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Inosine, an endogenous ligand of the brain benzodiazepine receptor, antagonizes pentylenetetrazole-evoked seizures

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ABSTRACT **Partially purified extracts of bovine brain were previously found to inhibit competitively the binding of [^{3}H]-diazepam to rat brain synaptosomal membranes. The purines inosine and hypoxanthine were subsequently identified as the compounds responsible for this inhibitory activity. Intracerebroventricular administration of inosine to mice of the C3H/HEN and NIH general purpose strains caused a dose- and time-dependent increase in the latency to clonicotonic seizures produced by intraperitoneal administration of the convulsant pentylenetetrazole. Intracerebroventricular administration of equimolar doses of 2′-deoxyinosine, which is more potent than inosine in inhibiting the binding of [^{3}H]diazepam *in vitro*, significantly increased pentylenetetrazole-evoked seizure latency. In contrast, both 7-methylinosine and thymidine were ineffective in inhibiting the *in vitro* binding of [^{3}H]diazepam and increasing the latency to pentylenetetrazole-induced seizures *in vivo*. These results suggest that endogenously occurring purines such as inosine exhibit diazepam like effects when administered intracerebroventricularly, and these effects may be related to the interaction of inosine and related compounds with benzodiazepine receptors in the central nervous system.**

The characterization of saturable, high affinity, and stereospecific benzodiazepine binding sites in the central nervous system has been described *in vitro* and *in vivo* (1–7). The good correlations obtained between the binding affinity of a series of benzodiazepines *in vitro* and their anticonvulsant (4), anxiolytic (8), and muscle relaxant properties (4) strongly suggests that these sites may be receptors mediating the pharmacologic actions of the benzodiazepines. However, neither the regional distribution of benzodiazepine binding sites within the central nervous system nor the screening of a large number of putative neurotransmitter substances (1, 8) has provided evidence that such receptor sites are associated with classical neurotransmitter pathways. These findings have prompted a search for an endogenously occurring compound(s) that binds to the benzodiazepine receptor and functions as a physiological ligand (9, 10).

Low molecular weight substances that are heat stable, dialyzable, and competitively inhibit [^{3}H]diazepam binding to synaptosomal membranes *in vitro* have been isolated from bovine brain (9, 11). These substances have been subsequently identified as the purines inosine and hypoxanthine, and it has been suggested that they may modulate the benzodiazepine receptor *in vivo* (11). Since the inhibition of pentylenetetrazole (PTZ)-induced seizures has been used as a sensitive measure for assessing benzodiazepinelike activity *in vivo*, the effects of inosine and related compounds have been examined by using this paradigm. We now report that the intracerebroventricular (ICV) administration of inosine elicits a dose- and time-dependent increase in the latency period between the intraperitoneal injection of PTZ and the onset of clonicotonic convulsions. 7-Methylinosine, an inosine derivative that is ineffective in inhibiting [^{3}H]diazepam binding *in vitro* ($K_i > 5000$ μM, unpublished observations), is also ineffective (at doses equimolar with inosine) in increasing PTZ-envoked seizure latency in mice. In contrast, 2′-deoxyinosine, which is more potent than inosine *in vitro* [$K_i = 395$ μM vs. 836 μM, respectively (unpublished observations)], elicits a larger increase in PTZ-induced seizure latency than does inosine. These data suggest that the alterations in PTZ-induced seizure latency produced by inosine and 2′-deoxyinosine may be related to their ability to bind to benzodiazepine receptors, and that inosine or a closely related compound(s) may serve as an endogenous benzodiazepinelike compound.

MATERIALS AND METHODS

Male mice of the C3H/HEN and NIH general purpose strains (16–21 g) were obtained from the Veterinary Research Branch, National Institutes of Health (Bethesda, MD). ICV injection into the right lateral ventricle was performed using a modification of the method of Noble *et al.* (12). Animals were lightly anesthetized with ether and an incision made in the scalp to expose the bregma. A burr hole large enough for insertion of a 27-gauge needle was made through the skull with a dissecting needle approximately 1 mm caudal and lateral to the bregma (cf. Noble *et al.*). Animals were placed in a holding cage and allowed to recover for 20 min prior to ICV injection of compounds.

After the recovery period, mice were injected ICV at timed intervals by restraining the animal with the palm of the hand and immobilizing the head between the thumb and forefinger. Compounds or vehicle (phosphate-buffered saline, pH 7.2) were introduced directly into the right lateral ventricle by using a 27-gauge needle fitted with a stainless steel jacket recessed to expose 3.0–3.5 mm of needle. The needle was inserted at right angles to the skull, and a 10-μl volume was injected. The needle was kept in place for 15 sec after injection. This system provided a rapid and reliable method for ICV injections to conscious mice. Random dye injections into mice by this technique resulted in the presence of dye in the ventricular system of more than 95% of the animals examined.

After ICV injections, animals were transferred to 15 × 18 × 12 cm plastic cages for observations. PTZ (100 mg/kg for C3H/HEN and 120 mg/kg for NIH general purpose mice) was injected intraperitoneally at specific intervals after ICV injection, and the animals were observed for the onset of clonicotonic seizures. Two trained observers were routinely used to verify onset of seizures. With these doses of PTZ, greater than 95% of

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Abbreviations: PTZ, pentylenetetrazole; ICV, intracerebroventricular(ly).

all animals injected experienced clonicotonic seizures and, subsequently, death.

Inosine was purchased from P-L Biochemicals. 2′-Deoxyinosine, 7-methylinosine, and thymidine were obtained from Sigma. PTZ was purchased from ICN-K & K Laboratories, Plainview, NY. Phosphate-buffered saline (sterile) was obtained from the National Institutes of Health Media Unit.

RESULTS

Effects of Injection Interval on PTZ-Induced Seizure Latency. A time-dependent increase in PTZ-elicited seizure latency was observed in C3H/HEN mice injected ICV with 150 μg of inosine (Fig. 1). Maximal increases in seizure latency (375%; $P < 0.001$) were observed after a 1-min interval between injection of inosine and PTZ. A 2-min interval resulted in a doubling of seizure latency ($P < 0.01$), whereas a 10-min interval resulted in no significant change in seizure latency between inosine- and vehicle-treated groups.

Dose-Dependence of Altered Seizure Latency Produced by Inosine. In a series of experiments designed to determine whether the inosine-induced change in seizure latency was dose-dependent, inosine (50, 100, or 150 μg) was administered ICV by using a fixed interval of 2 min between injection of inosine and the intraperitoneal challenge with PTZ. A graded increase in seizure latency was observed in this dose range; however, the increase in seizure latency produced by inosine was statistically significant ($P < 0.02$) only at 150 μg (Fig. 2). Mice that were neither anesthetized nor injected ICV with vehicle had a slightly lower latency than vehicle-injected animals, which may be indicative of residual amounts of ether retained by the animals. In a preliminary series of experiments, a 5-min recovery period following ether anesthesia resulted in an increased seizure latency [4.2 ± 0.3 (SEM) min, $n = 12$] compared with that of the 20-min recovery period routinely used or that observed in noninjected controls (see Fig. 2). Nonetheless, ICV administration of inosine (150 μg), even after a 5-min recovery period, resulted in a significant increase in PTZ-induced seizure latency when compared to saline controls [10.3 ± 1.8 min ($n = 15$) vs. 4.2 ± 0.3 min ($n = 12$) $P < 0.01$].

Effects of Inosine Derivatives on PTZ-Induced Seizure Latency. The effects of ICV administered 7-methylinosine and 2′-deoxyinosine were examined in NIH general purpose mice challenged with PTZ at 120 mg/kg. In this series of experiments, 150 μg (0.56 μmol) of inosine increased seizure latency by 76% ($P < 0.02$), whereas equimolar doses of 2′-deoxyinosine

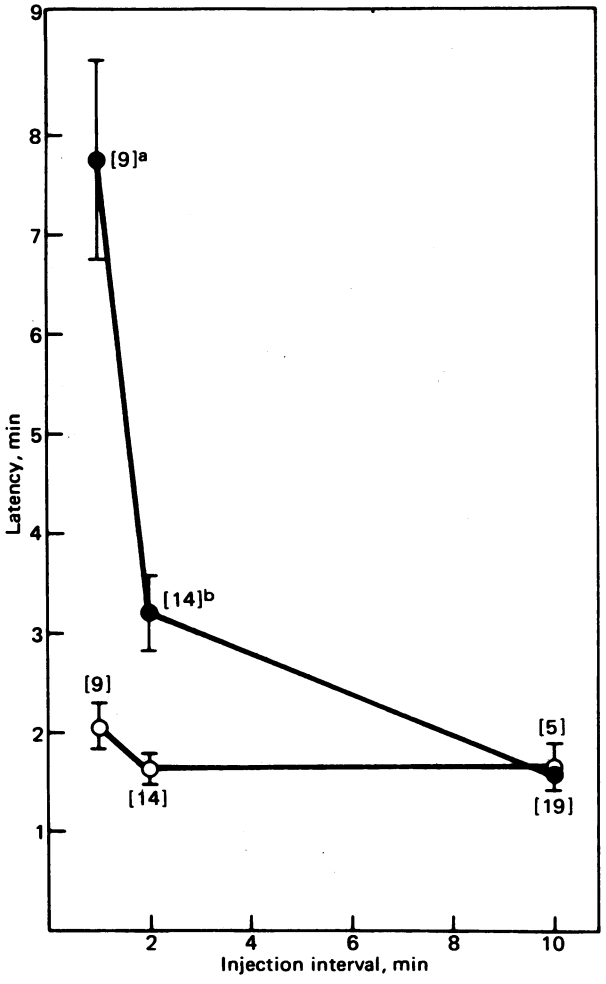

FIG. 1. Effects of injection interval on PTZ-induced seizure latency. Male C3H/HEN mice were injected ICV with inosine (150 μg) or vehicle followed by an intraperitoneal injection of PTZ (100 mg/kg) at specified intervals. The period between the injection of PTZ and the onset of seizures (latency) was noted. Values in brackets are the number of animals injected. ○, Vehicle; ●, inosine. a, $P < 0.001$; b, $P < 0.01$ compared to vehicle-injected animals.

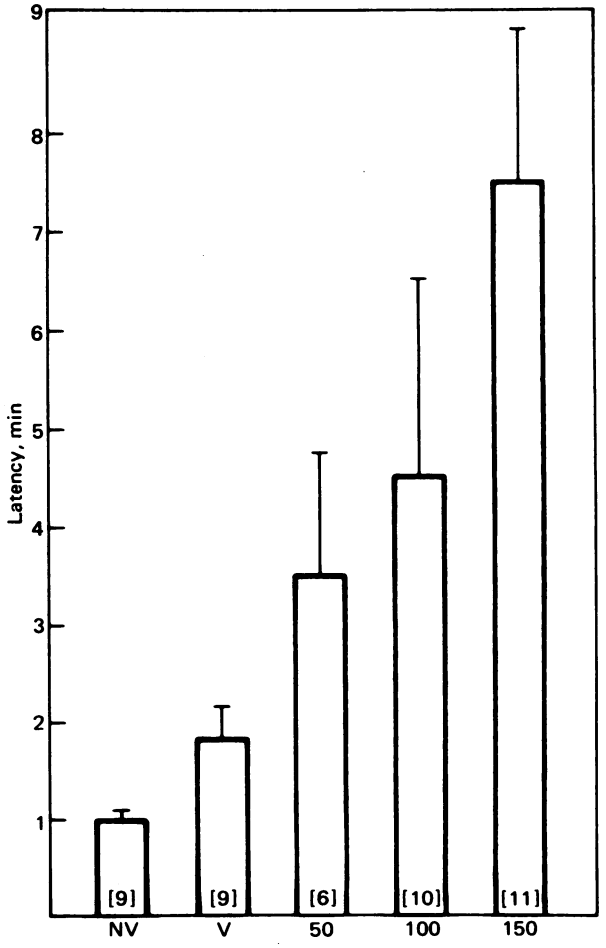

FIG. 2. Dose-dependence of alterations in seizure latency by inosine. Male C3H/HEN mice were injected ICV with 50, 100, or 150 μg of inosine, followed by an intraperitoneal injection of PTZ (100 mg/kg) 2 min later. The seizure latency was noted. NV, no vehicle; V, vehicle administered ICV. For animals injected with 150 μg of inosine, $P < 0.02$ compared to vehicle-injected animals.

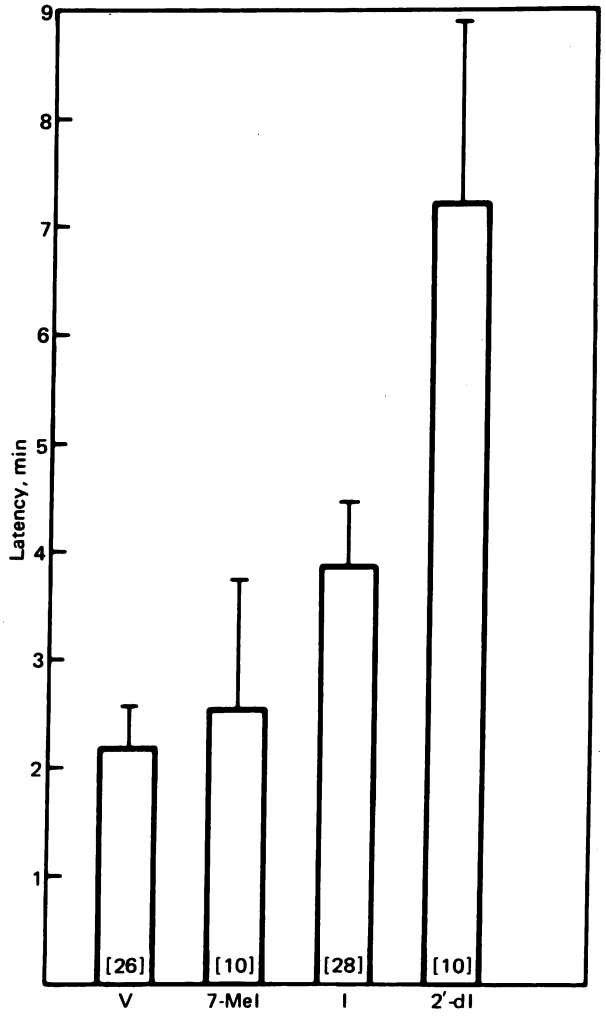

FIG. 3. Effects of inosine derivatives on PTZ-induced seizure latency. Male NIH general purpose mice were injected ICV with 0.56 μmol of compound or 10 liters of vehicle. One minute after injection of compound, the animals were injected intraperitoneally with 120 mg of PTZ per kg. V, vehicle; 7-MeI, 7-methylinosine; I, inosine; 2'-dI, 2'-deoxyinosine. $P < 0.001$ for 2'-deoxyinosine-injected animals and $P < 0.01$ for inosine-injected animals compared with vehicle-injected animals.

increased seizure latency by 330% ($P < 0.001$). In contrast, 7-methylinosine (0.56 μmol) was ineffective in increasing seizure latency (Fig. 3) as was administration of equimolar doses of thymidine [1.9 ± 0.2 min ($n = 19$) vs. 1.8 ± 0.3 ($n = 9$) for vehicle-injected mice]. No sedation or extraordinary behavior was noted after ICV injection of purines compared to vehicle controls.

DISCUSSION

The identification of high-affinity benzodiazepine binding sites in the central nervous system suggests the presence of endogenous substances capable of regulating the neurophysiological events subserved by these receptor sites. The characterization of low molecular weight, competitive inhibitors of [^{3}H]diazepam binding from bovine brain (9) and their subsequent identification as inosine and hypoxanthine (11) suggests that these or closely related substances may function as a ligand to the benzodiazepine receptor.

If these compounds interact with the benzodiazepine receptor *in vivo*, it is likely they will either mimic or antagonize the pharmacologic actions of the benzodiazepines. The effects of ICV administered inosine on PTZ-induced convulsions were examined because of the well-described techniques for assessment of convulsant/anticonvulsant properties of drugs and the observation that the anticonvulsant (anti-PTZ) effects of the benzodiazepines are well correlated with the anxiolytic potencies of these compounds (13). Furthermore, a pharmacologic action antagonistic to the benzodiazepines—*i.e.*, production of seizures—would also be readily detected by the use of these techniques.

The observation that inosine and 2'-deoxyinosine increase the latency to seizures produced by PTZ in a dose- and time-dependent fashion suggests a partial antagonism of the effects of PTZ, rather than a total blockade of seizures routinely observed with pharmacologic doses of many benzodiazepines. This finding may be explained by the relatively rapid loss of intraventricularly administered inosine from the central nervous system; an 80% loss of radioactivity was observed 2 min after ICV administration of 150 μg of [^{14}C]inosine (unpublished observations). Other explanations include the metabolic transformation of inosine to inactive purines or its translocation to sites distant from the benzodiazepine receptor. In contrast, when diazepam is administered, it is bound in significant amounts to the benzodiazepine receptor for many hours after a single anticonvulsant dose (unpublished results).

The *in vitro* affinities of inosine and hypoxanthine for the benzodiazepine receptor are weak when compared to the benzodiazepines themselves. This fact is difficult to reconcile with a proposed role of these compounds as endogenous modulators of the benzodiazepine receptor, particularly when whole brain concentrations of these purines have been estimated to be between 20 and 60 μM (14, 15). However, recent studies from this laboratory (unpublished results) have demonstrated that only a small fraction (20–30%) of benzodiazepine receptors need be occupied to afford full protection from PTZ-induced seizures, suggesting that the concentration of purines needed to inhibit 50% of [^{3}H]diazepam binding (IC_{50}) may not be a relevant measure of their physiologic properties *in vivo*. These observations are also supported by Lippa *et al.* (16) who demonstrated that only 10–20% of benzodiazepine receptors need be occupied to manifest a maximal antianxiety response in a standard conflict test for benzodiazepines.

The studies of McIlwain and associates (17, 18) have demonstrated that the levels of both inosine and hypoxanthine rise dramatically after electrical or chemical depolarization of brain slices, whereas tissue concentrations of these compounds at specific subcellular loci may be significantly greater. These findings, coupled with the additive inhibition of [^{3}H]diazepam binding, observed *in vitro* with inosine and hypoxanthine (11), support the hypothesis that these or related compounds may play a role in the function of benzodiazepine receptors *in vivo*.

An earlier report on the protective effects of adenosine against audiogenic seizures in mice (19) must also be re-evaluated in light of the present findings. These investigators observed a profound sedative effect after peripheral administration of large (130 mg/kg) doses of adenosine. Sedative effects of adenosine have also been noted following central administration of adenosine (20) or an inhibitor of adenosine deaminase (21). However, the protective effects of adenosine against audiogenic seizures are still manifest after the sedative effects are no longer apparent. In view of the action of ICV administered inosine on pentylenetetrazole-evoked seizures, the anticonvulsant effects of adenosine may result from the deamination of adenosine to inosine, because adenosine deaminase activity in the brain is high (21, 22) compared with peripheral tissues.

Nevertheless, our findings that inosine and closely related purines have pharmacological effects not unlike those expected of a short-acting benzodiazepine suggest that such purines may function as endogenous ligands for the benzodiazepine receptor. Additional investigation with a combination of neuropharmacologic, electrophysiologic, and behavioral techniques will be necessary to elucidate a definitive role for purines in the functions of the benzodiazepine receptor.

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