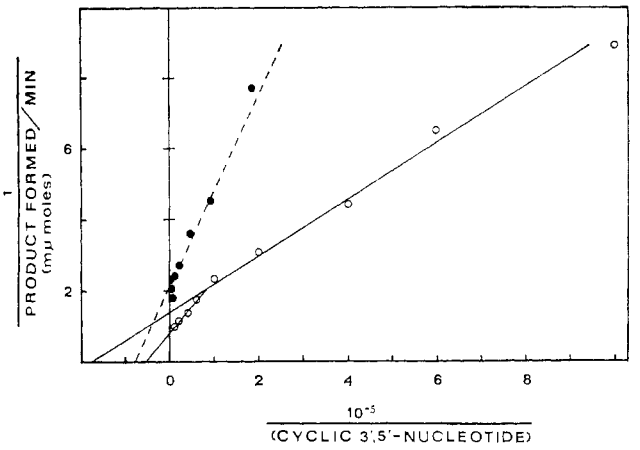

FIGURE 6: Activity of cyclic nucleotide phosphodiesterase in crude muscle extracts. The extract represents the 10,000*g* supernatant of the homogenate as described in the Results section. Assays were performed at pH 7.5 as described in the Materials and Methods with varying concentrations of cAMP or cGMP. The specific activity of the extract with cAMP as a substrate was 0.5 unit/mg of protein. Each point represents the average of two determinations: (○) cAMP; (●) cGMP. The line drawn through the cGMP data was determined from these data as well as activities at concentrations as low as 1 μM.

K_m increased by a factor of 3.3. As a result of the opposing effects of imidazole, that is, a higher V_{max} but a lower affinity, the stimulation by imidazole at physiological concentrations of cAMP was relatively small.

Hydrolysis of cGMP. The purified muscle phosphodiesterase preparation also hydrolyzed cGMP. A double-reciprocal plot of data obtained at concentrations of cGMP varying from 0.1 to 4 mM was linear and indicated a K_m of approximately 2.7 mM. The V_{max} was equal to about 90% of that obtained with cAMP as a substrate. The ability of the preparation to hydrolyze cGMP could have been due to a unique enzyme or to lack of absolute specificity of the cAMP phosphodiesterase. To examine these possibilities unlabeled cGMP was tested as an inhibitor of the hydrolysis of radioactive cAMP over a wide range of concentrations of cAMP. The results showed that the cGMP was indeed a competitive inhibitor of the hydrolysis of cAMP and the K_i calculated from these data was 3 mM in excellent agreement with the K_m determined by direct assay. It is thus concluded that the hydrolysis of cGMP is carried out by the cAMP phosphodiesterase.

K_m of Phosphodiesterase in Crude Extracts of Skeletal Muscle. As mentioned previously, kinetic analysis of crude extracts of a number of tissues had indicated the presence of phosphodiesterases with different affinities for cAMP. Crude rabbit muscle extracts were also assayed at varying concentrations of cAMP or cGMP and the results plotted as double reciprocals (Figure 6). The assays, carried out at pH 7.5, indicated the presence of an enzyme with a K_m for cAMP of about 5 μM, corresponding to the enzyme that was purified. At higher concentrations of cAMP (above 10 μM) an activity with a low affinity is suggested by the downward deviation of the experimental points from the extrapolated line (Figure 6). The K_m of this activity would appear to be about 20 μM but the accuracy of such measurements in crude extracts is certainly in doubt. The crude extract also contained an enzyme with a relatively high affinity for cGMP (Figure 6). The K_m of this enzyme (15 μM) is lower by a factor of 200 than the cGMP-hydrolyzing activity of purified cAMP phosphodiesterase. From the intercepts one can estimate the V_{max}'s which indicate that the low K_m cAMP phosphodiesterase, the high K_m cAMP phosphodiesterase, and the cGMP phosphodiesterase were present in ratio of 1.4:1.0:1.0, respectively. A small amount of enzyme with K_m above 0.2 mM probably would not have been detected by these assays. It is important to note that the analysis was carried out on a 10,000*g* supernatant of the low ionic strength extraction. The presence of a particulate enzyme such as the activity observed by Beavo *et al.* (1970) in bovine heart would not be included in the affinity analysis described here. However, a comparison of the total activity at 0.1 mM cAMP of the whole muscle homogenate to the activity of the 10,000*g* supernatant indicated that almost all of the phosphodiesterase activity was extracted into the low ionic strength buffer.

Discussion

It now appears likely that there exists a number of enzymes capable of hydrolyzing cAMP and cGMP. Skeletal muscle may be added to the growing list of tissues that display enzymatic activities with more than one affinity for these nucleotides. Partial separation of high- and low-affinity enzymes has been indicated for several tissues (Beavo *et al.*, 1970; Jard and Bernard, 1970), but the possibility existed that a single enzyme exhibited two K_m values due to negative cooperativity. This work demonstrates that at least in the case of muscle an enzyme is present that has a single low K_m value for cAMP and a single but much higher K_m for cGMP. Analysis of crude extracts, on the other hand, indicate the presence of a cGMP phosphodiesterase and a lower affinity cAMP phosphodiesterase in addition to the high-affinity enzyme that has been characterized in this report. Drummond and Perrott-Yee (1961) first surveyed tissues for the activity of cAMP phosphodiesterase and their results indicated that the enzymatic activity in skeletal muscle was low relative to that of a number of other tissues. It should be noted, however, that this activity was determined at high substrate concentrations. As shown here, much of the activity of skeletal muscle is of the low K_m type and at physiological concentrations of cAMP the muscle enzyme would have an activity more than 100 times greater than the same amount of a high K_m enzyme such as that isolated by Nair (1966) from dog heart.

The steady-state concentration of cAMP in tissues must obviously be influenced by the pertinent kinetic parameters of adenylyl cyclase and phosphodiesterase as well as the total activities of the two enzymes as determined by analyses carried out under optimal conditions. Skeletal muscle has been shown to have a concentration of cAMP that is one-tenth to one-fifth those found in adrenal gland, brain, and liver (Walton and Garren, 1970). This might well be reasonable if muscle is unique in that it contains relatively more of the high-affinity phosphodiesterase than other mammalian tissues. The results of Beavo *et al.* (1970) who studied the cAMP phosphodiesterase activities of rat tissues at high- and low-substrate levels support this contention. They observed that the ratio of activity at 1 μM *vs.* activity at 1 mM was higher for skeletal muscle than liver and brain. This suggests a relatively greater amount of high-affinity phosphodiesterase in rat skeletal muscle. Although the pH optimum of the enzyme as determined by assays at 0.1 mM cAMP was quite high (pH 9.0), the activity of the enzyme at physiological concentrations of cAMP (5 μM) is higher near pH 7.5. This, of course, is due to the opposing effects of increasing V_{max} but decreasing affinity as the pH is increased from 7 to 9.

An important difference between the low K_m muscle enzyme

and the high K_m enzymes described previously is the lesser sensitivity of the muscle enzyme to inhibition by the methylxanthines. The K_i's for these compounds are only two or three times higher than those reported for the high K_m enzymes (Honda and Imamura, 1968); but because of great difference in affinity for cAMP between the enzymes, the muscle enzyme is much less inhibited at comparable concentrations of substrate and inhibitor. This observation is of interest to investigators who assay adenylyl cyclase and isolate cAMP from tissues, because in these procedures theophylline is routinely employed to prevent phosphodiesterase action at concentrations that would inhibit a high K_m enzyme but will not inhibit an enzyme with kinetic parameters similar to those of the muscle phosphodiesterase. Furthermore, one might predict that muscle and any other tissue with a relatively large amount of the low K_m phosphodiesterase would be insensitive to the action of the methylxanthines *in vivo* if indeed the pharmacological actions of these drugs can be attributed solely to the inhibition of cAMP hydrolysis. The list of agents that modulate the level of cAMP is indeed impressive (Robison *et al.*, 1968) and most studies have suggested that adenylyl cyclase is the point of control. Recently, however, Loten and Sneyd (1970) have shown that the phosphodiesterase activity of adipose tissue homogenates was increased by brief treatment of the intact tissue with insulin. These workers noted a decrease in K_m of the high K_m activity of adipose homogenates and an increase in the V_{max} of the low K_m activity. It is obvious that the cyclic nucleotide phosphodiesterase could have an important regulatory function and the existence of multiple forms of the enzyme with different kinetic properties provides some substance for speculation on this possibility. If the forms were interconvertible, then the phosphodiesterase with the high K_m would represent a reservoir of enzyme capable of being converted into the low K_m form with its much greater activity at the physiological concentration of cAMP. The availability of both the low K_m enzyme free of high K_m activity and the previously described high K_m enzyme will permit an evaluation of the possibility of interconvertible forms in a cell-free preparation.

Acknowledgment

The authors are indebted to Mrs. Shiow-Luan Huang for excellent technical assistance.

References

Appleman, M. M., and Kemp, R. G. (1966), *Biochem. Biophys. Res. Commun. 24*, 564.

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

Brooker, G., Thomas, L. J., and Appleman, M. M. (1968), *Biochemistry 7*, 4177.

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

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

DeLange, R. J., Kemp, R. G., Riley, W. D., Cooper, R. A., and Krebs, E. G. (1968), *J. Biol. Chem. 243*, 2200.

Dousa, T., and Rychlik, I. (1970), *Biochim. Biophys. Acta 204*, 10.

Drummond, G. I., and Perrott-Yee, S. (1961), *J. Biol. Chem. 236*, 1126.

Honda, F., and Imamura, H. (1968), *Biochim. Biophys. Acta 161*, 267.

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

Jungas, R. L. (1966), *Proc. Nat. Acad. Sci. U. S. 56*, 757.

Levitzki, A., and Koshland, D. E. (1969), *Proc. Nat. Acad. Sci. U. S. 62*, 1121.

Loten, E. G., and Sneyd, J. G. T. (1970), *Biochem. J. 120*, 187.

Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951), *J. Biol. Chem. 139*, 265.

Nair, K. G. (1966), *Biochemistry 5*, 150.

Robison, G. A., Butcher, R. W., and Sutherland, E. W. (1968), *Annu. Rev. Biochem. 37*, 149.

Rosen, O. M. (1970), *Arch. Biochem. Biophys. 139*, 447.

Walton, G. M., and Garren, L. D. (1970), *Biochemistry 9*, 4223.

Weichselbaum, T. E. (1946), *Amer. J. Clin. Pathol. 10 (Suppl.)*, 40.