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DIFFERENTIAL EFFECTS OF VARIOUS PHOSPHODIESTERASE INHIBITORS, PYRIMIDINE AND PURINE COMPOUNDS, AND INORGANIC PHOSPHATES ON CYCLIC CMP, CYCLIC AMP AND CYCLIC GMP PHOSPHODIESTERASES*

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Abstract—The effects of various compounds on homogeneous cyclic CMP phosphodiesterase (cyclic CMP–PDE) from pig liver were compared with the effects on cyclic AMP phosphodiesterase (cyclic AMP–PDE) and cyclic GMP phosphodiesterase (cyclic GMP–PDE). Of the conventional inhibitors for AMP–PDE and cyclic GMP–PDE, only Sch 15280 was found to inhibit cyclic CMP–PDE. Nucleoside monophosphates, orthophosphate, and 2′:3′-cyclic nucleotides were rather specific and were more effective in inhibiting cyclic CMP–PDE, compared to their effects on cyclic AMP–PDE and cyclic GMP–PDE. On the other hand, nucleoside di- and triphosphates and pyrophosphate (PP_i) were less effective in inhibiting cyclic CMP–PDE and were without marked effect on cyclic AMP–PDE and cyclic GMP–PDE. Orthophosphate (P_i) was more potent than CMP, CDP and CTP in inhibiting cyclic CMP–PDE, with a rank order of inhibitory potency of $P_i > CMP > CDP > CTP$. Of the 3′:5′-cyclic nucleotides examined, cyclic UMP was more specific in inhibiting cyclic CMP–PDE compared to its effect on cyclic AMP–PDE and cyclic GMP–PDE. In all experiments similar results were obtained when either cyclic CMP or cyclic AMP was used as a substrate for this multifunctional cyclic CMP–PDE, supporting the contention that a single catalytic site on the enzyme is responsible for the hydrolysis of both cyclic CMP and cyclic AMP. The present studies further support our original suggestion that cyclic CMP–PDE is a unique enzyme that is distinguishable from the conventional enzymes for purine cyclic nucleotides.

We [1] and Cheng and Bloch [2] have reported, and partially purified from a number of rat tissues [1] and leukemia L-1210 cells [2], a new species of phosphodiesterase (PDE) that hydrolyzes cyclic CMP. By employing crude extracts of rat liver, or partially purified enzyme from the same tissue, we observed that cyclic CMP–PDE activity, compared to conventional cyclic AMP–PDE and cyclic GMP–PDE, is far more resistant to inhibition by classical PDE inhibitors such as papaverine [1, 3]. Conversely, the cyclic CMP hydrolyzing activity is especially sensitive to inhibition by phosphates [3, 4]. We have now purified cyclic CMP–PDE over 10,000-fold to homogeneity from pig liver extract [5]. The pure enzyme hydrolyzes both cyclic CMP and cyclic AMP almost equally well, and represents the first mammalian enzyme that utilizes both pyrimidine and purine cyclic nucleotides as substrates [5]. The present investigations were undertaken to clarify whether or not (1) the unusual effects of agents on cyclic CMP–PDE activity are artifacts due to the use of impure enzyme preparations, and (2) hydrolysis of cyclic CMP and cyclic AMP by the same enzyme would be similarly resistant to, or inhibited by, a variety of agents. The evidence obtained in these studies clearly substantiated our previous observations made with crude enzyme preparations for cyclic CMP hydrolysis [1, 3, 4] and supports the contention that cyclic CMP–PDE has unique catalytic sites common for cyclic CMP and cyclic AMP, with their hydrolysis being similarly resistant to, or inhibited by, given agents.

MATERIALS AND METHODS

Materials. Cyclic [5-^{3}H]CMP (18 Ci/mmole) was purchased from the Amersham Corp., Arlington Heights, IL; cyclic [G-^{3}H]AMP (28 Ci/mmole) and cyclic [G-^{3}H]GMP (40 Ci/mmole) were from the New England Nuclear Corp., Boston, MA; and AG1-X8 (Cl^- form, 100–200 mesh) was from the Bio-Rad Laboratories, Rockville Centre, NY. Snake venom (*Crotalus adamanteus*) was from the Sigma Chemical Co., St. Louis, MO. Various pyrimidine and purine compounds were purchased either from the Sigma Chemical Co. or Boehringer Mannheim Biochemicals, Indianapolis, IN. Caffeine, theophylline, 3-isobutyl-1-methylxanthine (IBMX) and papaverine were from the Sigma Chemical Co. or the Aldrich Chemical Co., Milwaukee, WI. Ro 7-2956 and Ro 20-1724 were provided by Dr. Herbert Sheppard (Hoffmann-La Roche Inc., Nutley, NJ), and Sch 15280 was a gift from Dr. William Kreutner (Schering Co., Kenilworth, NJ). SQ 20,009 was from Squibb and Sons, Inc., Princeton, NJ.

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Table 1. Effects of various phosphodiesterase inhibitors on cyclic CMP-PDE, cyclic AMP-PDE and cyclic GMP-PDE*

Inhibitor (1 mM)	Phosphodiesterase activity (% control)			
	Cyclic CMP-PDE		Cyclic AMP-PDE	Cyclic GMP-PDE
	cCMP	cAMP	cAMP	cGMP
None (control)	100	100	100	100
Papaverine	91	96	3	3
IBMX	84	108	4	2
Theophylline	102	106	32	47
Caffeine	90	115	59	87
SQ 20,009	95	100	10	12
Ro 7-2956	107	110	15	79
Ro 20-1724	100	108	12	48
Sch 15280	41	50	46	74

* All assays were carried out using 1 μM substrates and the one-step assay procedure. The control activities (pmoles hydrolyzed/min), which were taken as 100 per cent, for cyclic CMP-PDE (0.2 μg) were 2.2 and 4.8 using cyclic CMP or cyclic AMP, respectively; 4.5 for cyclic AMP-PDE (6.3 μg); and 5.2 for cyclic GMP-PDE (10.5 μg). The data presented are averages of two determinations, assay variations being less than ± 5 per cent.

Methods. Cyclic CMP-PDE was purified to homogeneity from pig liver as described eslewhere [5]. Cyclic AMP-PDE and cyclic GMP-PDE were partially purified from guinea pig lung through the DEAE-cellulose step as previously described [6]. All enzyme preparations were stabilized by the addition of bovine serum albumin (2 mg/ml) and were stored at −80°.

The assay methods for cyclic CMP, cyclic AMP, and cyclic GMP phosphodiesterase activities were similar to those described previously [1, 6]. The standard reaction mixture contained, in a final volume of 0.1 ml, Hepes [4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid] buffer (pH 7.0), 5.0 μmoles; 5'-nucleotidase (*C. adamanteus*, snake venom), 15–30 μg; $MgSO_4$, 1.0 μmole; 2-mercaptoethanol, 1.0 μmole; various amounts of cyclic [5-^{3}H]CMP, cyclic [G-^{3}H]AMP, or cyclic [G-^{3}H]GMP, containing approximately 80,000 cpm; and an appropriate amount of enzyme protein. The reaction was started by addition of either substrate or enzyme and was carried out at 37° for 1–10 min. In some experiments a two-step assay procedure, instead of the one-step procedure described above, was used. In these cases, the first step was carried out as described above in the absence of 5'-nucleotidase, and the reaction was stopped by boiling. The second step was then carried out at 37° in the presence of 5'-nucleotidase. Quantitatively similar results were obtained using either procedure. Appropriate amounts of enzyme activity and appropriate incubation times were used so that no more than 25 per cent of each substrate was hydrolyzed under the assay conditions. In all experiments reported herein, the enzyme activities were linear as a function of incubation time and enzyme protein. None of the compounds tested was shown to affect 5'-nucleotidase activity. All assays were performed in duplicate and the values were corrected for values obtained in the absence of phosphodiesterase. The pyrimidine and purine compounds and the inorganic phosphates were dissolved in water, and other compounds were dissolved in 10% dimethylsulfoxide, which was without significant effect on enzyme activities. In addition, all solutions were neutralized to pH 7. Protein was determined by the method of Bradford [7], using the Bio-Rad protein determination kit and ovalbumin as a standard.

RESULTS

The effects of various phosphodiesterase inhibitors on cyclic CMP-PDE, cyclic AMP-PDE and cyclic GMP-PDE are summarized in Table 1. Cyclic CMP-PDE was insensitive to all but one of the various inhibitors tested when assayed using either cyclic CMP or cyclic AMP as a substrate. Only Sch 15280 was found to produce marked inhibition (about 50–60 per cent) of the enzyme. In contrast, cyclic AMP-PDE and cyclic GMP-PDE were inhibited to various degrees (13–98 per cent) by the agents. For example, papaverine and IBMX

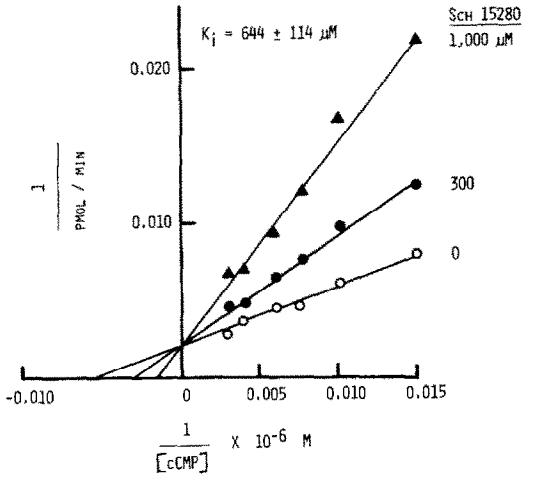

Fig. 1. Double-reciprocal plots of the inhibition of cyclic CMP-PDE by Sch 15280. Cyclic CMP-PDE (0.2 μg) was assayed using the one-step procedure. The concentrations of the cyclic CMP used ranged from 66.6 to 333.3 μM.

Table 2. Effects of pyrimidine and purine bases, their nucleosides and nucleotides, and inorganic phosphates, on cyclic CMP–PDE, cyclic AMP–PDE, and cyclic GMP–PDE*

Addition (1 mM)	Phosphodiesterase activity (% control)			
	Cyclic CMP–PDE		Cyclic AMP–PDE	Cyclic GMP–PDE
	cCMP	cAMP	cAMP	cGMP
None (control)	100	100	100	100
Cytosine	113	108	108	98
Cytidine	115	105	101	105
5′-CMP	30	36	78	97
5′-CDP	73	78	80	89
5′-CTP	85	86	92	105
3′-CMP	81	92	72	93
3′:5′-cCMP	21†	27	72	93
2′:3′-cCMP	16	16	74	110
CpC	87	87	82	100
Uracil	98	100	87	106
Uridine	100	100	86	107
5′-UMP	18	24	89	97
5′-UDP	57	66	100	88
5′-UTP	64	72	104	87
3′:5′-cUMP	33	41	87	98
2′:3′-cUMP	23	29	96	108
Adenine	96	104	67	87
Adenosine	95	104	64	93
5′-AMP	9	13	72	92
5′-ADP	28	32	87	90
5′-ATP	69	85	92	95
3′-AMP	18	19	78	91
3′:5′-cAMP	6	12†	6†	45
2′:3′-cAMP	3	7	60	101
2′-Deoxy-3′:5′-cAMP	1	3	6	65
ApA	84	88	67	79
Guanine	95	94	102	110
Guanosine	98	108	74	78
5′-GMP	6	14	77	46
5′-GDP	27	39	84	87
5′-GTP	75	92	91	90
3′-GMP	22	38	110	95
3′:5′-cGMP	14	23	40	3†
2′:3′-cGMP	5	8	67	69
2′-Deoxy-3′:5′-cGMP	1	13	62	11
GpG	62	60	93	55
P_i	10	12	85	111
PP_i	95	89	91	96

* Amounts and activities of enzyme used were as in Table 1. The two-step assay procedure was used. The concentration of substrates was 1 μM. Orthophosphate (P_i) and pyrophosphate (PP_i) used were Na_2HPO_4 and $Na_4P_2O_7$ respectively. The data presented are averages of two determinations, assay variations being less than ± 5 per cent.

† Decrease in the apparent activity was due to dilution of the radioactive substrate.

inhibited both cyclic AMP–PDE and cyclic GMP–PDE by more than 95 per cent. On the other hand, Ro 7-2956 and Ro 20-1724 were more effective inhibitors of cyclic AMP–PDE (> 80 per cent inhibition) than of cyclic GMP–PDE (< 52 per cent inhibition), as we reported earlier [8]. The mechanism of inhibition by Sch 15280 of cyclic CMP–PDE was determined, using cyclic CMP as a substrate (Fig. 1). Sch 15280 was found to be a competitive inhibitor of cyclic CMP hydrolysis with a K_i value of 644 ± 114 μM (three determinations).

The differential effects of various pyrimidine and purine compounds and of inorganic phosphates on cyclic CMP–PDE, cyclic AMP–PDE, and cyclic GMP–PDE are summarized in Table 2. The nucleoside monophosphates, orthophosphate, and 2′: 3′-cyclic nucleotides were rather specific and more effective in inhibiting cyclic CMP–PDE, compared to their effects on cyclic AMP–PDE and cyclic GMP–PDE. One exception was 3′-CMP which, unlike 3′-AMP, 3′-GMP and other nucleoside monophosphates, did not markedly inhibit cyclic CMP–PDE. On the other hand, the nucleoside di- and triphosphates and pyrophosphate were less effective than the nucleoside monophosphates in inhibiting cyclic CMP–PDE, and had little effect on the other two enzymes. The pyrimidine and purine bases and their corresponding nucleosides, had little effect on cyclic CMP–PDE or cyclic GMP–PDE. Adenine and adenosine, when compared with cytosine, cytidine, uracil, uridine, guanine and guanosine, were more specific in inhibiting cyclic AMP–PDE. Of the 3′:5′-cyclic nucleotides tested, cyclic UMP was more effective in inhibiting cyclic CMP–

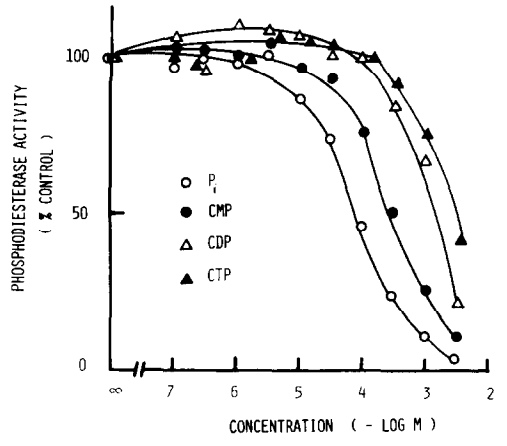

Fig. 2. Inhibition of cyclic CMP–PDE by orthophosphate (P_i) and 5′-CMP, 5′-CDP and 5′-CTP. Cyclic CMP–PDE (0.2 μg) was assayed using the two-step procedure in the presence of 1 μM cyclic CMP. Orthophosphate was added as Na_2HPO_4.

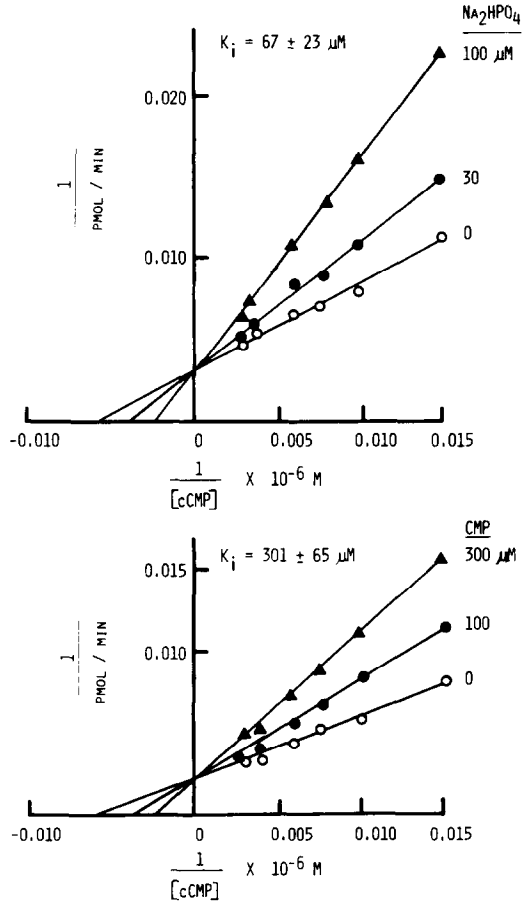

Fig. 3. Double-reciprocal plot of the inhibition of cyclic CMP–PDE by orthophosphate (Na_2HPO_4) and 5′-CMP. Cyclic CMP–PDE (0.2 μg) was assayed using the two-step procedure. The concentration of the cyclic CMP used ranged from 66.6 to 333.3 μM.

PDE, in comparison to its inhibition of cyclic AMP–PDE and cyclic GMP–PDE. On the other hand, 2′-deoxy cyclic AMP markedly inhibited cyclic AMP–PDE and cyclic CMP–PDE and less effectively inhibited cyclic GMP–PDE. Likewise, 2′-deoxy cyclic GMP was a more effective inhibitor of cyclic GMP–PDE and of cyclic CMP–PDE than of cyclic AMP–PDE. These results are in agreement with our previous reports that 2′-deoxy cyclic AMP and 2′-deoxy cyclic GMP are specific and potent inhibitors of cyclic AMP–PDE and cyclic GMP–PDE, respectively [3, 8], and that both are approximately equipotent in inhibiting cyclic CMP–PDE [3]. We suggested previously [3] that 2′-deoxy cyclic CMP (a compound not currently available) would be a specific inhibitor for cyclic CMP–PDE. In addition, 2′-deoxy cyclic UMP may also be a specific inhibitor of cyclic CMP–PDE. GpG was found to inhibit cyclic CMP–PDE (about 40 per cent) and cyclic GMP–PDE (45 per cent), with little effect on cyclic AMP–PDE.

Orthophosphate (P_i) was more potent in inhibiting cyclic CMP–PDE than either CMP, CDP, or CTP (Fig. 2), with a rank order of inhibitory potency of $P_i > CMP > CDP > CTP$, in agreement with the data presented in Table 2. Using cyclic CMP as substrate, the mechanism of the inhibition of cyclic CMP–PDE by orthophosphate and CMP was determined (Fig. 3). Orthophosphate competitively inhibited cyclic CMP hydrolysis with a K_i value of 67 ± 23 μM (three determinations). CMP also competitively inhibited cyclic CMP hydrolysis with a K_i value of 301 ± 65 μM (three determinations). The lower K_i value for orthophosphate, compared to that of CMP, is in agreement with the order of inhibitory potency (Fig. 2).

DISCUSSION

The present studies strengthen our original suggestion that cyclic CMP–PDE is a unique enzyme distinguishable from the conventional phosphodiesterases for purine cyclic nucleotides. The results obtained in these studies, using a homogeneous preparation of cyclic CMP–PDE, extend our earlier observations made with impure enzyme preparations of cyclic CMP–PDE with respect to the insensitivity to various pharmacologic agents and to inhibition by various nucleotides and inorganic phosphate [1, 3, 4]. We reported previously that cyclic CMP–PDE hydrolyzes cyclic CMP and cyclic AMP to comparable degrees and, based on kinetic analysis, that a single catalytic site on the enzyme is responsible for the hydrolysis of both cyclic CMP and cyclic AMP [5]. In the present studies, qualitatively and quantitatively similar results were obtained when either cyclic CMP or cyclic AMP was used as a substrate for cyclic CMP–PDE. These results further support the contention that a single catalytic site on the enzyme is responsible for the hydrolysis of both cyclic CMP and cyclic AMP.

Although we were originally interested in cyclic CMP–PDE with respect to its hydrolytic activity for cyclic CMP, the potential significance of its ability to hydrolyze cyclic AMP cannot be overlooked. The

relative insensitivity of this multifunctional cyclic CMP–PDE to the various pharmacologic agents could have important implications. For example, in studies where phosphodiesterase inhibitors are used to examine cyclic AMP-mediated events by inhibiting the degradation of cyclic AMP, the influence of cyclic CMP–DPE could be substantial. This may be especially true for tissues containing significant levels of cyclic CMP–PDE, such as liver, kidney, or intestine [1, 9]. It has been shown by others that cyclic CMP, but not cyclic AMP or cyclic GMP, promotes growth of leukemia L1210 cells in culture [10, 11], and that elevated cyclic CMP is associated with acute myelocytic leukemia [12] and regenerating liver [12, 13]. Although the natural occurrence [10, 12, 14, 15] and postulated role of cyclic CMP as a positive effector of cell proliferation [10–12] remain to be fully established, the means to increase the cellular cyclic CMP (and presumably to promote growth) are to depress the tissue level, or inhibit the activity, or both, of cyclic CMP–PDE. We observed previously that the enzyme levels are indeed lower in the fetal tissues [1, 9], fast growing Morris hepatoma 3924A [16] and regenerating liver [17], compared to corresponding control tissues of lower growth rates. From our previous [1, 3, 4] and present studies, it is also clear that conventional pharmacologic agents would be ineffective in altering intracellular levels of cyclic CMP by inhibition of cyclic CMP–PDE. Should the role of cyclic CMP in cell proliferation be established, a search for potent and specific inhibitors of cyclic CMP–PDE would be of interest. Based upon the inhibition produced by various compounds, which was observed in the previous [3] and present studies, 2′-deoxy cyclic CMP and perhaps 2′-deoxy UMP appear to be promising agents. Synthesis of these compounds and investigations on their possible biological effects seem worthwhile.

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