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THE ISOENZYME SELECTIVITY OF AH 21–132 AS AN INHIBITOR OF CYCLIC NUCLEOTIDE PHOSPHODIESTERASE ACTIVITY

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The smooth muscle relaxant, AH 21–132, was tested for its inhibitory effect on the cyclic nucleotide phosphodiesterase (PDE) activities fractionated from guinea-pig cardiac ventricle and bovine trachealis muscle. Both tissues yielded significant PDE-I and PDE-II activities. The cardiac ventricle also contained a significant amount of PDE-III whilst the trachealis contained PDE-IV. AH 21–132 inhibited PDE-III and PDE-IV selectively (K_i values 0.30–0.55 μM) compared with PDE-I and PDE-II (K_i values 20–140 μM).

KEY WORDS: Cyclic nucleotides, phosphodiesterase, trachea, heart.

INTRODUCTION

The compound AH 21–132 (($\pm$)cis-6-*p*-acetamidophenyl)-1,2,3,4,4a,10b-hexahydro-8,9-dimethoxy-2-methylbenzo-[c][1,6]naphthyridine)[1] has been reported to have non-specific relaxant activity in guinea-pig isolated trachealis and intestinal smooth muscle.[2,3,4] The tracheal relaxant effects do not involve β-adrenoceptor activation since they are not antagonised by propranolol.[2,3]

Tested on the soluble cyclic nucleotide phosphodiesterase (PDE) of rat brain, AH 21–132 has been shown to act as a competitive inhibitor with a K_i value of 6 μM.[1] Using crude tissue extracts of guinea-pig trachealis and ileal smooth muscle, it has been shown that AH 21–132 is an inhibitor of PDE showing approximately ten-fold selectivity in favour of cyclic AMP hydrolysis over cyclic GMP hydrolysis.[3,4] The concentration range over which AH 21–132 inhibits cAMP hydrolysis in tracheal homogenates encompasses that required to cause tracheal relaxation.[3] Furthermore the (-)-enantiomer of AH 21–132 is more potent than the (+)-enantiomer both in causing tracheal relaxation and inhibiting the hydrolysis of cAMP.[5] It may therefore be that the relaxant activity of AH 21–132 in smooth muscle is founded on its ability to inhibit PDE.

It is well documented that PDE exists in multiple forms which differ in their substrate specificity, their stimulation by Ca^{2+}/calmodulin and their inhibitor sensitivity.[6] Several isoenzyme-specific inhibitors have been developed and, as well as being used for isoenzyme identification, have significant potential for use as thera-

Abbreviation used: PDE, cyclic nucleotide phosphodiesterase (EC 3.1.4.17)
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peutic agents.[7] We now present results of a study seeking to assess the selectivity of AH 21–132 as an inhibitor of the PDE isoenzymes fractionated from guinea pig cardiac ventricle and bovine trachealis.

MATERIALS AND METHODS

Materials

[8-^{3}H]Adenosine 3′,5′-cyclic phosphate (26.1 Ci/mmole) and [8-^{3}H]guanosine 3′,5′-cyclic phosphate (23.7 Ci/mmole) were obtained from Amersham International (Amersham, Bucks, U.K.). DEAE-Sepharose CL-6B, Dowex 1 × 8, cyclic AMP, cyclic GMP, calmodulin and *Ophiophagus hannah* venom were obtained from Sigma Chemical Co. (Poole, Dorset, U.K.). Other reagents were from BDH (Poole, Dorset, U.K.).

AH 21–132 was provided by Sandoz A.G., Basel, Switzerland, M&B 22,948 (2-o-propyloxyphenyl-8-azapurin-6-one) was a gift from Rhône-Poulenc, Dagenham, Essex, U.K., Ro 20-1724 [4-(3-butoxy-4-methoxybenzyl)-2-imidazoline] was a gift from Roche Products Ltd., Welwyn, Herts., U.K. and SK&F 94120 {5-(4-acetamido-phenyl)pyrazin-2[1H]-one} was a gift from Smith, Kline and French, Welwyn Garden City, Herts., U.K.

Tricoloured guinea pigs (400–650g, of either sex) were supplied by the University of Manchester Animal House. Bovine tracheae were obtained from the local abattoir.

Methods

Separation of PDE activities Guinea pigs were killed by stunning and bleeding. The hearts were removed and placed on ice. The ventricles were freed of atrial tissue and either used immediately or rapidly frozen in liquid N_2 and stored at $-70°$ C until required. Bovine tracheae were placed in ice-cold Krebs-Henseleit solution for transport to the laboratory where the trachealis muscle was carefully dissected from other tracheal tissue, cut into pieces of approximately 1g and either used immediately or frozen in liquid N_2 and stored at $-70°$ C.

Approximately 1 g of tissue was homogenised in 10 volumes of buffer A (0.07 M-sodium acetate, pH 6.5, containing 5mM-2-mercaptoethanol, 0.1mM-phenylmethanesulphonyl fluoride, 2 μg/ml-leupeptin, 2 μg/ml-pepstatin and 2 μg/ml-antipain) using an Ilado tissue disperser. The homogenate was centrifuged at 25000 g for 20 min. Fractionation of the 25000 g supernatant was performed at 4° C on a DEAE-Sepharose CL-6B column (1.1 × 8 cm) by a modification of the methods of Thompson *et al.*,[8] and Reeves *et al.*,[9]. The extract was loaded onto the column, equilibrated with buffer A, and unbound protein washed off with 20 ml buffer A. Isoenzymes were eluted with an 80 ml linear gradient to 1.0 M-sodium acetate in buffer A with 1 ml fractions being collected. Fractions were assayed for PDE activity within 24 h and stored for longer periods at $-20°$ C after addition of ethylene glycol to a final concentration of 30% (v/v).

Isoenzymes are named as described by Reeves *et al.*,[9]. Rechromatography was performed where necessary to eliminate cross-contamination of fractionated isoenzymes. The identities of the isoenzymes were confirmed by their sensitivities to M&B 22,948 (PDE-I specific inhibitor), Ca^{2+}/calmodulin (activator of PDE-I), SK&F

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94120 (PDE-III specific inhibitor), Ro 20-1724 (PDE-IV specific inhibitor) and cGMP (activator of PDE-II and inhibitor of PDE-III but not PDE-IV). All isoenzymes used for kinetic analysis were at least 90% pure based on the sensitivities to the above agents.

Assay of PDE activity PDE activity was measured using the two step assay of Thompson and Appleman[10] as modified by Rutten *et al.*[11] in a final volume of 100 μl containing 40mM-Tris-HCl (pH 8.0), 2.5mM-$MgCl_2$ and 3.75mM-2-mercaptoethanol. Each assay contained 0.2 μCi [^{3}H]cyclic AMP or [^{3}H]cyclic GMP with 1 μM-cyclic nucleotide for analysis of column eluates.

For determination of K_m and K_i values at least 5 concentrations of cyclic nucleotide and 5 concentrations of inhibitor were used (in the range from approximately 1/10 to 10 times the observed K_m and K_i) for each determination. The data were analysed by fitting to the equation for competitive inhibition using the method of Walmsley and Lowe[12] to obtain K_m and K_i values.

RESULTS AND DISCUSSION

Representative elution profiles obtained from chromatography of the 25000 g supernatants from guinea-pig cardiac ventricle and bovine trachealis are shown in Figures 1 and 2 respectively. Both show a significant M&B 22,948-sensitive PDE-I peak (which was stimulated by Ca^{2+}/calmodulin, data not shown) and a smaller, cyclic GMP-stimulated PDE-II activity. The third major peaks eluting from the extracts from the two tissues differed in their inhibitor sensitivity despite showing similar chromatographic behaviour. The peak from cardiac ventricle was identified as predominantly PDE-III being profoundly (> 75%) inhibited by 10 μM-cyclic GMP and 50 μM-SK&F 94120 but only weakly (< 25%) inhibited by 25 μM-Ro 20-1724.[9] However, that from bovine trachealis was predominantly PDE-IV showing no significant inhibition by either 10 μM-cyclic GMP or 50 μM-SK&F 94120 but approximately 70% inhibition by 25 μM-Ro 20-1724.[9] These profiles are consistent with those obtained by other workers.[9,13,14] Both the PDE-III and PDE-IV peaks were markedly inhibited by 5 μM-AH 21-132.

The isoenzyme selectivity of AH 21-132 is shown in Table I along with the K_m values for both cyclic AMP and cyclic GMP. The K_m values for the fractionated isoenzymes are similar to those reported by others.[9,13] When cAMP hydrolysis is considered, it is clear that AH 21-132 preferentially inhibits both PDE-III and PDE-IV (K_i values less than 1 μM), showing greater than 80 fold selectivity compared with PDE-I and PDE-II (K_i values ranging from 35 μM to 140 μM).

This pattern of specificity of AH 21-132 was unexpected. Other PDE inhibitors have generally fallen into two categories,[7] either nonspecific (such as the methylxanthines) showing similar K_i values for all isoenzymes, or isoenzyme specific for only a single form (including SK&F 94120 and Ro 20-1724 which can differentiate between PDE-III and PDE-IV).[9] AH 21-132 is an analogue of papaverine (see Figure 3). Papaverine has been described as a nonspecific PDE inhibitor.[7] However a more recent report[13] indicates that papaverine may show some selectivity, inhibiting both PDE-III and PDE-IV with similar potencies, but being approximately 20-fold less potent in inhibiting PDE-I.

In homogenates of canine trachealis PDE-Ic accounts for the majority (85%) of the

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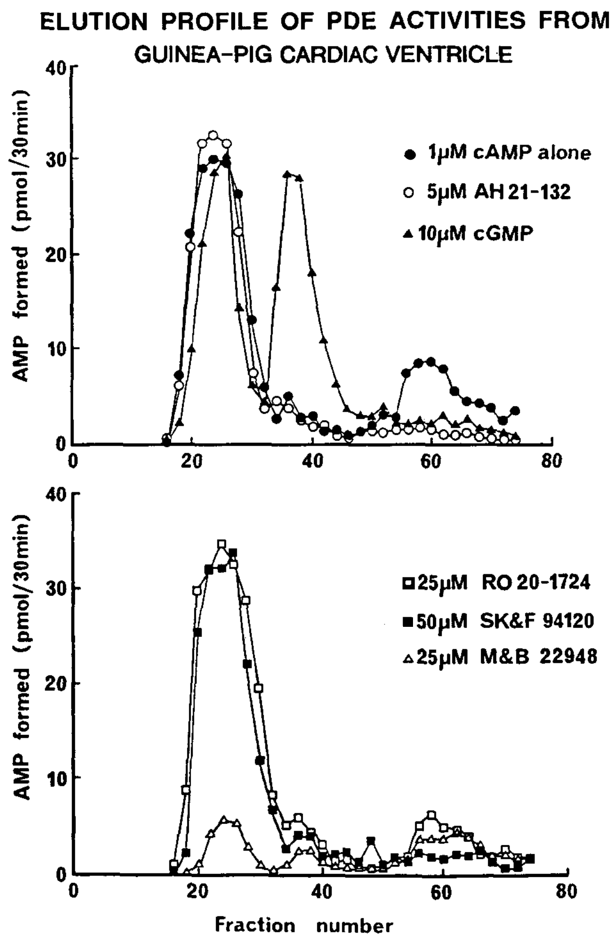

FIGURE 1 Elution profile of PDE activities from guinea-pig cardiac ventricle after DEAE-Sepharose CL-6B chromatography. Activities were assayed at 1 μM-cyclic AMP alone (●) or in the presence of 10 μM-cyclic GMP (▲), 5 μM-AH 21-132 (○), 25 μM-M&B 22,948 (△) 50 μM-SK&F 94120 (■) or 25 μM-Ro 20-1724 (□).

total cAMP hydrolytic activity. In contrast PDE-III and PDE-IV provide only 5% and 10% respectively of the total cyclic AMP hydrolytic activity. However, in the intact canine trachealis cell, PDE-III and PDE-IV are the important isoenzymes which act on the intracellular cyclic AMP controlling the mechanical behaviour of the tissue. An inhibitor of PDE-III or PDE-IV would therefore be expected to cause the intracellular accumulation of cAMP and thereby itself cause relaxation or potentiate relaxants which act to increase the production of cyclic AMP.[14] The ability of AH 21-132 to inhibit PDE-III and PDE-IV may therefore explain its tracheal relaxant activity.

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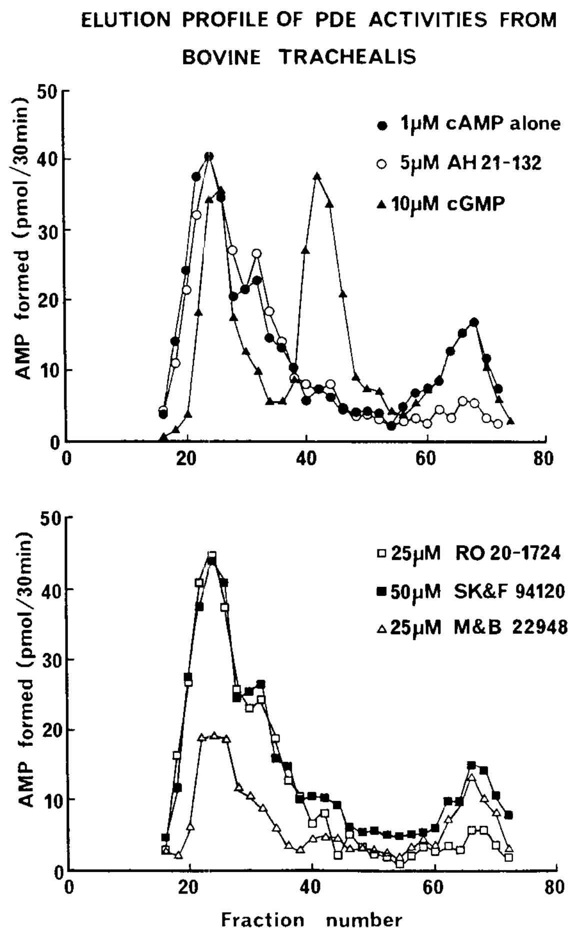

FIGURE 2 Elution profile of PDE activities from bovine trachealis after DEAE-Sepharose CL-6B chromatography. Activities were assayed at 1 μM-cyclic AMP alone (●) or in the presence of 10 μM-cyclic GMP (▲), 5 μM-AH 21-132 (○), 25 μM-M&B 22,948 (△), 50 μM-SK&F 94120 (■) or 25 μM-Ro 20-1724 (□).

Torphy and Undem[15] have presented evidence which suggests that inhibition of PDE-IV can lead to inhibition of mediator release from inflammatory cells *in vitro* and can protect against the inflammatory effects of arachidonic acid *in vivo*. Since AH 21-132 acts selectively to inhibit both PDE-III and PDE-IV it may be that this compound can produce both bronchodilator and anti-inflammatory effects in the lung. In accord with this, AH 21-132 has been reported not only to relax isolated airways smooth muscle[2,3] but also to inhibit the pulmonary eosinophilia induced by platelet activating factor or sensitisation to ovalbumin.[16,17]

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TABLE I
Kinetic characterisation of PDE isoenzymes.

Tissue	PDE Type	cyclic AMP K_m (μM)	cyclic AMP K_i (μM)	cyclic GMP K_m (μM)	cyclic GMP K_i (μM)
Guinea pig	I	2.7 ± 0.3	140 ± 20	4.1 ± 1.5	48 ± 16
cardiac	II	190*	35*	30*	20*
ventricle	III	0.82 ± 0.19	0.30 ± 0.08	8.3 ± 4.5	0.55 ± 0.16
Bovine	I	3.7 ± 1.6	42 ± 10	3.2 ± 0.7	120 ± 30
trachealis	II	220*	46*	40*	36*
	IV	0.89 ± 0.16	0.50 ± 0.14	#	#

PDE activities were separated as shown in Figures 1 and 2 and further purified by rechromatography as necessary. K_m values for cyclic nucleotides and K_i values for AH 21-132 are presented ± s.d.
*PDE-II showed positive cooperativity (Hill coefficient of 1.3 for cyclic AMP and 1.5 for cyclic GMP); values represent half-maximal substrate concentration and apparent K_i based on Dixon plots.
PDE-IV showed no significant hydrolysis of cyclic GMP.

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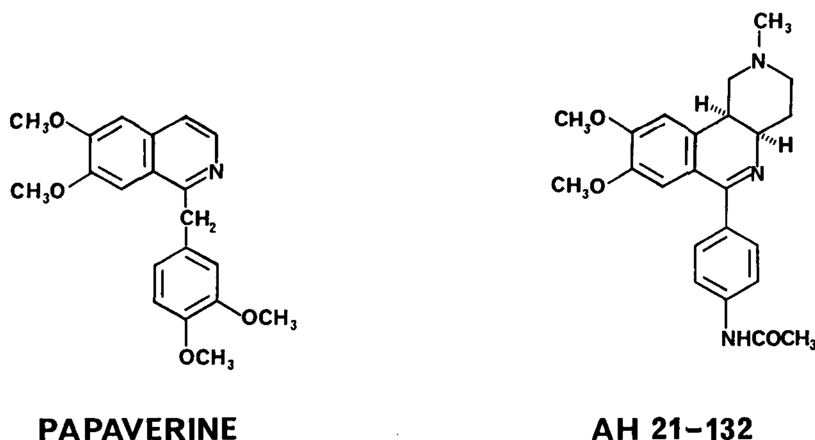

FIGURE 3 The structures of AH 21-132 and papaverine.

Acknowledgements

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