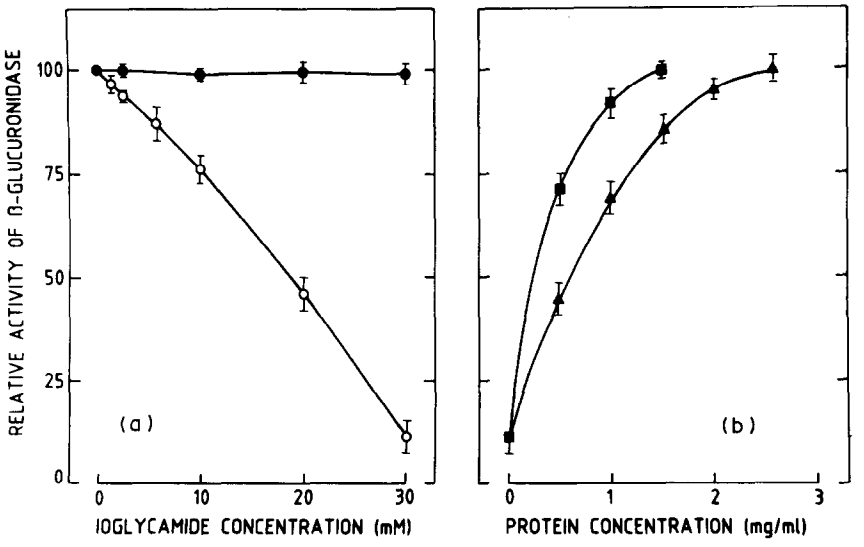

Fig. 1. (a) Effect of ioglycamide on the β-glucuronidase activity, (closed circles) in liver homogenates (final protein concentration of 4 mg/ml), or (open circles) in a purified enzyme preparation (final protein concentration of approximately 5 μg/ml). Mean values ±1 S.D. (N = 6) are given. (b) Effect of bovine serum albumin (closed squares) or liver cytosol (closed triangles) on the activity of a purified β-glucuronidase preparation, in the presence of ioglycamide (30 mM) as the inhibitory substance. Mean values ±1 S.D. are given.

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Purine effects on (^{3}H)-clonidine binding to rat brain

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A potentiation of the effects of noradrenaline by adenosine has been shown in electrophysiological experiments [1]. In the vas deferens Holck and Marks [2] demonstrated interactions of adenosine on noradrenaline evoked responses as well as the rate of resensitisation, findings which have recently been extended by Long and Stone [3] with the recognition that adenosine can potentiate the effects of noradrenaline at α_1 receptors while modifying the rate of resensitisation of noradrenaline receptors by an action at α_2 receptors.

Watanabe and colleagues [4–6] have recently demonstrated that incubation with these purines will induce binding to the vas deferens of radiolabelled clonidine. This latter compound is normally regarded as a selective ligand for α_2 receptors which are normally considered to be only presynaptic in the vas and which are not known to mediate any post-junctional effects of catecholamines in this system (but see ref 3). Such an induction or enhancement in the numbers of α_2 receptors by purines could be of major significance in the central nervous system in view of the importance of central α_2 sites in the regulation of sympathetic outflow to the cardiovascular system [7, 8], and certain aspects of behaviour [9, 10].

Membrane preparation

Male Wistar Porton rats weighing 200–250 g were killed by stunning and decapitation. The brain was then homogenized in 10 vol. ice-cold sucrose (800 r.p.m.; 10 strokes). The homogenate was diluted to 20 vol. and centrifuged at 1000 *g* for 10 min at 4°. The supernatant was then re-centrifuged at 40,000 *g* for 30 min at 4°, and the pellet from this spin was washed three times by resuspending in 25 vol. Tris buffer (50 mM, pH 7.4) and centrifuging at 40,000 *g* for 30 min.

The final pellets were resuspended in 12.5 vol. Tris buffer to produce 3.8 ml aliquots for the binding assays, plus 2 × 0.1 ml samples for protein determination. Samples were stored at −20° until used, but for no longer than 4 weeks.

(^3H)-clonidine binding

The binding characteristics of (^{3}H)-clonidine were normally determined at a range of final concentrations from 0.1 to 16 nM. Non-specific binding was estimated by displacement using 50 μM unlabelled clonidine. The reaction was carried out at room temperature by adding 0.1 ml of tissue suspension to 0.4 ml of reaction mixture. The reaction was stopped after 20 min by two additions of 2 ml Tris buffer and immediate filtering on Whatman GF/B filters. Filters were further washed by 3 × 5 ml aliquots of buffer and radioactivity measured by liquid scintillation spectrometry. In the preincubation experiments, the compounds of interest were incubated with tissue suspension for 30 min before the addition of (^{3}H)-clonidine.

Displacement by various ligands was determined at concentrations of ligand between 10 nM and 100 μM. When less than 50% displacement was obtained at the 100 μM level, the percentage displacement at this concentration has been entered in Table 1.

Protein was determined by the method of Lowry *et al.* [11], using bovine serum albumin as standard.

All tests were conducted in duplicate and results are expressed as the mean ± S.E.M. for N separate experiments. The K_d and B_{max} values were estimated by Scatchard analysis, and statistical analysis was performed using either Student's *t*-test for comparison of means, or a paired *t*-test.

Chemicals

[Benzene-^{3}H]-clonidine, sp.act. 52–66 Ci/mmol and N^6-[adenine-2,8-^{3}H]-cyclohexyladenosine, sp.act. 13–25 Ci/mmol were obtained from New England Nuclear.

The following drugs were gifts from the manufacturers: clonidine HCl and B-HT933 (2-amino-6-ethyl-5,65,7,8-tetrahydro-4H-oxazolo-[4,5d]-axepine) (Boehringer); 5′ *N*-ethylcarboxamide adenosine, NECA (Byk Gulden); prazosin (Pfizer); nifedipine (Bayer).

Drug treatments

Rats described as reserpinised were treated with reserpine, 5 mg/kg i.p. 48 and 24 hr before sacrifice.

Chronic treatment with L-N^6-phenylisopropyladenosine (L-PIA) or diazepam was carried out for 7 days using 0.5 mg/kg/day i.p. and 1 mg/kg/day respectively, the last dose being made 24 hr before sacrifice. L-PIA was dissolved initially in ethanol and then diluted with sterile saline for injection. Control animals in these experiments received an equivalent injection of ethanol in saline. Diazepam was administered as Valium for injection from commercially prepared sterile ampoules.

Clonidine binding to homogenates of whole rat brain was

Table 1. Displacement of specific (^{3}H)-clonidine binding

Compound	IC_{50} (μM)	Clonidine binding (% control) (100 μM compound)
Adenosine	–	91 ± 3.5 (4)*
2-chloroadenosine	–	89 ± 1.7 (6)†
CHA	–	90 ± 2.4 (3)
L-PIA	–	87 ± 3.45 (4)†
NECA	–	98 ± 4.0 (3)
Cyclic AMP	–	88 ± 5.8 (3)
ATP	–	79 ± 3.82 (6)†
β,γ-methylene ATP	–	70 ± 6.64 (8)†
α,β-methylene ATP	–	72 ± 4.0 (4)†
Theophylline	–	97 ± 6.4 (4)
Yohimbine	0.052	
B-HT 933	0.27	
Prazosin	8.2	
Isoprenaline	10.2	
Propranolol	–	77 ± 6.5 (4)†
Nifedipine	–	99 ± 2.1 (3)‡
Verapamil	9.2	
Inorganic phosphate	–	88 ± 5.7 (4)
Pyrophosphate	–	78 ± 3.4 (6)†
Trimetaphosphate	–	97 ± 6.2 (4)
Tripolyphosphate	–	73 ± 6.1 (4)†

* Mean ± S.E.M. (N).
† Significant displacement at $P < 0.05$ (paired *t*-test).
‡ At 50 μM nifedipine (limited by solubility).

found to be saturable, reversible and exhibited a K_d of 2.2 ± 0.13 (N = 10)nM and a B_{max} of 143.0 ± 18.25 (10) fmol/mg protein.

The effects of a variety of agents on the displacement of specific clonidine binding are summarized in Table 1, which confirms that the binding was most sensitive to α_2 compounds.

Of the various purine compounds tested the nucleotides, ATP, β,γ-methylene ATP and α,β-methylene ATP at the relatively high concentration of 10^{-4} M were able to produce a significant inhibition of clonidine binding. Of the nucleoside analogues of adenosine, only 2-chloroadenosine and L-PIA produced any significant effect (Table 1).

A series of phosphate complexes were also tested (Table 1). It will be seen that both the pyrophosphate and tripolyphosphate anions were as effective as ATP and its stable analogues in displacing clonidine binding, while inorganic phosphate and trimetaphosphate were largely inactive.

Scatchard analysis of the effects of β,γ-methylene ATP on the clonidine binding showed a significant increase of K_d and a lower mean B_{max} for the clonidine binding though the latter change was not statistically significant (Table 2). Verapamil produced a clearly significant increase in K_d of clonidine (Table 2).

Changing incubation conditions for example by pre-incubating with compounds of interest before adding labelled clonidine, using Krebs bicarbonate buffer instead of Tris, or using animals pretreated with reserpine had no significant effect on the results, although greater mean displacements of clonidine by purines were now recorded (Table 3).

The effects of treating animals chronically with diazepam or L-PIA administered intraperitoneally on clonidine binding are summarized in Table 4. While there was a reduction in both the K_d and B_{max} values after diazepam treatment, these did not reach statistical significance. There was, however, a significant increase in both parameters following chronic administration of L-PIA.

Discussion

While the triphosphate nucleotide analogues of adenosine were more effective than the nucleosides in displacing (^{3}H)-clonidine, there is some doubt as to the underlying mechanism, since some phosphate chains were equally effective. The importance of the configuration of the phosphate side chain in nucleotide actions has been emphasised previously [12–14]. Both nifedipine and verapamil can block the activation of postjunctional α_2 receptors in vascular muscle whereas nifedipine has no effect on the presynaptic α_2 sites on nerve terminals [15, 16]. The neuronal presynaptic α_2 sites are blocked by verapamil [15]. Since only verapamil affected clonidine binding here, this may imply that the clonidine binding sites examined in the present work, at least in the acute experiments, were entirely presynaptic in nature, or that the presynaptic receptors were present in such abundance that any induced changes of postjunctional sites were masked.

It is clear that neither adenine nucleosides or nucleotides have the same qualitative effects in acute experiments on [^{3}H]-clonidine binding in brain that were seen in vas deferens at the same temperature and using the same range of incubation conditions or reserpinisation [4–6]. Thus, while clonidine binding was enhanced in vas deferens, it was unchanged or slightly diminished in cerebral tissue.

Treatment with L-PIA for 7 days did, however, yield an elevation of both K_d and B_{max} values for clonidine.

Table 4. Effect of chronic treatments with diazepam (1 mg/kg/day for days) or L-PIA (0.5 mg/kg/day for 7 days) on specific (^{3}H)-clonidine binding

Treatment	(^{3}H)-clonidine binding	
	K_d	B_{max}
Vehicle controls	2.40 ± 0.15 (15)*	144 ± 8.96 (15)
Diazepam	2.29 ± 0.18 (6)	117 ± 7.9 (6)
L-PIA	2.92 ± 0.15 (13)†	170 ± 4.6 (14)‡

* Mean ± S.E.M. (N).
† $P < 0.05$ compared with control.
‡ $P < 0.02$ compared with control.

Table 2. Effects of β,γ-methylene ATP and verapamil on binding parameters of clonidine

Compound	Concentration (μM)	K_d (nM)	Clonidine binding B_{max} (fmol/mg protein)
Control	—	2.2 ± 0.13 (10)*	143 ± 18.25 (10)
β,γ-Methylene ATP	100	2.9 ± 0.32 (6)†	112 ± 10.4 (6)
Verapamil	5	5.2 ± 0.28 (5)†	137 ± 16 (5)
	10	7.9 ± 0.30 (4)†	120 ± 11 (4)
	100	27 ± 3.1 (6)†	110 ± 20 (6)

* Mean ± S.E.M. (N).
† $P < 0.05$ compared with control.

Table 3. Effect of pre-incubation with purines on [^{3}H]-clonidine binding

Compound	Concentration (μM)	Specific clonidine binding (% control)		
		Tris medium	Krebs medium	Tris (reserpinised animals)
Adenosine	100	90 ± 3.4 (4)*	92 ± 4.2 (3)	87 ± 4.6 (4)
2-Chloroadenosine	10	92 ± 1.8 (4)†	96 ± 3.9 (3)	89 ± 5.4 (3)
	100	67 ± 5.8 (5)†	78 ± 5.0 (3)†	55 ± 5.3 (4)†
β,γ-Methylene ATP	100	61 ± 3.2 (5)†	64 ± 4.5 (3)†	52 ± 4.9 (5)†

* Mean ± S.E.M. (N).
† $P < 0.05$ compared with controls (100%).

Peripherally administered L-PIA is known to cross the blood–brain barrier and produce behavioural changes including anti-convulsant effects and a depression of locomotor activity [17–19], the latter response being noted also in the present study. It is therefore possible that the observed increase of clonidine binding may be related to that reported in the vas deferens, but that either the change of clonidine receptor number occurs more slowly than in the vas, or that a larger change is required of a subpopulation of clonidine sites (e.g. postjunctional) because of the predominance of an unchanged subgroup (e.g. prejunctional). The problems of interpretation however, are much greater in chronic experiments, and it is not yet possible to eliminate other explanations of the present observation. For example, since purines, including L-PIA, are potent inhibitors of transmitter release [20], it is possible that the chronic suppression of presynaptic release by L-PIA has resulted secondarily in an up-regulation of α_2 receptors.

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Inhibition by benzodiazepines and β-carbolines of brief (5 seconds) synaptosomal accumulation of [^{3}H]-adenosine

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Adenosine is probably an important regulator of neuronal function in both the peripheral and central nervous systems [1, 2], and it has been proposed that a variety of compounds may owe at least part of their pharmacological activity to an effect on the uptake and thus inactivation of adenosine [3]. Much interest has centred on the benzodiazepines, which suppress significantly the uptake of adenosine into CNS preparations at therapeutically relevant concentrations [4–6]. However, it has been pointed out that with the incubation times usually used in such studies, the influence of metabolism by adenosine deaminase and adenosine kinase [7] and the loss of adenosine by efflux may reduce the reliability of the results as a true reflection of uptake [8, 9]. Effects on uptake attributed to compounds might for example be due to changes of metabolic processes, or, conversely, the potency of substances as uptake inhibitors may be under-estimated because the uptake process is not rate-limiting under the conditions of the assay. The present study was therefore undertaken to examine the effects of benzodiazepines on the uptake of adenosine into cerebral synaptosomes after an incubation period of only 5 seconds [10]. We have adopted the term "brief" accumulation to refer to this short time scale. A range of benzodiazepine related compounds have been tested, in order to assess the relevance of any observed effects to the behavioural actions of these substances.

Cerebral synaptosomes were prepared from male Wistar rats weighing 150–200 g as previously described [11, 12]. Briefly, animals were killed by stunning and cervical dislocation, and the cerebral cortex was dissected out and homogenised at 800 r.p.m. using a Potter Teflon/glass homogeniser on ice. All subsequent processing was performed on ice or at 2°. The homogenate was centrifuged at 900 *g* for 10 min. The 900 *g* supernatant was layered directly onto 1.2 M sucrose and centrifuged at 160,000 *g* for 15 min. The 0.32 M/0.8 M sucrose interphase was collected, diluted 1:3 with 0.32 M sucrose and layered onto 0.8 M sucrose. A further 15 min centrifugation at 16,000 *g* yielded the synaptosomal preparation (pelleted) which was resuspended in 0.32 M sucrose to form a suspension of 100–150 mg initial wet weight ml^{-1}.

For the brief accumulation of ^{3}H-adenosine into the synaptosomes a 250 μl aliquot of the synaptosomal suspension was added to oxygenated Krebs–Henseleit buffer containing test compounds (total volume = 2 ml) and preincubated for 1 hr at 25°. ^{3}H-Adenosine (1 μM, specific activity 1.04 TBq/mmol, Amersham International) was also maintained at 25°. A 250 μl aliquot of the radiolabelled solution and the synaptosomal preparation was then simultaneously transferred to the well of a filtration unit (millipore 12-port filtration system housing Whatman GF/B glass fibre filters) connected to a valved vacuum. After