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MODES OF ACTION OF HYPOXANTHINE, INOSINE AND INOSINE 5′-MONOPHOSPHATE ON CYCLIC NUCLEOTIDE PHOSPHODIESTERASE FROM BOVINE BRAIN

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Abstract—A purified bovine brain cyclic nucleotide phosphodiesterase catalyzed the hydrolysis of both cyclic AMP and cyclic GMP. Alternative substrate inhibition experiments indicated that cyclic AMP and cyclic GMP were hydrolyzed by the same enzyme and that they shared a common binding site. Inosine, inosine 5′-monophosphate and hypoxanthine competitively inhibited the hydrolysis of cyclic AMP and cyclic GMP by bovine brain cyclic nucleotide phosphodiesterase. This inhibition of cyclic nucleotide hydrolysis by the purines was affected by the pH of the mixture. The inhibition constants of inosine, inosine 5′-monophososphate and hypoxanthine when inhibiting enzymatic hydrolysis of cyclic AMP were 0.36 ± 0.05, 1.3 ± 0.2 and 3.2 ± 0.5 mM, respectively. With cyclic GMP as substrate, the inhibition constants were 0.50 ± 0.09, 1.8 ± 0.2 and 4.5 ± 0.7 mM for inosine, inosine 5′-monophosphate and hypoxantine respectively. The per cent inhibition by inosine, inosine 5′-monophosphate or hypoxanthine of the cyclic nucleotide phosphodiesterase activity was not altered by the addition of calmodulin (calcium-dependent protein activator of cyclic nucleotide phosphodiesterase) to the enzyme. The effect of calmodulin was not changed by these purine inhibitors. These results suggest that the binding site of calmodulin differed from that of the inhibitors and from that of cyclic AMP and cyclic GMP.

Cyclic AMP and cyclic GMP are intermediates for many vital cellular functions of hormones and biogenic amines [1–4]. They also appear to regulate the growth and differentiation of cells [5–8]. Cyclic nucleotide phosphodiesterase (EC 3.1.4.17) is the only intracellular enzyme or enzyme system known to catabolize the cyclic nucleotides. Multiple forms of cyclic nucleotide phosphodiesterase which differentially hydrolyze either cyclic AMP or cyclic GMP have been found in a wide variety of tissues [9–15]. Thus, selective inhibition of cyclic nucleotide phosphodiesterase in particular cells (or tissues) may increase either cyclic AMP or cyclic GMP and affect cellular functions [16].

Several factors are known to modulate cyclic nucleotide phosphodiesterase activity in cells. The best understood of these is calmodulin, a calcium-dependent protein activator of cyclic nucleotide phosphodiesterase which is found in a variety of cells [17–22]. In the presence of Ca^{2+}, this protein activates certain forms of cyclic nucleotide phosphodiesterase [23–26]. Recently, several proteins which inhibit the activity of cyclic nucleotide phosphodiesterase have been isolated from tissues [27–32]. Thus, it appears that cyclic nucleotide phosphodiesterase activity is subject to inhibitory regulators in the cells.

We have recently isolated from bovine brain homogenate a small molecular weight compound which inhibited the hydrolysis of cyclic AMP and of cyclic GMP by cyclic nucleotide phosphodiesterase. The compound has been identified by thin-layer chromatography and high-pressure liquid chromatography as hypoxanthine.* In this report, we have further investigated the specificity and the relative potency of hypoxanthine, its nucleoside, inosine, and its nucleotide, inosine 5′-monophosphate (IMP), when inhibiting the activities of the bovine brain cyclic nucleotide phosphodiesterase, and have characterized the modes of inhibition by enzyme kinetic analysis.

METHODS

Materials. Bovine brains were obtained freshly from the slaughterhouse, quickly frozen and stored at −20°; they were thawed immediately before use. UM-2 membranes were obtained from the Amicon Corp. (Lexington, MA). Diethylaminoethyl cellulose (DEAE-cellulose), inosine, IMP, hypoxanthine, snake venom (*Crotalus atrox*), ethyleneglycol-bis-(β-aminoethyl ether) *N,N′*-tetra acetic acid (EGTA), cyclic AMP and cyclic GMP were obtained from the Sigma Chemical Co. (St. Louis, MO). [^{3}H-G] adenosine 3′, 5′-cyclic phosphate, ammonium salt (cyclic [G-^{3}H]AMP; 41.1 Ci/mmole), [^{3}H-G]guanosine 3′,5′-cyclic phosphate, ammonium salt (cyclic [G-^{3}H]GMP; 9.8 Ci/mmole), and [8-^{14}C]adenosine 3′,5′-cyclic phosphate, ammonium salt (cyclic [8-^{14}C]AMP; 53.1 mCi/mmole) were obtained from New England Nuclear (Boston, MA) and shown to be >95 per cent pure by thin-layer chromatography on cellulose plates with isopropanol–ammonium hydroxide–water (7 : 1 : 2, v/v) as solvent. The cellulose plates used were Eastman Chromagram Sheets (160 μm thick) and were

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obtained from the Eastman Kodak Co. (Rochester, NY). Ion-exchange resin (Amberlite, IRP-58, 200–400 mesh) was obtained from the Chemical Dynamics Corp. (South Plainfield, NJ). The resin was washed successively with 0.5 N NaOH, glass-distilled water, 0.5 N HCl, and then thoroughly with glass-distilled water until the mixture was at pH 4.0. It was used as a slurry of one part resin to three parts water (v/v).

Preparation of bovine brain cyclic nucleotide phosphodiesterase. Cyclic nucleotide phosphodiesterase was purified from the bovine brain supernatant fraction by $(NH_4)_2SO_4$ fractionation, calcium phosphate absorption, and DEAE-cellulose chromatography [33]; the homogenous enzyme has been obtained by Klee *et al.* [34] and Mossill *et al.* [35]. The purified cyclic nucleotide phosphodiesterase had a specific activity of 900 pmoles cyclic AMP hydrolyzed per min per mg of protein at a 1 μM substrate concentration. The enzyme also degraded cyclic GMP and had a specific activity of 3000 pmoles cyclic GMP hydrolyzed per min per mg of protein at a 1 μM substrate concentration. The activity of the enzyme with either cyclic AMP or cyclic GMP as substrate was increased more than 3-fold by the addition of saturating amounts of calmodulin. Calmodulin was prepared as described previously [24].

Assay of cyclic nucleotide phosphodiesterase activity. The assay was a modification of that described by Thompson and Appleman [9]. The assay mixture contained 40 mM Tris–HCl (pH 7.8), 3 mM $MgSO_4$, 50 μM $CaCl_2$, 1 μM unlabeled cyclic AMP and about 1×10^5 cpm cyclic [G-^{3}H]AMP. Appropriate amounts of calmodulin and enzyme were added along with water to make a total volume of 100 μl. After a 10-min incubation at 30°, the reaction was terminated by placing the tubes in boiling water for 45 sec. Fifty micrograms of snake venom (*Crotalus atrox*) were added to convert 5′-AMP to adenosine. At the end of another 10-min incubation at 30°, 1 ml of the slurried anion-exchange resin (Amberlite IRP-58) was added to terminate the reaction. The mixture was centrifuged at 1000 *g* for 10 min and a fraction of the supernatant fluid which contained [G-^{3}H]adenosine was counted by liquid scintillation spectrometry. The assay of cyclic GMP hydrolysis was the same as that of cyclic AMP except that the substrate was cyclic GMP and cyclic [G-^{3}H]GMP. The recovery of ^{3}H nucleosides was greater than 90 per cent. The phosphodiesterase inhibitors used in the experiments did not affect the conversion of nucleotides to nucleosides from pH 3 to 10.

Measurement of protein. Protein was determined according to the method of Lowry *et al.* [36], with bovine serum albumin as a standard.

Enzyme kinetic analysis. The hydrolysis of cyclic nucleotides by bovine brain cyclic nucleotide phosphodiesterase followed Michaelis–Menten kinetics over a wide range of concentrations of either cyclic AMP (0.5 to 200 μM) or cyclic GMP (0.1 to 40 μM). The kinetic parameters (e.g. K_m, K_i and V_{max}) were obtained according to the method of Cleland [37]. The alternative substrate experiments were studied by employing cyclic [8-^{14}C]AMP and cyclic [G-^{3}H]GMP to indicate their respective rates

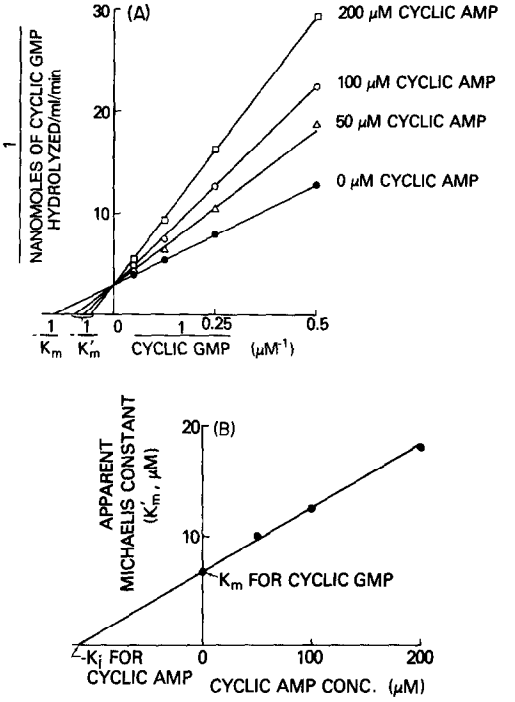

Fig. 1. Panel A: double-reciprocal plot of the effect of cyclic AMP on the hydrolysis of cyclic GMP. The purified cyclic nucleotide phosphodiesterase (1.0 μg) was derived from the DEAE-cellulose step (see Methods). The data shown represent the means of the assays in triplicate. Panel B: Replot of apparent Michaelis constant (K'_m) obtained from Fig. 1A vs the concentration of inhibitor, i.e. cyclic AMP.

of hydrolysis. In the experiment, the amount of substrate hydrolyzed was limited to less than 30 per cent by using suitable amounts of enzyme and appropriate incubation times. The assay was performed in triplicate.

RESULTS

Hydrolysis of cyclic AMP and cyclic GMP by bovine brain cyclic nucleotide phosphodiesterase. The cyclic nucleotide phosphodiesterase, isolated from bovine brain in our laboratory, hydrolyzed both cyclic AMP and cyclic GMP. The enzyme had a Michaelis constant (K_m) of 6.4 ± 1.4 μM for hydrolysis of cyclic GMP and of 55.0 ± 8.7 μM for hydrolysis of cyclic AMP. The maximum velocity (V_{max}) for hydrolysis of cyclic GMP was 36.8 ± 3.2 nmoles/min/mg of protein, approximately 54 per cent of that for cyclic AMP (68.4 ± 4.1 nmoles/min/mg of protein). Kinetic studies revealed that each cyclic nucleotide competitively inhibited the hydrolysis of the other (Fig. 1). The K_i value was 103 ± 22 μM for cyclic AMP and 4 ± 0.8 μM for cyclic GMP. A simple explanation for these results is that these two substrates are hydrolyzed by one enzyme and share a common substrate site. It is also possible, however, that the isolated enzyme fraction contained two enzymes, each of which hydrolyzed one substrate, and that each hydrolysis was competitively inhibited by the other substrate. We employed an alternative substrate method, first

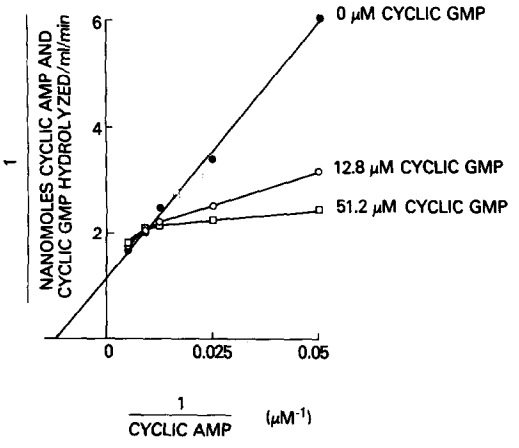

Fig. 2. Alternative substrate effect with cyclic AMP and cyclic GMP. The reciprocal of the sum of initial velocity of both cyclic AMP and cyclic GMP is plotted vs the reciprocal of the concentration of cyclic AMP. Cyclic [G-^{3}H]GMP and cyclic [8-^{14}C]AMP were used to indicate their respective rates of hydrolysis. The amount of cyclic nucleotide phosphodiesterase used was 1.7 μg. The data shown represent the means of the assays in duplicate.

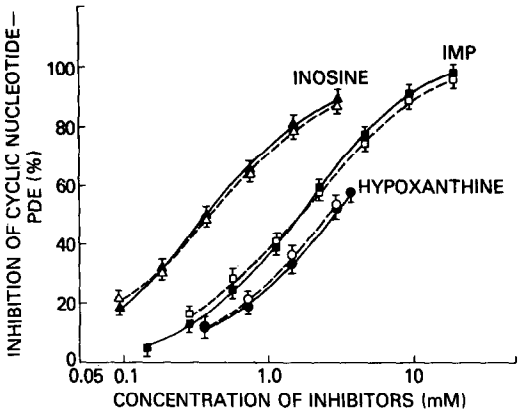

Fig. 3. Inhibitory effects of inosine, IMP and hypoxanthine on the hydrolysis of cyclic AMP by cyclic nucleotide phosphodiesterase (cyclic nucleotide-PDE). The per cent inhibition of the enzyme activity by the inhibitor is plotted vs the concentration of the inhibitor on a logarithmic scale. Open symbols represent the effects of the inhibitors on cyclic nucleotide-PDE (2.1 μg) in the absence of calmodulin, whereas closed symbols represent the effects of the inhibitors on the enzyme in the presence of calmodulin (2.2 μg). The concentration of cyclic AMP used was 1 μM. The points plotted are the mean values of at least three experiments. Vertical lines indicate S.E.

described by Ariëns *et al.* [38] in studying drug–receptor interactions and later modified by Reiner [39] and Cha [40] for enzymes, to differentiate between these two possibilities. Figure 2 is a double-reciprocal plot in which the initial velocity which was measured was the sum of the hydrolysis rates of cyclic AMP and cyclic GMP; the concentration of cyclic AMP was varied in the presence of two concentrations of cyclic GMP. The pattern observed is typical of that seen with competing substrates which have significantly different V_{max} values [40–42]. If the two substrates were hydrolyzed by two different enzymes, there should be no common point of intersection [40]. Our results, therefore, clearly indicate that the same enzyme reacts with both cyclic AMP and cyclic GMP and that they share a common binding site.

Effects of inosine, IMP and hypoxanthine on the activity of the bovine brain cyclic nucleotide phosphodiesterase. Inosine, IMP and hypoxanthine each inhibited the hydrolysis of cyclic AMP by the purified cyclic nucleotide phosphodiesterase. The concentration–response curves obtained by plotting per cent inhibition of enzyme activity vs the logarithm of inhibitor concentration were sigmoidal (Fig. 3). The order of potency of inhibitors was: inosine > IMP > hypoxanthine. Both inosine and IMP caused more than 90 per cent inhibition at higher concentrations. The effect of hypoxanthine, however, was limited by its solubility; only a 57 per cent reduction in enzyme activity could be obtained at a concentration of 3.7 mM.

We then examined the effects of inosine, IMP and hypoxanthine on the hydrolysis of cyclic GMP by cyclic GMP by cyclic nucleotide phosphodiesterase. The order of potency of the inhibitors was identical to that described for the inhibition of cyclic AMP hydrolysis.

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Influence of pH on the inhibitory effects of inosine, IMP and hypoxanthine. The activity of the purified bovine brain cyclic nucleotide phosphodiesterase was pH-dependent and had a pH optimum of 7.5. When the pH was changed by ±0.3 pH units from pH 7.5, the hydrolysis of cyclic GMP by the enzyme decreased by only 10 per cent; greater changes in pH, however, caused a more dramatic decline of the enzyme activity, as noted for the other mammalian cyclic nucleotide phosphodiesterase [43, 44]. Because of this phenomenon, we limited our study of the influence of pH on the inhibitory effect of purine derivatives to pH 7.2 to 7.8. When the pH of the reaction mixture was lowered from 7.8 to 7.2,

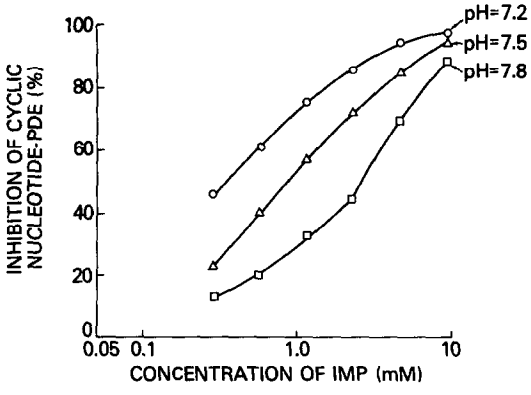

Fig. 4. Effect of pH on the inhibitory effect of IMP. The per cent inhibition of cyclic GMP hydrolysis by IMP is plotted vs the concentration of IMP on a logarithmic scale. Bovine brain cyclic nucleotide phosphodiesterase (cyclic nucleotide-PDE, 0.85 μg) was used. The concentration of cyclic GMP used was 1 μM. The points plotted are the mean values of two experiments.

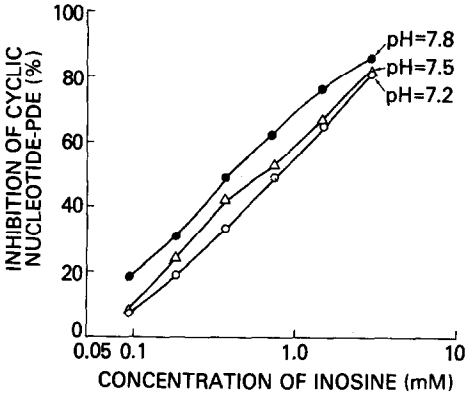

Fig. 5. Effect of pH on the inhibitory effect of inosine. The conditions were the same as cited in the legend of Fig. 4. The points plotted are the mean values of two experiments.

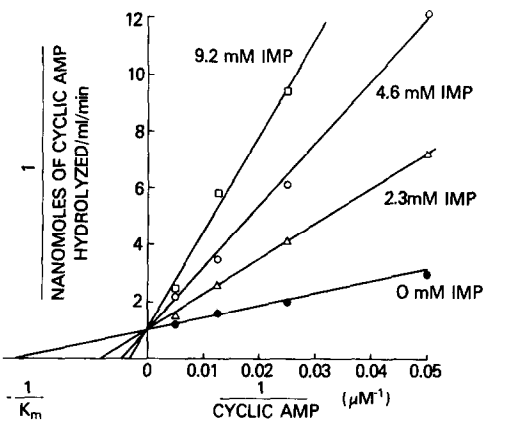

Fig. 6. Double-reciprocal plot of the effect of IMP in inhibiting the hydrolysis of cyclic AMP. The enzyme (2.1 μg) was derived from the DEAE-cellulose step. The data shown represent the mean of the assays in triplicate.

the potency of IMP when inhibiting the hydrolysis of cyclic AMP or cyclic GMP increased more than 7-fold (I_{50} decreased from 2.6 to 0.35 mM, Fig. 4). On the other hand, the inhibitory potency of inosine decreased 2-fold when the pH was decreased from 7.8 to 7.2 (Fig. 5). Thus, at pH 7.8, our regular assay pH, inosine was more potent than IMP (Fig. 3), whereas at pH 7.2, IMP was more potent than inosine in inhibiting the activity of bovine brain cyclic nucleotide phosphodiesterase (Figs. 4 and 5).

Modes of action of purine inhibitors. An enzyme kinetic approach was used to evaluate the modes of inhibition of bovine brain cyclic nucleotide phosphodiesterase by purine derivatives. Figure 6 is a double reciprocal plot showing the inhibition of cyclic AMP hydrolysis by IMP. The K_m, but not the V_{max}, changed with the changing IMP concentration, indicating that the mode of inhibition was competitive. The replot of apparent K_m vs concentration of IMP produced a straight line which indicated a pure one-site competitive inhibition. Hypoxanthine and inosine also competitively inhibited the binding of cyclic AMP to the enzyme. Using the method of Cleland [37], we found K_i values of 0.36 ± 0.05, 1.3 ± 0.2 and 3.2 ± 0.5 mM for inosine, IMP and hypoxanthine.

Inosine, IMP and hypoxanthine competitively inhibited the hydrolysis of cyclic GMP by cyclic nucleotide phosphodiesterase. The K_i values of inosine, IMP and hypoxanthine as inhibitors of cyclic GMP hydrolysis were 0.50 ± 0.09, 1.8 ± 0.2 and 4.5 ± 0.7 mM, respectively.

Relationships between calmodulin, cyclic nucleotides and purine inhibitors. The calcium ion is an essential factor for the activation effect of calmodulin, a heat stable protein activator, on cyclic nucleotide phosphodiesterase [23–26]. Inhibitors which chelate Ca^{2+} should decrease the calmodulin-dependent activity of cyclic nucleotide phosphodiesterase [45, 46]. We, therefore, evaluated the influence of Ca^{2+} on the inhibitory effects of inosine, IMP and hypoxanthine on bovine brain cyclic nucleotide phosphodiesterase. Addition of 0.1 mM EGTA to our reaction mixture caused only about a 10 per cent decrease of the isolated bovine brain cyclic nucleotide phosphodiesterase activity. Since EGTA in the amount used was able to chelate all the Ca^{2+} in the reaction mixture, the purified enzyme predominantly displayed Ca^{2+}–calmodulin-independent activity (i.e. basal activity). The inhibitory effects of inosine, IMP and hypoxanthine on the cyclic nucleotide phosphodiesterase were not affected by the addition of EGTA, and were, therefore, Ca^{2+}-independent.

The effect of calmodulin on cyclic nucleotide phosphodiesterase has received much attention [18–26], since its discovery by Cheung [17]. The influence of calmodulin on the kinetic parameters of cyclic nucleotide phosphodiesterase, however, shows many patterns. For example, calmodulin has been reported to increase the V_{max} alone [47], decrease the K_m alone [21], or increase the V_{max} and decrease the K_m [48]. These discrepancies may be due to the different sources and preparations of the enzymes and/or different assay conditions. We found that calmodulin increased the V_{max} but had little effect on the K_m value of bovine brain cyclic nucleotide phosphodiesterase as isolated in this study. Our results agree with those reported by Pitchard and Cheung [47]. The Hill plot for the hydrolysis of cyclic AMP by cyclic nucleotide phosphodiesterase gave a coefficient equal to 1, with or without the addition of calmodulin.* This result suggests that calmodulin does not increase the enzyme activity by causing a positive cooperativity between multiple enzyme substrate sites.

We found the potency of inosine, IMP and hypoxanthine when inhibiting the activity of the cyclic nucleotide phosphodiesterase was not affected by the addition of calmodulin (Fig. 3). Furthermore, these purines did not prevent the activating effect of calmodulin on the cyclic nucleotide phosphodiesterase (Fig. 7). These results suggest that the binding site of inosine (or IMP and hypoxanthine) on the enzyme is different from that of calmodulin.

* C-M. Liang, Y. P. Liu and B. A. Chabner, unpublished results.

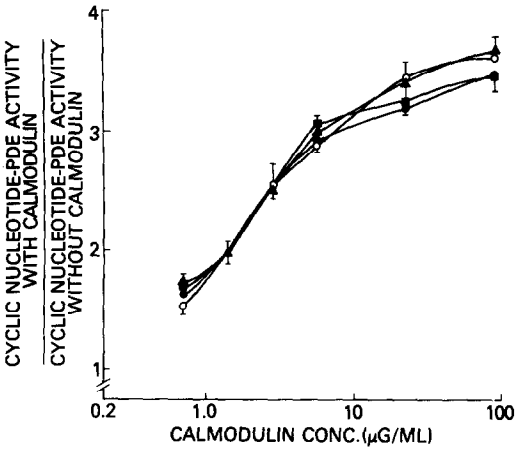

Fig. 7. Influence of inosine on the activity of calmodulin. The effect of calmodulin on cyclic nucleotide phosphodiesterase is plotted vs the concentration of calmodulin on a logarithmic scale. The cyclic nucleotide-PDE (2.1 μg) was used without the presence of inosine (○——○), with 0.093 mM inosine (▲——▲), with 0.186 mM inosine (■——■), or with 0.373 mM inosine (●——●). The concentration of cyclic AMP used was 1 μM. These points plotted are the mean values of three experiments. Vertical lines indicate S.E.

DISCUSSION

Cyclic nucleotide phosphodiesterase, as first isolated by Butcher and Sutherland [43] from bovine heart, hydrolyzed both cyclic AMP and cyclic GMP [49, 50]. The observations that (1) cyclic AMP competitively inhibited the hydrolysis of cyclic GMP and vice versa, with K_i values similar to the respective K_m values [20, 51], and (2) the cyclic nucleotide phosphodiesterase activities for cyclic AMP and cyclic GMP remain at a constant ratio throughout the purification process [20, 51] have been suggested as indications that a single enzyme catalyzes the hydrolysis of these two substrates. This proposal, however, required further experimental support. In this communication, we report the use of an alternative substrate method to support the single enzyme theory. Our results (Fig. 2) strongly indicate that the hydrolysis of both cyclic AMP and cyclic GMP by bovine brain cyclic nucleotide phosphodiesterase is catalyzed by a single enzyme. This conclusion is further supported by the observation that each of several purine inhibitors shows a similar K_i value regardless of whether cyclic AMP or cyclic GMP is used as a substrate.

Inosine inhibited the hydrolysis of cyclic AMP by cyclic nucleotide phosphodiesterase from rat skeletal muscle [52], rat adipose epithelium [53] and toad bladder epithelium [54]. Our observation that inosine inhibited the hydrolysis of cyclic AMP by bovine brain cyclic nucleotide phosphodiesterase (Fig. 3), therefore, is consistent with these reports. Furthermore, we found that inosine also inhibited the hydrolysis of cyclic GMP and that this inhibition was competitive in nature.

Lucacchini *et al.* [52] reported that hypoxanthine inhibited the hydrolysis of cyclic AMP by rat skeletal muscle phosphodiesterase. Huang and Kemp [44], on the other hand, found that hypoxanthine did not affect the activity of cyclic nucleotide phosphodiesterase from rabbit skeletal muscle. Goren and Rosen [55] reported that IMP did not affect the activity of cyclic nucleotide phosphodiesterase from beef heart. We found that IMP and hypoxanthine inhibited the hydrolysis of both cyclic AMP and cyclic GMP by bovine brain cyclic nucleotide phosphodiesterase and the inhibition was calmodulin-independent (Figs. 3 and 7). The discrepancy may be due to different sources and preparations of the enzymes and/or different assay conditions.

The mode of action of hypoxanthine or IMP on the cyclic nucleotide phosphodiesterase has not been reported previously. We found that these two purines, like inosine, competitively inhibited the enzymatic hydrolysis of cyclic nucleotides (e.g. Fig. 6). The observation that inosine was a more potent inhibitor than hypoxanthine (Fig. 3) may indicate that the binding between inosine and the enzyme substrate site(s) involves not only the purine nucleus but also the ribose moiety. The inhibitory effect of IMP was very dependent upon the pH of the reaction mixture; a small decrease in the pH (from 7.8 to 7.2) increased its inhibitory potency by more than 7-fold (Fig. 4). Under the same conditions, the activity of cyclic nucleotide phosphodiesterase was little affected, but the inhibitory effect of inosine decreased by about 2-fold (Fig. 5). Although the significance of these findings requires more extensive studies, these investigations raise interesting possibilities for pH-dependent modulation of cyclic nucleotide phosphodiesterase by purines.

In studying the protective effect of hypoxanthine on the toxicity caused by thiopurine cyclic nucleotides, Koontz and Wicks [56] observed that higher concentrations of hypoxanthine (>100 μM) inhibited the cellular growth of Reuber H 35 (rat hepatoma cell line). Since the pharmacologic inhibition of cyclic nucleotide phosphodiesterase activity can cause the inhibition of cell growth [6, 8], this finding could be explained by the inhibitory effect of the purines on cyclic nucleotide phosphodiesterase. Recently, inosine has been demonstrated to increase coronary blood flow to the heart [57]. Noting that adenosine is a good coronary dilator as well as a cyclic nucleotide phosphodiesterase inhibitor, we speculate that the coronary dilating effect of inosine is related to the inhibition of cyclic nucleotide phosphodiesterase activity.

Inosine, IMP and hypoxanthine are all naturally occurring purine derivatives. During exercise or ischemia, the plasma levels of hypoxanthine and inosine are elevated [58]. The intracellular levels of these purines can be even higher. The inhibitory effects of inosine, IMP and hypoxanthine on cyclic nucleotide phosphodiesterase are additive with each other.* It is possible that these intracellular purine derivatives, either individually or jointly, may regulate the activity of cyclic nucleotide phosphodiester-

* C-M- Liang, Y. P. Liu and B. A. Chabner, unpublished results.

ase. Elevated hypoxanthine concentrations are found in the cerebrospinal fluid of people with Lesch–Nyhan syndrome [59], and it will be interesting to investigate whether the neurological damage of this syndrome is related to the effect of these purine derivatives on the cyclic nucleotide phosphodiesterase.

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