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Enhancement of ^{3}H-Diazepam Binding by SQ 65,396: A Novel Anti-Anxiety Agent[1]

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BEER, B., C. A. KLEPNER, A. S. LIPPA AND R. F. SQUIRES. *Enhancement of ^{3}H-diazepam binding by SQ 65,396: A novel anti-anxiety agent.* PHARMAC. BIOCHEM. BEHAV. 9(6) 849–851, 1978.—SQ 65,396, a clinically active anti-anxiety agent, enhanced the binding of ^{3}H-diazepam at 1.5 nM. This effect was due to an increase in the affinity for the ligand, without a change in the number of ^{3}H-diazepam binding sites. This action of SQ 65,396 may mediate its anti-anxiety effects by affecting the action of an endogenous modulator of the "benzodiazepine receptor." Several other substances and treatments increase the affinity of ^{3}H-diazepam for its receptors by mechanisms which may be related to the effect produced by SQ 65,396.

Anti-anxiety drugs Benzodiazepine receptors Endogenous ligands

SQ 65, 396 is one of a series of pyrazolo(3,4-6)pyridines with cyclic AMP phosphodiesterase inhibitory properties, which correlates with their ability to restore behavior suppressed by punishment in animal conflict procedures [1]. In six clinical trials, from which placebo responders were excluded, SQ 65,396 was found effective in reducing anxiety symptoms in 85% of the cases studied (Z. P. Horovitz, personal communication). Unlike diazepam, SQ 65,396 was shown to be without sedative effects in animals and failed to protect against convulsions induced by electroshock and strychnine, and was only weakly effective in protecting against pentylenetetrazole convulsions.

Brain-specific benzodiazepine receptors which selectively recognize pharmacologically and clinically active benzodiazepines *in vitro* appear to mediate their anti-anxiety action in man [11]. All of these benzodiazepines displace ^{3}H-diazepam or ^{3}H-flunitrazepam from their binding sites on brain membrane fragments [2]. Recently, a new series of triazolopyridazines, which produce anxiolytic-like effects in animals, have also been reported to inhibit ^{3}H-diazepam binding in rat brain [4,12].

Since the mechanism of action of SQ 65,396 remains unknown, we wanted to determine the effect of this drug on the binding of ^{3}H-diazepam to its brain-specific receptors. We now report that SQ 65,396 significantly increases the affinity of ^{3}H-diazepam for binding sites on rat brain membrane fragments.

METHOD

Male, albino rats (100–200 g; Royalhart Farms) were housed 4–6 per cage with food and water available ad lib. After sacrificing by decapitation, frontal cortex was separated from the rest of the brain at the anterior border of the caudate nucleus. This tissue was weighed and gently homogenized (Potter-Elvehjem, teflon-glass homogenizer) in 20 volumes of ice cold 0.32 M sucrose solution. Homogenates were centrifuged twice at 1000 g for 10 min at 4°C. The pellets were discarded and supernatants re-centrifuged at 30,000 g for 20 min at 4°C. The crude P_2 pellets thus formed were resuspended in twice the original homogenizing volume of cold 50 mM Tris·HCl buffer, pH 7.4. Three hundred μl of P_2 suspension (approximately 0.4 mg protein) was added to ice cold glass tubes containing Tris·HCl buffer and incubated with [N-Methyl] ^{3}H-diazepam (1.5 nM, S.A. 39 Ci/mmole, New England Nuclear) and SQ 65,396 (or deionized water) in a final volume of 2 ml. Non-specific binding was determined in the presence of 3 μM of unlabeled diazepam. After incubation at 0°C for 20 min, the reaction was terminated by rapid filtration under vacuum through Whatman GF/C filters. Following two 5 ml washes with iced Tris·HCl buffer, the filters were placed into scintillation vials, and radioactivity determined by conventional scintillation counting. Protein was determined by the method of Lowry [5]. Data are presented as specific binding, calculated by subtracting binding in the presence of 3 μM unlabeled diazepam from total binding determined in the absence of unlabeled diazepam.

RESULTS AND DISCUSSION

In the first experiment, we determined the effects of various concentrations of SQ 65,396 on the binding of 1.5 nM ^{3}H-diazepam. The data in Fig. 1 represent a typical experiment obtained by pooling frontal cortices from 6 animals. The data points are the means of triplicate determinations. SQ 65,396 increased the binding of ^{3}H-diazepam across a wide range of concentrations with the maximal effect occurring at 1–2 μM concentrations of SQ 65,396.

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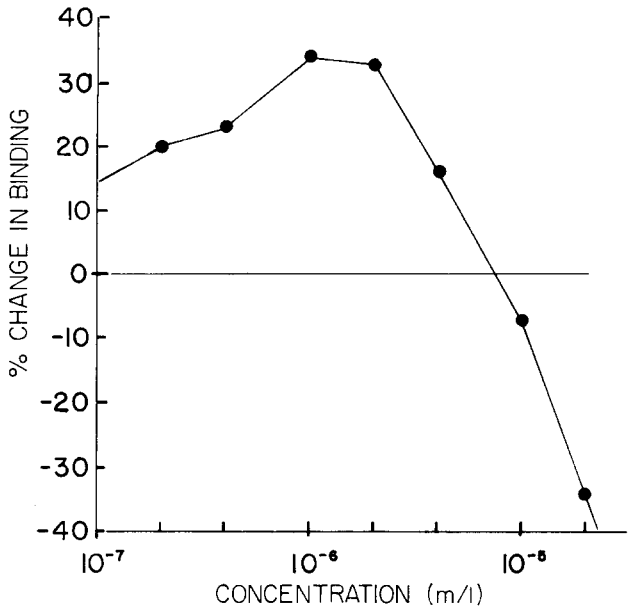

FIG. 1. Concentration effects of SQ 65,396 on ^{3}H-diazepam binding in rat brain.

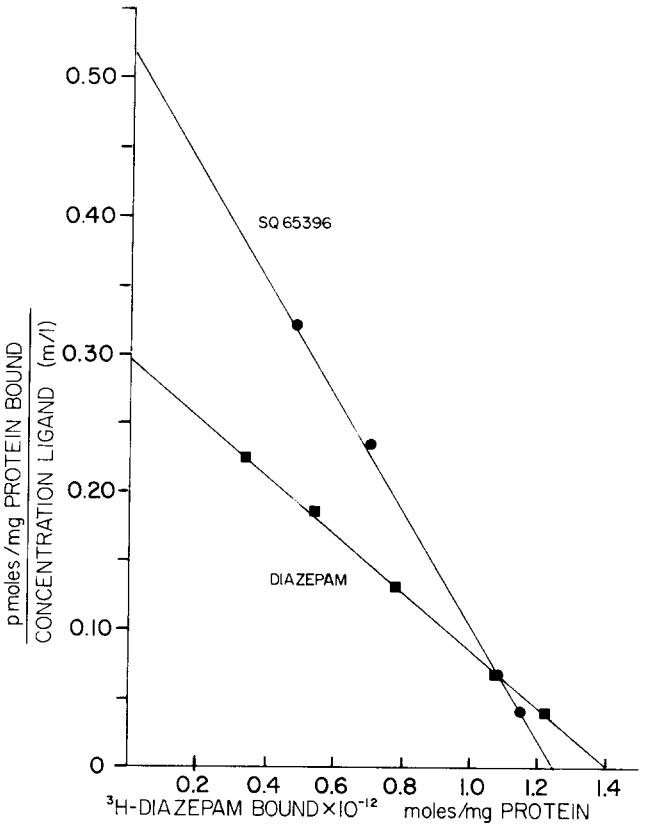

FIG. 2. Scatchard analysis of the effect of SQ 65,396 on ^{3}H-diazepam binding in the rat brain.

Additional experiments were conducted to determine the cause of the increased ^{3}H-diazepam binding. P_2 suspensions were incubated with various concentrations of ^{3}H-diazepam (1.5 to 30 nM) in the presence or absence of 1 μM SQ 65,396. K_D and B_{max} were determined by linear regression of the Scatchard plot [10]. As can be seen in Fig. 2, SQ 65,396 reduced the K_D from 5.27 to 2.45 nM without affecting the B_{max} (1.40 and 1.24 pmoles/mg protein for control and SQ 65, 396, respectively). These results indicate that the increased binding of ^{3}H-diazepam observed with SQ 65,396 was due to an increased affinity of ^{3}H-diazepam for its binding sites rather than to any change in the number of binding sites.

The effect of SQ 65,396 on the K_D for ^{3}H-diazepam is abolished by preincubating the brain membranes at 37°C for 30 min. It has recently been shown that heat pretreatment (50°C for 10 min) also increases the affinity of ^{3}H-diazepam for its binding sites without changing the maximal number of binding sites (C. Braestrup, personal communication).

The only substances so far reported which inhibit ^{3}H-diazepam binding are the pharmacologically and clinically active benzodiazepines, a new series of triazolopyridazines [4,12] and the endogenous purines, inosine and hypoxanthine [7]. The fact that SQ 65,396 significantly enhanced binding suggests the possibility of a novel mode of action for this clinically effective anti-anxiety drug.

Since SQ 65,396 increased affinity of ^{3}H-diazepam for its receptor, it may exert anxiolytic effects by enhancing the binding of an endogenous ligand for that receptor. Alternatively, the increased affinity may be due to an interaction with an endogenous inhibitor or modulator of the brain-specific benzodiazepine receptors. An endogenous, competitive inhibitor of ^{3}H-diazepam binding to rat brain membranes was recently described [3]. This substance is a heat stable, acid protein with an apparent molecular weight of 15,000, which also inhibits high affinity ^{3}H-GABA binding in a non-competitive fashion.

In agreement with the proposed interactions between ^{3}H-diazepam and ^{3}H-GABA binding sites, both GABA and the GABA-mimetic substance muscimol increase the binding of ^{3}H-diazepam (2 nM) to rat brain membranes, an effect which was reversed by the GABA antagonist bicuculline [13]. The enhancing effects of GABA and muscimol on ^{3}H-diazepam binding were also shown to be mainly on the affinity constant (K_D), and not on the maximal number of ^{3}H-diazepam binding sites, analogous to the effect produced by SQ 65,396.

Interestingly, it was recently shown that 5 mM Ni^{++} ion also greatly enhances the binding of subsaturating concentrations of ^{3}H-diazepam (3.4 nM) by increasing affinity without an effect on total binding sites [6]. One may speculate that Ni^{++} ion forms an inactive complex with an endogenous inhibitor of the benzodiazepine receptor.

In conclusion, it is tempting to speculate that Ni^{++}, heat pretreatment, GABA, muscimol and SQ 65,396 may all act through the common mechanism of reducing the activity of an endogenous inhibitor or modulator of benzodiazepine receptors.

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