Diazepam and flurazepam inhibit adenosine uptake by rat brain synaptosomes

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The suggestion has been made in earlier publications that benzodiazepines may exert some of their therapeutic effects by potentiating the pharmacological actions of endogenously released adenosine (Phillis 1979; Phillis et al 1979a). Adenosine and the adenine nucleotides are highly potent depressants of the firing of many central neurons. Diazepam potentiates the depressant actions of adenosine and 5′-adenosine monophosphate (5′-AMP) on the spontaneous firing of cerebral cortical neurons and in larger amounts it evokes a similar depression of neuronal firing. The depressant actions of another benzodiazepine, flurazepam, are inhibited by the adenosine antagonist, theophylline. Both sets of observations are consistent with the postulate that benzodiazepines exert their actions on cortical neurons through a purinergic link.

Mah & Daly (1976) have shown that diazepam inhibits the uptake of adenosine by rat brain slices but in their experiments high concentrations (5×10^{-5}–2×10^{-4} M) of diazepam were required to elicit such an inhibition. These concentrations are well above those which are likely to occur in the brain or in plasma (up to 10^{-6} M) during the therapeutic administration of diazepam to man (Hillestad et al 1974; Skolnick et al 1979). To validate our hypothesis that diazepam may elicit at least some of its therapeutic actions by inhibiting adenosine uptake, it is necessary to demonstrate that this agent, in therapeutically relevant concentrations, can in fact significantly affect such uptake. The results presented in this communication show that diazepam, at concentrations as low as 10^{-8}–10^{-7} M, reduces adenosine uptake. A second benzodiazepine, flurazepam, which is less potent than diazepam as a therapeutic agent (Möhler & Okada 1978), also inhibited uptake, but somewhat less effectively.

Synaptosomes were prepared by the method of Gray & Whittaker (1962) as modified by White (1975). Briefly, 4 male Wistar rats were killed by cervical dislocation. The cerebral cortices were removed, weighed and homogenized in 10 volumes (w/v) of ice-cold 0·32 M sucrose solution, pH 7·5. The homogenate was centrifuged at 1000 *g* for 10 min. The debris was discarded and the remaining supernatant was centrifuged again at 12 000 *g* for 20 min at 4°C. The resultant pellet was suspended in 12 ml of ice-cold 0·32 M sucrose solution, pH 7·5, and transferred to a discontinuous sucrose gradient and centrifuged at 150 000 *g* for 60 min. The synaptosomal fraction was collected and diluted to 40 ml with 0·32 M sucrose solution and then centrifuged at 20 000 *g* for 20 min at 4°C. The synaptosomal pellet was resuspended in 4 ml of ice cold 0·32 M sucrose solution. The protein content of each preparation was determined by the method of Lowry et al (1951).

The incubation solution contained (in final concentration mM) NaCl 120, $MgSO_4$ 1·18, $NaHCO_3$ 26, KH_2PO_4 1·2, $CaCl_2$ 1·77, glucose 5·5 and sucrose 58·5 (White 1975). This solution was first gassed with 95% O_2 and 5% CO_2 for at least 30 min (or until pH 7·5) at 37°C. Benzodiazepine solution (10 μl), at varying concentrations, was added to the medium (980 μl) which also contained 50 μl (approximately 0·1 mg protein) of the synaptosomal preparation. The mixture was preincubated for 2 min at 37°C before the addition of [^{3}H]-adenosine (10 μl, specific activity 1 μCi $nmol^{-1}$, ICN, Calif. U.S.A.) to a final concentration of 2×10^{-7} M. Following the addition of [^{3}H]adenosine, incubation was allowed for 30 s and the reaction was stopped by diluting the substrate, [^{3}H]adenosine, in the medium with 5·0 ml of washing solution (0·26 M sucrose, 0·002 M $CaCl_2$, 0·002 M $MgCl_2$ and 20 mM Tris-HCl, pH 7·4) at room temperature (20°C) and the suspension was filtered through a Whatman microfiber glass filter with suction. The particles retained on the microfilter were subsequently washed twice with 5 ml of washing solution at room temperature. The filter was then transferred to a scintillation counting vial. To this were added 4 ml of PCS (Amersham) and 10 ml Omnifluor-Toluene solution. Radioactivity was determined in a Nuclear Chicago Isocap 300 spectrophotometer.

Diazepam was tested as an inhibitor of adenosine uptake into rat brain cortical synaptosomes. The results presented in Table 1 show that at a concentration of 10^{-8} M, diazepam caused a 13·6% reduction in the rate of uptake and that at higher concentrations there was an increasingly significant reduction in the rate of adenosine uptake. The concentration of diazepam required for a 20% reduction in the rate of uptake was 1×10^{-7} M, and that for a 50% inhibition was 1×10^{-4} M.

Flurazepam was clearly less potent than diazepam; the concentrations required for 20% and 50% inhibition of uptake being 8×10^{-6} and 5×10^{-4} M respectively. Flurazepam appears to inhibit adenosine uptake into rat brain synaptosomes competitively with a K_i value of 151 μM (unpublished observations).

In vivo experiments on rat cerebral cortical neurons have demonstrated the importance of adenosine uptake as a factor in the regulation of extracellular adenosine levels. Adenosine uptake inhibitors greatly potentiate the magnitude and duration of the depressant effects of iontophoretically applied adenosine and when applied in larger amounts they usually depress cell firing (Phillis & Kostopoulos 1975; Phillis et al 1979b, c). The uptake

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Table 1. 50 μl of rat cortical synaptosomal preparation was incubated with 2×10^{-7} M [^{3}H]adenosine for 30 s at 37°C. At the end of the incubation period, the incubation mixture was filtered through a GF/C Whatman fiber glass filter. The filter was subsequently washed twice with isotonic washing solution (for composition of the washing solution see text).

Drug (Concn) M	Adenosine uptake pmol mg^{-1} prot./30s	Inhibition %	Number of experiments
Control	15·76±0·70	—	29
Diazepam			
10^{-8}	13·61±1·17	13·6	9
10^{-7}	12·61±0·82***	20·0	9
10^{-6}	11·39±0·50**	27·7	6
10^{-5}	11·08±0·64**	30·0	9
10^{-4}	7·77±0·66*	50·7	9
Flurazepam			
10^{-8}	15·90±1·30	0	9
10^{-7}	15·70±1·49	0	9
10^{-6}	14·27±0·76	9·4	9
10^{-5}	11·70±1·16**	25·7	6
10^{-4}	9·66±1·22*	38·5	9

Results are expressed as mean ± s.e.m.
* $0·001 > P$
** $0·01 > P > 0·001$
*** $0·05 > P > 0·01$

inhibitors used in these experiments included dipyridamole, hexobendine, lidoflazine, papaverine and nitrobenzylthioguanosine and the effects of diazepam were observed to be very similar to the other uptake inhibitors. Further evidence that diazepam potentiates the effects of adenosine by inhibiting its uptake has been forthcoming from experiments on intestinal smooth muscle (Clanachan & Marshall 1980) in which diazepam (10^{-6} M) enhanced the depressant actions of adenosine but not those of 2-chloroadenosine, an adenosine analogue which does not readily cross cell membranes (Daly 1979).

The experiments reported in this communication give further support to the proposal that the therapeutic actions of benzodiazepines may involve a potentiation of the effects of endogenously released adenosine or other adenine nucleotides. Diazepam, in concentrations which are therapeutically relevant (10^{-8}–10^{-6} M), caused a 13·6–27·7 % inhibition of adenosine uptake (with an IC20 value of 1×10^{-7} M). Uptake into neuronal or glial cells constitutes a major route for the removal of extracellular adenosine and reductions of this order of magnitude would be expected to lead to enhanced extracellular adenosine concentrations. Diazepam (0·2 mg kg^{-1}) has in fact been shown to increase the rate of release of adenosine from the rat cerebral cortex (Phillis et al 1980).

Flurazepam had comparable actions to diazepam but was rather less potent. As the dose levels of flurazepam used therapeutically are higher than those for diazepam, our findings with this agent are consistent with the postulate that benzodiazepines may act by inhibiting adenosine uptake. The correlations between the clinical efficacy of a range of benzodiazepines and their effectiveness as synaptosomal adenosine uptake inhibitors are currently under investigation.

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