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Benzodiazepines modulate the A_2 adenosine binding sites on 108CC15 neuroblastoma × glioma hybrid cells

C.R. Snell[1] & P.H. Snell

MRC Neuroendocrinology Unit, Newcastle General Hospital, Westgate Road, Newcastle upon Tyne NE4 6BE.

1 We have demonstrated high affinity diazepam binding sites of the Ro5–4864 benzodiazepine receptor subtype on 108CC15 neuroblastoma × glioma hybrid cells. These cells were previously shown to have purinoceptors of the A_2 adenosine subtype and we have now found that [^{3}H]-adenosine can be displaced from this binding site by the benzodiazepines and related compounds that can also bind to the Ro5–4864 site.

2 Diazepam was found to have no intrinsic activity at the A_2-receptor as measured by the stimulation of adenosine 3′:5′-cyclic monophosphate (cylic AMP) production in this cell line.

3 At concentrations sufficient to compete for the A_2-receptor, diazepam was shown to facilitate, by approximately 2 fold, the stimulation of cyclic AMP by adenosine.

4 These effects are not due to inhibition of adenosine uptake or phosphodiesterase activity, but are probably a consequence of modulation of the coupling of the A_2-receptor to cyclic AMP production in this hybrid cell line.

Introduction

After the initial demonstration of benzodiazepine binding sites in mammalian brain (Braestrup & Squires, 1977; Squires & Braestrup, 1977), subsequent workers have shown the presence of two principle populations of binding sites for diazepam in the central nervous system (Klepner *et al.*, 1979). In addition, a third binding site for diazepam has recently been found in mammalian brain that selectively binds the diazepam analogue, Ro5–4864 (Schoemaker *et al.*, 1981; Marangos *et al.*, 1982). This binding site was previously demonstrated in peripheral tissue (Braestrup & Squires, 1977; Regan *et al.*, 1981) and also found in some cultured cell lines (Syapin & Skolnick, 1979).

The anxiolytic actions of the benzodiazepines are thought to be mediated via the diazepam binding sites as a result of facilitation of γ-aminobutyric acid (GABA) neurotransmission in the CNS by allosteric modulation of the affinity of the GABA receptor (e.g. see Olsen, 1981). However, the relationship between the benzodiazepine receptor subtypes in the CNS and the multiple effects of benzodiazepines *in vivo* is not understood. A variety of endogenous ligands for the benzodiazepine binding sites have been proposed (Skolnick *et al.*, 1978; Marangos *et al.*, 1979; Braestrup *et al.*, 1980; Massotti & Guidotti, 1980; Davies & Cohen 1980; Woolf & Nixon 1981) ranging in nature from purines to high molecular weight proteins. An involvement of purines in the actions of benzodiazepines has been suggested and Phillis and his coworkers (Bender *et al.*, 1980b; Phillis *et al.*, 1980; Wu *et al.*, 1981); have proposed that inhibition of adenosine uptake could be involved in some of the pharmacological effects of benzodiazepines.

In this paper we demonstrate and characterize the binding site for Ro5–4864 on 108CC15 neuroblastoma × glioma hybrid cells and examine the relationship of this binding site to the purinoceptors we have previously shown on this cell line (Snell *et al.*, 1982; Snell & Snell, 1983). We also demonstrate an overlap in specificity between the two binding sites and show that, at the A_2-purinoceptor, benzodiazepines potentiate the ability of adenosine to stimulate adenosine 3′:5′-cyclic monophosphate (cyclic AMP) production.

[1] Author for correspondence.

Methods

Cell culture

The 108CC15 neuroblastoma × glioma hybrid cells were grown in monolayer in 75 cm^2 flasks at 37 °C in Dulbecco's modified Eagle's medium supplemented with 10% foetal calf serum, hypoxanthine 100 μM, aminopterin 1 μM and thymidine 16 μM in a humidified atmosphere of 10% CO_2: 90% air. Cells with passage number between 14 and 20 were harvested at 80% confluency (approx 6×10^6 cells per flask), washed twice with washing buffer (137 mM NaCl, 5.4 mM KCl, 0.17 mM Na_2HPO_4, 0.22 mM KH_2PO_2, 5.5 mM glucose) and resuspended in assay buffer (50 mM Tris, 0.1% bovine serum albumin, pH7.4) at a concentration of 10^6 cells per ml for use in the binding assays. Cells were counted in a haemocytometer.

Receptor binding assay

Displacement using constant label concentration 108CC15 hybrid cells (5×10^5 cells per tube) were maintained at 1 °C for 15 min with [2, 5, 8-^{3}H]-adenosine (Amersham International plc; 42 Ci $mmol^{-1}$, 1.8 pmol per tube), [N-methyl-^{3}H]-diazepam (Amersham International plc; 71 Ci $mmol^{-1}$, 0.5 pmol per tube) or [N-methyl-^{3}H]-Ro5–4864 (New England Nuclear Inc., 72.4 Ci $mmol^{-1}$; 2 pmol per tube) before addition to a serial dilution of unlabelled ligand under test in a final volume of 1ml. After a further 30 min (for [^{3}H]-adenosine assay) or 60 min (for [^{3}H]-diazepam or [^{3}H]-Ro5–4864 assay) the bound ligand was separated from free by centrifugation at 16,000 *g* for 3 min. The supernatants were aspirated off and the pellets superficially washed with ice cold buffer (0.2 ml). The tips of the tubes were cut off and the pellets suspended in water (0.5 ml). After addition of Packard 299 scintillant (4 ml) the radioactivity in the pellet was measured on a Packard 460 CD scintillation counter with on-line d.p.m. correction. All assays were performed in triplicate. Specific binding was taken as the difference between that bound in the absence and presence of excess cold ligand; 1×10^{-3} M adenosine, 1×10^{-6} M diazepam or 1×10^{-6} M Ro5–4864.

The homogeneous displacement data were analysed by the method of Scatchard (1949) to give the equilibrium dissociation constant, K_d and the receptor density, B_{max}.

Receptor saturation with increasing label concentration 108CC15 hybrid cells (5×10^5 cells) were added to assay tubes containing [N-methyl-^{3}H]-diazepam (0.5–40 nM) in a final volume of 1 ml of buffer (0.05 M Tris, 0.1% BSA, pH 7.4). A parallel series of tubes were incubated in the presence of diazepam. 1×10^{-6} M. After 60 min at 1 °C the tubes were centrifuged at 16,000 *g* for 3 min to separate bound from free label. The radioactivity in the pellet was measured as described for the displacement assay. All binding assays were performed in triplicate and the specific binding taken as the difference between the amounts bound in the absence and presence of excess unlabelled ligand.

Stimulation of cyclic AMP in the hybrid cells

108CC15 hybrid cells were cultured in 4 well Limbro plates (55 mm diameter) as described above. When approx 70% confluent, the cells were preincubated for 30 min at 37 °C in Dulbecco's modified Eagle's medium. Test compounds were added and the cells incubated for a further 30 min at 37 °C, after which the medium was removed and the cells washed twice at room temperature with 5 ml of phosphate buffered saline. To each well was added 1 ml of ethanolic HCl (1 M HCl/ethanol; 1 : 99 v/v) and after incubation for 30 min at room temperature the ethanol extract from each well was combined with a further 0.5 ml wash of the lysed cells with ethanolic HCl. The ethanol extracts were dried *in vacuo* and the residue dissolved in 0.5 ml of cyclic AMP assay buffer (Tris HCl 50 mM, pH 7.4, containing theophylline 8 mM and 2-mercaptoethanol 6 mM). The cells remaining in the wells were digested with 3 M sodium hydroxide (2 ml) at 70 °C and assayed for protein by the method of Lowry *et al.* (1951). All assays were carried out in triplicate. Some incubations included rolipram, ZK 62711, 10 μM to inhibit degradation of cyclic AMP by endogenous phosphodiesterase activity (Schwabe *et al.*, 1976). Diazepam was added in 3% ethanol.

Cyclic AMP assay

Cyclic AMP levels were measured by the cyclic AMP binding protein method with binding protein isolated from bovine adrenal cortices and [^{3}H]-cyclic AMP (Amersham International plc, 28 Ci $mmol^{-1}$) as label in the soluble binding assay as described by Brown *et al.*, (1971). Briefly, each assay tube contained 50 μl of either a known amount of cyclic AMP (0–15 pmol) as standard or of an unknown sample, 100 μl of [^{3}H]-cyclic AMP (0.5 pmol), 100 μl of a suitable dilution of binding protein and buffer to a final volume of 300 μl. After 1.5 h at 4 °C, 100 μl of a suspension of 10% Norit GSX charcoal in buffer containing 2% bovine serum albumin was added to each tube and the tubes briefly agitated. After centrifugation (1200 *g* for 15 min at 4 °C) a 100 μl aliquot of the supernatant was taken for measurement of radioactivity. Calibration curves were plotted as percentage

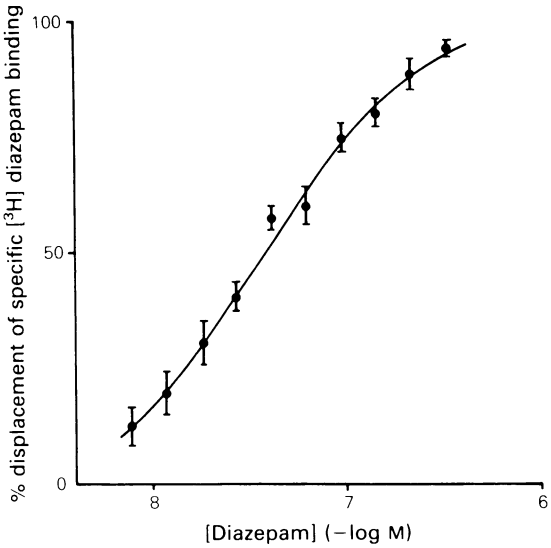

Figure 1 Displacement of specific [^{3}H]-diazepam bound to 108CC15 hybrid cells by unlabelled diazepam. Cells (5×10^5 per tube) were incubated with 0.5 nM [^{3}H]-diazepam at 1 °C for 60 min in the presence of a range of concentrations of unlabelled diazepam as described in the Methods. Non specific binding was defined as that not displaceable by 1×10^{-6} M diazepam. Each point represents the average of eight experiments conducted in triplicate, error bars represent s.e.mean.

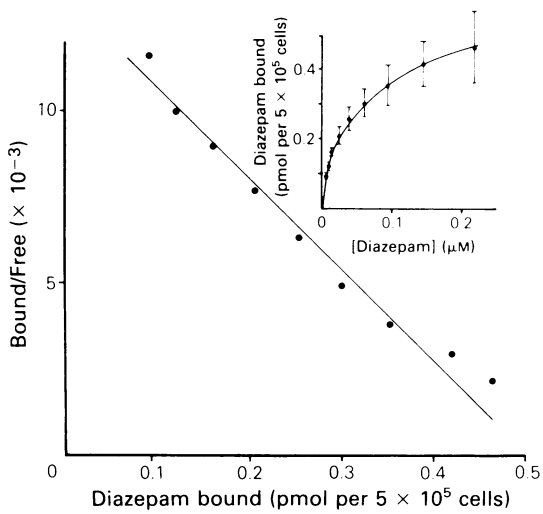

Figure 2 Scatchard plot and saturation curve for the displacement of specific [^{3}H]-diazepam binding to 108CC15 hybrid cells by diazepam. Experiments were conducted as described in the legend to Figure 1. The points represent the average of six experiments in triplicate. The best fit was obtained by linear regression ($r = 0.96$). Inset: saturation curve for [^{3}H]-diazepam labelled binding site ± s.e.mean

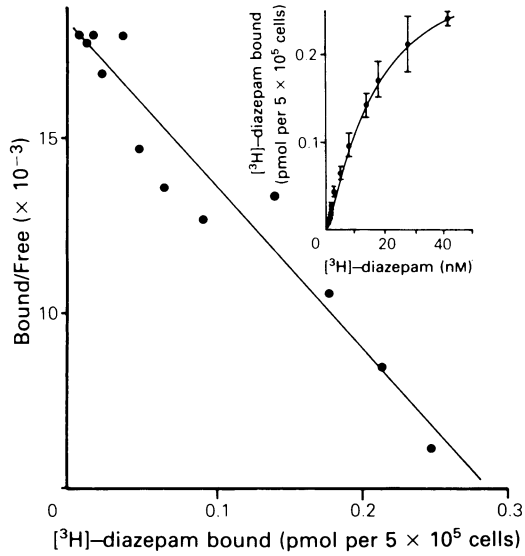

Figure 3 Scatchard plot and saturation curve of [^{3}H]-diazepam binding as a function of increasing concentration of [^{3}H]-diazepam. Cells (5×10^5 per tube) were incubated at 1 °C for 60 min with various concentrations of [^{3}H]-diazepam as described in the Methods. Non specific binding was determined in the presence of 1×10^{-6} M diazepam. Points represent the average of six experiments in triplicate. The best fit straight line was obtained by linear regression ($r = 0.96$). Inset: saturation curve for[^{3}H]-diazepam specifically bound as a function of [^{3}H]-diazepam concentration ± s.e.mean

radioactivity bound against amount of standard cyclic AMP added. Amounts of cyclic AMP in unknown samples were determined by reference to the calibration curve. All measurements were conducted in triplicate.

Results

The [^{3}H]-diazepam binding sites on 108CC15 hybrid cells

Specific binding of [^{3}H]-diazepam to intact 108CC15 neuroblastoma × glioma hybrid cells could be displaced by diazepam with an IC_{50} of 41.75 ± 5.8 nM ($n = 8$) (Figure 1). These same data were plotted by the method of Scatchard (Figure 2) to show a single binding site with B_{max} of 503 ± 85 fmol per 5×10^5 cells and a K_d of 38.1 ± 8.7 nM ($n = 6$) in good agreement with the IC_{50} value obtained from the displacement curve.

A Scatchard plot of binding site saturation by increasing concentrations of [^{3}H]-diazepam (Figure 3) showed a single binding site with B_{max} of 318 ± 27

Table 1 Inhibition of specific [^{3}H]-diazepam binding to 108CC15 hybrid cells by ligands selective for different receptor sybtypes

Compound	*IC_{50} vs [^{3}H]-diazepam* (nM)	
Diazepam	41.8 ± 5.8	(n = 8)
β-Carboline-3-carboxylic acid ethylester	4025 ± 25	(n = 2)
Cl 218,872	> 50000	(n = 2)
Clonazepam	> 100000	(n = 3)
Ro5-4864	1.8 ± 0.1	(n = 3)

Hybrid cells were preincubated with [^{3}H]-diazepam (0.5 pmol per tube) for 15 min at 1 °C before addition to a serial dilution of ligand under study in a final volume of 1 ml. After 60 min at 1 °C the bound [^{3}H]-diazepam was measured and the data analysed as described in Methods.

fmol per 5×10^5 cells and K_d of 23.5 ± 4.1 nM (n = 6) (Figure 3) in excellent agreement with the displacement data. The nature of the benzodiazepine binding site was examined by use of ligands with specificity for particular subtypes of benzodiazepine receptor site. The IC_{50} values for β-carboline-3-carboxylic acid ethylester, Cl 218,872, clonazepam and Ro5-4864 against [^{3}H]-diazepam are shown in Table 1. The binding site had a high affinity for Ro5-4864, an analogue which is specific for the so called 'peripheral' receptor type. This specificity was confirmed from displacement of [^{3}H]-Ro5-4864 by unlabelled Ro5-4864. Scatchard analysis of these data is shown in Figure 4; one binding site was found with B_{max} of 616 ± 51 fmol per 5×10^{-5} cells and K_d of 3.2 ± 0.7 nM (n = 3). Diazepam had an IC_{50} of 0.45 ± 0.03 μM (n = 3) for the [^{3}H]-Ro5-4864 binding site, and adenosine did not displace [^{3}H]-Ro5-4864 from this site even at 1 mM concentration.

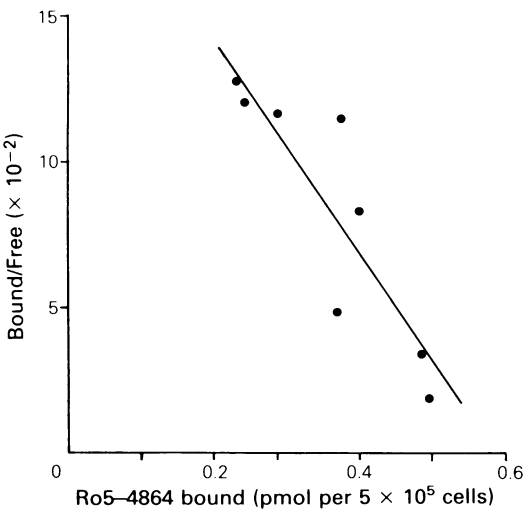

Figure 4 Scatchard plot of displacement of specific [^{3}H]-Ro5-4864 binding to 108CC15 hybrid cells by unlabelled Ro5-4864. Cells (5×10^5 per tube) were incubated with 2 nM [^{3}H]-Ro5-4864 at 1 °C for 60 min in the presence of various concentrations of Ro5-4864. Non-specific binding was defined as that bound in the presence of 10^{-6} M Ro5-4864. Points represent the average of three experiments in triplicate. The best fit straight line was obtained by linear regression (r = 0.88).

Comparison of the ligand requirement of the [^{3}H]-diazepam and [^{3}H]-adenosine binding sites on 108CC15 hybrid cells

The ligand specificities of the [^{3}H]-diazepam and [^{3}H]-adenosine binding sites were studied by displacement of the relevant tritiated ligand from the binding sites on 108CC15 hybrid cells by a range of benzodiazepines and purine related compounds. Table 2 shows the IC_{50} values obtained for the ligands when displacing [^{3}H]-diazepam and [^{3}H]-adenosine from their respective binding sites. Those benzodiazepine ligands that had affinity for the [^{3}H]-diazepam binding sites on these cells, i.e. diazepam, β-carboline-3-carboxylic acid ethylester and Ro5-4864, were also capable of displacing [^{3}H]-adenosine from the A_2-purinoceptor with affinities of the same magnitude as adenosine itself, whereas clonazepam and Cl-218,872 had very low affinity for both the [^{3}H]-diazepam and [^{3}H]-adenosine binding sites. Adenosine had an IC_{50} of 2.5 μM at the [^{3}H]-adenosine site but had an affinity of less than 1 mM at the [^{3}H]-diazepam site. Nevertheless a pyrazolopyridine, SQ20,009 structurally related to adenosine, had a remarkably high affinity for both binding sites. SQ20,006, an analogue of SQ20,009 showed lower affinity for both binding sites. Clearly adenosine at micromolar concentration will bind only to the A_2-purinoceptor on these cells; however, diazepam will bind to the benzodiazepine binding site of the Ro5-4864 type in the concentration range of 40 nM and will also bind to the A_2-purinoceptor at micromolar concentrations.

Effect of adenosine and diazepam on cyclic AMP levels in the hybrid cells

Table 3 shows the results obtained when adenosine and diazepam were tested for stimulation of cyclic AMP levels in the hybrid cells. The response of the cells to adenosine was partly dependent on the density of cells and their time in culture. Nevertheless the results represent means and respective standard errors for all data regardless of the culture history.

Table 2 IC_{50} values for a range of benzodiazepine and purinergic ligands when displacing [^{3}H]-diazepam and [^{3}H]-adenosine from 108CC15 hybrid cells

Compound	*IC_{50} vs [^{3}H]-diazepam* (μM)		*IC_{50} vs [^{3}H]-adenosine* (μM)	
Diazepam	0.042 ± 0.006	(n = 8)	5.61 ± 1.35	(n = 3)
β-Carboline-3-carboxylic Acid ethylester	4.025 ± 0.025	(n = 2)	13	(n = 1)
CL-218,872	> 50	(n = 2)	> 50	(n = 2)
Ro5-4864	0.0018 ± 0.0001	(n = 3)	10.88 ± 7.19	(n = 3)
Clonazepam	> 100	(n = 3)	100	
Adenosine	> 1000	(n = 2)	2.5 ± 0.1	(n = 3)
SQ20009	4.2 ± 0.2	(n = 2)	4.37 ± 1.09	(n = 4)
SQ20006	76	(n = 1)	21	(n = 1)
Rolipram, ZK62711	42.6 ± 1.7	(n = 3)	165 ± 2.9	(n = 3)

Hybrid cells were preincubated with either [^{3}H]-diazepam, (0.5 pmol per tube), or [^{3}H]-adenosine, (1.8 pmol per tube), for 15 min at 1 °C before addition to a serial dilution of ligand under study in a final volume of 1 ml. After 60 min at 1 °C for [^{3}H]-diazepam or 30 min at 1 °C for [^{3}H]-adenosine, displacement of bound labelled ligand was measured and data analysed as described in the Methods.

Diazepam 10^{-5} M has no effect on the basal level of cyclic AMP production in the presence of the phosphodiesterase inhibitor, rolipram and produced a slight elevation of cyclic AMP levels in the absence of the inhibitor. Adenosine gave a dose-dependent elevation of cyclic AMP production in both the presence and absence of rolipram. Adenosine 10^{-5} M, the standard test concentration, was found to produce a 5 fold increase over basal cyclic AMP levels in the absence of rolipram and a 20 fold increase in its presence. When adenosine and diazepam were added together, both at 10^{-5} M, there was a considerable potentiation of cyclic AMP production compared to adenosine alone at this concentration in the presence and absence of rolipram. Exposure of test compound and controls in each experiment were conducted under the same conditions of cell density and time in culture and, when each experiment was analysed, cyclic AMP production was potentiated by 51 ± 11% and 119 ± 37% in the presence and absence of rolipram respectively. When 10^{-7} M N^6-[L-2-phenylisopropyl]-adenosine (PIA) was substituted for adenosine in these experiments a similar potentiation of cyclic AMP production was also observed (Snell & Snell, unpublished observation).

Discussion

The diazepam binding site we have demonstrated on the hybrid cells represents a single homogenous population of benzodiazepine receptors with K_d of 38.1 nM for [^{3}H]-diazepam and a density of 503 fmol per 5×10^5 cells (see Figure 2). Structure-activity studies clearly show this binding site to be highly specific for the Ro5-4864 benzodiazepine analogue and not the central type I and II benzodiazepine receptor ligands. The receptor density using [^{3}H]-Ro5-4864 (Figure 4) was in good agreement (616 fmol per 5×10^5 cells) with that found for [^{3}H]-diazepam, suggesting that these two ligands are binding to the same site on these cells. The Ro5-4864

Table 3 Effect of adenosine and diazepam on cyclic AMP levels in 108CC15 hybrid cells in the presence and absence of the phosphodiesterase inhibitor, rolipram

Compound	*Cyclic AMP production* (pmol mg^{-1} protein 30 min^{-1}) + rolipram 10 μM		*Cyclic AMP production* (pmol mg^{-1} protein 30 min^{-1}) − rolipram 10 μM	
(1) None (Basal)	92.4 ± 8.6	(n = 12)	18.33 ± 2.36	(n = 9)
(2) Diazepam 10^{-5} M	97.3 ± 8.99	(n = 3	28.6 ± 6.3	(n = 9)
(3) Adenosine 10^{-5} M	1894 ± 339	(n = 10)	96 ± 16	(n = 5)
(4) Adenosine 10^{-5} M + diazepam 10^{-5} M	2809 ± 445	(n = 10)	225 ± 68	(n = 5)
Increase in cyclic AMP levels for matched cultures (control = 1)	1.51 ± 0.11	(n = 10)	2.19 ± 0.37	(n = 5)

108CC15 hybrid cells were grown in 4 well plates and treated with test compounds as described in Methods. Intracellular cyclic AMP levels were measured using the cyclic AMP binding protein assay as described by Brown *et al.* (1971).

binding site is of considerable interest as it is not only present in peripheral tissue such as the kidney, but has recently been demonstrated in the CNS and accounts for up to 25% of all central diazepam binding sites (Marangos *et al.*, 1982; Schoemaker *et al.*, 1981).

The suggestions by other workers (Bender *et al*, 1980b; Phillis *et al.*, 1980; Wu *et al.*, 1981) that purines could be involved in the action of the benzodiazepines led us to compare the specificity of this benzodiazepine binding site and the A_2-purinoceptors we have previously characterized on these cells (Snell & Snell, 1983). Structure-activity studies using benzodiazepine related compounds (Table 2) showed that those analogues with affinity for the [^{3}H]-diazepam binding site also had affinity for the [^{3}H]-adenosine receptor. This would suggest that the diazepam and adenosine binding sites have similar structural requirements for benzodiazepine ligands. Adenosine, had no detectable affinity for the diazepam binding site, therefore the overlap in specificity between the two sites cannot be completely mutual. Nevertheless, some purine-related compounds were capable of displacing both ligands with similar affinity e.g. the pyrazolopyridines SQ20,009 (Beer *et al.*, 1972) and SQ20,006 (Placheta & Karobath, 1980).

Clearly benzodiazepines and other related compounds with affinity for the Ro5–4864 binding sites are capable of displacing [^{3}H]-adenosine from the A_2-adenosine receptor. However, clarification of the pharmacological nature of this interaction can only be obtained from the study of events subsequent to the binding process. Previous workers (Sharma *et al.*, 1975; Daly, 1977) have shown that the stimulation of the A_2-adenosine receptors produces an elevation of intracellular cyclic AMP levels. Therefore we have examined the effects of diazepam on cyclic AMP production associated with the A_2-adenosine receptors on these cells. Cyclic AMP levels were measured in both absence and presence of rolipram, a compound that has been shown to be a potent phosphodiesterase inhibitor and to have low affinity for adenosine receptors (see Schwabe *et al.*, 1976 and Table 2). The inhibitor was used at 10 μM, at which concentration maximal inhibition of phosphodiesterase was observed. Higher concentrations of rolipram produced no further statistically significant enhancement in the magnitude of cyclic AMP accumulaton. Diazepam at 10 μM, which from the displacement studies should ensure over 50% occupancy of the A_2-adenosine binding site, had no effect on cyclic AMP levels when compared to basal levels in the presence of rolipram. In the absence of rolipram, diazepam produced a small but significant increase in cyclic AMP production which could be a result of the reported ability of diazepam to inhibit phosphodiesterase activity (Schultz, 1974; Propst *et al.*, 1979). Adenosine at 10 μM, produced an increase in cyclic AMP accumulation in both the absence and presence of rolipram. Surprisingly, although having no intrinsic activity at the A_2-receptor, diazepam at 10 μM dramatically potentiated adenosine-stimulated cyclic AMP production. The effect was observed in both the absence and presence of rolipram. The susceptibility of the cells to stimulation of cyclic AMP production by adenosine was dependent on the time in culture and on their density in the monolayer. Despite this, the increase in cyclic AMP levels induced by the inclusion of diazepam is clearly seen. Each experiment included controls under the same conditions of culture. When comparison was made within an experiment between cells stimulated by adenosine in the absence and presence of diazepam, the potentiation was $51 \pm 11\%$ and $119 \pm 37\%$ in the presence and absence of rolipram respectively. At diazepam concentrations of 10^{-6} and 10^{-7} M, potentiation was not observed. Therefore this effect is probably associated with the ability of diazepam to bind to the adenosine A_2-receptor and not from occupation of the 40 nM diazepam binding site.

Phillis and his coworkers (Bender *et al.*, 1980a,b; Phillis *et al.*, 1980; Wu *et al.*, 1981) have shown that benzodiazepines can inhibit adenosine uptake in the mammalian CNS. At 10 μM, diazepam inhibits adenosine uptake by 33% in rat cortical synaptosomes (Phillis *et al.*, 1980). If the uptake sites on the cell line are similar to those on rat synaptosomes, i.e., with a V_{max} of 6.8 pmol mg^{-1} protein min^{-1} (Bender *et al.*, 1980a), the extracellular adenosine concentration would be increased during the incubation by at most 0.68% in the presence of diazepam. This would be insufficient to explain the observed elevation in cyclic AMP production. In addition, a similar potentiation by diazepam was observed when phenylisopropyl adenosine, an adenosine analogue that is not taken up by synaptosomes, was used to stimulate the A_2-receptor. It is also unlikely that the effects of diazepam are attributable to the reported ability of diazepam to inhibit phosphodiesterase activity (Schultz, 1974; Propst *et al.*, 1979) as the effects are observed in both the presence and absence of the phosphodiesterase inhibitor, rolipram.

From the displacement studies, at 10 μM, diazepam and adenosine will be competing for the A_2-adenosine binding site. Diazepam has no intrinsic activity at this A_2-receptor as measured by its effect on cyclic AMP production but is clearly not an antagonist. It would appear to be a competitive modulator of the efficacy of adenosine at this receptor, possibly by some allosteric interaction between adjacent binding sites.

We conclude that 108CC15 neuroblastoma × glioma hybrid cells possess ben-

zodiazepine binding sites of the Ro5-4864 subtype. Those benzodiazepines that bind to this site also compete for [^{3}H]-adenosine at the A_2-purinoceptors present on these cells. At concentrations required to compete for this binding site, diazepam has no intrinsic activity but acts to facilitate the ability of adenosine to stimulate cyclic AMP production. Ro5-4864 does not have the anxiolytic properties of diazepam but has been found to be a proconvulsant and an anxiogenic agent when assayed in the rat (File & Lister, 1983). It is unlikely therefore that this benzodiazepine receptor subtype is associated with the principal clinical properties of diazepam but could be involved in some of the other effects of the benzodiazepines. Adenosine A_2 binding sites are present in the CNS (Snell & Snell, 1983) and are associated with stimulation of cyclic AMP production (Sattin & Rall, 1970; Daly, 1977; Fredhom *et al.*, 1982), it is possible, therefore, that central Ro5-4864 binding sites could also be associated with adenosine binding sites in a similar manner to that observed in the hybrid cells.

References

BEER, B., CHASIN, M., CLODY, D.E., VOGEL, J.R. & HOROVITZ, Z.P. (1972). Cyclic adenosine monophosphate phosphodiesterase in brain: effect on anxiety. *Science*, **176**, 428-430.

BENDER, A.S., PHILLIS, J.W. & WU, P.H. (1980a). Diazepam and flurazepam inhibit adenosine uptake by rat brain synaptosomes. *J. Pharm. Pharmac.*, **32**, 293-294.

BENDER, A.S., WU, P.H. & PHILLIS, J.W. (1980b). The characterization of [^{3}H] adenosine uptake into rat cerebral cortical synaptosomes. *J. Neurochem.*, **35**, 629-640.

BRAESTRUP, C., NIELSEN, M. & OLSEN, C.E. (1980). Urinary and brain β-carboline-3-carboxylates as potent inhibitors of brain benzodiazepine receptors. *Proc. natn. Acad. Sci. U.S.A.*, **77**, 2288-2292.

BRAESTRUP, C. & SQUIRES, R.F. (1977). Specific benzodiazepine receptors in rat brain characterized by high affinity [^{3}H] diazepam binding. *Proc. natn. Acad. Sci. U.S.A.*, **74**, 3805-3809.

BROWN, B.L., ALBANO, J.D.M., EKINS, R.P., SGHERZI, A.M. & TAMPION, W. (1971). A simple and sensitive saturation assay method for the measurement of adenosine 3':5'-cyclic monophosphate. *Biochem. J.*, **121**, 561-562.

DALY, J.W. (1977). *Cyclic Nucleotides in the Central Nervous System*. New York: Plenum Press.

DAVIES, L.G. & COHEN, R.K. (1980). Identification of an endogenous peptide ligand for the benzodiazepine receptor. *Biochem. biophys. Res. Commun.*, **92**, 141-148.

FILE, S.E. & LISTER, R.G. (1983). The anxiogenic action of Ro5-4864 is reversed by phenytoin. *Neurosci. Letts*, **35**, 93-96.

FREDHOLM, B.B., JONZON, B., LINDGREN, E. & LINDSTROM, K. (1982). Adenosine receptors mediating cyclic AMP production in the rat hippocampus. *J. Neurochem.*, **39**, 165-175.

KLEPNER, C.A., LIPPA, A.S., BENSON, D.I., SANO, M.C. & BEER, B. (1979). Resolution of two biochemically and pharmacologically distinct benzodiazepine receptors. *Pharmac. Biochem. Behav.*, **11**, 457-462.

LOWRY, O.H., ROSEBROUGH, N.J., FARR, A.L. & RANDALL, R.J. (1951). Protein measurements with the folin phenol reagent. *J. biol. Chem.*, **193**, 205-275.

MARANGOS, P.J., PATEL, J., BOULENGER, J.-P. & CLARK-ROSENBERG, R. (1982). Characterization of peripheral-type benzodiazepine binding sites in brain using [^{3}H] Ro5-4864. *Mol Pharmac.*, **22**, 26-32.

MARANGOS, P.J., PAUL, S.M., GOODWIN, F.K. & SKOLNICK, P. (1979). Putative endogenous ligands for the benzodiazepine receptor. *Life Sci.*, **25**, 1093-1108.

MASSOTTI, M. & GUIDOTTI, A (1980). Endogenous regulators of benzodiazepine recognition sites. *Life Sci.*, **27**, 847-854.

OLSEN, R. W.(1981). GABA-benzodiazepine-barbiturate receptor interactions. *J. Neurochem.*, **37**, 1-13.

PHILLIS, J.W., BENDER, A.S. & WU, P.H. (1980). Benzodiazepines inhibit adenosine uptake into rat brain synaptosomes. *Brain Res.*, **195**, 494-498.

PLACHETA, P. & KAROBATH, M. (1980). In vitro modulation by SQ20009 and SQ65396 of GABA receptor binding in rat CNS membranes. *Eur. J. Pharmac.*, **62**, 225-228.

PROPST, F., MORODER, L., WUNSCH, E. & HAMPRECHT, B. (1979). The influence of secretin, glucagon and other peptides, of amino acids, prostaglandin endoperoxide analogs and diazepam on the level of adenosine 3',5'-cyclic monophosphate in neuroblastoma × glioma hybrid cells. *J. Neurochem.*, **32**, 1495-1500.

REGAN, J.W., YAMAMURA, H.I., YAMADA, S. & ROESKE, W.R. (1981). High affinity renal [^{3}H] flunitrazepam binding: characterization, localization and alteration in hypertension. *Life Sci.*, **28**, 991-998.

SATTIN, A. & RALL, T.W. (1970). The effect of adenosine and adenine nucleotides on the cyclic AMP content of guinea pig cerebral cortex slices. *Mol. Pharmac.*, **6**, 13-23.

SCATCHARD, G. (1949). The attraction of proteins for small molecules and ions. *Ann. N.Y. Acad. Sci.*, **51**, 660-665.

SCHOEMAKER, H., BLISS, M. & YAMAMURA, H.I. (1981). Specific high-affinity saturable binding of [^{3}H] Ro5-4864 to benzodiazepine binding sites in the rat cerebral cortex. *Eur. J. Pharmac.*, **71**, 173-175.

SCHULTZ, J. (1974). Adenosine 3'-5'-monophosphate in guinea pig cerebral cortical slices: effect of benzodiazepines. *J. Neurochem.*, **22**, 685-690.

SCHWABE, U., MIYAKE, M., OHGA, Y. & DALY, J.W. (1976). 4-(3-cyclopentyloxy-4-methoxyphenyl)-2-pyrrolidone (ZK 62711): a potent inhibitor of adenosine cyclic 3', 5'-monophosphate phosphodiesterases in

homogenates and tissue slices from rat brain. *Mol. Pharmac.*, **12,** 900–910.

SHARMA, S.K., KLEE, W.A. & NIRENBERG, M. (1975). Dual regulation of adenylate cyclase accounts for narcotic dependence and tolerance. *Proc. natn. Acad. Sci. U.S.A.*, **72,** 3092–3096.

SKOLNICK, P., MARANGOS, P.J., GOODWIN, F.K., EDWARDS, M. & PAUL, S. (1978). Identification of inosine and hypoxanthine as endogenous inhibitors of [^{3}H] diazepam binding in the central nervous system. *Life Sci.*, **23,** 1473–1480.

SNELL, P.H., MOSES, M.A. & SNELL, C.R. (1982). The binding of [^{3}H] adenosine to cells of the 108CC15 neuroblastoma × glioma hybrid line. *Biochem. Soc. Trans.*, **10,** 55–56.

SNELL, P.H. & SNELL, C.R. (1983). [^{3}H] adenosine binding sites on 108CC15 neuroblastoma × glioma hybrid cell line and rat brain membranes. *Neurochemistry Int.*, **5,** 245–249.

SQUIRES, R.F. & BRAESTRUP, C. (1977). Benzodiazepine receptors in rat brain. *Nature*, **266,** 732–734.

SYAPIN, P.J. & SKOLNICK, P. (1979). Characterization of benzodiazepine binding sites in cultured cells of neural origin. *J. Neurochem.*, **32,** 1047–1051.

WOOLF, J.H. & NIXON, J.C. (1981). Endogenous effector of the benzodiazepine binding site: purification and characterisation. *Biochemistry*, **20,** 4263–4269.

WU, P.H., PHILLIS, J.W. & BENDER, A.S. (1981). Do benzodiazepines bind at adenosine uptake sites in CNS? *Life Sci.*, **28,** 1023–1031.

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