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Effects of benzodiazepine administration on A_1 adenosine receptor binding in-vivo and ex-vivo

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Abstract—The adenosine receptor has been implicated in the central mechanism of action of benzodiazepines. The specific binding of an A_1-selective adenosine antagonist radioligand, [^{3}H]8-cyclopentyl-1,3-dipropylxanthine, was measured in-vivo in mice treated with alprazolam (2 mg kg^{-1}, i.p.), lorazepam (2 mg kg^{-1}, i.p.) and vehicle. Binding studies were performed in-vivo and ex-vivo in mice receiving continuous infusion of alprazolam (2 mg kg^{-1} day^{-1}), lorazepam (2 mg kg^{-1} day^{-1}) and vehicle by mini-osmotic pumps for 6 days. Continuous infusion of alprazolam and lorazepam significantly decreased specific binding by 34 and 53%, respectively, compared with vehicle treatment ($P < 0.01$). Single doses of alprazolam and lorazepam induced a similar trend in specific binding in-vivo ($P = 0.07$). There were no alterations in A_1-receptor density (B_{max}) or affinity (K_d) in cortex, hippocampus or brainstem in ex-vivo studies. Benzodiazepine treatment may diminish A_1- receptor binding in-vivo by inhibiting adenosine uptake or by direct occupancy of the A_1 adenosine receptor recognition site.

The central effects of benzodiazepines are mediated by molecular mechanisms which have not been completely elucidated. Benzodiazepines are known to bind to a high-affinity benzodiazepine receptor site on a supramolecular $GABA_A$ complex, enhancing binding of γ-aminobutyric acid (GABA) to its recognition site and altering coupling to its effector. However, benzodiazepine-induced enhancement of GABA-ergic synaptic transmission or its antagonism do not explain all of the clinical effects of benzodiazepines. Other mechanisms of action have been considered including the interaction of benzodiazepines with the neuromodulator adenosine. Adenosine is an endogenous neuromodulator which binds to its own recognition sites, A_1 and A_2, and like benzodiazepines, adenosine agonists are known to be sedating, motor-depressant and anticonvulsant (Phillis & O'Regan 1988).

Benzodiazepines reverse the behavioural effects of adenosine receptor antagonists, such as caffeine. Diazepam reverses caffeine-induced restlessness, tension, alertness and arousal in man (Roache & Griffiths 1987). In animal models, interactions between benzodiazepine and adenosine agonist and antagonist agents on locomotor activity, suppressed behaviours and conflict behaviours have been demonstrated (Crawley et al 1981; Polc et al 1981; Barraco et al 1984; Kaplan et al 1990). However, a lack of interaction between diazepam and adenosine agonists was shown in another behavioural study (Commissaris et al 1990). Consistent interactions have been seen with convulsant effects, since adenosine agonists suppress the induction of seizures by bicuculline, a GABA-ergic proconvulsant agent (Franklin et al 1989), and benzodiazepines inhibit caffeine-induced seizures (Marangos et al 1981; Chweh et al 1986).

Functional responses of the adenosine receptor system have been examined by measuring adenosine-induced depression of evoked activity in neurons. Alterations of adenosine-evoked depression by benzodiazepine have been consistently found, suggesting their role in modulating adenosinergic responses (Phillis 1979; Mally et al 1990).

Various alterations of the specific components of the adenosine receptor system by benzodiazepines have shown that benzodiazepines inhibit the uptake and release of adenosine at a specific high-affinity site, resulting in increased concentrations of extracellular adenosine (Phillis et al 1980a, b; Bender et al 1980). Adenosine-uptake-inhibiting agents and benzodiazepines similarly block adenosine uptake (Bender & Hertz 1986). A correlation between inhibition of benzodiazepine binding and inhibition of adenosine uptake has been found with various drugs (Wu et al 1980). Additionally, a correlation between adenosine-uptake inhibition and inhibition of seizure-induction by the GABA-ergic agent, pentetrazol, has been described (Chweh et al 1984). These correlations suggest relationships between adenosine-uptake-inhibition and benzodiazepine receptor binding and function.

Adenosine and diazepam are structurally similar with a flat, fused heterocyclic ring structure and neutral charge, suggesting that cross-reactivity of drugs could occur between the two receptor systems. Benzodiazepines bind weakly to adenosine receptors in-vitro at micromolar concentrations while binding to

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benzodiazepine receptors at nanomolar concentrations (Bruns et al 1983). Diazepam enhances the stimulation of adenosine-induced second messenger accumulation (cAMP) which can be reversed by addition of the adenosine antagonist, theophylline, or by adenosine uptake inhibitors (York & Davies 1982). Chronic administration of benzodiazepines decreased A_2 adenosine receptor binding and diminished A_2-receptor-mediated cAMP stimulation (Hawkins et al 1988a, b, 1989). These findings establish a possible adenosinergic mechanism of action for benzodiazepine anxiolytics.

In this study, we have examined the effects of single doses and continuous infusions of alprazolam and lorazepam on specific binding in-vivo of the A_1 adenosine antagonist radioligand, [^{3}H]8-cyclopentyl-1,3,-dipropylxanthine (DPCPX). Additionally, we have evaluated the effects of continuous benzodiazepine infusions on A_1 adenosine receptor affinity and density using an ex-vivo binding technique and provided more evidence for the role of the adenosine receptor in the molecular mechanism of benzodiazepines.

Materials and methods

Materials. Male CD-1 mice (30 g; 6–8 weeks old) from Charles River Laboratories (Wilmington, MA) were housed under a 12 h light/dark cycle with free access to laboratory chow and water.

Alprazolam and lorazepam were generously donated by the Upjohn Company (Kalamazoo, MI) and Wyeth-Ayerst Research (Princeton, NJ), respectively. Solutions were prepared in polyethylene glycol 400 (PEG; J. T. Baker, Inc, Phillipsburg, NJ). For continuous infusions, solutions were placed into Alzet Model 2001 mini-osmotic pumps (Alza Corp, Palo Alto, CA) that deliver at constant rates of 1 μL h^{-1}.

8-Cyclopentyl-1,3-dipropylxanthine (DPCPX) was obtained from Research Biochemicals Inc. (Natick, MA). Adenosine deaminase from calf intestine, type IX (calf spleen), was obtained as a liquid suspension from Sigma (St Louis, MO).

Cyclopentyl-1,3-dipropylxanthine, 8-[dipropyl-2,3-^{3}H(n)] or [^{3}H]DPCPX (sp. act. 120 Ci mmol^{-1}), was utilized for all binding studies, and was obtained from New England Nuclear Products, Dupont (Boston, MA). [^{3}H]DPCPX for in-vivo binding studies was dissolved in equivalent amounts of PEG and then prepared as a 1:10 dilution in sterile 0.9% NaCl (saline). Solvable, a tissue solubilizer, was also obtained from New England Nuclear. Scintillation fluid (Ecoscint H) was obtained from National Diagnostics (Manville, NJ). Other reagents were obtained from standard commercial sources.

Drug administration in mice. Two different treatment conditions were investigated using in-vivo binding methods: acute benzodiazepine administration (alprazolam 2 mg kg^{-1}, lorazepam 2 mg kg^{-1} and PEG, i.p.) 1 h after i.p. administration; continuous benzodiazepine infusion, after 6 days of drug administration. For continuous dose infusion, a single osmotic pump was implanted s.c. in each mouse to deliver alprazolam or lorazepam doses of 2 mg kg^{-1} day^{-1} or vehicle. Additionally, ex-vivo binding studies were performed in mice receiving drug for six days.

Receptor binding in-vivo. An in-vivo receptor binding technique utilizing the high affinity, A_1-selective, adenosine antagonist radioligand, [^{3}H]DPCPX, and unlabelled DPCPX has been previously described (Kaplan et al 1992). A parenteral solution of [^{3}H]DPCPX (10 μCi) in PEG-saline solution was administered i.p. to mice at time zero. Time zero corresponds to 40 min after benzodiazepine administration in the single dose study, or on day 6 of the continuous dose study. Nonspecific binding was measured in groups of mice which received a saturating dose (10 mg kg^{-1}, i.p.) of unlabelled DPCPX-PEG solution, 10 min after radioligand administration. Total binding was measured in groups of mice which received PEG only, 10 min after radioligand administration. Twenty min after radioligand injection, mice were killed by cervical displacement and decapitated. For mice receiving single benzodiazepine doses, radioligand was injected 40 min after drug administration. Brains were removed and were dissected on ice. Cortices from each mouse were solubilized in scintillation vials using 2 mL of Solvable at 40°C for 24 h. Scintillation fluid (10 mL) was added to each vial and samples were counted by conventional scintillation spectrometry, 24 h later. Individual counts were corrected by brain weight and specific binding was defined as total minus nonspecific counts. Total and nonspecific binding was measured in each treatment group.

Receptor binding ex-vivo. Tissue preparations and radioligand incubations were as previously described in detail (Kaplan et al 1992). Briefly, brains were removed immediately after decapitation and cortical, hippocampal and brainstem regions were dissected on ice. Brain regions from each treatment group were pooled and homogenized in 5 mL of 50 mM Tris buffer by a Polytron unit (Brinkmann, Westbury, NY) at a setting of 6 for 10 s. Homogenates were centrifuged at 30000 *g* for 20 min at 4°C. The pellet was resuspended in 5 mL of 50 mM Tris containing 5 units mL^{-1} of adenosine deaminase for 30 min at 30°C, which metabolizes excess adenosine released by tissues. Membrane suspensions were centrifuged at 30000 *g* for 20 min at 4°C and resuspended in 5 mL of Tris buffer. Samples were frozen at −70°C for later use.

Suspensions were thawed, centrifuged at 30000 *g* for 20 min at 4°C and resuspended in 5 mL Tris buffer (protein concentration between 0·1 and 0·5 mg mL^{-1}). Incubations were in triplicate on ice (4°C) for 60 min, using 50 μL of radioligand (at varying final concentrations between 0·05 and 10 nM), 300 μL Tris with adenosine deaminase (5 units mL^{-1}), and unlabelled DPCPX in dimethylsulphoxide (final concentration 10 μM) or dimethylsulphoxide only, and finally 100 μL of membrane. All incubations were terminated by filtration through Whatman GF/B filters on a Brandel 48R cell harvester by rapid washing of the filters. Wet filters were added to scintillation vials with 10 mL of scintillation fluid. Radioactivity in vials was measured using conventional scintillation spectrometry. Each assay was evaluated by Scatchard analysis utilizing linear regression to obtain estimates of K_d and B_{max}. Data were included if the correlation coefficient of the regression line of the plot was statistically significant ($P < 0·05$). For each treatment, at least 3 experiments were performed.

Results

Effects of acute alprazolam, lorazepam and vehicle administration on in-vivo specific binding of [^{3}H]DPCPX in cortex are shown in Fig. 1A. Specific binding (means ± s.d.) after acute drug treatment were as follows: vehicle, 39·2 (6·3), alprazolam, 25·4 (8·8) and lorazepam, 23·0 (8·3) counts min^{-1} (mg tissue)$^{-1}$. Analysis of variance showed a trend towards differences among the three values, which did not reach significance ($F = 3·17$, $P = 0·07$). Effects of continuous benzodiazepine infusions over 6 days on specific binding are shown in Fig. 1B. Specific binding values were as follows: vehicle, 78·7 (27·1), alprazolam 52·1 (3·6) and lorazepam 36·7 (12·9) counts min^{-1} (mg tissue)$^{-1}$. Analysis of variance demonstrated that these specific binding means were significantly different from each other ($F = 7·38$, $P < 0·01$). When the Newman-Keuls joint probability test was applied, significant differences between alprazolam-vehicle and lorazepam-vehicle were found ($P < 0·05$) but not for alprazolam-lorazepam.

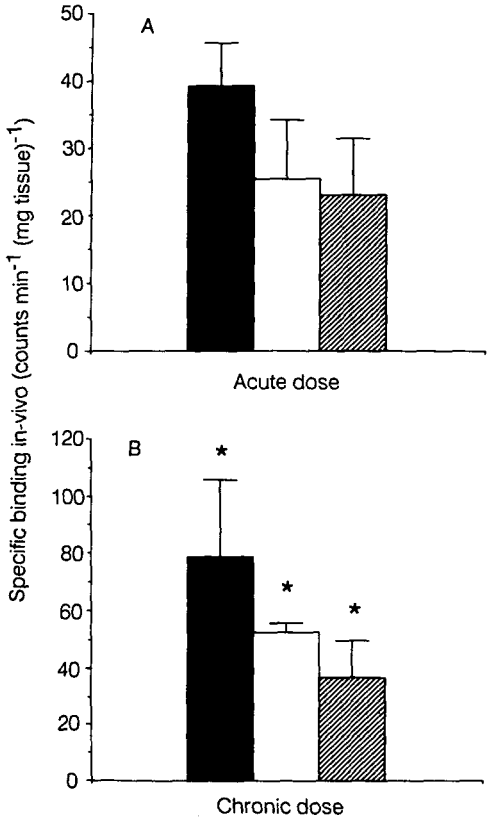

FIG. 1. A. Effects of acute administration of alprazolam (2 mg kg^{-1}), lorazepam (2 mg kg^{-1}) and vehicle on specific [^{3}H]DPCPX binding in cortices of mice, 60 min after drug injection. B. Effects of a 6 day continuous infusion of alprazolam (2 mg kg^{-1} day^{-1}), lorazepam (2 mg kg^{-1} day^{-1}) and vehicle on specific binding. Values (counts min^{-1} (mg tissue)$^{-1}$) are given as means ± s.d., n = 8 for each group. ■ Vehicle, □ alprazolam, ■ lorazepam. *$P < 0.05$ compared with values in the acute dose experiments.

Table 1. Effects of continuous alprazolam, lorazepam or vehicle infusions on A_1 adenosine receptor binding ex-vivo.

	K_d (nM)*	B_{max} (fmol mg^{-1})*	n
Cortex			
vehicle	1·65 (0·51)	155 (17)	10
alprazolam	1·93 (0·46)	157 (20)	8
lorazepam	1·90 (0·41)	158 (15)	6
Hippocampus			
vehicle	1·74 (0·48)	197 (29)	5
alprazolam	1·86 (0·55)	200 (13)	5
lorazepam	1·64 (0·40)	186 (34)	3
Brainstem			
vehicle	1·59 (0·20)	84 (7)	3
alprazolam	2·11 (0·57)	108 (10)	3
lorazepam	1·78 (0·46)	98 (23)	5

* Values given as mean (± s.d.).

To determine effects of continuous benzodiazepine infusions on A_1 adenosine receptor affinity and density, ex-vivo binding studies were performed in cortical, hippocampal and brainstem membranes. Table 1 presents the mean K_d and B_{max} values derived from Scatchard transformations. No significant differences between treatment groups were found. In summary, continuous infusion of benzodiazepines decreased specific A_1 adenosine receptor binding in-vivo but did not alter K_d or B_{max} measured ex-vivo.

Discussion

Benzodiazepines have consistently been found to reverse the behavioural and proconvulsant effects of adenosine antagonists, alter adenosine-induced electrophysiological responses, inhibit adenosine uptake and downregulate A_2 adenosine receptor binding. We utilized a novel in-vivo receptor binding technique which allows for the quantitation of specific, high-affinity radioligand occupancy of A_1-receptors under physiological conditions. Single equipotent doses of alprazolam and lorazepam diminished specific binding by 35% and 41% (vs vehicle), respectively, 1 h after treatment. With acute administration, there was a trend towards significant differences between drug treatments on specific binding ($P = 0.07$). After six days continuous infusion, alprazolam and lorazepam decreased specific binding by 34 and 53%, respectively, and the mean binding values of the treatment groups were significantly different from each other ($P < 0.01$). Differences in specific binding values between single and continuous dosage of vehicle may reflect physiological alterations produced by pump implantation.

One explanation for the decrease in specific binding in-vivo after drug treatment is that benzodiazepines bind to the adenosine receptor recognition site and displace radioligand. This is possible since the concentration of alprazolam 1 h post-injection (2 mg kg^{-1}) in a previous study (Kaplan et al 1990) and the A_1-receptor binding affinity for benzodiazepines in rat brain membranes are both in the micromolar range (Bruns et al 1983). However, the ability of benzodiazepines to bind to A_1 adenosine receptor sites is controversial (Williams & Risley 1981). An alternative hypothesis is that benzodiazepines inhibit adenosine uptake in-vivo and that these relatively greater concentrations of adenosine displace radioligand at the A_1 adenosine receptor binding site. The concentrations of benzodiazepine required for inhibition of adenosine uptake are also in the micromolar range (Bender & Hertz 1986) and could account for the in-vivo A_1-receptor changes. The excess adenosine concentrations may be metabolized by adenosine deaminase in ex-vivo studies, accounting for the lack of receptor alterations in this study.

Since no alterations of A_1-receptor density or affinity in brain regions were noted, benzodiazepines do not regulate adenosine receptor function as do adenosine agonists or antagonists. Desensitization of the A_1-receptors has been demonstrated with prolonged adenosine agonist exposure and sensitization found with prolonged antagonist treatment (Stiles 1990). Our receptor binding results are in agreement with those of Hawkins et al (1988a, b, 1989) in which no changes in B_{max} or K_d of A_1-receptors were found in cortex or hippocampus. Those investigators did find decreases in striatal A_2 B_{max} with prolonged diazepam treatment, which correlated with functional alterations in the rat sleep-wake cycle.

Our study provides more evidence for the role of the adenosine receptor in the molecular mechanism of benzodiazepines. Since continuous infusions of 2 mg kg^{-1} day^{-1} of alprazolam or lorazepam produce steady-state plasma concentrations similar to those found in man given high therapeutic doses (Miller et al 1988, 1989), our findings may apply also to man. Benzodiazepine treatment may diminish A_1-receptor occupancy in-vivo indirectly by inhibiting adenosine uptake or directly occupying the A_1 recognition site. Follow-up studies are necessary to further address the question of benzodiazepine effects on adenosine-mediated second messenger formation and adenosine-mediated functional alterations.

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