Biochemical Pharmacology, Vol. 42, No. 3, pp. 663–677, 1991.
Printed in Great Britain.

0006-2952/91 $3.00 + 0.00

SELECTIVE INHIBITION OF A HIGH AFFINITY TYPE IV CYCLIC AMP PHOSPHODIESTERASE IN BOVINE TRACHEALIS BY AH 21-132

RELEVANCE TO THE SPASMOLYTIC AND ANTI-SPASMOGENIC ACTIONS OF AH 21-132 IN THE INTACT TISSUE

MARK A. GIEMBYCZ* and PETER J. BARNES

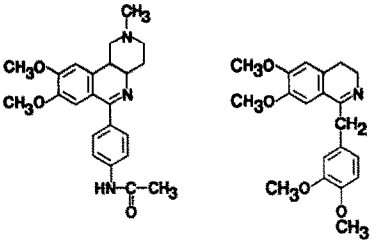
Department of Thoracic Medicine, National Heart and Lung Institute, Royal Brompton and National Heart and Lung Hospital, Dovehouse Street, London SW3 6LY, U.K.

(*Received* 7 *February* 1991; *accepted* 3 *April* 1991)

Abstract—The ability of a papaverine-derived bronchodilator, AH 21-132, to inhibit cyclic nucleotide hydrolysis and to increase the cAMP content and the activity of cAMP-dependent protein kinase (A-kinase) was evaluated in bovine tracheal smooth muscle (BTSM) and related to the mechanical effects elicited by this compound *in vitro*. AH 21-132 (100 nM–1 mM) produced a concentration-related relaxation of BTSM pre-contracted with methacholine (MCh) that was subject to marked functional antagonism. AH 21-132 (100 μM) also displayed anti-spasmogenic activity preventing the generation of tone induced by low, but not high, concentrations of MCh. Neither the spasmolytic nor anti-spasmogenic effects of AH 21-132 were antagonized by the β_2-adrenoceptor blocking drug ICI 118551 (50 nM). Three Ca^{2+}- and calmodulin-*independent* cyclic nucleotide phosphodiesterases (PDE) were resolved from the soluble fraction of BTSM homogenates by Q-Sepharose anion exchange chromatography. These PDEs were identified by kinetic and inhibitor sensitivity criteria as the Type II (cGMP-stimulated), Type IV (Ro 20-1724-inhibited) and Type V (cGMP-specific) isoenzymes. A small amount (~5%) of a Type III PDE seemed to be present but this was not identified with certainty. AH 21-132 selectively inhibited Type IV PDE in a competitive manner with an IC_{50} and K_I of 3.7 and 2.7 μM, respectively. AH 21-132 similarly increased the cAMP content (from 5.3 to 23.1 pmol/mg protein after 1 mM AH 21-132) and activated A-kinase (from 29.6% to 53.5% after 1 mM AH 21-132) in intact BTSM over the same concentration range at which this compound influenced tone. In addition, AH 21-132 in high concentrations (>100 μM), while exerting no direct effect on A-kinase itself, markedly potentiated (*ca.* four-fold at 3 mM AH 21-132) the ability of cAMP to activate A-kinase *without* affecting the affinity of cAMP for this enzyme. It is concluded that the spasmolytic and anti-spasmogenic effects of AH 21-132 in BTSM may be related, in part, to its ability to inhibit Type IV PDE, increase the intracellular cAMP content and so activate A-kinase. A cyclic nucleotide-dependent mechanism is therefore proposed. In addition, the ability of AH 21-132 to augment cAMP-dependent phosphorylation in a *cell-free* system, when Type IV PDE is inhibited fully, provides the possibility that the observed relaxation elicited by high concentrations of AH 21-132, while cAMP-dependent, does not require any further increase in the intracellular cAMP concentration.

AH 21-132 [1] ((±) *cis*-6-(*p*-acetamidophenyl)-1,2,3,4,4*a*,10*b*-hexahydro-8,9-dimethoxy-2-methyl-[*c*][1,6]-naphthyridine bis hydrogen maleinate) is a benzonaphthyridine derivative similar structurally to the non-selective cyclic nucleotide phosphodiesterase (PDE) inhibitor papaverine (Fig. 1). AH 21-132 differs from papaverine, however, in at least two ways. First, it does not contain the dopamine moiety in its structure and second it is fully ionized (positively charged) at physiological pH (Fig. 1).

In vitro, AH 21-132 is an effective relaxant of both human [2] and guinea-pig [3] airway smooth muscle preparations and is also active at preventing spasmogen-induced tension generation [3]. In the guinea-pig *in vivo* AH 21-132 is a bronchodilator [2], inhibits platelet activating factor (PAF)- and isoprenaline-induced airways hyper-reactivity [4] and displays appreciable efficacy at preventing PAF-induced lung eosinophilia [5].



AH 21-132 **Papaverine**

Fig. 1. Chemical structure of AH 21-132 and papaverine.

Although previous studies have established that AH 21-132 is not a β-adrenoceptor agonist [2,3] relatively little is known about the mechanism(s) that underlies the mechanical effects of AH 21-132 in airways smooth muscle. Preliminary data indicate

* Author to whom all correspondence should be addressed.

that the relaxation evoked by AH 21-132 resembles that elicited by alkylxanthines suggesting that a cyclic nucleotide-dependent process may mediate, at least in part, the anti-spasmogenic and/or spasmolytic actions of this compound. We have addressed this possibility by comparing the potential inhibitory action of AH 21-132 on the soluble cyclic nucleotide PDE isoenzymes resolved from bovine tracheal smooth muscle (BTSM) with its effect on the cyclic AMP (cAMP) content and on the activity of cAMP-dependent protein kinase (A-kinase) in intact tissues.

A preliminary account of some of these data has been presented to the British Pharmacological Society [6].

MATERIALS AND METHODS

Isolation and preparation of bovine tracheal smooth muscle. Tracheae from young cows (*ca.* 18 months) were obtained from a local abbatoir and transported to the laboratory in ice-cold Krebs–Henseleit (KH) solution. The cervical trachealis was isolated, stripped free of the epithelium and all other extrinsic connective tissue and used immediately for tension, cAMP or A-kinase activity ratio studies as described below. For chromatographic resolution of PDE and A-kinase isoenzymes tracheal smooth muscle was blotted on absorbant tissue, snap-frozen in liquid nitrogen and stored at −80°.

Tension measurements. Strips (*ca.* 2 mm × 2 mm × 10 mm) of tracheal smooth muscle were cut and mounted vertically, under an initial stretching tension of 20 mN, in 5-mL tissue baths containing oxygenating KH solution maintained at 37°. Each strip was left to equilibrate for 60 min after which methacholine (MCh; 40 nM, 400 nM, 4 μM or 40 μM) was added. When the increase in tone had stabilized the relaxant activity of isoprenaline or AH 21-132 was examined according to the method of Van Rossum [7]. In some tissues the effect of the β_2-adrenoceptor blocking drug ICI 118551 (50 nM) on relaxations induced by isoprenaline and AH 21-132 was examined. In these experiments the tracheal smooth muscle strips were incubated for a further 60 min in the presence of ICI 118551 so as to attain equilibrium β-adrenoceptor blockade before the addition of MCh. Estimates of antagonist affinity (pK_b) were calculated using the following equation: $\log_{10} K_b = \log_{10} [A]/(B'/B) - 1$ where $[A]$ is the concentration of antagonist, B' is the EC_{50} of the agonist in the presence of antagonist and B is the EC_{50} of the agonist alone. The irreversible inhibitor of cyclo-oxygenase, flurbiprofen (8 μM), was present in the KH solution throughout the tension experiments to prevent the spontaneous and drug-stimulated release of prostanoids from the trachealis. Changes in tension resulting from drug treatment were measured isometrically using Grass FT03.c force-displacement transducers and were displayed on a Grass 7D ink-writing, curvi-linear polygraph. The composition of the KH solution was as follows (mM); NaCl 118, KCl 4.7, $MgSO_4 \cdot 7H_2O$ 1.2, KH_2PO_4 1.2, $NaHCO_3$ 25, glucose 11.7, $CaCl_2 \cdot 6H_2O$ 1.4.

Measurement of cAMP content. Strips (*ca.* 2 mm × 2 mm × 10 mm) of bovine trachealis were incubated free-floating in oxygenating KH solution at 37°. After an equilibration period of 60 min, during which time the KH solution was replaced four times, tissues were exposed to vehicle or AH 21-132 (100 nM to 1 mM) for 10 min after which they were rapidly removed, blotted on dry absorbent paper and snap-frozen by submersion in liquid nitrogen. The time elapsed between removal of the tissue and freezing was less than 5 sec. Tracheal strips treated in this way were then stored at −80°. Cyclic nucleotides were extracted into 6% (w/v) trichloroacetic acid (TCA), neutralized and acetylated [8]. cAMP content was subsequently estimated by radioimmunoassay (RIA) using [^{125}I]cyclic AMP as tracer [8]. The RIA procedure used was identical to that previously described for the estimation of cGMP content in BTSM slices [8]. The detection limit and sensitivity (IC_{50}) of this assay was 10 and 145 fmol cyclic AMP, respectively.

Preparation of crude cyclic nucleotide PDE. Frozen BTSM was homogenized (Polytron PCU, Kinematica GmbH, Switzerland) for 2 × 10 sec bursts at setting 8 in 20 vol. (w/v) of ice-cold Buffer A [20 mM triethanolamine HCl (TEA) (pH 8.0), 2 mM ethyleneglycolbis(aminoethylether)tetraacetic acid (EGTA), 2 mM (±) 1,4-dithiothreitol (DTT)]. The resulting homogenate was centrifuged (Sorval R5C5) at 45,000 g_{max} for 30 min at 4° in a fixed-arm SS34 rotor (r_{av} 9 cm) to form soluble and particulate fractions. The soluble enzyme was then diluted 45-fold in Buffer A supplemented with bovine serum albumin (BSA) (2 mg/mL) and immediately assayed for cyclic nucleotide PDE activity as described below.

Resolution of cyclic nucleotide PDE isoenzymes by Q-Sepharose anion-exchange chromatography. Unless otherwise stated all chromatographic procedures were performed at 4°. Approximately 5 g of frozen BTSM were homogenized as described above in 10 vol. (w/v) of Buffer B [20 mM bis-Tris (pH 6.5), 50 mM sodium acetate, 2 mM DTT, 1 mM EDTA, 5 mM glucose] supplemented with a proteinase inhibitor mixture consisting of 2 mM benzamidine, 100 μM phenylmethylsulphonylfluoride (PMSF), 100 μg/mL bacitracin, 10 μg/mL soybean trypsin inhibitor and 50 μM leupeptin. The resulting homogenate was centrifuged as above to form soluble and particulate fractions. The pellet was resuspended by hand in 3 vol. (w/v) of Buffer B containing 300 mM KCl to remove any electrostatically bound PDE, and re-centrifuged. The supernatant fractions from both spins were combined, filtered through two layers of cotton gauze and made 30% (v/v) with ethylene glycol [9]. Following dilution of the soluble fraction in Buffer C [20 mM bis-Tris (pH 6.5) 1 mM EDTA, 30% ethylene glycol, 5 mM glucose], to lower the conductivity to <4 mS (at 4°), the enzyme was applied, at a flow rate of 1 mL/min, to a column (Bio-Rex; 1.5 cm i.d., 18 cm bed volume) of Q-Sepharose pre-equilibrated in Buffer C. The column was subsequently washed with Buffer B until the absorbance (at 280 nm) of the eluate returned to baseline and then eluted with a linearly increasing sodium acetate gradient running from 50 to 960 mM in a total volume of 440 mL. The flow rate was adjusted to 500 μL/min and 44 × 10 mL fractions

were collected. Each fraction was assayed for cAMP and cGMP hydrolytic activity as described below. In addition, cAMP hydrolysis was measured in the presence of 10 μM cGMP, to aid identification of cGMP-stimulated (Type II) and cGMP-inhibited (Type III) PDE isoforms and in the presence of calmodulin (25 units plus 2 mM $CaCl_2$) an activator of Ca^{2+}- and calmodulin-dependent PDE isoenzyme(s). Greater than 82% of the PDE activity applied to the column was recovered. Fractions comprising each peak of activity were pooled and stored at −20° for several months without significant loss of activity.

Assay of cyclic nucleotide PDE. Cyclic nucleotide PDE activity was measured using a modification [10] of the method originally described by Thompson and Appleman [11]. The reaction is based upon the phosphodiesteratic cleavage of [^{3}H]cAMP or [^{3}H]cGMP to its corresponding labelled nucleoside 5′-monophosphate which is subsequently dephosphorylated by alkaline phosphatase. Assays were performed in triplicate at 37° and initiated by the addition of 30 μL of enzyme or of the desired column fraction to 270 μL of a reaction medium containing (final concentration): 40 mM TEA (pH 8.0), 2 mM DTT, 5 mM $Mg(CH_3COO)_2$, 500 μg/mL BSA, 0.25 units alkaline phosphatase and 1 μM cAMP supplemented with *ca.* 250,000 dpm of [8-^{3}H]cAMP and *ca.* 5000 dpm [8-^{14}C]adenosine to estimate recovery. The reaction was terminated after 10 min by the addition of 1 mL of a mixture of methanol:Dowex AG 1-X8:water (2:1;1), vortex mixed and then left for 10 min in an ice bath before centrifugation at 12,000 g_{max} for 4 min at 4° in a Hettich microfuge. The radioactivity in aliquots (800 μL) of the resulting supernatants was then measured by liquid scintillation spectrometry in 2 mL ACS II (Amersham) at a counting efficiency of *ca.* 60%. One unit of enzyme activity is defined as that amount of PDE which catalysed the formation of 1 pmol 5′-AMP in 1 min per milligram of protein at 37° after correction for the recovery (routinely 65–85%) of [8-^{14}C]adenosine. All assays were performed over the linear part of the reaction under conditions where less than 20% of the substrate was utilized.

cGMP hydrolysis was assayed exactly as described above using 1 μM cGMP as substrate.

Protein content was determined according to Lowry *et al.* [12] using BSA as standard.

Determination of kinetic constants. Values of K_m and V_{max} were determined by varying the amount of unlabelled cAMP or cGMP in the reaction medium in the presence of a fixed concentration of the corresponding [^{3}H]cyclic nucleotide tracer. Appropriate corrections were made for the changes in specific activity of the substrate. Double reciprocal plots were constructed and analysed by regression using the method of least squares. Values of K_I were derived from secondary plots of these data as described by Dixon [13] using substrate and inhibitor concentrations that spanned the K_m and estimated K_I.

Categorization of cyclic nucleotide PDE isoenzymes. Calcium and calmodulin-*independent* PDE isoenzymes are categorized according to the nomenclature of Beavo and Reifsnyder [14]. Thus, Type I, Type II, Type III, Type IV and Type V PDEs refer to the Ca^{2+}- and calmodulin-dependent, cGMP-stimulated, cGMP-inhibited, Ro 20-1724-inhibited and cGMP-specific isoforms, respectively (see Ref. 15).

Resolution of A-kinase isoenzymes by DEAE-Biogel A agarose anion-exchange chromatography. The Type I and Type II A-kinase isoenzymes from the soluble and particulate fractions of bovine tracheal smooth muscle were separated by anion exhange chromatography using the method described previously for the separation of guinea-pig lung A-kinase isoenzymes [16] except that DEAE-Biogel A agarose was used as the exchange matrix and that 5 mM 3-(*N*-morpholino)propanesulphonic acid (MOPS) (pH 7.2) was used to buffer the pH. Approximately 79% of the total A-kinase activity applied to the column was recovered.

Determination of the A-kinase activation state. Free-floating tracheal smooth muscle strips were treated with AH 21-132 or its respective vehicle and snap-frozen in liquid nitrogen exactly as described above for the cAMP experiments. Each frozen tissue was then homogenized (Polytron) for 1 × 15 sec burst at setting 8 in 20 vol. (w/v) of ice-cold Buffer D [10 mM MOPS (pH 7.2), 10 mM EDTA, 10 mM DTT, 500 μM 3-isobutyl-1-methyl-xanthine (IBMX)]. Since bovine tracheal smooth muscle contains primarily the Type II isoenzyme of A-kinase (see Results) Buffer D was supplemented with 400 mM KCl to prevent the inactivation of the Type II isoenzyme during tissue processing [17]. The resulting homogenate was centrifuged at 31,000 g_{max} for 15 min at 4° in a fixed-arm rotor to form soluble and particulate fractions. The soluble enzyme containing >99% of the total A-kinase activity (see Results) was diluted 16-fold in Buffer D immediately before being assayed for phosphotransferase activity.

Soluble A-kinase activity was measured using a modification [18, 19] of the method originally described by Witt and Roskoski [20] as described by us previously using Kemptide as substrate [21]. The activation state of A-kinase is expressed as an activity ratio which is the ratio of the specific activity obtained in the absence of exogenous cAMP divided by the specific activity of A-kinase obtained in the presence of a concentration of cAMP required to maximally activate the enzyme.

Quantitation of regulatory subunits. Regulatory subunits in the post-DEAE-Biogel A agarose fractions were quantified by the method described by Sugden and Corbin [22]. Briefly, assays were performed at 25° and were initiated by adding 25 μL of each column fraction to 75 μL of a reaction medium containing (final concentration): 25 mM KH_2PO_4, 25 mM K_2HPO_4 (pH 6.8), 1 mM EDTA, 2 M NaCl, 10 μg/mL histone IIa, 10 mM $Mg(CH_3COO)_2$ and 500 nM [5,8-^{3}H]cAMP. Non-specific binding was determined in the presence of 50 μM unlabelled cAMP. Reactions were allowed to proceed for 45 min and then quenched with 6 mL ice-cold Buffer E (5 mM KH_2PO_4, 5 mM K_2HPO_4; pH 6.8) followed by rapid filtration under negative pressure through Millipore HAWP nitrocellulose filter discs (pore size = 0.45 μm). Filters were then extensively washed with a further 20 mL of Buffer E, dried and dissolved in 5 mL ACS II. The tritium

trapped by the discs was determined by liquid scintillation spectrometry.

Chemicals, drugs and analytical reagents. The following were purchased from the Sigma Chemical Co. (Poole, Dorset): alkaline phosphatase (P-4252), bacitracin, benzamidine, BSA (grade III), calmodulin (P-2277), cAMP, anticyclic AMP antibody, cGMP, DTT, EGTA, EDTA, flurbiprofen (free acid), histone IIa, A-kinase inhibitor peptide (IP_{20}; Thr-Thr-Tyr-Ala-Asp-Phe-Ile-Ala-Ser-Gly-Arg-Thr-Gly-Arg-Arg-Asn-Ala-Ile-His-Asp), IBMX, (−)isoprenaline-D-bitartate, Kemptide, magnesium acetate, MOPS, PMSF, sodium acetate, soybean trypsin inhibitor and TEA. All other chemicals, drugs and analytical reagents were obtained from the following sources: [8-^{14}C]adenosine (1.85–2.2 GBq/mmol), [γ-^{32}P]-ATP (0.74–1.5 TBq/mmol), [8-^{3}H]cAMP (0.74–1.1 TBq/mmol), [5,8-^{3}H]cAMP (1.5–2.2 TBq/mmol), adenosine 3′,5′-cyclic phosphoric acid 2′-*O*-succinyl-3-[^{125}I]iodotyrosine methyl ester (~74 TBq/mmol) and [8-^{3}H]cGMP (0.37–1.1 TBq/mmol; Amersham International, Bucks), ICI 118551 (*erythro*-DL-1-(7-methylindan-4-yloxy)-3-(isopropyl amino-butan-2-ol) HCl; Imperial Chemical Industries, Macclesfield, Cheshire), AH 21-132 ((±)-*cis*-6-(*p*-acetamidophenyl)-1,2,3,4,4*a*,10*b*-hexahydro-8,9-dimethoxy-2-methyl-benzo-[*c*] [1,6] napthyridine; Sandoz AG, Basle, Switzerland), SK&F 94120 ([5-(4-acetamidophenyl)pyrazine-2[1*H*]-one]; Smith-Kline Beecham, Welwyn, Hertfordshire), rolipram (ZK-62711; 4-(3-cyclopentyloxy-4-methoxyphenyl)-2-pyrrolidine; Schering AG, Hounslow, Middlesex), zaprinast (M & B 22948 2-*O*-propoxyphenyl-8-azapurin-6-one; Rhone-Poulenc Rorer Inc., Dagenham, Essex), denbufylline (1,3-di-*n*-butyl-7-(2-oxo-propyl) xanthine; Smith-Kline Beecham, Betchworth, Surrey), Dowex AG 1 X8 (200–400 mesh; acetate form) and DEAE-Biogel A agarose (BioRad), Q-Sepharose (Pharmacia) and salts for buffers and constituents of the assay cocktail (BDH, Poole, Dorset).

Dissolution of drugs. Drugs were made up as stock solutions of 100 mM in ethanol (rolipram, denbufylline), 100 mM NaOH (zaprinast, flurbiprofen, SK&F 94120) and distilled/de-ionized water (AH 21-132, ICI 118551). Each drug was then diluted to the desired working concentration in the appropriate assay buffer.

Data and statistical analysis. Concentration–response curves were analysed by least-squares non-linear iterative regression with the 'Inplot' curve fitting program (GraphPad Software; San Diego, CA, U.S.A.). Values in the text represent the mean ± SEM of N independent determinations. IC_{50} values refer to the concentration of drug required to cause 50% inhibition while EC_{50} values represent the concentration of drug needed to produce a response that is 50% of the maximum attained by that drug. Where statistical evaluation was required data were analysed non-parametrically using the statistical package 'Number Cruncher Statistical System' (written by Dr J.H. Hintze, 865 East 400 North Kaysville, UT, U.S.A.). Mann–Whitney U-tests were employed to assess significance between unpaired variates. Where three or more groups of data were compared the Kruskal–Wallace analysis of variance (ANOVA) was used and where significance was indicated determined by multiple comparisons. The null hypothesis was rejected when $P < 0.05$.

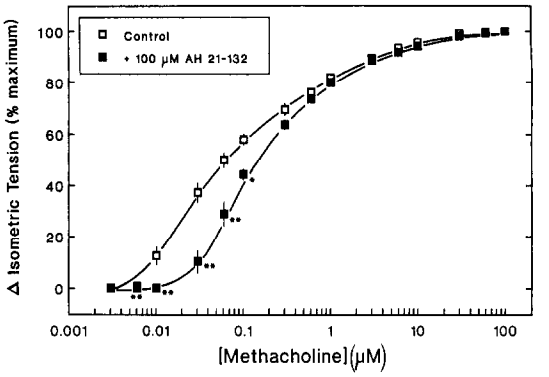


Fig. 2. Effect of AH 21-132 on the MCh concentration–response relationship in BTSM. Flurbiprofen- (8 μM) treated smooth muscle strips were set up for isometric tension recording as described in Materials and Methods and incubated for 10 min with either 100 μM AH 21-132 (■) or its respective vehicle (□). Cumulative concentration–response curves were then constructed to MCh. Each data point represents the mean ± SEM of seven independent determinations. * and ** indicate significant ($P < 0.05$ and $P < 0.01$, respectively) inhibition of MCh-induced tone with respect to responses obtained with time-matched control tissues (Mann–Whitney U-test).

RESULTS

Mechanical effects of AH 21-132 in bovine tracheal smooth muscle

Anti-spasmogenic activity. Methacholine (MCh; 3 nM–100 μM) elicited concentration-dependent contractions of BTSM strips with an EC_{50} and T_{max} of 82.3 ± 15.3 nM (N = 7) and 401.6 ± 35.1 mN (N = 7) respectively (Fig. 2). Iterative, non-linear curve fitting revealed that the mean slope of the MCh concentration–response curve was shallow with a Hill coefficient ($n_H = 0.75$) significantly less than unity. Pre-treatment of BTSM strips with AH 21-132 (100 μM for 10 min) antagonized the spasmogenic action of low (6–100 nM), but not high (300 nM–100 μM), concentrations of MCh which resulted in a non-parallel rightwards shift in the MCh concentration–response curve without reducing the T_{max} (424.5 ± 44.7 mN, N = 7; Fig. 2). AH 21-132 thus, effectively reduced the potency of MCh to 162.8 ± 16.8 nM (N = 7) and markedly increased the mean slope of the concentration–response curve ($n_H = 0.98$).

Spasmolytic activity. To determine whether AH 21-132 possessed inherent spasmolytic activity, BTSM strips were first pre-contracted to a sustained level of tone with MCh (400 nM; *ca.* EC_{70}), and then challenged with increasing concentrations of AH 21-132 (100 nM–1 mM). As shown in Fig. 3, AH 21-132 relaxed BTSM strips in a concentration-dependent manner with an estimated EC_{50} of *ca.*

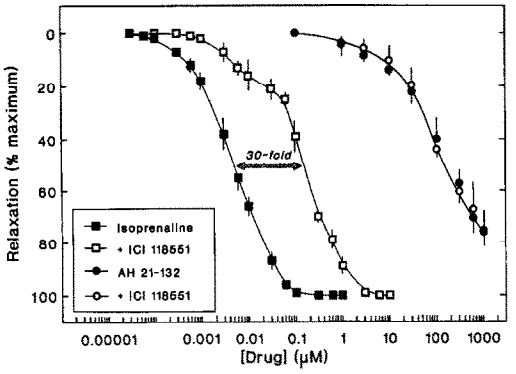


Fig. 3. Relaxant action of AH 21-132 and isoprenaline on MCh-induced tone in BTSM. Flurbiprofen- (8 μM) treated smooth muscle strips were set up for isometric tension recording as described in Materials and Methods, pre-treated for 60 min with ICI 118551 (50 nM; open symbols) or is respective vehicle (filled symbols) and challenged with 400 nM MCh (~EC_{70}). When the increase in tone had plateaued concentration–response curves were constructed to either AH 21-132 (circles) or isoprenaline (squares). Each data point represents the mean ± SEM of six independent determinations.

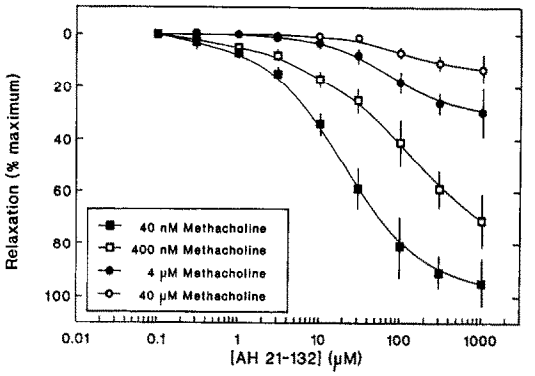


Fig. 4. Functional antagonism of AH 21-132-induced BTSM relaxation. Flurbiprofen- (8 μM) treated smooth muscle strips were set up for isometric tension recording as described in Materials and Methods and pre-contracted with MCh [40 nM (■), 400 nM (□), 4 μM (●), 40 μM (○)]. When the increase in tone had plateaued concentration–response curves were constructed to AH 21-132. Each data point represents the mean ± SEM of six independent determinations.

120 μM. Pre-treatment (50 nM for 60 min) of tissues with the selective β_2-adrenoceptor blocking drug ICI 118551 [23], before the addition of MCh, did not antagonize either the spasmogenic action of MCh or the ability of AH 21-132 to promote relaxation (Fig. 3). For comparative purposes, the relaxation induced by the β-adrenoceptor agonist, isoprenaline, was studied under conditions identical to those described above for the AH 21-132 experiments. Challenge of MCh- (400 nM)-contracted BTSM strips with isoprenaline (30 pM–1 μM) reversed completely the induced tone (Fig. 3). This effect was concentration-dependent (EC_{50}: 8.3 ± 1.2 nM, N = 6) and was antagonized by ICI 118551 (50 nM). It is apparent from Fig. 3, however, that the isoprenaline concentration–response curve was biphasic in ICI 118551-pre-treated tissues due, presumably, to the interaction of isoprenaline with the minor population (*ca.* 30%) of β_1-adrenoceptors that co-exist in BTSM for which ICI 118551 has a lower affinity than for adrenoceptors of the β_2-subtype [24, 25]. At the EC_{50}, ICI 118551 (50 nM) produced a 30-fold rightwards shift in the isoprenaline concentration–response curve from which an estimated affinity (pK_b) of 8.76 was calculated for this antagonist (Fig. 3).

Under the conditions employed in this experiment isoprenaline was *ca.* 9600-fold more potent than AH 21–132 at promoting relaxation of BTSM.

It is well established in airway smooth muscle that the relaxation induced by β-adrenoceptor agonists is subject to marked functional antagonism both *in vitro* and *in vivo*. It was considered of interest, therefore, to determine if AH 21-132-induced relaxation was similarly subject to functional antagonism. Shown in Fig. 4 is the effect of AH 21-132 on tone in BTSM pre-contracted with 40 nM, 400 nM, 4 μM and 40 μM MCh. Both the magnitude of relaxation and the EC_{50} for AH 21-132 were inversely related to the degree of MCh-induced tone (Fig. 4; Table 1). Indeed, when BTSM was contracted with 40 μM MCh, AH 21-132 was almost inactive at eliciting relaxation.

Table 1. Functional antagonism of AH 21-132-induced relaxation of BTSM by methacholine

[Methacholine]	Relaxation (% Maximum)	EC_{50} (μM)
40 nM	97.8 ± 6.1	21.5 ± 4.3
400 nM	>73%*	~120*
4 μM	28.3 ± 10.6	93.3 ± 10.7
40 μM	17.2 ± 6.2	168.3 ± 26.40

* Neither IC_{50} nor maximum relaxation could be determined accurately due to incomplete concentration–response relationship.

Values represent the mean ± SEM of six independent determinations.

Biochemical effects of AH 21-132 *in bovine tracheal smooth muscle*

Given that AH 21-132 is structurally related to the non-selective PDE inhibitor papaverine (see Fig. 1), does not contain the dopamine moiety in its structure (cf. papaverine) and is not a β-adrenoceptor agonist in BTSM (see above), it was considered possible that the relaxant mechanism of action of AH 21-132 in this tissue may, in part, be cyclic nucleotide-mediated through its ability to inhibit cAMP and/or cGMP hydrolysis. Studies were, therefore, performed to determine if AH 21-132 is an inhibitor of cyclic nucleotide PDE in BTSM.

Studies on crude PDE. At a substrate concentration of 1 μM, the 45,000 g_{max} × 30 min supernatant (soluble) fraction of BTSM homogenates readily

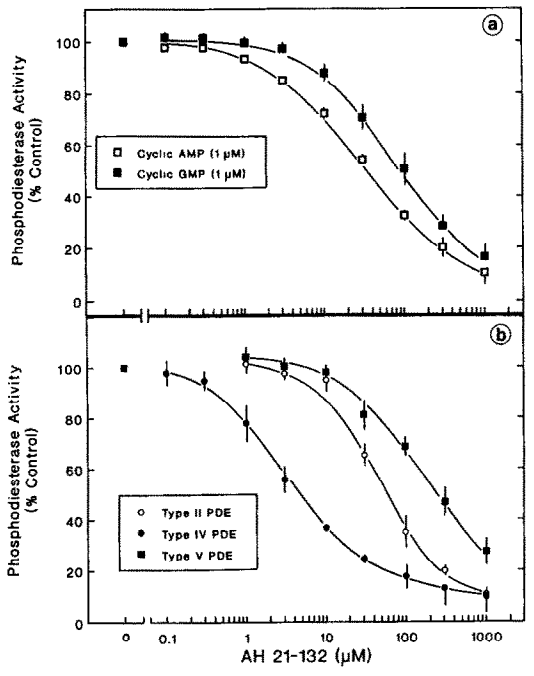


Fig. 5. Inhibition by AH 21-132 of BTSM soluble cyclic nucleotide PDE. Panel (a) shows the effect of AH 21-132 on the hydrolysis of cAMP [1 μM (□)] and cGMP [1 μM (■)] on crude BTSM PDE preparations. In panel (b) the inhibitory effect of AH 21-132 on the activity of BTSM soluble Type II (○), Type IV (●) and Type V (■) PDE isoenzyme resolved by Q-Sepharose anion-exchange chromatography is shown (see Fig. 6). Data represent the mean ± SEM of five independent determinations.

hydrolysed both cAMP (483.9 ± 54.9 pmol/min/mg protein, N = 10) and cGMP (728.5 ± 136.4 pmol/min/mg protein, N = 6). Inclusion of AH 21-132 (100 nM–1 mM) in the reaction cocktail resulted in a concentration-dependent inhibition of cAMP and cGMP hydrolysis with Hill coefficients ($n_H^{cyclic\ AMP}$: 0.74; $n_H^{cyclic\ GMP}$: 0.83) significantly less than unity (Fig. 5a). AH 21-132 was a significantly (P = 0.006, Mann–Whitney U-test) more potent (*ca.* 2.4-fold) inhibitor of cAMP (IC_{50}: 37.9 ± 3.9 μM, N = 10) hydrolysis than of the hydrolysis of cGMP (IC_{50}: 90.5 ± 22.3 μM, N = 6; Fig. 5a).

Resolution of cyclic nucleotide PDE isoenzymes in BTSM by Q-Sepharose anion-exchange chromatography. Figure 6 shows a typical elution profile of soluble cyclic nucleotide PDEs from BTSM following their resolution by Q-sepharose anion-exchange chromatography. In the presence of EGTA (2 mM) three peaks (denoted I, II and III in Fig. 6a) of Ca^{2+}- and calmodulin-*independent* activity were resolved eluting at 200, 450 and 640 mM sodium acetate, respectively. At a substrate concentration of 1 μM, the PDE isoenzyme(s) in peak I and in peak II fractions preferentially hydrolysed cGMP with respective K_m values of 6 and 51 μM; cAMP was a poor substrate for the PDE in both of these peaks ($K_m^{peak\ I}$: 126 μM; $K_m^{peak\ II}$: 56 μM). In contrast, the PDE isoenzyme(s) in peak III fractions preferred cAMP over cGMP as substrate with apparent K_m values of 2.6 and 30 μM, respectively). Analysis of cGMP hydrolysis by peak I fractions and of cAMP hydrolysis by peak III fractions revealed simple Michaelis–Menten kinetics while peak II fractions displayed positive cooperativity with respect to both cAMP (n_H: 1.8) and cGMP (n_H: 1.29) hydrolysis (data not shown). Inclusion of cGMP (10 μM) in the reaction cocktail significantly increased (*ca.* four-fold) peak II-catalysed cAMP hydrolysis suggesting the presence of a Type II PDE isoform (Fig. 6c). In addition, cGMP (10 μM) very slightly inhibited cAMP hydrolysis catalysed by peak III fractions thus tentatively identifying a small amount of a Type III PDE isoform together with a cGMP-insensitive species (Type IV) which made up most of the peak III activity (Fig. 6c).

In the presence of $CaCl_2$ (2 mM) and calmodulin (25 units) cyclic AMP hydrolysis in peak I fractions and in fractions 15–22 (where little cyclic nucleotide PDE activity was detected), was augmented *ca.* four- to eight-fold. Since Ca^{2+}- and calmodulin-dependent, cyclic nucleotide PDEs are unlikely to be active in contracted BTSM (where $[Ca^{2+}]_i$ is close to, or at, resting levels) and the likely fact that they do not regulate the cAMP content in intact airway smooth muscle [26] these isoenzyme(s) were not studied further in these experiments.

Inhibitor sensitivity of cyclic nucleotide PDE in peak I, peak II and peak III fractions. The effect of several compounds identified previously as isoenzyme-selective PDE inhibitors was examined on the PDE activity associated with peaks I, II and III. Zaprinast, a selective inhibitor of the Type V PDE isoenzyme [27], produced a concentration-dependent inhibition of the PDE activity in all three peaks but exhibited markedly selectivity (>100-fold) for the isoenzyme(s) in peak I fractions (Table 2). In contrast, the selective Type III PDE inhibitor, SK&F 94120 [28], did not inhibit cGMP hydrolysis catalysed by either peak I or peak II fractions at concentrations up to 100 μM. SK&F 94120 did, however, produce a small (*ca.* 5%) inhibition of cAMP hydrolysis catalysed by peak III fractions at a concentration (30 μM) that has no effect on soluble tracheal smooth muscle Type IV PDE [29]. Due to the small inhibition of peak III activity seen with SK&F 94120, determination of its IC_{50} was not possible. Whereas SK&F 94120 was essentially inactive on peak III PDE, the selective Type IV PDE inhibitors rolipram [28] and denbufylline [30] inhibited cAMP hydrolysis with marked (>100-fold) selectivity for the SK&F 94120-resistant isoenzyme(s) in peak III fractions (Table 2).

Collectively, these kinetic data together with the sensitivity of each peak of PDE activity to selective PDE inhibitors suggests the presence of three soluble, Ca^{2+}- and calmodulin-*independent*, cyclic nucleotide PDE isoenzymes in BTSM namely Types II, IV and V (see Ref. 14 for nomenclature). In addition, a small amount of Type III PDE may also be present.

Effect of AH 21-132 on Type II, Type IV and Type V PDE activity and kinetic analysis of Type IV PDE inhibition. Having identified and partially characterized the soluble PDE isoenzymes in BTSM the potential inhibitory effect of AH 21-132 on the

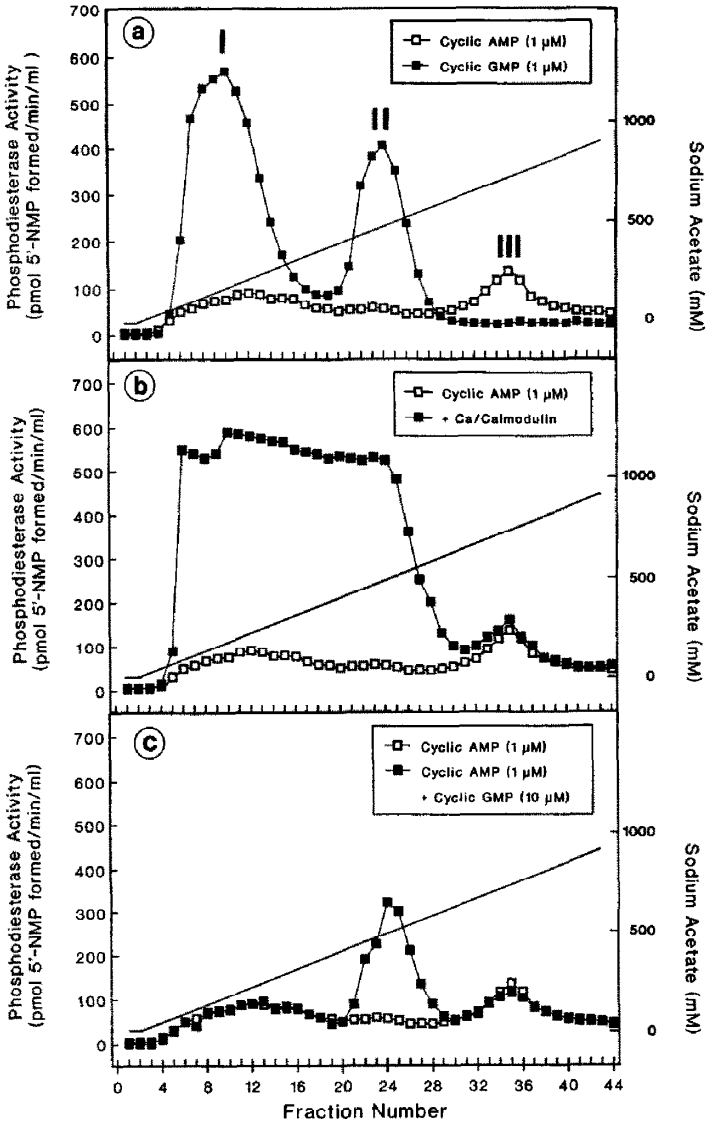


Fig. 6. Elution profile of BTSM soluble cyclic nucleotide PDE activities from Q-Sepharose anion-exchange columns. Supernatants (45,000 g_{max} × 30 min) from tissue homogenates were applied to the column, washed and eluted with a linearly increasing sodium acetate gradient as described in Materials and Methods. Panel (a): PDE activity in the presence of 1 μM cAMP and 1 μM cGMP. Panel (b): cAMP (1 μM) hydrolysis in the absence and presence of $CaCl_2$ (2 mM) and calmodulin (25 units). Panel (c): cAMP (1 μM) hydrolysis in the absence and presence of 10 μM cGMP. These data are typical of five independent determinations.

PDEs was assessed. As shown in Fig. 5b, AH 21-132 selectivity inhibited Type IV PDE (IC_{50}: 3.7 ± 1.2 μM, N = 10) over both the Type II (IC_{50}: 53.1 ± 6.9 μM, N = 6) and Type V (IC_{50}: 306.5 ± 54.2 μM, N = 6) PDE isoenzymes (Table 2). Kinetic analysis of the inhibition by AH 21-132 of Type IV PDE activity (conducted in the presence of 10 μM SK&F 94120 to limit cAMP hydrolysis catalysed by the small amount of Type III PDE) showed it to be competitive in nature (Fig. 7a) with a K_I, derived from secondary (Dixon [13]) plots of the data, of 2.7 ± 0.4 μM (N = 3; Fig. 7b).

Effect of AH 21-132 *on the cAMP content of intact BTSM.* The observation that AH 21-132 selectively inhibited soluble Type IV PDE in a *cell-free* system prompted studies to determine if AH 21-132 could, in addition, increase the cAMP content in the *intact* tissue.

In free-floating strips of BTSM pre-treated with the cyclo-oxygenase inhibitor flurbiprofen (8 μM), the cAMP content was 5.3 ± 1.6 pmol/mg protein (N = 6). Treatment for 10 min with AH 21-132 (100 nM–1 mM) evoked an ICI 118551-insensitive, concentration-dependent increase in tissue cAMP with a *ca.* four-fold increase (to 23.1 ± 5.1 pmol/mg protein, N = 6) observed at 1 mM AH 21-132 (Fig.

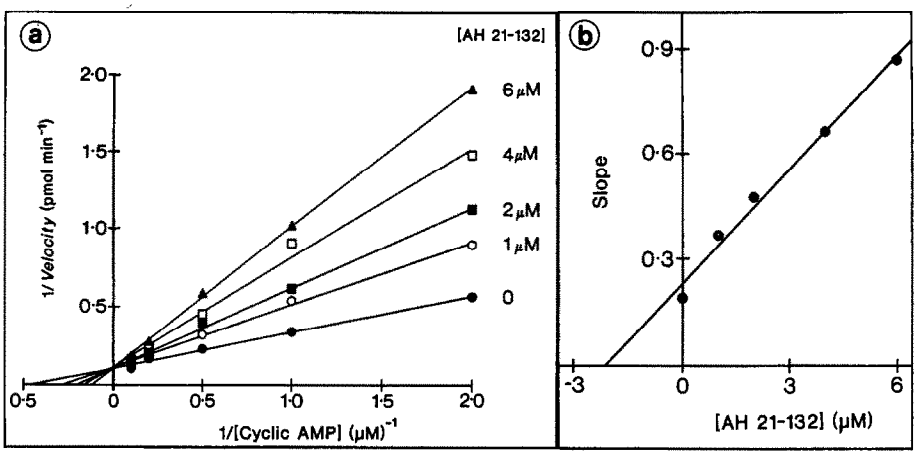


Fig. 7. Kinetic analysis of the inhibition of soluble Type IV PDE in BTSM by AH 21-132. Panel (a) represents Lineweaver–Burk plots of cAMP (1 μM) hydrolysis catalysed by PDE peak III fractions (32 to 38 inclusive in Fig. 6) in the absence and presence of AH 21-132 (1–6 μM). Panel (b) shows a secondary (Dixon [13]) plot of these data from which a K_I of 2.4 μM was obtained in this experiment. These data are typical of three independent determinations. To eliminate the contribution of Type III PDE to cAMP hydrolysis these experiments were performed in the presence of 10 μM SK&F 94120.

Table 2. Sensitivity of soluble Type II, Type IV and Type V PDE isoenzymes to selective PDE inhibitors

	IC_{50} (μM)*		
Inhibitor	II	IV	V
AH 21-132	53	3.6	>300
Zaprinast	93	197	0.9
SK&F 94120	>100	>100	>100
Rolipram	>100	1.6	>100
Denbufylline	ND	0.7	86

Given below are the mean IC_{50} values of a number of isoenzyme-selective PDE inhibitors on Type II, Type IV and Type V PDEs resolved from the soluble fraction of BTSM homogenates. PDE activity was measured in the presence of 2 mM EGTA at a substrate concentration of 1 μM cGMP (Type II and Type V PDE) or 1 μM cAMP (Type IV PDE). Type IV PDE activity was assessed in the presence of 10 μM SK&F 94120 to limit any contribution that the small amount of Type III PDE activity may make to total cAMP hydrolysis.

* IC_{50} values refer to the concentration of inhibitor that reduced cyclic nucleotide hydrolysis by 50% of that activity observed in the absence of inhibitor.

ND, not determined.

8a). Increases in tissue cAMP content thus occurred at concentrations where both anti-spasmogenic and spasmolytic effects of AH 21-132 were seen (see above).

Resolution of cAMP-dependent protein kinase isoenzymes in BTSM by DEAE-Biogel A agarose anion-exchange chromatography. It is now well recognized that there are at least two charged species (Type I and Type II) of A-kinase that co-exist in some cell types so denoted by the order from which they elute from anion-exchange columns (see Ref. 31 for review). These isoenzymes differ in a number

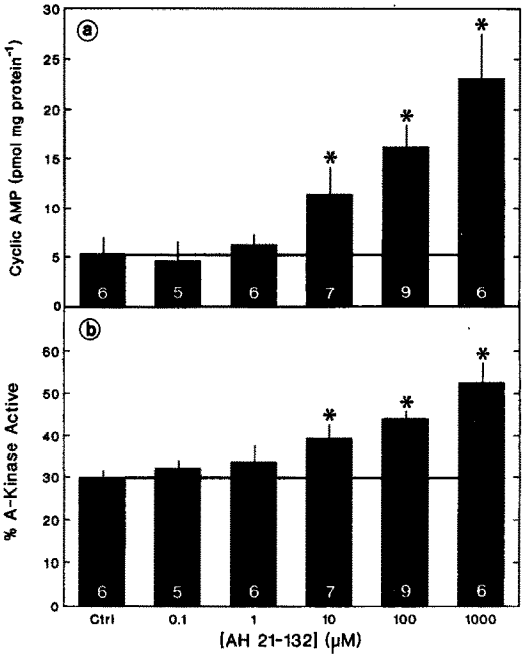


Fig. 8. Effect of AH 21-132 on the cAMP content (panel a) and on the activity of A-kinase (panel b) in BTSM. Free-floating, flurbiprofen (8 μM)-pre-treated strips of BTSM were challenged with AH 21-132 for 10 min and immediately snap-frozen in liquid nitrogen. The cAMP content and A-kinase activity ratio were then determined as described in Materials and Methods. Values at the base of each histogram refer to the number of independent determinations. * Indicates significant ($P < 0.05$) increase over control tissues (Kruskal–Wallace ANOVA with multiple comparisons).

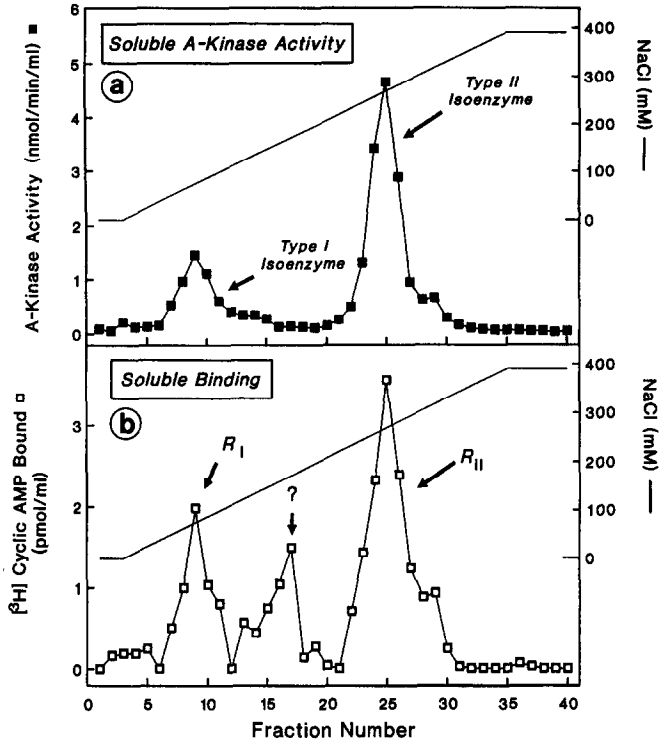


Fig. 9. Elution profile of BTSM soluble A-kinase (panel a, ■) and specific [^{3}H]cAMP binding (panel b, □) from DEAE-Biogel A agarose columns. Supernatants (31,000 g_{max} × 20 min) from tissue homogenates were applied to the column, washed and eluted with a linearly increasing NaCl gradient. See Materials and Methods for further details.

of potentially functional ways not least in their sensitivity to changes in ionic strength. This latter characteristic must be appreciated fully if accurate estimates of the *in vivo* activation state of A-kinase are to be made. Thus, in tissues which contain predominantly the Type I isoenzyme, homogenization buffers of low ionic strength must be employed for this condition prevents artifactual enzyme activation. In contrast, high salt-containing homogenization buffers must be used for tissues which express predominantly the Type II isoform since high ionic strength is necessary to limit enzyme inactivation. It is clear, therefore, that the A-kinase isoenzymes present in a tissue need to be established before the activation state of this enzyme can be accurately estimated.

Subcellular distribution studies revealed that >99% of the total A-kinase activity and >96% specific [^{3}H]cAMP binding was recovered in the 31,000 g_{max} × 20 min supernatant fraction of BTSM homogenates. The particulate activity was, therefore, not examined further in these studies.

Two peaks of soluble A-kinase activity eluted from the DEAE-Biogel A agarose column at 100 and 280 mM NaCl, respectively, indicating the presence of both A-kinase isoenzymes in this tissue (Fig. 9a). In five independent experiments Type I A-kinase represented 25.3 ± 2.8% and Type II A-kinase 71.9 ± 3% of the total cAMP-dependent phosphotransferase activity. Consistent with these enzymatic data was the observation that three peaks of specific [^{3}H]cAMP binding were resolved from the column, two of which eluted at exactly the same ionic strength as A-kinase activity and which comprised 18.5 ± 2.5% (co-eluting the Type I A-kinase) and 60.8 ± 5% (co-eluting with Type II A-kinase) of the total specific binding, respectively (Fig. 9b). If the third peak of specific binding was subtracted from the total specific binding the percentage specific [^{3}H]cAMP bound in those fractions where Type I and Type II A-kinase co-eluted was 23.6 ± 3.8% (N = 5) and 76.4 ± 3.8% (N = 5), respectively, a distribution quantitatively identical to the Type I:Type II A-kinase isoenzyme activity ratio (see above).

The third peak of specific [^{3}H]cAMP binding activity (denoted by a question mark in Fig. 9b) eluted from the column at intermediate ionic strength (170 mM NaCl) and represented 19.4 ± 2.4% (N = 5) of the total specific [^{3}H]cAMP bound. This was not, however, associated with detectable A-kinase activity when Kemptide (or histone IIa, unpublished observation) was used as phosphate acceptor (cf. Fig. 9a with Fig. 9b). The nature of this specific [^{3}H]cAMP binding is, at present, unknown but it may represent free regulatory subunits derived predominantly from the Type I holoenzyme which would tend to dissociate under the initial low ionic strength conditions employed for isoenzyme fractionation.

Effect of AH 21-132 *on cAMP-dependent protein kinase activity in intact BTSM.* In free-floating, flurbiprofen- (8 μM) pre-treated strips of BTSM 29.6 ± 2.2% (N = 6) of soluble A-kinase was active (Fig. 8b). Treatment of BTSM with AH 21-132 (100 nM–1mM) for 10 min resulted in a concentration-dependent, ICI 118551-*insensitive* increase in the A-kinase activity ratio (Fig. 8b). The proportion of the total soluble enzyme active was thus increased by *ca.* 24% to 53.5 ± 6.7% (N = 6) after treatment with 1 mM AH 21-132. Activation of A-kinase thus occurred at concentrations where both spasmolytic and anti-spasmogenic effects of AH 21-132 were observed (see above). The magnitude of this increase in A-kinase activity produced by AH 21-132 was similar to that promoted by isoprenaline (10 μM for 5 min) in BTSM (from 29.8 ± 0.8%, N = 12 to 50.5 ± 1.4%, N = 12).

It is noteworthy that the activation of A-kinase by both AH 21-132 and isoprenaline was attributable solely to an increase in the concentration of free catalytic subunits since total phosphotransferase was unchanged (data not shown).

Effect of AH 21-132 *on cAMP-dependent protein kinase activity in a cell-free system.* The effect of cAMP on crude soluble A-kinase isolated from BTSM is shown in Fig. 10. Under the conditions of the assay cAMP produced a concentration-dependent activation of A-kinase with a K_{act} of 211.3 ± 25.9 nM (N = 6); maximal activation was achieved at 10 μM cAMP. AH 21-132 did not affect directly Kemptide phosphorylation over the concentration range (100 nM–100 μM) at which it inhibited Type IV PDE (Fig. 5b) nor did it modify the activation of A-kinase by cAMP. Higher concentrations (>100 μM) of AH 21-132 similarly exerted no direct stimulatory (or inhibitory) action on Kemptide phosphorylation nor did this drug affect the binding of [γ-^{32}P]ATP to the P81 cation-exchange paper in the absence of enzyme. In contrast, phosphotransferase activity was significantly ($P < 0.01$; Kruskal–Wallace ANOVA with multiple comparisons) augmented by AH 21-132 at all concentrations of cAMP that activated A-kinase resulting in a concentration-dependent upwards shift of the cAMP activation curve (Fig. 10a). Kinetic analysis showed that this effect occurred without an apparent change in the K_{act} of cAMP for A-kinase (slopes of Eadie-Hoftsee-transformed data not significantly different from one another; see Fig. 10b) and that the phosphotransferase did not exhibit positive cooperatively with respect to cAMP-induced phosphorylation of Kemptide in the presence of AH 21-132.

The augmentation of cAMP (10 μM)-stimulated Kemptide phosphorylation by all concentrations of AH 12-132 was sensitive (>97%) to inihibition by IP_{20} (1 μM), a specific eicosapeptide inhibitor of A-kinase ([32]; data not shown).

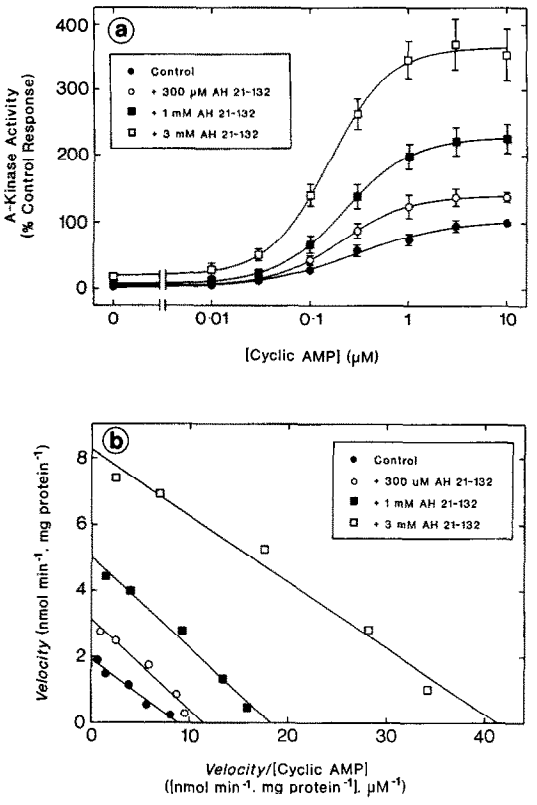


Fig. 10. Effect of AH 21-132 on the activation of A-kinase by cAMP. Strips of BTSM were homogenized in ice-cold Buffer D, centrifuged and the supernatant diluted as described in Materials and Methods. Panel (a) shows the ability of cAMP (10 nM–10 μM) to activate soluble A-kinase in the absence (●) and in the presence of 300 μM (○), 1 mM (■) and 3 mM (□) AH 21-132. In panel (b) Eadie–Hoftsee transformation of these data are illustrated highlighting the fact that AH 21-132 does not affect significantly the K_{act} of cAMP for A-kinase. Each data point represents the mean ± SEM of five independent determinations. Error bars in panel (b) are omitted for clarity.

DISCUSSION

The results of these studies demonstrate that AH 21-132 exhibits both spasmolytic and anti-spasmogenic activity in BTSM. The ability of AH 21-132 to selectively inhibit cAMP hydrolysis in a *cell-free* system together with the observation that AH 21-132 increased the cAMP content and activated A-kinase in *intact* BTSM, over the same concentration range that it affected tone, suggests that a cAMP-dependent mechanism(s) may underlie, at least in part, the inhibitory effects of AH 21-132 in this tissue.

Spasmolytic and anti-spasmogenic effects of AH 21-132 *in BTSM*

AH 21-132 produced a concentration-dependent relaxation of MCh-contracted BTSM that was not antagonized by the selective β_2-adrenoceptor blocking drug ICI 118551 [23]. Agonism of β_2-adrenoceptors, therefore, cannot explain the relaxant mechanism of action of AH 21-132 in this tissue. This conclusion is supported by the apparent ineffectiveness of propranolol at antagonizing the relaxant action of AH 21-132 in both human [2] and guinea-pig [2,3] isolated airways smooth muscle. In contrast, the relaxation elicited by isoprenaline

in BTSM was potently antagonized by ICI 118551 with an affinity ($pK_b = 8.76$) consistent with an interaction at β_2-adrenoceptors.

Although AH 21-132 was, indeed, an effective relaxant of BTSM, this was seen only when tissues were pre-contracted with relatively low concentrations (<300 nM) of MCh. Indeed, both the potency of AH 21-132 and the ability of this drug to elicit relaxation were inversely related to the initial degree of MCh-induced tone demonstrating that the mechanical actions of AH 21-132 are subject to marked functional antagonism. This finding is in keeping with that reported for both β-adrenoceptor agonists [33–35] and Type III PDE inhibitors [36] in canine and bovine trachealis and has been attributed, in part, to the suppression by MCh of drug-induced A-kinase activation [35, 36].

In addition to relaxing BTSM, AH 21-132 effectively inhibited the *generation* of tension induced by low, but *not* high, concentrations of MCh (i.e. the anti-spasmogenic activity of AH 21-132 was reduced as the MCh concentration was increased). This result is exactly what would be predicted in a system where functional antagonism between two opposing influences operates. Similar data have been reported previously for the effect of AH 21-132 on acetylcholine-induced tone in guinea-pig isolated trachealis [3]. However, in this study AH 21-132 produced a concentration-dependent rightwards shift in the acetylcholine concentration–response curve *and* significantly reduced the maximum response attained. The reason(s) for these partly inconsistent results is not clear but species difference, presence of the epithelium in the study of Small *et al.*, [3] or the fact that AH 21-132 can inhibit muscarinic cholinoceptor-mediated inositol polyphosphate biosynthesis [37] may be responsible.

AH 21-132 *as an inhibitor of cyclic nucleotide hydrolysis in BTSM*

In the soluble fraction of BTSM homogenates, AH 21-132 selectively inhibited cAMP hydrolysis in a concentration-dependent manner, a finding consistent with that recently published by Small *et al.* [3] in smooth muscle-rich strips isolated from guinea-pig tracheae. Since the concentration range over which AH 21-132 inhibited PDE paralleled closely that required for both spasmolytic and anti-spasmogenic activity the possibility exists that the inhibition of PDE and the mechanical effects of AH 21-132 are causally related.

The observation that the concentration–response curves for inhibition of cyclic nucleotide hydrolysis by AH 21-132 were shallow suggests the existence of multiple PDE isoenzymes in BTSM as has been reported in canine trachealis [29]. In addition, the more potent inhibition of cAMP hydrolysis with respect to the hydrolysis of cGMP suggests that AH 21-132 may selectively inhibit a PDE isoenzyme which preferentially hydrolyses cAMP. To evaluate these possibilities experiments were performed to resolve and partially characterize the soluble PDE isoenzymes in BTSM and to assess the sensitivity of the species so identified to AH 21-132.

Soluble cyclic nucleotide PDE isoenzymes in BTSM: resolution, identification and selective inhibition by AH 21-132

In the presence of EGTA three peaks of cyclic nucleotide hydrolysing activity were resolved from the soluble fraction of BTSM homogenates by Q-Sepharose anion-exchange chromatography. The first peak of activity that eluted from the column was identified as a cGMP-specific [15] or Type V [14] PDE, since it selectively hydrolysed cGMP over cAMP and resembled, in both kinetic terms and sensitivity to the selective Type V PDE inhibitor zaprinast, the cGMP-specific PDE isolated from canine trachealis [29], bovine lung [38], bovine photoreceptors [39] and human platelets [40, 41].

cAMP hydrolysis in fractions 6 to 22 was stimulated four- to eight-fold by Ca^{2+} and calmodulin indicating the presence of a Ca^{2+}- and calmodulin-requiring PDE isoenzyme(s) that co-elutes, in part, with Type V PDE in this tissue. Such co-purification of Ca^{2+}- and calmodulin-dependent PDE isoenzyme(s) with Type V PDE is a consistent finding in the separation of PDEs from many tissues including those from tracheal smooth muscle [29]. The observation that this peak of PDE activity was broad may indicate the presence of multiple forms of Ca^{2+}- and calmodulin-dependent PDEs in BTSM as reported for human trachealis [42].

The second peak of PDE activity that eluted from the column hydrolysed cAMP and cGMP with kinetics similar to that reported for a cGMP-stimulated [15] or Type II [14] PDE isolated from bovine heart [43], canine trachealis [29] and calf liver [44]. Thus, this peak of activity exhibited low affinity for cAMP and cGMP, displayed positive cooperativity with respect to both substrates and was poorly inhibited by zaprinast. Furthermore, the hydrolysis of cAMP by fractions in this peak was augmented four-fold in the presence of 10 μM cGMP.

The final peak of PDE activity that eluted from the column was identified as predominantly (>95%) a Ro 20-1724-sensitive [15] or Type IV [14] PDE isoenzyme and displayed kinetic characteristics and inhibitor sensitivity similar to the soluble cAMP-selective PDE in canine trachealis [29], porcine endothelial cells [45] and rat cerebral cortex [30], and the particulate cAMP-selective PDE in human neutrophils [46]. Evidence to support this conclusion was the greater affinity that cAMP displayed for this PDE over cGMP, its sensitivity to inhibition by drugs (rolipram and denbufylline) categorized previously as Type IV-selective PDE inhibitors and its almost complete insensitivity to cGMP and the Type III-selective PDE inhibitor SK&F 94120 (but see below). This finding in BTSM contrasts to that found in the soluble fraction of canine trachealis where appreciable Type III PDE activity was found [29].

Although the hydrolysis of cAMP by peak III fractions apparently displayed simple Michealis–Menten kinetics (see Results), studies with cGMP and SK&F 94120 suggested that *ca.* 5% of peak III hydrolysing activity (at 1 μM cAMP) was catalysed by a Type III [15] or cGMP-inhibited [14] PDE isoenzyme (see Fig. 1c). Presumably, the high Type

IV:Type III PDE isoenzyme activity ratio obscured the deviation in the kinetics of cAMP hydrolysis that would be predicted when two enzymes which catalyse the same reaction act on the same substrate.

Thus, using both kinetic and inhibitor sensitivity criteria three soluble, Ca^{2+}- and calmodulin-*independent* cyclic nucleotide PDE isoenzymes (Type II, cGMP-stimulated; Type IV, Ro 20-1724-inhibited; and Type V, cGMP-specific) are co-expressed in BTSM. In addition, a small amount of soluble Type III PDE *may* be present but appears to make negligible contribution to peak III-catalysed cAMP hydrolysis at 1 μM substrate.

It is noteworthy that, if present, this small amount of soluble Type III PDE may play an important role in regulating the cAMP content in intact BTSM. This supposition is suggested by preliminary reports which document the ability of SK&F 94120 to relax cholinomimetic-contracted BTSM [47, 48] at concentrations at which this PDE inhibitor retains selectivity for the Type III PDE isoenzyme [28]. Alternatively, the relaxation elicited by SK&F 94120 may occur predominantly through the inhibition of a *particulate* Type III PDE which following differential centrifugation, is located in the 45,000 g_{max} × 30 min pellet. Such a possibility would be consistent with that found in canine trachealis where the particulate material is enriched in Type III PDE relative to the Type IV PDE isoenzyme [29]. It is pertinent to mention in this respect that while the physiological signifcance of the subcellular location of cyclic nucleotide PDEs in airway smooth muscle is currently unclear, Weishaar *et al.* [49] have reported that positive inotropic effects in the heart are produced only when the particulate Type III PDE isoenzyme is inhibited; inhibition of the same isoform in the soluble fraction does not affect contractility.

Tested on the resolved PDE isoenzymes, AH 21-132 was a selective and competitive (with respect to cAMP) inhibitor of Type IV PDE with an IC_{50} similar to that recently reported for inhibition of Type IV PDE in guinea-pig eosinophils [50, 51] and a K_I in close agreement with that observed for inhibition of a soluble crude PDE preparation isolated from rat cerebral cortex [1].

Phosphodiesterase inhibition as a mechanism of action of AH 21-132 *in intact BTSM*

Although AH 21-132 preferentially inhibited Type IV PDE in a *cell-free* system (see above) it was necessary to determine if AH 21-132 elevated the cAMP content in the *intact* tissue before a cyclic nucleotide-dependent mechanism could be postulated to underlie its anti-spasmogenic and/or spasmolytic activity. This was especially relevant given the highly charged nature of AH 21-132 at pH 7.4 which would tend to prevent its entry into cells. The observation that AH 21-132 did, indeed, produce a concentration-dependent increase in the tissue cAMP content over the concentration range for which it inhibited Type IV PDE and exhibited anti-spasmogenic and spasmolytic activity, is consistent with this hypothesis.

Since changes in intracellular cAMP are considered to be of physiological significance only if a corresponding alteration in the activity of A-kinase is also observed, experiments were conducted to determine what A-kinase isoforms are present in BTSM and to assess the effect of AH21-132 on the activation state of this enzyme in the intact tissue.

Subcellular distribution studies revealed that A-kinase was almost exclusively (>99%) recovered from the soluble fraction of BTSM homogenates indicating that in this tissue A-kinase is predominantly a cytosolic enzyme.

Chromatography of soluble extracts of BTSM over DEAE-Biogel A agarose resolved two peaks of cAMP-dependent phosphotransferase activity and specific [^{3}H]cAMP binding that eluted at ionic strengths consistent with them being the so-called Type I and Type II A-kinase isoenzymes. In agreement with that reported for guinea-pig lung [16], guinea-pig trachealis [52] and human trachealis (M.A. Giembycz, C.D. Stretton and P.J. Barnes, unpublished observations), the Type II A-kinase was the predominant isoenzyme in this tissue.

Consistent with the cAMP results, AH 21-132 increased the A-kinase activity ratio in a concentration-dependent manner over the same concentration range that it affected tone. Although these biochemical actions of AH 21-132 correlated well with the mechanical actions of this compound, it must be borne in mind that this apparent association may be purely fortuitous. It is clear, therefore, that before a *causal* association between AH 21-132-induced PDE inhibition and relaxation can be proposed with any certainty experiments, similar to those described by Torphy *et al.* [36], must be performed examining the interaction between AH 21-132 and β-adrenoceptor agonists. Thus, if AH 21-132 does mediate relaxation of BTSM by inhibiting a PDE (Type IV?) isoform then it should potentiate β-adrenoceptor-mediated relaxation, cAMP accumulation and A-kinase activation. This possibility is currently under investigation. Since submission of this manuscript, Small *et al.* [53] reported that AH 21-132 potentiates isoprenaline-induced relaxation and cAMP accumulation in guinea-pig tracheal strips suggesting that PDE inhibition does, indeed, represent a primary mechanism by which AH 21-132 evokes its mechanical effects in the airways.

AH 21-132 *and cAMP-dependent protein kinase in a cell-free system*

The finding that AH 21-132 augmented markedly the activation of A-kinase by cAMP in a cell-free system was unexpected and deserves discussion. That AH 21-132 neither directly stimulated A-kinase in the absence of exogenous cAMP nor increased the binding of [γ-^{32}P]-ATP to the cation-exchange (P81) paper in the absence of enzyme effectively eliminates the possibility that these data arose from some methodological artifact brought about by the highly positive charged nature of AH 21-132 at physiological pH. Indeed, the observation that cAMP (10 μM)-stimulated Kemptide phosphorylation in the presence of 3 mM AH 21-132 was essentially abolished by IP_{20} indicates that the observed phosphorylation was attributable entirely to A-kinase.

The question that now arises is whether this

anomalous effect of AH 21-132 contributes to the mechanical actions of the drug in *intact* BTSM. It is clear from the data presented in Fig. 4 that concentrations of AH 21-132 greater than 100 μM are required to relax BTSM fully especially under high levels of MCh-induced tone. This additional effect of AH 21-132 could, therefore, be relevant pharmacologically. Inconsistent with this hypothesis, however, was the finding that the specific activity of soluble A-kinase in *intact* BTSM strips pre-treated with 1 mM AH 21-132 (a concentration which doubled the specific activity in a *cell-free* system) was not increased. These seemingly conflicting results are likely to be explained by the fact that homogenization will markedly dilute the AH 21-132 present in the intact tissue and, unless this potentiating effect of AH 21-132 is irreversible, will no longer be present at an effective concentration.

Another intriguing question that arises from these data relates to where and how AH 21-132 potentiates A-kinase activity. That inhibition of cAMP hydrolysis accounts for these results can be discounted for at least three reasons. First, the concentrations of AH 21-132 which potentiated cAMP-stimulated Kemptide phosphorylation were in excess of those required to abolish Type IV PDE activity in a cell free system. Second, the non-selective PDE inhibitor, IBMX, was present in the reaction cocktail at a concentration (500 μM) which, in preliminary studies, abolished cAMP hydrolysis in crude BTSM supernatants [6]. Third, potentiation was observed when A-kinase was saturated with cAMP.

Given that PDE does not represent the site of action where AH 21-132 potentiates A-kinase activation, at what intracellular locus could AH 21-132 act to produce this effect? The finding that Kemptide phosphorylation was augmented by fully active (+10 μM cAMP) A-kinase together with the fact that affinity of cAMP for activation of this phosphotransferase was unchanged suggests that AH 21-132 probably acts directly on A-kinase at the level of the catalytic subunit. One possibility is that an endogenous allosteric activator exists *in vivo* (which may be inactivated or diluted during the preparation of the enzyme) for which AH 21-132 can substitute albeit with poor affinity. There is, however, no evidence to our knowledge for such a regulatory molecule. Conversely, AH 21-132 may displace and endogenous inhibitor of A-kinase which survives tissue preparation and dilution. Although unlikely, studies with IP_{20}, an eicospeptide inhibitor of A-kinase [32], may permit the testing of this hypothesis.

Whatever the mechanism these unexpected observations may provide the impetus for experiments designed to understand further the molecular process by which cAMP activates A-kinase. Furthermore, it is conceivable that these data with AH 21-132 could lead to a unique therapeutic approach aimed towards the treatment of many disease states (e.g. asthma, congestive heart failure) where activation of cAMP-dependent pathways is regarded as being beneficial.

Conclusion

The data presented herein provide evidence that the mechanical effects elicited by AH 21-132 in BTSM may be related to its ability to selectively inhibit Type IV PDE, increase the cAMP content and so activate A-kinase. In addition, a novel mechanism may contribute to AH 21-132-induced relaxation in BTSM whereby this drug potentiates, by some unknown mechanism, cAMP-induced A-kinase activation but without increasing further the tissue cAMP content.

Acknowledgements—The authors thank Judith C.W. Mak for performing the binding studies and Sandoz AG, Basle, Switzerland (AH 21-132), Schering AG, Berlin, Germany (rolipram), SmithKline-Beecham, Harlow (SK&F 94120, denfubylline) and Rhone-Poulenc Rorer Ltd, Dagenham (zaprinast) for the gifts of drugs. We acknowledge the Medical Research Council for financial support.

REFERENCES

1. Markstein R, Digges K, Marshall NR and Starke K, Forskolin and the release of noradrenaline in cerebrocortical slices. *Naunyn Schmiedebergs Arch Pharmacol* **325**: 17–24, 1984.
2. Bewley JS and Chapman ID, AH 21-132, a novel relaxant of airway smooth muscle. *Br J Pharmacol* **93**: 52P, 1988.
3. Small RC, Boyle JP, Duty S, Elliott KRF, Foster RW and Watt AJ, Analysis of the relaxant effects of AH 21-132 in guinea-pig isolated trachealis. *Br J Pharmacol* **97**: 1165–1173, 1989.
4. Kristersson A, Morley J and Schaeublin E, Properties of AH 21-132 that could contribute to prophylactic activity in asthma. *Br J Pharmacol* **93**: 53P, 1988.
5. Aoki S, Boubekeur K and Sanjar S, The effect of AH 21-132 and other anti-asthma drugs on PAF-induced eosinophilia in the guinea-pig. *Br J Pharmacol* **93**; 54P, 1988.
6. Giembycz MA, Mak JCW, Ukena D, Chilvers ER and Barnes PJ, AH 21-132 selectively inhibits Type III/IV cyclic nucleotide-dependent phosphodiesterase activity in bovine airway smooth muscle. *Br J Pharmacol* **97**: 559P, 1989.
7. Van Rossum JM, Cumulative dose–response curve II. Technique for the making of dose–response curves in isolated organs and the evaluation of drug parameters. *Arch Int Pharmacodyn Ther* **143**: 298–330, 1963.
8. Chilvers ER, Giembycz MA, Challiss RAJ, Barnes PJ and Nahorski SR, Lack of effect of zaprinast on methacholine-induced tone and inositol (1,4,5)trisphosphate accumulation in bovine tracheal smooth muscle. *Br J Pharmacol* **103**: 1119–1125, 1991.
9. Van Inwegen RG, Pledger WJ, Strada SJ and Thompson WJ, Characterization of cyclic nucleotide phosphodiesterases with multiple separation techniques. *Arch Biochem Biophys* **175**: 700–709, 1976.
10. Schwartz JP and Passonneau JV, Cyclic AMP-mediated induction of cyclic AMP phosphodiesterase of C_6 glioma cells. *Proc Natl Acad Sci USA* **71**: 3844–3848, 1974.
11. Thompson WJ and Appleman MM, Multiple cyclic nucleotide phosphodiesterase activities from rat brain. *Biochemistry* **10**: 311–316, 1971.
12. Lowry OH, Rosebrough NJ, Farr AL and Randall RJ, Protein measurement with the Folin phenol reagent. *J Biol Chem* **193**: 265–275, 1951.
13. Dixon M, The determination of enzyme inhibitor constants. *Biochem J* **55**: 170–171, 1953.
14. Beavo JA and Reifsnyder DH, Primary sequence of cyclic nucleotide phosphodiesterase isozymes and the design of selective inhibitors. *Trends Pharmacol Sci* **11**: 150–155, 1990.

15. Beavo JA, Multiple isozymes of cyclic nucleotide phosphodiesterase. *Adv Second Messenger Phosphoprotein Res* **22**: 1–38, 1988.
16. Giembycz MA and Diamond J, Partial characterization of cyclic AMP-dependent protein kinases in guinea-pig lung employing the synthetic heptapeptide substrate, Kemptide: *In vitro* sensitivity of the soluble enzyme to isoprenaline, forskolin, methacholine and leukotriene D_4. *Biochem Pharmacol* **39**: 1297–1312, 1990.
17. Cherrington AD, Assimacopoulos FD, Harper SC, Corbin JD, Park CR and Exton JH, Studies on the α-adrenergic activation of hepatic glucose output. II. Investigation of the roles of adenosine 3′:5′-monophosphate- and guanosine 3′:5′-monophosphate-dependent protein kinase in the actions of phenylephrine in isolated hepatocytes. *J Biol Chem* **251**: 5209–5218, 1976.
18. Cook PF, Neville ME, Vrana KE, Hartl FT and Roskoski RR, Adenosine cyclic 3′,5′-monophosphate-dependent protein kinase: kinetic mechanism for the bovine skeletal muscle catalytic subunit. *Biochemistry* **21**: 5794–5799, 1982.
19. Glass DB, Masarcchia RA, Feramisco JR and Kemp BE, Isolation of phosphorylated peptides and proteins on ion exchange papers. *Anal Biochem* **87**: 566–575, 1978.
20. Witt JJ and Roskoski R, Rapid protein kinase assay using phosphocellulose-paper absorption. *Anal Biochem* **66**: 253–258, 1975.
21. Giembycz MA and Diamond J, Evaluation of Kemptide, a synthetic serine-containing heptapeptide, as a phosphate acceptor for the estimation of cyclic AMP-dependent protein kinase activity in respiratory tissues. *Biochem Pharmacol* **39**: 271–283, 1990.
22. Sugden PH and Corbin JD, Adenosine 3′:5′-cyclic monophosphate-binding proteins in bovine and rat tissues. *Biochem J* **159**: 423–437, 1976.
23. O'Donnell SR and Wanstall JC, Evidence that ICI 118,551 is a potent, highly beta$_2$-selective adrenoceptor antagonist and can be used to characterize beta-adrenoceptor populations in tissues. *Life Sci* **27**: 671–677, 1980.
24. Kaumann AJ and Lemoine H, Separation of catecholamine binding and relaxation in bovine tracheal smooth muscle dependent upon cyclic AMP. *J Physiol* **348**: 47P, 1984.
25. Lemione H, Novotny GEK and Kaumann AJ, Neuronally released (−)-noradrenaline relaxes smooth muscle of calf trachea mainly through β_1-adrenoceptors: comparison with (−)-adrenaline and relation to adenylate cyclase stimulation. *Naunyn Schmiedebergs Arch Pharmacol* **339**: 85–98, 1989.
26. Torphy TJ, Action of mediators on airway smooth muscle: functional antagonism as a mechanism for bronchodilator drugs. In: *Directions for New Anti-Asthma Drugs. Agents and Actions Supplements* (Eds. O'Donnell SR and Persson CGA), Vol. 23, pp. 37–53. Birkhauser Verlag, Basle, 1988.
27. Weishaar RE, Cain MH and Bristol JA, A new generation of phosphodiesterase inhibitors: multiple molecular forms of phosphodiesterase and the potential for drug selectivity. *J Med Chem* **28**: 537–545, 1985.
28. Reeves ML, Leigh BK and England PJ, The identification of a new cyclic nucleotide phosphodiesterase in human and guinea-pig cardiac ventricle. *Biochem J* **241**: 535–541, 1987.
29. Torphy TJ and Cieslinski LB, Characterization and selective inhibition of cyclic nucleotide phosphodiesterase isozymes in canine tracheal smooth muscle. *Mol Pharmacol* **37**: 206–214, 1990.
30. Nicholson CD, Jackman SA and Wilke R, The ability of denbufylline to inhibit cyclic nucleotide phosphodiesterase and its affinity for adenosine receptors and the adenosine re-uptake site. *Br J Pharmacol* **97**: 889–897, 1989.
31. Beebe SJ and Corbin CD, Cyclic nucleotide-dependent protein kinases. *The Enzymes* **17**: 43–111, 1986.
32. Cheng H-C, Kemp BE, Pearson RB, Smith AJ, Misconi L, van Patten SM and Walsh DA, A potent synthetic peptide inhibitor of the cyclic AMP-dependent protein kinase. *J Biol Chem* **261**: 989–992, 1986.
33. Offermeier J and van den Brink FG, The antagonism between cholinomimetic agonists and β-adrenoceptor stimulants. The differentiation between functional and metaffinoid antagonism. *Eur J Pharmacol* **27**: 206–213, 1974.
34. van den Brink FG, The model of functional interation. II. Experimental verification of a new model: The antagonism of β-adrenoceptor stimulants and other agonists. *Eur J Pharmacol* **22**: 279–286, 1973.
35. Torphy TJ, Rinard GA, Rietow MG and Mayer SE, Functional antagonism in canine tracheal smooth muscle: Inhibition by methacholine of the mechanical and biochemical responses to isoproterenol. *J Pharmacol Exp Ther* **227**: 694–699, 1983.
36. Torphy TJ, Burman M, Huang LBF and Tucker SS, Inhibition of the low Km cyclic AMP phosphodiesterase in intact canine trachealis by SK&F 94836: mechanical and biochemical responses. *J Pharmacol Exp Ther* **246**: 843–850, 1988.
37. Giembycz MA, Chilvers ER and Barnes PJ, Biochemical and pharmacological interactions between the papaverine derivative AH 21-132 and methacholine in bovine tracheal smooth muscle. *Eur J Pharmacol* **183**: 2157–2158, 1990.
38. Thomas MK, Francis SH and Corbin JD, Characterization of a purified bovine lung cGMP-binding phosphodiesterase. *J Biol Chem* **265**: 14964–14970, 1990.
39. Gillespie PG and Beavo JA, Inhibition and stimulation of photoreceptor phosphodiesterases by dipyridamole and M & B 22,948. *Mol Pharmacol* **36**: 773–781, 1989.
40. Hidaka H and Asano T, Human blood platelet 3′,5′-cyclic nucleotide phosphodiesterase. *Biochim Biophys Acta* **429**: 485–497, 1976.
41. Simpson AWM, Reeves ML and Rink TJ, Effects of SK&F 94120, an inhibitor of cyclic nucleotide phosphodiesterase Type III, on human platelets. *Biochem Pharmacol* **37**: 2315–2320, 1988.
42. Cieslinski LB, Reeves ML and Torphy TJ, Cyclic nucleotide phosphodiesterases (PDEs) in canine and human Tracheal Smooth Muscle (TSM). *FASEB J* **2**: A4474, 1988.
43. Martins TJ, Mumby MC and Beavo JA, Purification and characterization of a cyclic GMP-stimulated cyclic nucleotide phosphodiesterase from bovine tissues. *J Biol Chem* **257**: 1973–1979.
44. Yamamoto T, Manganello VC and Vaughan M, Purification and characterization of cyclic GMP-stimulated cyclic nucleotide phosphodiesterase from calf liver. Effects of divalent cations. *J Biol Chem* **258**: 12526–12533, 1983.
45. Souness JE, Diocee BK, Martin W and Moodie SA, Pig aortic endothelial-cell cyclic nucleotide phosphodiesterases. Use of phosphodiesterase inhibitors to evaluate their roles in regulating cyclic nucleotide levels in intact cells. *Biochem J* **266**: 127–132, 1990.
46. Nielson CP, Vestal RE, Sturm RJ and Heaslip R, Effects of selective phosphodiesterase inhibitors on the polymorphonuclear leukocyte repiratory burst. *J Allergy Clin Immunol* **86**: 801–808, 1990.
47. Hall IP, Walker D, Hill SJ and Tattersfield AE, Effect of isoenzyme selective phosphodiesterase inhibitors on bovine tracheal smooth muscle. *Eur J Pharmacol* **183**: 1096–1097, 1990.
48. Nicholson CD, Shahid M, van Amsterdam RGM and

Zaagsma J, Cyclic nucleotide phosphodiesterase (PDE) isoenzymes in bovine tracheal smooth muscle, their inhibition and the ability of isoenzyme inhibitors to relax pre-contracted preparations. *Eur J Pharmacol* **183**: 1097–1098, 1990.

49. Weishaar RW, Kobylarz-Singer DC and Kaplan HR, Subclasses of cyclic AMP phosphodiesterase in cardiac muscle. *J Mol Cell Cardiol* **19**: 1025–1036, 1987.
50. Dent G, Giembycz MA, Rabe KF and Barnes PJ, Inhibition of eosinophil cyclic nucleotide PDE activity and opsonised zymosan-stimulated respiratory burst by 'type IV'-selective PDE inhibitors. *Br J Pharmacol*, in press.
51. Souness JE, Turner NC, Diocee BK, Hassal GA and Wood LJ, Characterization of guinea-pig eosinophil cyclic AMP phosphodiesterase (PDE). *Br J Pharmacol* **102**: 38P, 1991.
52. Langlands JM and Rodger IW, Determination of soluble cyclic AMP-dependent protein kinase activity in guinca-pig tracheal smooth muscle. Preferential use of kemptide as a phosphorylating substrate. *Biochem Pharmacol* **39**: 1365–1374, 1990.
53. Small RC, Berry JL, Boyle JP, Chapman ID, Elliott KRF, Foster RW and Watt AJ, Biochemical and electrical aspects of the tracheal relaxant action of AH 21-132. *Eur J Pharmacol* **192**: 417–426, 1991.