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cAMP-PHOSPHODIESTERASE INHIBITORS AND TRACHEAL SMOOTH MUSCLE RELAXATION

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Abstract—A series of compounds drawn from a wide variety of chemical and pharmacologic classes were tested for their ability to act as inhibitors of a guinea pig tracheal cAMP-phosphodiesterase. Active compounds, defined as equal to or more potent than 1,3-dimethylxanthine (theophylline), were subsequently tested for guinea pig tracheal smooth muscle relaxant activity *in vitro*. A strong correlation ($r = 0.988$ with 95 per cent confidence limits of 0.933 to 0.998) was found between the two activities, suggesting that, under these conditions, smooth muscle relaxation was due to elevation of tracheal cAMP levels.

The classical bronchodilators include β-adrenergic agents (i.e. isoproterenol) and the phosphodiesterase inhibitors (i.e. methyl xanthines such as theophylline). The recognition that β-agonists produced increases in cellular cAMP levels in smooth muscle preparations from intestine [1, 2], uterus and blood vessels [3] provided the basis for the hypothesis that the bronchodilating effects of β-adrenergics and methyl xanthines were mediated by the level of cAMP in tracheobronchial smooth muscle [4]. Several indirect studies supported this hypothesis. For instance, catecholamines increased cAMP levels in preparations from rat lung [5], and Lugnier *et al.* [6] were able to show a correlation between the ability of drugs to inhibit beef heart phosphodiesterase and their ability to inhibit the barium-induced contraction of rat aortic strips. In addition, in the guinea pig tracheal chain preparation commonly used to examine bronchodilating agents [7], the addition of dibutyryl-cAMP to the bath induced smooth muscle relaxation which, unlike the response to epinephrine, could not be antagonized by β-adrenergic blocking agents [8]. Recently, Murad and Kimura [4, 9] provided additional evidence for the cAMP-mediation hypothesis and also suggested that the histamine- and acetylcholine-induced bronchial smooth muscle contractions may be due to varying cGMP levels in the tissue.

Based on the cAMP hypothesis, inhibition of the cAMP-phosphodiesterase enzyme should lead to tracheal smooth muscle relaxation (i.e. bronchodilation), as a result of the increased cAMP levels within the tissue. To further investigate this hypothesis, we tested a series of compounds (previously reported to show phosphodiesterase inhibitory activity in other tissues) to determine whether a correlation existed between their activity as inhibitors of a cAMP-dependent phosphodiesterase from tracheal tissue and their subsequent relaxant activity on tracheal smooth muscle *in vitro*. The isolation and methods of assay for both these test systems are described below.

METHODS

Enzyme studies

Isolation of cAMP-phosphodiesterase(s). All isolation procedures were carried out at 4° unless otherwise stated. Tracheae were removed from twenty to fifty Hartley-strain guinea pigs (weighing 300–500 g) after sacrificing the animals by a blow to the head. After removal of as much cartilage as possible, the muscle strips were cut and homogenized in 50 mM Tris–HCl, pH 6.0, containing 3.75 mM mercaptoethanol, using a Tekmar tissumizer at a tissue to buffer ratio of 1:5 to 1:10. The suspension was centrifuged at 20,000 *g* for 20 min. The supernatant solution was decanted and further centrifuged at 100,000 *g* for 90 min. The final concentration to approximately 1 mg ml^{-1} protein was performed using an Amicon UM10 ultrafilter and was followed by storage in 250-μl aliquots at −70° until used. Throughout the isolation procedure, the activity was followed by the luciferin–luciferase assay of Weiss *et al.* [10] with 300 μM cAMP as the substrate and using a DuPont biometer to measure light output.

Kinetic determinations and inhibition studies

Kinetic data on the enzyme(s) were generated by the use of the Thompson and Appleman [11] radioactive assay for cAMP-phosphodiesterase, after preliminary studies as to saturating substrate concentrations and linearity (with respect to time and protein concentration) using the luciferase technic. In all kinetic runs, substrate concentrations covered the range of 4–500 μM with at least 15 separate concentrations in triplicate being used in any one run. Apparent K_m and V_{max} values were determined by the use of a least-squares weighted program utilizing the Taylor expansion [12]. In this particular transform, the regression coefficient given is that calculated from the unweighted points.

Inhibition data were collected by running a conven-

tional Thompson and Appleman assay for 30 min at 30° in the presence of varying levels of the compound under study. Final concentrations ranged from 1 to 5000 μM (except where otherwise noted), with suitable controls run concomitantly. Where solubilities permitted, seven concentrations were studied (each concentration at least in triplicate). Logarithmic dose–response curves were drawn by eye, from which ID_{50} values (i.e. that concentration producing 50 per cent inhibition) were obtained.

In all radioactive assays, the final counting was performed in a Nuclear Chicago Mark II scintillation counter, using 10 ml of a dioxane-based scintillator (Instabray, Yorktown Research), and the raw counts were transformed into disintegrations per min by use of a suitable computer program, thus eliminating any errors due to differential quenching of samples. In all of the above assays, reaction rates were linear with respect to time and enzyme concentrations.

Isolated tissue studies

Tracheal smooth muscle relaxation. Tracheae were excised from guinea pigs of the same strain and weight as in the enzyme studies. Tracheal chains were prepared as described previously [13] and suspended in tissue baths containing Krebs–bicarbonate solution maintained at 37.5° and aerated with 95% O_2–5% CO_2. Relaxations of spontaneous tone of the tracheal chains were recorded isotonically. Test agents were added to the bath fluid using a cumulative method of drug administration. Responses were expressed as a percentage of the maximum possible relaxation of each tracheal chain preparation. This value was determined by the addition to the bath at the end of each experiment of a supramaximal relaxant dose of papaverine (10 μg ml^{-1}). Each compound was studied using three to five separate tracheal chain preparations, and each preparation was used only once. Individual ED_{50} values (concentration producing 50 per cent maximum response) were obtained from cumulative log concentration–response lines drawn by eye to the data from each tissue. Mean ED_{50} values for each test agent were than calculated with 95 per cent confidence limits using direct assay technics [14].

The Krebs–bicarbonate solution contained the following (per liter): NaCl, 6.6 g; KCl, 0.35 g; $CaCl_2$, 0.28 g; KH_2PO_4, 0.162 g; $MgSO_4 \cdot 7H_2O$, 0.294 g; $NaHCO_3$, 2.1 g; dextrose, 2.08 g. The solution contained 0.02% ascorbic acid to retard oxidation of the compounds. Drug solutions were prepared each day in 0.9% saline containing 0.20% ascorbic acid, and were stored at 0° and protected from light until used.

Compounds used

The compounds selected for these experiments represented a wide variety of chemical classes, and all had been reported to possess phosphodiesterase inhibitory activity in one or more of a variety of tissue preparations. The compounds, their source, and the literature reference are as follows: 6,7-dimethoxy-1- veratrylisoquinoline (Papaverine, Sigma Chemical Co.) [15]; 7-chloro-4-(4[ethyl(2-hydroxyethyl)amino]-1-methyl-butylamino)quinoline (Hydroxychloroquin, Sterling Drug Co.) [15]; 4-3-[2(trifluoromethyl)-phenothiazin-10-yl]propyl-1-piperazine ethanol (Fluphenazine, Squibb) [15]; 6-chloro-2 H-1,2,4-benzothiadiazine-7-sulfonamide 1,1-dioxide (Chlorothiazide, Merck, Sharp & Dohme) [16]; 7-chloro-1,3-dihydro-1-methyl-5-phenyl-2 H-1,4-benzodiazepin-2-one (Diazepam, Hoffmann-LaRoche) [15]; 3-acetamido-6-methyl-8-*n*-propyl-*s*-triazolo[4,3-a]pyrazine (ICI 58301, Imperial Chemical Industries, UK) [17]; 1-(*p*-chlorobenzoyl)-5-methoxyl-2-methylindole-3-acetic acid (Indomethacin, Merck, Sharp & Dohme) [15]; 1-Methyl-2′,6′.-pipecoloxylidide (Mepivacaine, Winthrop) [18]; 1,3-dimethylxanthine (Theophylline, Sigma Chemical Co.) [15]; 4-(3-butoxy-4-methoxybenzyl)-2-imidazolidinone (Ro 20-1724, courtesy of Dr. H. Sheppard, Hoffmann-LaRoche) [19]; 3-pyridinecarboxylic acid amide (Nicotinamide, Sigma Chemical Co.) [20]; 4-ethyl-6,7-dimethoxyquinazoline (Quazodine, Mead Johnson) [21]; and 1-(*tert*-butyliminomethyl)-2-(3-indolyl)indoline (MJ-8952-1, Mead Johnson) [16].

RESULTS

Kinetic parameters of guinea pig tracheal cAMP-phosphodiesterase

Four separate preparations of this enzyme were isolated and assayed. Individual "apparent" kinetic constants are given in Table 1 and, as found with other cAMP-phosphodiesterases from a variety of tissues [22], the guinea pig tracheal enzyme(s) exhibit biphasic kinetics with (a) a low affinity, high K_m enzyme (type 1); and (b) a high affinity, low K_m enzyme (type 2) [23].

Inhibition of the high K_m cAMP-phosphodiesterase

The assays were run at a substrate concentration of 300 μM cAMP (i.e. twice the average K_m) under the following conditions: (a) 30° water bath oscillating at 100 rpm; (b) 30 min of incubation with 10 μg protein; and (c) a maximum usage of 3 per cent of the available substrate. A Dixon plot [24] of the data

Table 1. Apparent kinetic parameters of tracheal cAMP-phosphodiesterase enzymes

Preparation	K_{mI}*	V_{maxI}†	K_{mII}*	V_{maxII}†
A	137.6	8.0	40.6	4.3
B	103.6	7.4	43.9	4.7
C	201.5	9.4	57.4	4.1
D	154.7	4.7	33.0	3.7
Mean ± S. E. M.	149.4 ± 20.4	7.4 ± 2.0	43.7 ± 5.1	4.2 ± 0.2

* Micromolar.
† Millienzyme units; nmoles mg^{-1} min^{-1}.

Table 2. Inhibition of tracheal cAMP-phosphodiesterase vs tracheal smooth muscle relaxation

Compound	Tracheal enzyme inhibition ID_{50} (M)	Tracheal muscle relaxation ED_{50} (M) (95% confidence limits)
Ro 20-1724	1.0×10^{-6}	2.0×10^{-6} ($0.3\text{-}10.2 \times 10^{-6}$)
Quazodine	2.0×10^{-5}	1.1×10^{-5} ($0.95\text{-}1.3 \times 10^{-5}$)
Papaverine	2.9×10^{-5}	9.0×10^{-7} ($4.0\text{-}23.0 \times 10^{-7}$)
Indomethacin	8.4×10^{-5}	2.5×10^{-6} ($0.7\text{-}8.4 \times 10^{-6}$)
ICI 58301	2.6×10^{-4}	1.2×10^{-6} (45%)*
Theophylline	4.1×10^{-4}	2.3×10^{-5} ($1.8\text{-}2.9 \times 10^{-5}$)
Diazepam	$0.5\text{-}1.0 \times 10^{-3}$†	3.2×10^{-5} ($0.99\text{-}10.0 \times 10^{-5}$)
MJ-8952-1	2.1×10^{-3}	1.4×10^{-4} (45%)*
Chlorothiazide	$>5 \times 10^{-3}$	Not tested
Hydroxychloroquin	‡	Not tested
Mepivacaine	‡	Not tested
Nicotinamide	‡	Not tested
Fluphenazine	§	Not tested

* Where ED values were not determined, results are given as the per cent response at the indicated concentration.
† Corrected for severe inhibition by the ethanol used to solubilize the drug.
‡ Insignificant inhibition at concentrations up to 5×10^{-3} M; solubility limitations precluded testing at higher concentrations.
§ Insignificant inhibition at concentrations up to 2×10^{-4} M; solubility limitations precluded testing at higher concentrations.

for papaverine, the primary standard in this series of experiments, indicated that this compound is a competitive inhibitor of the high K_m enzyme ($K_i = 6\ \mu M$). The ID_{50} values for the thirteen compounds are given in Table 2. As indicated, five compounds exhibited insignificant inhibitory activity in this preparation at the concentrations listed, and solubility limitations precluded further evaluation. Consequently, these latter five compounds were not tested for relaxant activity in the tracheal chain preparation.

Tracheal chain relaxation

The eight compounds listed in Table 2 that exhibited significant inhibition of the cAMP-phosphodiesterase were tested for their ability to cause relaxation of spontaneously contracted guinea pig tracheal chains. The ED_{50} values plus 95 per cent confidence limits for tracheal relaxation were calculated and are also listed in Table 2.

DISCUSSION

Statistical analysis of the data for the first eight compounds in Table 2 demonstrated a significant correlation between their activities *in vitro* as inhibitors of the high K_m (low affinity) cAMP-phosphodiesterase and their concomitant tracheal relaxant activities. The correlation coefficient found was 0.998 with 95 per cent confidence limits of 0.933 to 0.998, suggesting that tracheal relaxation under these conditions is cAMP mediated. We should take note of the possibility that the low K_m (high affinity) enzyme system may be more representative of the activity *in vivo* in this species and/or tissue. However, until the levels *in vivo* of tracheal cAMP under suitable experimental conditions are available, it is debatable which is the more appropriate enzyme to use in these studies; in addition, refinement of the comparision by using cAMP substrate levels in the assay nearest to those found *in vivo*, as suggested by other workers in this field [23], will also have to await such data.

Inspection of Table 2 shows that five of the compounds chosen for study in the cAMP-phosphodiesterase system were inactive in our hands. Since all compounds in Table 2 were chosen for testing on the basis of their previously reported activity against the phosphodiesterase present in a variety of other, non-tracheal tissue preparations [15, 16, 18, 20], their lack of activity in our system might be due to either the method of enzyme preparation or species and tissue differences. Similar differences in specificity have been reported by other workers [15, 23] for a variety of tissues.

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