MOLECULAR PHARMACOLOGY, 13, 38–43

Effects of Antiallergic Agents, Compound 48/80, and Some Reference Inhibitors on the Activity of Partially Purified Human Lung Tissue Adenosine Cyclic 3′,5′-Monophosphate and Guanosine Cyclic 3′,5′-Monophosphate Phosphodiesterases

HÅKAN BERGSTRAND,[1] JAN KRISTOFFERSSON, BRITTA LUNDQUIST, AND ANNIKA SCHURMANN

Research and Development Laboratories, AB Draco, Fack, S-221 01 Lund, Sweden

(Received May 4, 1976)

(Accepted August 23, 1976)

SUMMARY

BERGSTRAND, HÅKAN, KRISTOFFERSSON, JAN, LUNDQUIST, BRITTA & SCHURMANN, ANNIKA (1977). Effects of antiallergic agents, compound 48/80, and some reference inhibitors on the activity of partially purified human lung tissue adenosine cyclic 3′,5′-monophosphate and guanosine cyclic 3′,5′-monophosphate phosphodiesterases. *Mol. Pharmacol.*, 13, 38–43.

Various compounds, some of which influence immediate allergic reactions in different ways, were examined for inhibitory effects on partially purified human lung tissue cyclic nucleotide phosphodiesterases. Some of the compounds reported to possess antiallergic properties were found to be more potent as inhibitors of a low-K_m, cyclic 3′,5′-GMP-specific phosphodiesterase than of a corresponding low-K_m, cyclic 3′,5′-AMP-specific enzyme. For 2-*o*-propoxyphenyl-8-azapurin-6-one (M & B 22,948), 6-*n*-butyl-2,8-dicarboxy-4,10-dioxo-1,4,7,10-tetrahydro-1,7-phenanthroline (ICI 74,917), and disodium cromoglycate this selectivity was remarkable; to produce 50% inhibition of enzyme activity, a 10–100-fold lower concentration of these agents was required with the former enzyme than with the latter. Compound 48/80, on the other hand, showed high selectivity as an inhibitor of the cyclic AMP-specific, low-K_m phosphodiesterase. Among the reference phosphodiesterase inhibitors examined, theophylline, 3-isobutyl-1-methylxanthine, and papaverine inhibited both low-K_m enzymes to a comparable degree, whereas 4-(3-butoxy-4-methoxybenzyl)-2-imidazolidinone (Ro 20-1724) was pronouncedly more active toward the cyclic AMP-specific enzyme. Most compounds examined also inhibited the activity of a high-K_m phosphodiesterase which hydrolyzes cyclic AMP and cyclic GMP at comparable rates. Pronounced inhibitory potency was recorded for Dicumarol, doxantrazole, compound 48/80, and papaverine, whereas M & B 22,948 was only slightly effective with this enzyme.

INTRODUCTION

In recent years it has become increasingly clear that cyclic nucleotide catabolism is controlled by more than one enzyme form. The pattern of these phosphodiesterases seems to vary from one tissue to another (1, 2), and even between different cell lines (3). We recently examined the phosphodiesterase activity of crude human lung tissue (4). Three major enzyme forms were identified and partially purified. One (fraction I) showed a high appar-

[1] To whom correspondence should be addressed.

ent affinity and specificity for cyclic 3',5'-GMP (apparent $K_m \sim 0.30$ μM); the second (fraction IIIb) showed corresponding selectivity for cyclic 3',5'-AMP (apparent $K_m \sim 0.35$ μM); whereas the third (fraction II) was characterized as a low-affinity enzyme with comparable specificity (apparent $K_m \sim 25$ μM) for both cyclic AMP and cyclic GMP. Although the cellular origin of these partially purified phosphodiesterases has still to be established, we found it of interest to examine their activity in the presence of some antiallergic agents and compound 48/80. The antiallergic agents were chosen for test because it has been suggested that the antiallergic asthma drug disodium cromoglycate exerts its pharmacological effect by inhibiting a mast cell cyclic AMP phosphodiesterase activity (5–8). Compound 48/80, which nonimmunologically induces release of histamine from rat mast cells, was examined because it has been reported to enhance the activity of a crude rat peritoneal mast cell phosphodiesterase preparation at a high, but not at a low, substrate concentration (9).

MATERIALS AND METHODS

Chemicals. Cyclic [^{3}H]AMP (20–40 Ci/mmole) and cyclic [^{3}H]GMP (2–10 Ci/mmole) were purchased from New England Nuclear Corporation. Sigma Chemical Company supplied snake venom (*Ophiophagus hannah*), bovine serum albumin, compound 48/80, and unlabeled cyclic nucleotides. A specimen of compound 48/80 was also obtained from AB Leo. Antiallergic agents[2] were kindly supplied by the following companies (numbers in parentheses refer to publications where data on the antiallergic effects and pharmacological actions of these compounds can be found): Fisons, Ltd., disodium cromoglycate (8, 10–21); Beecham Pharmaceuticals, 5,6-dimethyl-2-nitroindane-1,3-dione (BRL 10833) (13); ICI, Ltd., 6-*n*-butyl-2,8-dicarboxy-4,10-dioxo-1,4,7,10-tetrahydro-1,7-phenanthroline (ICI 74,917) (14, 15); May & Baker, Ltd., 2-*o*-propoxyphenyl-8-azapurine-6-one (M & B 22,948) (16); Wellcome Research Laboratories, 3-(5-tetrazolyl)thioxanthone 10,10-dioxide (doxantrazole) (17–19); Allen & Hanbury Research, Ltd., 7-(2-hydroxyethoxy)-9-oxoxanthene-2-carboxylic acid (AH 7725) (20, 21); AB Leo, 2,3,5,6-tetrahydro-6-phenylimidazole[2,1-*b*]thiazole (levamisole) (22); American Cyanamid, diethylcarbamazine (23); Sigma, Dicumarol (17). Dr. G. Kjellin, AB Draco, synthesized 3-isobutyl-1-methylxanthine and 8-azatheophylline (24), and Dr. L. Å. Svensson of AB Draco synthesized 3-(*p*-chlorophenoxy)-1,2-propanediol (chlorphenesin) (25). A local drugstore supplied papaverine hydrochloride (6, 17), theophylline (6, 7, 17, 26), and 4-(3-butoxy-4-methoxybenzyl)-2-imidazolidinone; Ro 20-1724 (27, 28) was obtained from Hoffmann-La Roche.

Buffer A, referred to below, consisted of 0.04 M Tris-HCl, pH 8 (4°), made 5 mM in $MgCl_2$ and 3.75 mM in 2-mercaptoethanol.

Assay of phosphodiesterase activity. The procedure of Thompson and Appleman (29) was followed with some modifications, described in detail elsewhere (4). Reactions were performed as follows: 50 μl of a suitable dilution of the enzyme preparation were mixed in a disposable plastic tube with 400 μl of buffer A (see above) made 0.5% in BSA[3] and containing the appropriate concentration of the inhibitor to be examined. The reaction was initiated by the addition of 50 μl of the appropriate concentration of cyclic [^{3}H]nucleotide in buffer A. After incubation for a suitable time (usually 12 min) at 30°, the reaction was terminated by immersing the tube in a boiling water bath for 2 min. After cooling to approximately 30°, 100 μl of snake venom (1 mg/ml in buffer A) were added, and the incubation was continued for 15 min before addition of 1 ml of a suspension of Bio-Rad AG1-X2 in water (1:3, v/v). The tubes were shaken for approximately 10 min and centrifuged, and 0.5 ml of the supernatant, mixed with 10 ml of Instagel (Packard), was assayed for radioactivity. Blanks were run under identical conditions, with the appropriate buffer replacing the enzyme addition. Specific activity

[2] For simplicity, some antiallergic compounds are referred to be their code numbers.

[3] The abbreviations used are: BSA, bovine serum albumin; DSCG, disodium cromoglycate; IBMX, 3-isobutyl-1-methylxanthine.

was determined on an aliquot of the substrate preparation. Results were corrected for adsorption of reaction product to the ion-exchange resin (AG1-X2) (see ref. 4). None of the agents listed in Table 1 as inhibitors of either phosphodiesterase activity was found to influence the nucleotidase activity of the snake venom or the efficiency of the ion-exchange adsorption (i.e., blank values and correction factors for loss of adenosine or guanosine were not affected) at concentrations at which the phosphodiesterase-inhibitory effect was recorded. The initial velocity of the phosphodiesterase reaction, v, was expressed as nanomoles of cyclic AMP or cyclic GMP hydrolyzed per minute per milligram of protein. The use of limited reaction times and buffer supplemented with 0.5% BSA for dilution of enzyme and for the assay ensured reasonable linearity of v with enzyme concentration and time until approximately 20% of the substrate had been hydrolyzed (see ref. 4).

Preparation of partially purified phosphodiesterase. The procedure for preparing phosphodiesterases was recently given in detail (4). Briefly, human lung tissue,

TABLE 1

Inhibitory effects of various compounds[a] on activity of different cyclic nucleotide phosphodiesterases purified from human lung tissue

Compound	I_{50}[b]		
	Fraction I, 0.12 μM cyclic GMP	Fraction II, 6.01 μM cyclic AMP	Fraction III, 0.13 μM cyclic AMP
	μM	μ⁻]M	μM
Compounds acting like DSCG			
AH 7725	100 (70)	250 (140)	350 (120)
BRL 10833	340 (90)	250 (130)	510 (180)
Doxantrazole	120 (4.0)	85 (4.5)	150 (8.0)
DSCG	250 (130)	360 (275)	3,500 (3,000)
ICI 74,197	16 (4.0)	80 (30)	280 (150)
M & B 22,948	1.1 (0.3)	170 (40)	230 (120)
Other antiallergic agents			
8-Azatheophylline[c]	1,500	330	1,700
Chlorphenesin[c]	1,600	>5,000	3,500
Dicumarol	>250 (35)	400 (6.0)	>250 (13)
Diethylcarbamazine[c]	3,500	>3,500	2,600
Levamisole[c]	>4,000	>10,000	>4,000
Histamine releaser			
Compound 48/80[c,d]	>5,000	12	11
Reference phosphodiesterase inhibitors			
IBMX[c]	3.4	12	6.9
Papaverine	9.0 (2.6)	17 (5.5)	6.6 (2.0)
Ro 20-1724[c]	250	390	9.1
Theophylline[c]	160	170	220

[a] Grouping is somewhat arbitrary; Dicumarol might be grouped as a compound acting like DSCG (17), and theophylline and papaverine show antiallergic activity (-, &, +&, ×-(.

[b] The I_{50} value is that concentration of each compound which produces 50% inhibition of the activity of the pertinent enzyme. Substrate concentrations employed were: 0.12 μM cyclic GMP (fraction I), 6 μM cyclic AMP (fraction II), and 0.13 μM cyclic AMP (fraction III). Results recorded when assays were performed in the absence of BSA are given in parentheses.

[c] No difference between figures obtained in the presence and absence of BSA.

[d] Figures given for compound 48/80 represent the concentration in micrograms per milliliter for the preparation supplied by Sigma.

obtained a few hours postoperatively from patients suffering from carcinoma of the lung, was freed from gross contamination of cancerous tissue and blood. The tissue was lyophilized and stored at −20°. After reconstitution of the tissue with cold water, it was homogenized in buffer A containing 0.1 M NaCl (2 g of buffer per gram of reconstituted tissue) with a Sorvall OmniMixer. After centrifugation at 200,000 × *g* for 30 min, the supernatant was fractionated on a DEAE-Sephadex A-50 ion-exchange column equilibrated with the homogenizing buffer and developed with a linearly increasing gradient of NaCl in the same buffer. Four main fractions of phosphodiesterase activity were regularly collected. Fraction IV probably constituted an enzymatically active fragment of fraction III. Fractions I–III were further purified by Sephadex G-200 gel filtration in buffer A–0.1 M NaCl. Active tubes were pooled, concentrated by Diaflo ultrafiltration, lyophilized, and stored at −20° until used. Fraction IIIb (see ref. 4) is presently referred to as fraction III.

RESULTS

Initially the inhibitory capacity of the compounds was examined by determining the concentration (I_{50}) which produced 50% inhibition of the activity of fraction I at 0.12 μM cyclic GMP, of fraction II at 6 μM cyclic AMP, and of fraction III at 0.13 μM cyclic AMP. Figure 1 shows the results obtained with M & B 22,948. The selective inhibitory effect of this compound for the cyclic GMP-specific, low-K_m phosphodiesterase is evident. Table 1 summarizes the results obtained with the compounds employed. DSCG and some antiallergic compounds with a mode of action similar to that of DSCG inhibited cyclic nucleotide phosphodiesterases, mostly with preference for cyclic GMP-hydrolyzing enzymes. Compound 48/80 showed inhibitory selectivity for cyclic AMP-hydrolyzing enzymes.

DSCG, compound 48/80, theophylline, papaverine, and IBMX were then examined for their effects on cyclic GMP hydrolysis by fraction I at substrate concentrations between 0.52 and 0.10 μM and on cyclic AMP hydrolysis by fraction II at substrate concentrations exceeding 1.2 μM. For each inhibitor and phosphodiesterase fraction, hydrolysis was examined with six different substrate concentrations at each of seven inhibitor concentrations (including zero concentration). Employing previously described methods (4), apparent K_i values were calculated and were found to correspond reasonably with the I_{50} values given in Table 1 (results not shown). In every case when significant inhibition was recorded in this way, it

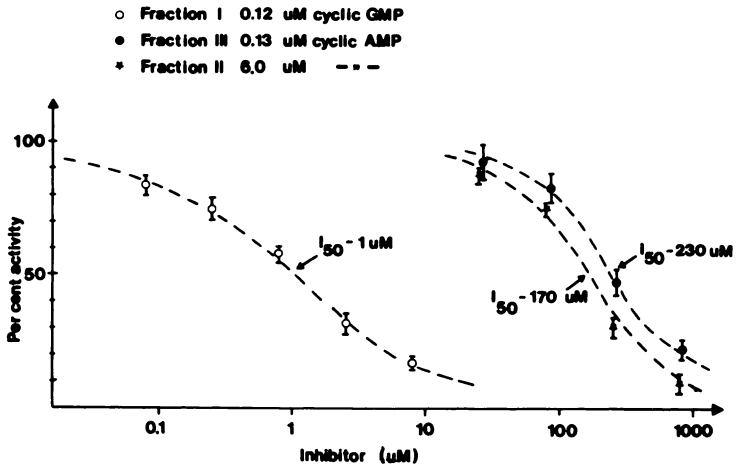

FIG. 1. *Inhibitory effect of various concentrations of M & B 22,948 on activity of partially purified human lung tissue phosphodiesterases*

Each point represents the mean ± standard error of four experiments, each performed in duplicate.

proved competitive irrespective of compound and enzyme employed.

To ensure linearity of reaction velocity with enzyme concentration and time, we normally supplement assay media with BSA (see MATERIALS AND METHODS). Binding to BSA could influence the free concentration of a compound and thus its inhibitory potency. Therefore the effect of each compound was also examined in the absence of BSA. Table 1 shows in parentheses the results recorded under these circumstances. The apparent inhibitory potency of many compounds seemed to be increased 2-3-fold in the absence of BSA. Dicumarol and doxantrazole showed a markedly increased inhibitory effect in the absence of BSA.

Substrate specificity of fraction II. Phosphodiesterase fraction II, which showed high apparent K_m values for hydrolysis of both cyclic AMP and cyclic GMP, could possibly be devoted biologically to hydrolysis of another, as yet unidentified, cyclic nucleotide. Therefore the cyclic AMP-hydrolyzing activity of this fraction was examined in the presence of various concentrations of some synthetic cyclic nucleotides. No effect, either stimulatory or inhibitory, was observed with cyclic IMP, cyclic TMP, cyclic UMP, or cyclic CMP at concentrations between 0.1 and 100 μM (results not shown).

DISCUSSION

The asthma drug DSCG has been suggested to exert its antiallergic effect through inhibition of a mast cell cyclic AMP phosphodiesterase activity (5–8). The present study primarily examined whether the ability to inhibit cyclic AMP phosphodiesterase activities is a general property of antiallergic agents (see ref. 19). Therefore the activities of different cyclic nucleotide phosphodiesterases, partially purified from crude human lung tissue, were examined at fixed, arbitrarily chosen substrate concentrations in the presence of various compounds of relevance to the issue. I_{50} values were determined for each combination of inhibitor and purified enzyme. Five of the compounds were then studied in more detail by kinetic analysis of their inhibitory properties. The previously determined I_{50} values were found to correspond reasonably well with their apparent K_i values. This suggested that the I_{50} values could indeed be used for provisional comparisons, either of the inhibitory potency of different compounds for a specified enzyme, or of the selectivity of a given compound for different phosphodiesterases. We then found that the inhibitory effect of a specified compound could differ remarkably from one enzyme to another. Thus three antiallergic agents—M & B 22,948, ICI 74,917, and DSCG—showed a pronounced selectivity for the cyclic GMP-specific, low-K_m phosphodiesterase, whereas compound 48/80 and Ro 20-1724 preferably inhibited the corresponding cyclic AMP-specific enzyme. Other antiallergic compounds (e.g., 8-azatheophylline and Dicumarol) showed some selectivity for a high-K_m phosphodiesterase hydrolyzing both cyclic AMP and cyclic GMP.

Thus, with the presently employed enzymes, we found no tendency of those antiallergic agents that presumably mimic the action of DSCG to act as potent cyclic AMP phosphodiesterase inhibitors. On the contrary, inhibition of the low-K_m, cyclic GMP-specific phosphodiesterase is a property of some of these agents which seems to merit consideration in relation to their pharmacological mode of action. However, the cellular origin of the enzymes has still to be established, and the mast cell phosphodiesterases have yet to be characterized [although a cyclic AMP-hydrolyzing activity exhibiting nonlinear kinetics has been observed in rat mast cell extracts (26)]. Therefore the relevance of the present results to the pharmacological action of DSCG and similarly acting compounds is unclear.

The cyclic AMP phosphodiesterase inhibition recorded with compound 48/80 and human lung tissue enzymes contrasts with the stimulatory effect observed with low concentrations of compound 48/80 on rat peritoneal mast cell phosphodiesterase activity (9). The reason for these seemingly opposite results has not so far been determined.

The apparent inhibitory potency of

many of the compounds examined seemed to increase when BSA was omitted from the assay medium. We believe that this depends on increased availability of the compounds rather than on increased lability of the partially purified enzymes in the absence of BSA. This belief is based on two observations: (a) some drugs showed no difference in inhibitory potency, whether or not BSA was present, and (b) with most compounds, the potency differences recorded were of the same magnitude irrespective of which enzyme activity was being examined. However, no studies that directly show binding of the pertinent compounds to BSA have been performed.

The present results merit consideration from a general biochemical standpoint. Phosphodiesterases from tissues other than lung could show specificity characteristics similar to those of the presently employed enzymes. Then some antiallergic compounds—for example, M & B 22,948 and ICI 74,197—and compound 48/80 might find application as probes either to inhibit specifically or to characterize the activity of a given phosphodiesterase. Some other compounds have recently been reported to show remarkable selectivity for specified phosphodiesterases, for example, trifluoperazine (30), dipyridamole (31), and, as confirmed in the present study, Ro 20-1724 (27, 28).

ACKNOWLEDGMENTS

We thank our colleagues at Draco for valuable discussions, and Drs. H. Schüller and H. Henriksson, who kindly provided human lung tissue specimens.

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