The ability of denbufylline to inhibit cyclic nucleotide phosphodiesterase and its affinity for adenosine receptors and the adenosine re-uptake site

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1   Denbufylline has been examined for its ability to inhibit cyclic nucleotide phosphodiesterase isoenzymes from rat cardiac ventricle and cerebrum, as well as for its affinity for adenosine A_1 and A_2 receptors and the re-uptake site. For comparison, SK&F 94120, theophylline and 3-isobutyl-1-methyl-xanthine (IBMX) were examined as phosphodiesterase inhibitors whilst N^6-cyclohexyladenosine, $R(-)$-N^6-(2-phenylisopropyl)-adenosine, 5'-N-ethylcarboxamido-adenosine, 2-nitrobenzylthioinosine, theophylline and IBMX were examined for their affinity for adenosine binding sites.

2   This investigation confirmed the presence of four phosphodiesterase activities in rat cardiac ventricle; in rat cerebrum only three were present.

3   Denbufylline selectively inhibited one form of Ca^{2+}-independent, low K_m cyclic AMP phosphodiesterase. The form inhibited was one of two present in cardiac ventricle and the sole one in cerebrum. This form was not inhibited by cyclic GMP. The inotropic agent SK&F 94120 selectively inhibited the form of cyclic AMP phosphodiesterase which was inhibited by cyclic GMP present in cardiac ventricle. Theophylline and IBMX were relatively non-selective phosphodiesterase inhibitors.

4   Denbufylline was a less potent inhibitor of ligand binding to adenosine receptors than of cyclic AMP phosphodiesterase. This contrasted with theophylline, which had a higher affinity for adenosine receptors, and IBMX which showed no marked selectivity. Denbufylline, theophylline and IBMX all had a low affinity for the adenosine re-uptake site.

5   Denbufylline is being developed as an agent for the therapy of multi-infarct dementia. The selective inhibition of a particular low K_m cyclic AMP phosphodiesterase may account for the activity of this compound.

Introduction

This investigation was carried out to characterise further the activity profile of the alkylxanthine denbufylline (1,3-di-n-butyl-7-(2'-oxopropyl)-xanthine). This compound has previously been shown to increase the oxygen tension (Po_2) and function of partially ischaemic skeletal muscle (Angersbach & Ochlich, 1984), to enhance the retention of information (Nicholson *et al.*, 1988) and to improve the flow properties of blood (Jukna & Nicholson, 1987). It is presently undergoing clinical evaluation in dementia (see existing data generated by O'Connolly *et al.*, 1986; 1988).

The ability of alkylxanthines to inhibit the effects of adenosine and cyclic nucleotide phosphodiesterase has been known since the work of Ther and collaborators (1957) and Sutherland and coworkers (Sutherland & Rall, 1958; Butcher & Sutherland, 1962), respectively. It has more recently become apparent that alkylxanthines are competitive antagonists at extracellular adenosine receptors (Londos *et al.*, 1978; Burnstock & Meghji, 1981; Fredholm & Persson, 1982; Nicholson, 1982; Schwabe *et al.*, 1983) and may inhibit the cellular re-uptake of adenosine (Stefanovich, 1983; Fredholm & Lindstrom, 1986). Many alkylxanthines are more potent adenosine receptor antagonists than inhibitors of cyclic nucleotide phosphodiesterase (Smellie *et al.*, 1979; Wu *et al.*, 1982).

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In this study, the ability of denbufylline to inhibit cyclic nucleotide phosphodiesterase from rat cerebral cortex and cardiac ventricle was examined. In mammalian tissue cells, several distinct molecular forms of cyclic nucleotide phosphodiesterase exist (Thompson & Appleman, 1971; Weishaar *et al.*, 1985; Reeves *et al.*, 1987). Accordingly, we examined the ability of denbufylline to inhibit separated phosphodiesterase isoenzymes. For comparison, theophylline, 3-isobutyl-1-methyl-xanthine (IBMX) and the inotropic agent SK&F 94120 (Gristwood *et al.*, 1986a) were also evaluated.

On the basis of their ability to mediate inhibition or activation of adenylate cyclase and agonist order of potency, extracellular adenosine receptors have been subdivided into A_1 and A_2 receptors (van Calker *et al.*, 1979; Bruns *et al.*, 1986). We have therefore determined the affinity of denbufylline for both A_1 and A_2 adenosine receptors as well as the adenosine re-uptake site, using ligand binding techniques. In these assays, theophylline, IBMX, $R(-)$-N^6-(2-phenylisopropyl)-adenosine (R-PIA), N^6-cyclohexyladenosine (CHA), 5'-N-ethylcarboxamidoadenosine (NECA) and 2-nitrobenzylthioinosine (NBI) were additionally examined. Part of this work was presented at the British Pharmacological Society (Nicholson & Wilke, 1987).

Methods

The study was performed using tissue obtained from adult male Wistar rats killed by cervical dislocation. Tissues were rapidly removed and placed in the relevant buffer; all preparative procedures were performed at 4°C.

Separation of cyclic nucleotide phosphodiesterase activities

Cyclic nucleotide phosphodiesterase activities were separated by use of a modification of the methods of Reeves *et al.* (1987). Rat cerebral cortex and cardiac ventricle were homogenized, with a Potter S homogenizer, in 8–10 volumes of buffer (composition in mM: Bis-Tris 20, 2-mercaptoethanol 5, benzamidine 2, EDTA 2, phenylmethanesulphonylfluoride (PMSF) 0.05, sodium acetate 50; pH 6.5). Genapol x 0.15% (v/v) was included in the cerebral cortex assays to aid extraction of membrane bound phosphodiesterase (Strada *et al.*, 1984). The cerebral homogenates were filtered through glass wool, sonicated three times for 20 s at 100 W and filtered through a Millex GS 0.22 μm filter unit. This step was omitted for the ventricular homogenates. The

homogenates were then centrifuged (15,000 g for 15 min cardiac tissue; 100,000 g for 60 min cerebral tissue) and the supernatants applied to columns (17 cm × 1.6 cm) of Q sepharose Fast Flow (Pharmacia) pre-equilibrated with homogenization buffer. The flow rate was 70 ml h⁻¹. The column was washed with 100 ml of buffer and phosphodiesterase activities eluted with a linear gradient of 0.05–1.0 M sodium acetate in buffer. The eluate was collected in 7 ml fractions and analysed for phosphodiesterase activity. For storage, ethylene glycol was added to a final concentration of 30% (v/v) and fractions were stored at −20°C.

Assay of phosphodiesterase activity

Phosphodiesterase activity was assayed by the radiochemical procedure described by Arch & Newsholme (1976). The incubation medium consisted of 50 mM Tris/HCl, 6 mM $MgCl_2$, 2.5 mM dithiothreitol, 0.05 mg ml⁻¹ 5'nucleotidase, 0.23 mg ml⁻¹ bovine serum albumin and the relevant concentrations of [³H]-adenosine 3':5'-cyclic monophosphate ([³H]-cyclic AMP), [³H]-guanosine 3':5'-cyclic monophosphate ([³H]-cyclic GMP), cyclic GMP, calmodulin and Ca^{2+}; pH 7.5. The reaction was initiated by the addition of 30 μl of enzyme preparation and tubes were incubated for 10 min at 30°C. The reaction was terminated by the addition of 1.2 ml of an anion-exchange resin slurry (BIO-RAD AG 1 × 8, 200–400 mesh). The tubes were centrifuged (200 g for 15 min) and a portion (0.25 ml) of supernatant was added to 5 ml of scintillation fluid (Aqualuma). Radioactivity was measured in a liquid scintillation counter.

The total radioactivity in the substrate at zero time was measured in the supernatant when all the substrate had been converted into product by addition of commercial phosphodiesterase (10 μg). Assays were conducted in the linear reaction range, where less than 50% of the initial substrate was hydrolysed. All assays were repeated at least three times.

Determination of V_{max} and K_m for the phosphodiesterase activities and K_i values for inhibitors

For determination of V_{max} and K_m the concentration of label ([³H]-cyclic AMP or [³H]-cyclic GMP) was kept constant in the presence of increasing amounts of unlabelled substrate. K_i values were obtained by using substrate and inhibition concentrations in the range of K_m and K_i values, respectively. Data were analysed by least squares linear regression analysis. Results are expressed as means ($\pm$ s.e.mean).
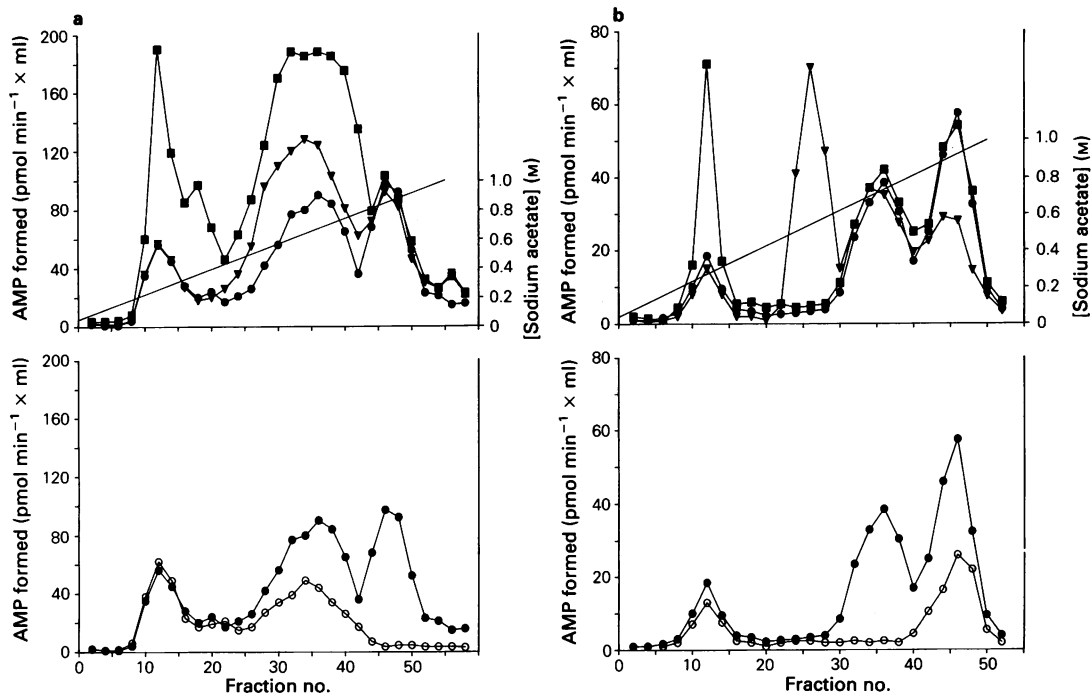
Figure 1 Separation profiles of cyclic nucleotide phosphodiesterase activities from rat cerebral cortex (a) and cardiac ventricle (b). Fractions were assayed with 1 μM cyclic AMP (●), 1 μM cyclic AMP and Ca^{2+}/calmodulin (0.1 mM per 75 u) (■), 1 μM cyclic AMP and 1 μM cyclic GMP (▼) and 1 μM cyclic AMP and 100 μM denbufylline (○).

Adenosine receptor and re-uptake site ligand binding assays

The A_1, [^{3}H]-CHA, binding assays and the A_2, [^{3}H]-NECA, binding assays were performed as described by Bruns *et al.* (1986). The [^{3}H]-NBI binding assays to the adenosine re-uptake site were carried out according to Marangos *et al.* (1982).

Brains were removed and one gram of the relevant tissues (whole brain minus cerebellum, for the [^{3}H]-CHA and [^{3}H]-NBI assays, and striata, for the [^{3}H]-NECA assays) were homogenized in 50 mM Tris-HCl (pH 7.4) supplemented with 10 μM PMSF for 30 s with a Potter S homogenizer, 10 strokes at 1500 r.p.m. The homogenate was diluted and centrifuged at 42,000 g for 18 min. The pellet was re-

Table 1 Kinetic properties of cyclic nucleotide phosphodiesterases from rat cerebrum and cardiac ventricle

Tissue	PDE activity	K_m cyclic AMP (μM)	K_m cyclic GMP (μM)	Effector
Cerebrum	Peak I	35	5	Ca^{2+}/calmodulin
	Peak II	40	4	Ca^{2+}/calmodulin, cyclic GMP
	Peak III	2	50	None
Cardiac ventricle	Peak I	2	2	Ca^{2+}/calmodulin
	Peak II	50*	40*	Cyclic GMP stimulated
	Peak III	1.6	>50	None
	Peak IV	0.8	5	Cyclic GMP inhibited

* Positive cooperativity.

suspended with the Potter S homogenizer in Tris-HCl buffer and the centrifugation step repeated. The pellet was then resuspended in Tris-HCl buffer, which contained 1 mM EDTA, and stored in plastic vials in liquid nitrogen. The membranes were used within one week.

All incubations were performed in triplicate in 12×75 mm polycarbonate tubes in a shaking water bath. Tubes contained 1 ml of 50 mM Tris-HCl (pH 7.4) and 2.5 or 0.1 units ml^{-1} of adenosine deaminase in the [^{3}H]-CHA and [^{3}H]-NECA assays, respectively. Adenosine deaminase was omitted from the [^{3}H]-NBI assays. Membranes obtained from 2 mg of tissue in 400 μl buffer were used in the [^{3}H]-CHA assay whilst for the [^{3}H]-NECA or [^{3}H]-NBI assays the membranes from 5 mg of original tissue were used suspended in 400 μl of buffer, respectively. The radiolabelled ligand concentrations were 2.5 nM [^{3}H]-CHA, 4 nM [^{3}H]-NECA and 0.6 nM [^{3}H]-NBI. Non-specific binding was determined by the addition of 50 μM PIA, 100 μM N^6-cyclopentyladenosine (CPA) and 5 μM NBI in the A_1, A_2 and re-uptake site assays, respectively. In the [^{3}H]-NECA assays, 50 nM CPA was included in all assays to eliminate A_1 binding (Bruns et al., 1986; this was confirmed by us in preliminary experiments).

Incubations were for 90 min at 37°C for the [^{3}H]-CHA assays and at 25°C for 60 min and 10 min for [^{3}H]-NECA and [^{3}H]-NBI assays, respectively. Preliminary experiments had shown that incubation times were sufficient to ensure an equilibrium in receptor occupation. Incubations were terminated by the addition of 3.8 ml ice cold buffer (50 mM Tris-HCl, 10 mM MgCl$_2$; pH 7.4) followed by rapid filtration through 2.4 cm GF/B filters under reduced pressure using a Millipore 1225 sampling manifold. Filters were washed with three 3.8 ml portions of buffer. The damp filters were placed in scintillation vials and 5 ml of Aqualuma added. The vials were left overnight, shaken and radioactivity counted in a liquid scintillation counter. All assays were repeated at least three times.

Data analysis

Specific receptor binding was defined as total binding minus non-specific binding. Composite K_D values for receptor sites occupied by radioligand were calculated from IC$_{50}$ values for displacement of unlabelled ligand using the equation: $K_D = IC_{50} - L$ where L = ^{3}H-ligand concentration. Kinetic constants for the inhibition of ligand binding to A_1 and A_2 receptors and the re-uptake site were determend as described by Bruns et al. (1986). Results are expressed as means ($\pm$ s.e.mean).

Materials

Denbufylline was synthesized in these laboratories by Dr J. Göring and SK&F 94120 ((5-acetimidophenyl)pyrazin-(1H)-one) was a gift from Smith Kline & French. The sources for the remaining compounds were as follows: [^{3}H]-CHA and [^{3}H]-NBI (New England Nuclear); [^{3}H]-NECA (Amersham/ Buchler); CHA (Boehringer, Mannheim); CPA (Research Biochemicals); R-PIA, NECA, NBI, IBMX, theophylline, adenosine deaminase (Type IV from bovine spleen) and PMSF (Sigma). GF/B filters were from Whatman and the Aqualuma scintillation fluid was from J.T. Baker.

Apart from NBI, all compounds were dissolved in buffer. NBI was dissolved in DMSO, appropriate quantities of the vehicle were added to the binding assay controls in these experiments.

Results

The phosphodiesterase activity profile obtained from rat cerebral and cardiac ventricle homogenates is shown in Figure 1. The kinetic properties of the separated activities and their modulation by Ca^{2+}/calmodulin and cyclic GMP are shown in Table 1.

Three activities were separated from cerebral tissue. The first two peaks eluted were Ca^{2+}/calmodulin-dependent and had a greater affinity for cyclic GMP than cyclic AMP. Hydrolysis of cyclic AMP by the second peak was activated by cyclic GMP. The third peak isolated from rat cerebral tissue had a lower K_m for cyclic AMP than for cyclic GMP and was not inhibited by cyclic GMP. The hydrolysis of cyclic nucleotides by all phosphodiesterase activities from cerebral tissue followed simple Michaelis-Menten kinetics.

Four peaks of phosphodiesterase activity were isolated from cardiac tissue. In contrast to cerebral tissue, only peak one was Ca^{2+}/calmodulin-dependent. In cardiac tissue, this form of phosphodiesterase had high and equal affinity for cyclic AMP and cyclic GMP. The second peak was dependent upon cyclic GMP for activation, showed positive cooperativity and had relatively low affinity for both cyclic GMP and cyclic AMP. Peaks III and IV both had a higher affinity for cyclic AMP than cyclic GMP, although the selectivity was much greater for peak III than peak IV. The hydrolysis of cyclic AMP by peak IV was inhibited by cyclic GMP. Peaks I, III and IV followed linear Michaelis-Menten kinetics.

The inhibition of the phosphodiesterase activities is shown in Table 2. Theophylline and IBMX were relatively non-selective inhibitors of all phosphodiesterase (PDE) activities, IBMX being more potent

Table 2 Inhibition constants of inhibitors of cyclic nucleotide phosphodiesterases from rat cerebrum and cardiac ventricle

Tissue	PDE activity	Denbufylline K_i (μM)	SK&F 94120 K_i (μM)	IBMX K_i (μM)	Theophylline K_i (μM)
Cerebrum	Peak I	N.I.	N.I.	44 ± 3	442 ± 28
	Peak II	(20)	N.I.	18 ± 5	240 ± 10
	Peak III	0.7 ± 0.2	N.I.	14 ± 6	142 ± 13
Cardiac ventricle	Peak I	(30)	N.I.	7 ± 2	108 ± 22
	Peak II	N.I.	N.I.	11 ± 2	220 ± 34
	Peak III	0.8 ± 0.3	N.I.	12 ± 4	176 ± 20
	Peak IV	(40)	1.5 ± 0.5	3.4 ± 0.8	58 ± 9

Data shown are mean $\pm$ s.e.mean ($n = 3$). (% inhibition) at 100 μM. N.I. = no inhibition up to 100 μM.

than theophylline. Denbufylline was a potent and selective inhibitor of peak III (cyclic AMP-specific PDE) from both tissues. The competitive nature of the inhibition of denbufylline is shown in Figure 2. SK&F 94120 selectively inhibited peak IV (cyclic GMP-inhibited PDE) eluted from cardiac tissue. This low K_m cyclic AMP phosphodiesterase was only weakly inhibited by denbufylline; it was absent in cerebral tissue.

The inhibition of [³H]-CHA, [³H]-NECA and [³H]-NBI binding is shown in Figure 3. The relative potencies of CHA, NECA, R-PIA and NBI served to characterize the [³H]-CHA as the A_1 receptor, the [³H]-NECA site as the A_2 receptor and the [³H]-NBI site as the re-uptake site. The orders of potency were CHA $\geq$ R-PIA > NECA for the A_1 site, NECA > R-PIA > CHA for A_2 binding and NBI $\gg$ R-PIA, CHA and NECA for the re-uptake site. Denbufylline displaced [³H]-CHA from A_1 and

Figure 3 The inhibition of [³H]-N⁶-cyclohexyladenosine ([³H]-CHA) (a) and [³H]-2-nitrobenzylthioinosine ([³H]-NBI) (c) binding to rat cortical membranes and of [³H]-5′-N-ethylcarboxamidoadenosine ([³H]-NECA) (b) binding to rat striatal membranes. Compounds examined as inhibitors of [³H]-CHA and [³H]-NECA binding were CHA (○), R(−)-N⁶-(2-phenylisopropyl)adenosine (△), NECA (□) and NBI (●). These compounds and additionally dipyridamole (■) were examined as inhibitors of [³H]-NBI binding. Results are presented as means with vertical lines showing s.e.mean ($n \geq 3$).
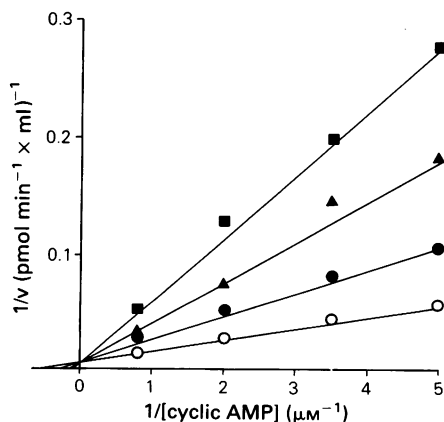
Figure 2 Competitive inhibition of fraction III cyclic nucleotide phosphodiesterase from rat cerebral cortex by denbufylline 1 (●), 3 (▲) and 10 (■) μM. (○) Control in absence of denbufylline.
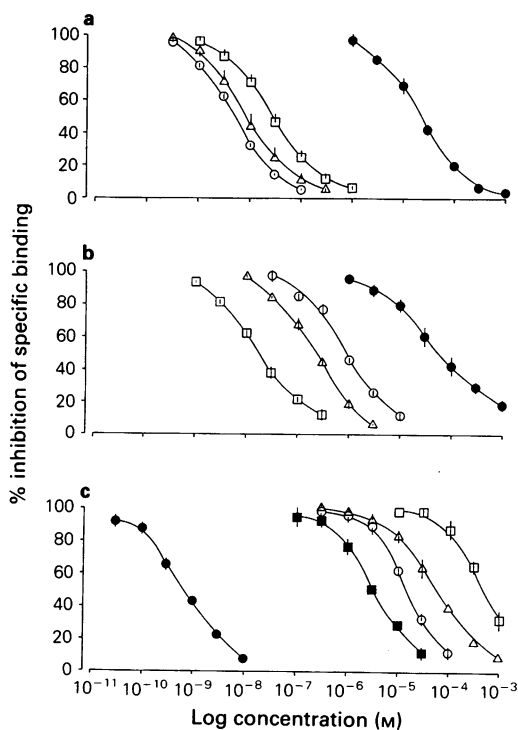
Table 3 The affinity of alkylxanthines for extra-cellular adenosine receptors and the adenosine re-uptake site (rat brain)

Compound	A_1 [³H]-CHA K_i (μM)	A_2 [³H]-NECA K_i (μM)	Uptake [³H]-NBI K_i (μM)
Denbufylline	20 ± 5	46 ± 2	200 ± 22
IBMX	1.8 ± 0.7	10 ± 3	690 ± 82
Theophylline	14 ± 1	24 ± 2	1100 ± 57

[³H]-NECA from A_2 receptors (Table 3). However, the affinity of denbufylline for the adenosine receptors was markedly lower than for cerebral low K_m cyclic AMP phosphodiesterase (Table 2). IBMX had a greater affinity for adenosine receptors than denbufylline, its affinity for A_1 receptors being greater than for A_2. IBMX was a less effective inhibitor of phosphodiesterase than of ligand binding to adenosine A_1 receptors. Theophylline had greater affinity for A_1 receptors than for A_2 receptors and for both extracellular adenosine receptors than for cyclic nucleotide phosphodiesterase. All three xanthines had a low affinity for the adenosine re-uptake site.

Discussion

The present study has demonstrated the presence of four cyclic nucleotide phosphodiesterase isoenzymes in rat heart. As judged by their kinetic properties and their sensitivity to the modulators Ca^{2+}/calmodulin and cyclic GMP and the selective inhibitor SK&F 94120, the phosphodiesterase activities in rat myocardium appear similar to those previously found in human and guinea-pig myocardium (Reeves et al., 1987). The previous study of Reeves et al. (1987) was the first demonstration of two forms of Ca^{2+}-independent cyclic nucleotide phosphodiesterase with a high affinity for cyclic AMP in cardiac muscle. These two isoenzymes may, however, be present in many tissues. Two such low K_m cyclic AMP phosphodiesterases have previously been separated from calf liver homogenates (Yamamoto et al., 1984) and adipocytes (Weber & Appleman, 1982). Their detection has been made possible by the improved separation properties of modern chromatographic medium (Reeves et al., 1987). The nomenclature used for the classification of phosphodiesterase isoenzymes varies between groups. In this paper, we have based our descriptions of the phosphodiesterase isoenzymes upon their order of elution by QAE-Sepharose with an increasing salt gradient, their substrate affinities, effector characteristics and the activity of inhibitors. Thus in our hands, cardiac peak III is a Ca^{2+}-independent low K_m cyclic AMP

phosphodiesterase which is not inhibited by cyclic GMP. This is similar to the third elution peak obtained by Reeves et al. (1987) from guinea-pig heart and the fourth elution peak obtained by this group from human heart. Reeves et al. (1987) based their nomenclature on data obtained from human heart and termed this phosphodiesterase PDE IV. The cardiac peak IV isoenzyme, in the present study, was termed PDE III by Reeves et al. (1987), because of its order of elution from human tissue. In our opinion, the most useful classification of phosphodiesterase isoenzymes presently available is that recently described by Beavo (1988). This classification assumes there are families of phosphodiesterase isoenzymes. According to this nomenclature, cardiac peak I phosphodiesterase and cerebral peak I and II phosphodiesterase belong to the calmodulin-stimulated family. Cardiac peak II phosphodiesterase is a cyclic GMP-stimulated phosphodiesterase. Cerebral peak III and cardiac peak III belong to the cyclic AMP-specific phosphodiesterase family. This is the isoenzyme that most investigators have referred to in the past as the 'low K_m' cyclic AMP PDE. It is selectively inhibited by Ro 20-1724 and by rolipram and, as shown in this study, by denbufylline. Cardiac peak IV is a member of the cyclic GMP-inhibited phosphodiesterase family. This isoenzyme is selectively inhibited by SK&F 94120 and by a number of positive inotropic agents (Weishaar et al., 1985).

The present investigation has shown that rat cerebral tissue, in contrast to cardiac ventricle, contains not more than three phosphodiesterases, only one of which is a Ca^{2+}-independent isoenzyme with a high affinity for cyclic AMP. The detection of two forms of Ca^{2+}/calmodulin-stimulated phosphodiesterase in cerebral tissue confirms previous findings (Kincaid et al., 1984; Strada et al., 1984). Both Ca^{2+}/calmodulin-stimulated phosphodiesterases have low affinity for cyclic AMP. This contrasts with the cardiac ventricle, where the Ca^{2+}/calmodulin-stimulated enzyme, as shown in the present study and previously by others (Reeves et al., 1987) has a high affinity for cyclic AMP.

With the presently available separation techniques it is not possible to ensure that all forms of phosphodiesterase will be resolved, that the isoenzymes will be separated in their physiological state or that the relative recovery of the different phosphodiesterase isoenzymes will be identical between tissues (see Appleman et al., 1982, for further discussion of these points). However, there is strong evidence that two Ca^{2+}-independent phosphodiesterases with a high affinity for cyclic AMP do co-exist in tissues and that they are not simply separation artefacts. The two such cyclic AMP low K_m isoenzymes identified by Reeves et al. (1987) and ourselves display simple

Michaelis-Menten kinetics, suggesting that the hyperbolic kinetics for cardiac Ca^{2+}-independent low K_m cyclic AMP phosphodiesterase observed previously (Weishaar et al., 1985; 1986) were due to the incomplete separation of two isoenzymes. This suggestion is supported by the findings of ourselves and others (Yamamoto et al., 1984; Reeves et al., 1987) that selective inhibitors of the two cyclic AMP phosphodiesterase subtypes can be identified. The presence of two forms of cyclic AMP phosphodiesterase, which differ in their tissue distribution and which can be selectively inhibited, suggests that compounds can be developed to influence selectively tissue function.

In contrast to theophylline and IBMX, which are non-selective inhibitors of phosphodiesterase isoenzymes (Davis, 1984; Lugnier et al., 1986; Weishaar et al., 1986), denbufylline selectively inhibits one form of Ca^{2+}-independent cyclic AMP phosphodiesterase in both rat heart and brain. This is the first account of an alkylxanthine selectively inhibiting a low K_m cyclic AMP phosphodiesterase. Interestingly, denbufylline has weak inotropic activity (Nicholson, unpublished information) and little effect on arterial blood pressure (Angersbach & Ochlich, 1984; Nicholson & Angersbach, 1986). In contrast, compounds such as SK&F 94120, which selectively inhibit the denbufylline insensitive, cyclic GMP inhibited low K_m cyclic AMP phosphodiesterase, produce positive inotropy (Gristwood et al., 1986b) and have marked vasodilator activity (Gristwood et al., 1986a). These data support the hypothesis that selective inhibitors of the different Ca^{2+}-independent low K_m cyclic AMP phosphodiesterases can selectively affect tissue function. Denbufylline improves recollection of information in mice and gerbils in which a learning or memory deficit has been induced by forebrain ischaemia (Nicholson et al., 1988) and, in this regard, cyclic AMP has been postulated to influence synaptic efficacy and memory formation (Goelet et al., 1986; Gray & Johnston, 1987). Consequently, selective inhibition of a cerebral cyclic AMP phosphodiesterase may account for the enhancing effect of denbufylline on learning and memory.

In addition to their ability to inhibit the hydrolysis of cyclic nucleotides, alkylxanthines are potent adenosine receptor antagonists (Londons et al, 1978; Burnstock & Meghji, 1981; Schwabe et al., 1985). The present study has shown that denbufylline does bind to adenosine A_1 and A_2 receptors. However, it is a more potent inhibitor of cerebral, high-affinity cyclic AMP phosphodiesterase than of ligand binding to adenosine receptors. Selective adenosine receptor antagonists, such as theophylline and caffeine, reduce cerebral blood flow (Mathew et al., 1983; Grome & Stefanovich, 1986). Denbufylline, on the other hand, increases the blood flow and oxygen tension of the oligaemic cortex (Nicholson & Angersbach, 1986) at doses which enhance retention of information (Nicholson et al., 1988), which indicates that it is not effectively antagonising the effects of adenosine at these doses. Further evidence for this is that denbufylline, at doses greater than those which enhance learning or memory formation, neither potentiates nor attenuates ischaemia-induced neuronal damage (Jukna & Nicholson, unpublished information). Adenosine antagonists and uptake inhibitors potentiate (Jarrott & Domer, 1980; Rudolphi et al., 1987a) and reduce (Rudolphi et al., 1987b) ischaemic neuronal damage, respectively. The lack of an effect of denbufylline is presumably a reflection of the relatively low affinity of denbufylline for neuronal adenosine receptors and the re-uptake site.

In summary, this investigation has confirmed previous findings (Reeves et al., 1987) of four cyclic nucleotide phosphodiesterase isoenzymes in cardiac tissue. The same separation techniques reveal only three isoenzymes in cerebral tissue. Denbufylline is an alkylxanthine which selectively inhibits one form of Ca^{2+}-independent low K_m cyclic AMP phosphodiesterase. This is one of two such isoenzymes in cardiac tissue and the sole one in the cerebrum. Denbufylline is a weak inhibitor of other forms of cyclic nucleotide phosphodiesterase and has a relatively low affinity for extracellular adenosine receptors and the re-uptake site. The selective inhibition of a cyclic AMP-specific phosphodiesterase may largely be responsible for the pharmacological effects of this compound, particularly as regards the increase in nutrition and metabolism of the oligaemic cortex and the enhancement of the retention of information.

References

ANGERSBACH, D. & OCHLICH, P. (1984). The effect of 7-(2' oxo-propyl)-1,3-di-n-butyl-xanthine (BRL 30892) on ischaemic skeletal muscle pO_2, pH and contractility in cats and rats. Arzneim. Forsch., 34, 1274–1278.

APPLEMAN, H.M., ARIANO, M.A., TAKEMOTO, D.J. &

WHITSON, R.H. (1982). Cyclic nucleotide phosphodiesterases. In Handb. Exp. Pharmacol., Vol. 58: Cyclic Nucleotides. ed. Nathanson, J.A. & Kebabian, J.W., pp. 261–300. Berlin, Heidelberg: Springer-Verlag.

ARCH, J.R.S. & NEWSHOLME, E.A. (1976). Activities and

some properties of adenylate cyclase and phosphodiesterase in muscle liver and nervous tissue from vertebrates and invertebrates in relation to the control of the concentration of adenosine 3'-5'-cyclic monophosphate. *Biochem. J.*, **158**, 603–622.

BEAVO, J.A. (1988). Multiple isoenzymes of cyclic nucleotide phosphodiesterase. In *Advances in Second Messenger and Phosphoprotein Research*, Vol. 22, ed. Greengard, P. & Robeison, G.A., pp. 1–38. New York: Raven Press.

BRUNS, R.F., LU, G.H. & PUGSLEY, T.A. (1986). Characterization of the A_2 adenosine receptor labelled by [^{3}H]-NECA in rat skeletal membranes. *Molec. Pharmacol.*, **29**, 331–346.

BURNSTOCK, G. & MEGHJI, P. (1981). Distribution of P1 and P2 purinoceptors in the guinea-pig and frog heart. *Br. J. Pharmacol.*, **73**, 879–885.

BUTCHER, R.W. & SUTHERLAND, E.W. (1962). Adenosine 3',5'-phosphate in biological materials. *J. Biochem.*, **237**, 1244–1250.

DAVIS, C.W. (1984). Assessment of selective inhibition of rat cerebral cortical calcium-independent and calcium-dependent phosphodiesterases in crude extracts using deoxycyclic AMP and potassium ions. *Biochem. Biophys. Acta*, **797**, 354–362.

FREDHOLM, B.B. & PERSSON, C.G.A. (1982). Xanthine derivatives as adenosine receptor antagonists. *Eur. J. Pharmacol.*, **81**, 673–676.

FREDHOLM, B.B. & LINDSTROM, K. (1986). The xanthine derivative 1-(5'-oxohexyl)-3-methyl-7-propyl-xanthine (HWA 285) enhances the action of adenosine. *Acta Pharmacol. Toxicol.*, **58**, 187–192.

GOELET, P., CASTELLUCCI, U.F., SCHACHER, S. & KANDEL, E.R. (1986). The long and the short of long-term memory—a molecular framework. *Nature*, **322**, 419–422.

GRAY, R. & JOHNSTON, D. (1987). Noradrenaline and β-adrenoceptor agonists increase activity of voltage-dependent calcium channels in hippocampal neurons. *Nature*, **327**, 620–622.

GRISTWOOD, R.W., EDEN, N.J., OWEN, D.A.A. & TAYLOR, E.M. (1986a). Pharmacological studies with SK&F 94120, a novel inotropic agent with vasodilator activity. *J. Pharm. Pharmacol.*, **38**, 452–459.

GRISTWOOD, R.W., ENGLISH, T.A.H., WALLWORK, J., SAMPFORD, K.A. & OWEN, D.A.A. (1986b). Analysis of responses to a selective phosphodiesterase III inhibitor, SK&F 94120, on isolated myocardium, including human ventricular myocardium from "endstage" failure patients. *J. Cardiovasc. Pharmacol.*, **9**, 719–727.

GROME, J.J. & STEFANOVICH, V. (1986). Differential effects of methylxanthines on local cerebral blood flow and glucose utilization in the conscious rat. *Naunyn-Schmiedebergs Arch. Pharmacol.*, **333**, 172–177.

JARROTT, D.M. & DOMER, F.R. (1980). A gerbil model of cerebral ischemia suitable for drug evaluation. *Stroke*, **11**, 203–209.

JUKNA, J.J. & NICHOLSON, C.D. (1987). The effect of denbufylline on the viscosity of rat whole blood and on the deformability (filterability) of rat blood cell suspensions. *Naunyn-Schmiedebergs Arch. Pharmacol.*, **335**, 445–448.

KINCAID, R.L., MANGANIELLO, V.C., ODYA, C.E., OSBORNE, JR., J.C., STITH-COLEMAN, I.E., DANELLO, M.A. & VAUGHAN, M. (1984). Purification and proper-

ties of calmodulin-stimulated phosphodiesterase from mammalian brain. *J. Biol. Chem.*, **259**, 5158–5166.

LONDOS, C., COOPER, D.M.F., SCHLEGEL, W. & RODBELL, M. (1978). Adenosine analogs inhibit adipocyte cyclase by a GTP-dependent process: Basis for actions of adenosine and methylxanthines on cyclic AMP production and lipolysis. *Proc. Natl. Acad. Sci. U.S.A.*, **75**, 5362–5366.

LUGNIER, C., SCHOEFFLER, P., LeBEC, A., STROUTHOU, E. & STOCLET, J.C. (1986). Selective inhibition of cyclic nucleotide phosphodiesterase of human, bovine and rat aorta. *Biochem. Pharmacol.*, **35**, 1743–1751.

MARANGOS, P.J., PATEL, J., CLARK-ROSENBERG, R. & MARTINO, A.M. (1982). [^{3}H]nitrobenzylthioinosine binding by a probe for the study of adenosine uptake sites in the brain. *J. Neurochem.*, **39**, 184–191.

MATHEW, R.J., BARR, D.L., WEINMAN, M.L. (1983). Caffeine and cerebral blood flow. *Br. J. Phsychiat.*, **143**, 604–608.

NICHOLSON, C.D. (1982). The effect of reserpine pretreatment, hexobendine and methylxanthines on the inotropic response to adenosine of guinea-pig isolated atria. *Naunyn-Schmiedebergs Arch. Pharmacol.*, **319**, R38.

NICHOLSON, C.D. & ANGERSBACH, D. (1986). Denbufylline (BRL 30892) – A novel drug to alleviate the consequences of cerebral ischaemia. In *Pharmacology of Cerebral Ischaemia*. ed. Krieglstein, J., pp. 331–396. Amsterdam: Elsevier.

NICHOLSON, C.D. & WILKE, R. (1987). The selective inhibition of a low K_m cyclic AMP phosphodiesterase from rat cerebral cortex by denbufylline. *Br. J. Pharmacol.*, **92**, 680P.

NICHOLSON, C.D., JUKNA, J.J., WILKE, R. & ANGERSBACH, D. (1988). The effect of denbufylline in passive avoidance trials in gerbils – following transient forebrain ischaemia – and in mice. *Drug Develop. Res.*, **14**, 349–352.

O'CONNOLLY, M., MAYER, M.-E., WOLF, D., BRETT, M. & GREB, W.H. (1986). Efficacy and tolerance of denbufylline (BRL 30892) in patients with cerebrovascular disease – an investigational study with a new agent. In *Pharmacology of Cerebral Ischaemia*. ed. Krieglstein, J., pp. 440–444. Amsterdam: Elsevier.

O'CONNOLLY, M., DIERDORFF, D., GREB, W.H., MAYER, M.-E. & WOLF, D. (1988). Efficacy of denbufylline in patients with multi-infact dementia. *Drug Develop. Res.*, **14**, 195–198.

REEVES, M.L., LEIGH, .B.K. & ENGLAND, P.J. (1987). The identification of a new cyclic nucleotide phosphodiesterase acitivity in human and guinea-pig cardiac ventricle. *Biochem. J.*, **241**, 535–541.

RUDOLPHI, K.A., KEIL, M. & HINZE, H.-J. (1987a). Effect of theophylline on ischemically induced hippocampal damage in mongolian gerbils: A behavioural and histopathological study. *J. Cereb. Blood Flow Metab.*, **7**, 74–81.

RUDOLPHI, K.A., KEIL, M. & WESTHÖFER, U. (1987b). Effects of phenobarbital and propentophylline on ischemically induced hipocampal damage in gerbils. *J. Cereb. Blood Flow Metab.*, **7**, S 78.

SCHWABE, U., UKENA, D. & LOHSE, M.J. (1985). Xanthine derivatives as antagonists at A_1 and A_2 adenosine receptors. *Naunyn-Schmiedebergs Arch. Pharmacol.*, **330**, 212–221.

SMELLIE, F.W., DAVIS, C.W., DALY, J.W. & WELLS, J.N. (1979). Alkylxanthines: Inhibition of adenosine elicited accumulation of cyclic AMP in brain slices and brain phosphodiesterase activity. *Life Sci.*, **24**, 2475–2482.

STEFANOVICH, V. (1983). Uptake of adenosine by isolated bovine cortex microvessels. *Neurochem. Res.*, **8**, 1459–1469.

STRADA, S.J., MARTIN, M.W. & THOMPSON, W.J. (1984). General properties of multiple molecular forms of cyclic nucleotide phosphodiesterase in the nervous system. In *Advances in Cyclic Nucleotide and Protein Phosphorylation Research*, Vol. 16. ed. Strada, S.J. & Thompson, W.J. pp. 13–29. New York: Raven Press.

SUTHERLAND, E.W. & RALL, T.W. (1958). Fractionation and characterization of a cyclic adenosine nucleotide formed by tissues particles. *J. Biol. Chem.*, **232**, 1077–1091.

THER, L., MUSCHAWECK, R. & HERGOTT, J. (1957). Antagonismus zwischen Adenosin und Methyl-xanthin am Reizleitungssystem des Herzens. *Arch. Exp. Pathol. Pharmacol.*, **231**, 586–590.

THOMPSON, W.J. & APPLEMAN, M.M. (1971). Characterization of cyclic nucleotide phosphodiesterases of rat tissues. *J. Biol. Chem.*, **246**, 3145–3150.

VAN CALKER, D., MÜLLER, M. & HAMPRECHT, B. (1979). Adenosine regulates via two different types of receptors, the accumulation of cyclic AMP in cultured brain cells. *J. Neurochem.*, **33**, 999–1005.

WEBER, H.W. & APPLEMAN, M.M. (1982). Insulin-dependent and insulin-independent low K_m cyclic AMP phosphodiesterase from rat adipose tissue. *J. Biol. Chem.*, **257**, 5539–5541.

WEISHAAR, R.E., CAIN, M.H. & BRISTOL, J.A. (1985). A new generation of phosphodiesterase inhibitors: Multiple molecular forms of phosphodiesterase and its potential for drug selectivity. *J. Med. Chem.*, **28**, 537–545.

WEISHAAR, R.E., BURROWS, S.D., KOBYLARZ, D.C., QUADE, M.M. & EVANS, D.B. (1986). Multiple molecular forms of cyclic nucleotide phosphodiesterase in cardiac and smooth muscle and in platelets. *Biochem. Pharmacol.*, **35**, 781–800.

WU, P.H., PHILLIS, J.W. & NYE, M.J. (1982). Alkylxanthines as adenosine receptor antagonists and membrane phosphodiesterase inhibitors in central nervous tissue: Evaluation of structure-activity relationships. *Life Sci.*, **31**, 2857–2867.

YAMAMOTO, T., LIEBERMAN, F., OSBORNE, J.C., MANGANIELLO, V.C., VAUGHAN, M. & HIDAKA, H. (1984). Selective inhibition of two soluble adenosine cyclic 3′,5′-phosphate phosphodiesterases partially purified from calf liver. *Biochem.*, **23**, 670–675.