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INHIBITION OF HUMAN LUNG CYCLIC GMP AND CYCLIC AMP PHOSPHODIESTERASES BY CERTAIN NULEOSIDES, NUCLEOTIDES, AND PHARMACOLOGICAL PHOSPHODIESTERASE INHIBITORS

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Abstract—The effects of various known inhibitors on the cyclic GMP phosphodiesterase and cyclic AMP phosphodiesterase enzymatic activities found in human lung tissue were investigated. Of the five compounds studied, 1-methyl-3-isobutylxanthine was the most specific for cyclic GMP hydrolysis, while 1-ethyl-4-(isopropylidine-hydrazino-1H-pyrazolo-(3, 4-b)-pyridine-5-carboxylic acid, ethyl ester hydrochloride (SQ 20009) was the most potent for inhibiting cyclic AMP hydrolysis. Common non-cyclic nucleotides were tested for their inhibitory abilities only. ATP, GMP and IMP revealed significant inhibition of both phosphodiesterase activities. Certain nucleosides and cyclic nucleotides were also tested at 1 μM substrate concentrations for their ability to inhibit hydrolysis. Cyclic IMP inhibited cyclic GMP activity, but it had no effect on the cyclic AMP phosphodiesterase. The 2′-deoxy nucleosides (2′-deoxy guanosine and 2′-deoxy inosine) inhibited both enzymes; 2′-deoxy GMP inhibited mainly the cyclic GMP acitivity, while 2′-deoxy adenosine inhibited only the cyclic AMP activity. The observation that 2′-deoxy guanosine was a non-competitive inhibitor of cyclic GMP hydrolysis suggested that a site on the enzyme, distinct from the active site, might be involved in the regulation of the human lung phosphodiesterase.

Intracellular cyclic nucleotide levels can be controlled through regulation of either synthesis via respective cyclases or degradation via the phosphodiesterase enzyme(s) [1, 2]. Although much is known about adenyl cyclase activation [3–5], little is known about the regulation of phosphodiesterase activity. While synthetic inhibitors of cyclic AMP and cyclic GMP phosphodiesterases are numerous [6–8], fewer studies have involved physiologically available compounds that may act as inhibitors [9–12].

Human lung tissue contains cyclic AMP and cyclic GMP phosphodiesterases [13–15] which reveal three apparent K_m activities for cyclic AMP and two apparent activities for cyclic GMP. Human lung tissue has been implicated as a source of pharmacological mediators in asthma and immediate hypersensitivity reactions [16–18], and, consequently, it has been a target for pharmacological research into compounds which decrease mediator release [19]. Since excellent biochemical studies by Austen *et al.* [20, 21] and Bourne *et al.* [22] have shown cyclic AMP and cyclic GMP to be closely related to mediator release, inhibition studies of the human lung phosphodiesterase may provide useful information regarding the allergic release of these mediators.

EXPERIMENTAL PROCEDURES

Materials. Cyclic [G-^{3}H]AMP (20 Ci/m-mole) and cyclic [G-^{3}H]GMP (10 Ci/m-mole) were purchased from Schwarz Mann, Orangeburg, NY. All nucleotides, cyclic nucleotides, 2′-deoxy nucleosides, xanthosine, inosine, cordycepin, and N^6-adenosine oxide, were obtained from the Sigma Chemical Co., St. Louis, MO. Snake venom (*Crotalus atrox*), aminophylline, and caffeine were also obtained from the Sigma Chemical Co. The phosphodiesterase inhibitor *dl*-4-(3-butoxy-4-methoxybenzyl)-2′-imidazolidione (Ro 20-1742) was a gift from Hoffmann-LaRoche, Nutley N.J.; E. R. Squibb & Sons, Inc., Prineton, N.J., graciously supplied the phosphodiesterase inhibitor 1-ethyl-4-(isopropylidine-hydrazino-1H-pyrazolo-(3, 4-b)-pyridine-5-carboxylic acid, ethyl ester hydrochloride (SQ 20009). Aldrich supplied 3-isobutyl-1-methylxanthine (MIX). Adenosine and guanosine were obtained from the Eastman Chemical Co.; Bio-Rad Laboratories (Richmond, CA) furnished the AG1-X2 ion exhange resin (chloride form, 200–400 mesh). Brinkmann Instruments provided the PEI-cellulose thin-layer sheets (0.1 mm thickness). All other chemicals were purchased from standard sources in the highest available grade.

Enzyme preparation and assay of the cyclic nucleotide phosphodiesterase activities. The phosphodiesterase enzymes used in this study were obtained as described earlier [14]. Briefly, human lung tissue, obtained surgically and frozen immediately, was trimmed of excess fat and connective tissue before homogenization in cold (4°) Tris–HCl buffer (pH 7.5, 0.05 M) containing 0.01 M $MgCl_2$ and 0.004 M β-mercaptoethanol. After centrifugation (25,000 *g*, 30 min), the supernatant fraction (containing > 93 per cent of the total enzymatic activity) was taken to 55% saturation with solid ammonium sulfate, stirred for 30 min at 4°, and centrifuged. The pellet was re-dissolved and dialyzed in the homogenizing buffer, and frozen (−20°) until use. Two assay procedures were utilized for monitoring the phosphodiesterase activities. The two-step method by Green *et al.* [14] and Appleman [23] was used for assaying the known phosphodiesterase inhibitors (i.e. caffeine, aminophylline, Ro 20-1724, MIX and SQ 20009) by the addition of substrate (either cyclic AMP or cyclic GMP) at the desired concentration containing

250,000–300,000 cpm of the tritiated compound. Sufficient enzyme was used to convert 5–15 per cent of the substrate to product during the reaction time. Ethanol (5.0%) was used to attain the highest concentration (2.5 mM) of Ro 20-1724; this concentration of solvent did not interfere with any of the determinations. After the addition of resin, the samples were centrifuged at 1500 *g* for 15 min, and an aliquot (0.20 ml) was removed and counted in a scintillation counter. The blanks (absence of enzyme or boiled enzyme) were usually 4–6% of added cyclic nucleotide.

The second assay procedure was developed from the results described by Trifilo and Dobson [24]. In addition to enzyme, substrate and nucleotide (or nucleoside), each tube contained 0.05 M Tris–HCl, pH 7.5, 0.02 M $MgCl_2$, and 0.004 M β-mercaptoethanol in a total volume of 0.20 ml. The enzymatic reaction was terminated by boiling for 0.5 min. After centrifugation (1500 *g*, 10 min) in a clinical centrifuge, 6 μl of the solution was applied (3 × 2 μl) to a thin-layer PEI impregnated cellulose (0.1 mm thick) plastic sheet. Each experimental aliquot was spotted at 1-cm intervals along an origin line 3.0 cm from the bottom edge. After allowing the solvent phase to run approximately $\frac{3}{4}$ of the length, the sheet was dried and the radioactive product (5′-AMP or 5′-GMP) was cut out, placed into a scintillation vial, and eluted from the sheet with 0.10 ml of 1 M potassium phosphate (pH 3.4). This assay method was necessary to eliminate any undesirable effects of the nucleotides on the snake venom nucleotidase used in the two-step procedure. This thin-layer method offers excellent reproducibility, a low blank of 40–70 cpm, and it eliminates the use of a second enzyme step that can yield erroneous results in inhibition studies.

RESULTS

In our earlier study [14] of the human lung phosphodiesterase enzyme(s), the relative potencies of aminophylline, caffeine and MIX in inhibiting the cyclic AMP and cyclic GMP enzymatic activities at an elevated substrate concentration of 100 μM were determined. However, since multiple enzyme forms (i.e. apparent K_m values for cyclic AMP of 0.4, 3 and 40 μM; apparent K_m values for cyclic GMP of 3 and 40 μM) are observed, lower substrate concentration are needed to include all of the phosphodiesterase activities. In order to establish more clearly the relative capabilities of these inhibitor compounds to inhibit

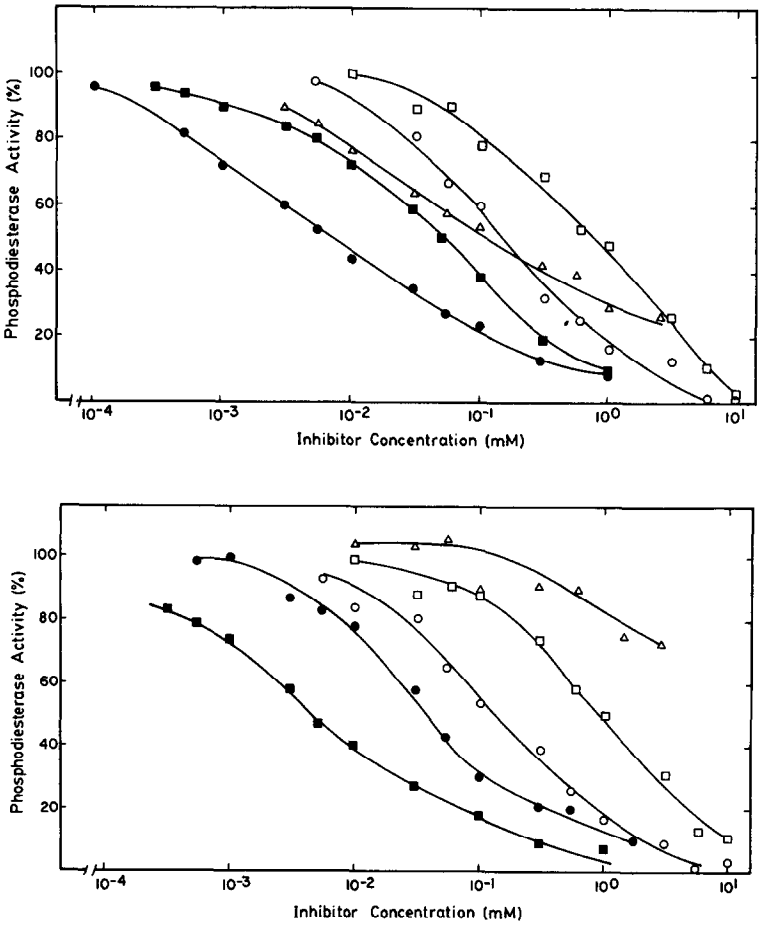

Fig. 1. Inhibition by various phosphodiesterase inhibitors of cyclic AMP phosphodiesterase (panel A) and cyclic GMP phosphodiesterase (panel B) from human lung. In both cases, the substrate concentration was 1 μM. Key: SQ 20009 (●); MIX (■); Ro 20-1724 (△); aminophylline (○); and caffeine (□).

Table 1. Inhibition of human lung cyclic AMP and cyclic GMP phosphodiesterase activities at various substrate concentrations*

	I_{50} (mM)					
	Cyclic AMP			Cyclic GMP		
Inhibitor	1 μM	10 μM	100 μM	1 μM	10 μM	100 μM
Aminophylline	0.160	0.32	1.80	0.138	0.35	1.59
Caffeine	0.800	1.41	2.40	1.0	1.2	1.7
Ro 20-1724	0.113	1.50	0.85	>2.5	>2.5	>2.5
SQ 20009	0.007	0.022	0.180	0.040	0.005	0.20
MIX (methylisobutylxanthine)	0.050	0.070	0.10	0.005	0.010	0.05

* The assay procedure utilized the Thompson and Appleman method. The concentrations of the inhibitors ranged from 10^{-6} to 10^{-2}M; all inhibitors (except Ro 20-1724 as indicated in Experimental Procedures) were dissolved in the assay buffer. All assays were performed in quadruplicate, and the means of two separate determinations are presented.

these phosphodiesterases at a more physiological concentration of substrates, aminophylline, caffeine, MIX, and two other well-established inhibitors, SQ 20009 and Ro 20-1724, were tested at lower substrate concentrations of 10 and 1 μM. Figs 1A and 1B illustrate that inhibition results at 1 μM cyclic AMP and 1 μM cyclic GMP, respectively, for the five inhibitors. For cyclic AMP (Fig. 1A), SQ 20009 reveals an extremely potent ability to inhibit enzymatic hydrolysis. Significant inhibition is seen at the low inhibitor concentration—0.0005 to 0.001 mM (0.50 to 1.0 μM). MIX is the next most potent inhibitor. Aminophylline and Ro 20-1724 are essentially equivalent inhibitors of cyclic AMP hydrolysis; however, both are, in most instances, approximately 1/10–1/20 as potent as SQ 20009. Caffeine is the least effective inhibitor.

The inhibition patterns for cyclic GMP are illustrated in Fig. 1B. The xanthine derivative, MIX, reveals the most potent inhibition of cyclic GMP hydrolysis. This inhibition patter differs from its cyclic AMP potency (Fig. 1A). SQ 20009 shows the next strongest inhibition of enzymatic activity; however, the inhibition appears to be less for cyclic GMP than for cyclic AMP. Aminophylline and caffeine have similar inhibition patterns for both cyclic GMP and cyclic AMP. The compound Ro 20-1724, in contrast to its ability to inhibit cyclic AMP hydrolysis, is a very poor inhibitor of cyclic GMP hydrolysis. Although solubility problems with this compound (Ro 20-1724) limit its use at concentrations greater than 4–5 mM, little inhibition (*ca.* 20 per cent) is noted at 2–3 mM.

The data observed at higher substrate (cyclic AMP and cyclic GMP) concentrations, as well as 1 μM, are summarized in Table 1. The I_{50} data at the different substrate levels resemble those seen at a 1.0 μM concentration. For cyclic AMP hydrolysis at a 1.0 μM concentration, the inhibitor potencies are; SQ 20009 > MIX > Ro 20-1724 > aminophylline > caffeine. However, for cyclic GMP hydrolysis (at 1.0 μM), the potency order is MIX >> SQ 20009 > aminophylline > caffeine >> Ro 20-1724. One possible explanation for the slight variations in the order of potency of the three substrate concentrations is that the different enzyme forms (i.e. the different apparent K_m values observed) have distinct specificities for the inhibitors. In data not shown, the type of inhibition for caffeine, MIX and aminophylline for the lowest apparent K_m enzymatic activity (cyclic AMP and cyclic GMP) is competitive.

With the possible exception of caffeine, the five previously described inhibitors are not normally found within human tissues. In an effort to investigate the possible regulatory role that certain metabolites might have on phosphodiesterase activity, nucleotides were studied as inhibitors. Table 2 reveals that most of the mono-, di- and triphosphates are inactive as inhibitors. The results with ATP are in general agreement with those reported earlier on human lung phosphodiesterase [14]. In these studies, the Mg^{2+} ion concentration is 20 mM and the pH is maintained at 7.5 by first neutralizing the nucleotides before addition to the assay mixture. Although ATP produces slight inhibition, GMP and IMP produce approximately 25 per cent inhibition with cyclic AMP as a substrate. With cyclic GMP as the substrate, similar results are obtained except that stronger inhibition is produced by GMP and IMP; ATP produces 30 per cent inhibition, while GMP and IMP produce approximately 50 per cent inhibition.

Table 2. Effect of nucleotides on cyclic AMP and cyclic GMP phosphodiesterase activities*

	Activity (%)	
Inhibitor	Cyclic AMP	Cyclic GMP
AMP	95± 7	89± 9
ADP	106± 8	95± 5
ATP	86± 9	66± 7
GMP	73± 7	44± 5
GDP	91± 9	82± 10
GTP	95± 6	93± 8
IMP	78± 8	58± 6
IDP	97± 5	95± 7
ITP	95± 5	89±.9
XMP	98± 4	82± 8
XTP	94± 5	89± 9
UMP	106± 5	91± 8
UDP	100± 8	86± 5
UTP	97± 3	86± 7

* The assay procedure utilized the thin-layer chromatography method outlined in Experimental Procedures. The concentration of substrate was 10^{-6} M; the inhibitor concentration was 3 mM in the assay buffer containing 20 mM $MgCl_2$. All assays were performed in duplicate, and the means of two separate determinations are presented.

Table 3. Inhibition of cyclic AMP and cyclic GMP phosphodiesterase activities by various nucleoside and nucleotide analogs*

Compound	I_{50} (mM) Cyclic GMP	Cyclic AMP
Cyclic XMP	0.18	0.95
Cyclic IMP	0.008	> 1.0
Adenosine	> 1.0	> 1.0
Guanosine	0.91	> 1.0
Inosine	> 1.0	> 1.0
Xanthosine	> 1.0	> 1.0
2′-Deoxy adenosine	> 1.0	0.40
2′-Deoxy guanosine	0.15	0.30
2′-Deoxy inosine	0.18	0.52
5′-GMP	> 1.0	> 1.0
2′-Deoxy GMP	0.10	0.90
2′, 3′-Cyclic AMP	> 1.0	> 1.0
2′, 3′-Cyclic UMP	> 1.0	> 1.0
8-Bromo cyclic IMP	0.56	> 1.0
N′-adenosine oxide	> 1.0	> 1.0
3′-Deoxy adenosine (cordycepin)	> 1.0	0.44

* The assay procedure utilized the Thompson and Appleman method. The concentrations of the various compounds ranged from 10^{-6} to 10^{-3} M. All inhibitors were dissolved in the assay buffer (with gentle heating when necessary). All assays were performed in triplicate, and the means of two separate determinations are presented.

Since GMP and IMP produced significant inhibition, other nucleotide and nucleoside analogs were tested for their ability to inhibit cyclic AMP and cyclic GMP hydrolysis. Table 3 shows the I_{50} (mM) for these compounds. Inosine 3′:5′-monophosphate (cyclic IMP) is very potent inhibitor of cyclic GMP hydrolysis, with an I_{50} of less that 10 μM. Interestingly, the next three most active inhibitors of cyclic GMP hydrolysis are 2′-deoxy derivatives: 2′-deoxy GMP, 2′-deoxy guanosine and 2′-deoxy inosine. These deoxy compounds are at least 6, 10 and 5 times, respectively, more potent than their 2′-hydroxy analogs. Xanthosine 3′:5′-monophosphate (cyclic XMP) and 8-bromo cyclic IMP also inhibit cyclic GMP hydrolysis, with an approximate I_{50} of 0.50 mM.

Three 2′-deoxy compounds (2′-deoxy adenosine, 2′-deoxy guanosine and 2′-deoxy inosine) are the most potent inhibitors of cyclic AMP activity. However, 2′-deoxy adenosine is the only compound which shows a specificity for cyclic AMP hydrolysis (i.e. it does not inhibit cyclic GMP acitivity, while 2′-deoxy guanosine and 2′-deoxy inosine do inhibit cyclic GMP). Although cyclic IMP is a good inhibitor of cyclic GMP hydrolysis, it is a very poor inhibitor of cyclic AMP hydrolysis (I_{50} > 1.0 mM). Cordycepin (3′-deoxy adenosine) shows some inhibition of cyclic AMP but is relatively inactive with cyclic GMP as a substrate.

Using cyclic GMP as the substrate, cyclic IMP (the most potent inhibitor) and 2′-deoxy guanosine reveal different modes of inhibition when plotted on a typical Lineweaver–Burk diagram (Fig. 2). Cyclic IMP shows competitive inhibition, with an apparent K_i of 6 μM. This type of inhibition by cyclic IMP has been seen by

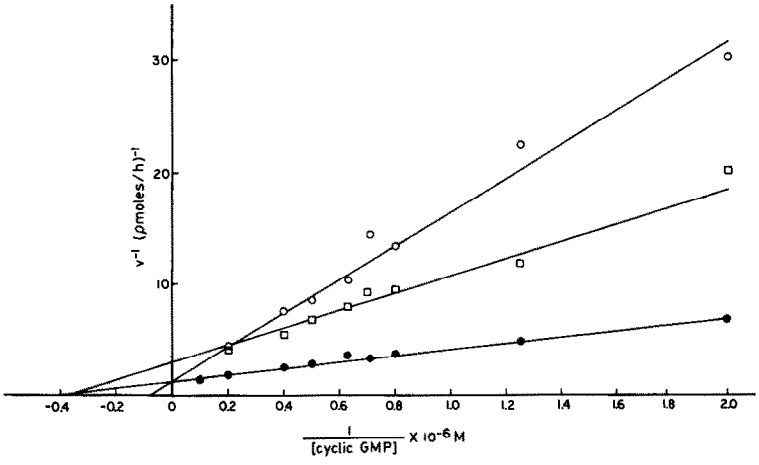

Fig. 2. Double-reciprocal plot of the inhibition of cyclic GMP phosphodiesterase (no inhibitor, ●) by 125 μM 2′-deoxy guanosine (□) and 30 μM cyclic IMP (○). The data represent the mean of two determinations performed in duplicate.

Davis and Kuo [25]. However, 2′-deoxy guanosine reveals a typical non-competitive inhibition of cyclic GMP hydrolysis.

DISCUSSION

Cyclic AMP and cyclic GMP phosphodiesterase enzymatic activities exist in human lung tissue. Comparative inhibition studies with five known phosphodiesterase inhibitors reveal important differences in their abilities to inhibit either cyclic AMP or cyclic GMP hydrolysis. The inhibitor potencies for cyclic AMP at 1.0 μM concentrations are: SQ 20009 > MIX > Ro 20-1724 > aminophylline > caffeine. For cyclic GMP, the order is MIX >> SQ 20009 > aminophylline > caffeine >> Ro 20-1724. These observed comparisons are similar to those reported by Davis and Kuo [26] for guinea pig lung phosphodiesterase. The xanthine derivative MIX is (at 1 μM substrate concentration) ten times more potent in inhibiting cyclic GMP phosphodiesterase activity than the cyclic AMP enzymatic activity. These results are similar to those reported by others [27, 28] using different sources of enzyme. The Ro 20-1724 (a substituted imidazolidinone) compound, however, reveals a higher specificity for inhibiting cyclic AMP hydrolysis than cyclic GMP. Since elevated intracellular cyclic AMP levels inhibit mediator release and elevated cyclic GMP levels enhance mediator release [21], the observed selectivity of Ro 20-1724 for inhibiting cyclic AMP activity could be of potential clinical usefulness in asthma and other allergic diseases. On the contrary, aminophylline, one of the currently available drugs for asthma, may have limited efficacy since it inhibits nearly equally the two (cyclic GMP and cyclic AMP) hydrolytic activities.

The inhibition of phosphodiesterases by nucleotides has been investigated in many reports dealing with enzymes from several sources [29–32]. ATP has been shown to be a potent inhibitor of several enzymes [29, 30], while having no effect on others [31]. These experiments confirmed our initial results [14] that ATP reveals significant inhibition of both cyclic AMP and cyclic GMP hydrolysis. A stronger inhibition by ATP of cyclic GMP hydrolysis is noted. The close monitoring of pH is essential when studying the nucleotides since the pH can be lowered to levels which slow enzymatic activity. Also, the requirement of Mg^{2+} for enzymatic activity necessitates the use of higher Mg^{2+} concentrations to compensate for any nucleotide chelation. The use of a nucleotidase step in the Thompson and Appleman procedure also necessitates careful interpretation of data involving nucleotides [33]. Consequently, we used a thin-layer chromatography procedure that allows one to isolate and measure the product of the phosphodiesterase reaction, the 5′-monophosphate nucleotide [24]. This method has a low blank (40–70 cpm), is highly reproducible, and its use eliminates the need for a second nucleotidase step.

The analogs of nucleotides and nucleosides showed varying potencies when inhibiting cyclic AMP and cyclic GMP hydrolysis. The strong inhibition by cyclic IMP of cyclic GMP hydrolysis has been seen by others [26, 34]. The finding that 2′-deoxy nucleosides inhibit phosphodiesterase activity has been noted in several reports [35, 36]. The observation that, at low substrate concentrations of cyclic GMP, 2′-deoxy guanosine is a non-competitive inhibitor is intriguing. Since the enzyme preparation is impure and multienzymatic activities are present, our data are preliminary. The most obvious interpretation, though, is the presence of a second site on the phosphodiesterase enzyme that interacts in some way with the active site. However, before a conclusion on the exact nature of this second site can be drawn, more extensive inhibitor studies must be made on a more homogeneous enzyme preparation. Our laboratory is involved currently in this project.

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