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Laudanosine Does Not Displace Receptor-specific Ligands from the Benzodiazepinergic or Muscarinic Receptors

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The present study was designed to investigate whether D,L-laudanosine (a breakdown product of the neuromuscular relaxant atracurium besylate) interacts with benzodiazepinergic receptors or muscarinic receptors, both of which are involved in epilepsy and other types of seizures. The ability of D,L-laudanosine (10^{-10} to 5×10^{-5} M) to displace ligands specific for these receptors from their binding sites was tested. D,L-Laudanosine failed to inhibit the binding of [^{3}H]flunitrazepam to central benzodiazepine receptors in the cerebral cortex, the binding of [^{3}H]PK 11195 to peripheral benzodiazepine binding sites in the cerebral cortex and kidney, the binding of [^{3}H]Ro 5-4864 to peripheral benzodiazepine binding sites in the kidney, or the binding of [^{3}H]quinuclidinyl benzilate to muscarinic receptors in the cerebral cortex. These results suggest that laudanosine does not exert its convulsive effect *via* interaction with benzodiazepinergic or muscarinic receptors. (Key words: Complications: seizures. Neuromuscular relaxants: atracurium; laudanosine. Receptors: benzodiazepine; isoquinoline; muscarinic.)

BENZODIAZEPINES are widely used for premedication and induction of anesthesia and during the perioperative period because of their anxiolytic and hypnotic properties. In general practice they are also used as central acting muscle relaxants and anticonvulsants. Many studies have suggested that benzodiazepines produce these pharmacologic effects *via* high-affinity, stereospecific central benzodiazepine receptors located in the central nervous system.[1,2] Central benzodiazepine receptors are coupled to γ-aminobutyric acid and to the chloride ionophore[3,4] and exhibit high affinity for diazepam and clonazepam. In addition, another type of recognition site for benzodiazepines has been identified in peripheral tissues as well as in the brain.[5,6] The exact function of these so-called peripheral benzodiazepine binding sites is unknown, although a relationship between the potent convulsant actions of parenterally administered Ro 5-4864 (4-chlorodiazepam) and the binding of this compound to peripheral benzodiazepine binding sites in brain has been reported.[7] Ro 5-4864 is utilized for studies of peripheral benzodiazepine binding sites because it is recognized only by peripheral benzodiazepine binding sites and not by central benzodiazepine receptors.

Central benzodiazepine receptors and peripheral benzodiazepine binding sites also bind the nonbenzodiazepine ligands quinolines and isoquinolines, respectively, with high affinity.[8,9] Laudanosine (N-methyltetrahydropapaverine), one of the breakdown products of the neuromuscular relaxant atracurium besylate, is structurally in the general class of quinolines or isoquinolines (fig. 1). When administered to dogs in repetitive high doses, laudanosine can produce convulsive seizures, which are suppressed by diazepam.[10] The purpose of the present study was to assess whether D,L-laudanosine is bound by and mediates its convulsant effect *via* the benzodiazepinergic system. Another receptor, the muscarinic cholinergic receptor, was also studied because of its involvement in experimental models of epilepsy and other types of seizures.[11]

Materials and Methods

MATERIALS

[^{3}H]Flunitrazepam (FNZ) (77 Ci/mmol), [^{3}H]PK 11195 (74.3 Ci/mmol), Ro 5-4864 (78.9 Ci/mmol), and [^{3}H]quinuclidinyl benzilate (QNB) (39.4 Ci/mmol) were purchased from New England Nuclear (Boston, Massachusetts). Unlabeled clonazepam was donated by Drs. H. Gutman and E. Kyburz, Hoffmann-La Roche (Basel, Switzerland). Unlabeled PK 11195 was a gift from Dr. G. Le Fur, Pharmuka Laboratories (Gennevilliers, France). D,L-Laudanosine was purchased from Sigma Chemical (St. Louis, Missouri). Lumax was obtained from Lumac (Schaesberg, The Netherlands). All other chemicals were obtained from commercial sources.

PREPARATION OF MEMBRANES

The study was approved by the Animal Care and Use Committee of the Technion Faculty of Medicine. Sprague-Dawley rats (200–250 g) were killed by decapitation. Their cerebral cortex and kidneys were rapidly removed and stored at −20° C until use. Prior to the binding assay,

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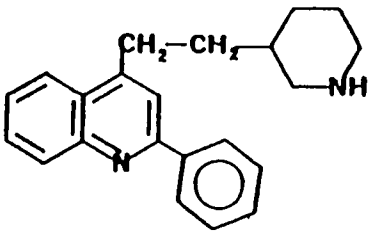
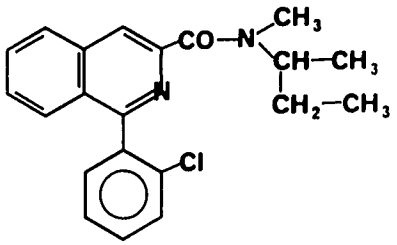
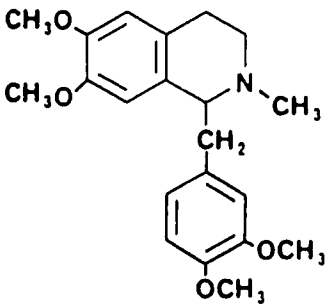


PK 9084



PK 11195



DL-Laudanosine

FIG. 1. The chemical structure of the central benzodiazepine ligand PK 9084 (a quinoline derivative); the peripheral benzodiazepine ligand PK 11195 (an isoquinoline carboxamide derivative); and laudanosine.

tissues were thawed and homogenized in 25 volumes of 50 mM Tris-HCl buffer, *p*H 7.4, and centrifuged at 49,000 *g* for 15 min. The cerebral cortex pellet was resuspended in 50 volumes Tris-HCl buffer and used for [^{3}H]PK 11195 binding. This homogenate was further diluted 1:4 for [^{3}H]FNZ and [^{3}H]QNB binding. The kidney pellet was suspended in 200 volumes of Tris-HCl buffer and used for [^{3}H]PK 11195 and [^{3}H]Ro 5-4864 binding.

BINDING ASSAYS

For central benzodiazepine receptor studies, cerebral cortical membranes (350 μl) were incubated with 25 μl [^{3}H]FNZ (1.5 nM final concentration) in the absence (total binding) or presence of 10^{-10} to 5×10^{-5} M (final concentration) unlabeled clonazepam or D,L-laudanosine. Clonazepam at a concentration of 10 μM was used to determine nonspecific binding. Specific binding was determined as total binding minus nonspecific binding and was used as control. After incubation for 60 min at 4° C, samples were filtered under vacuum over Whatman GF/B filters and washed three times with 5 ml of ice-cold Tris-HCl buffer. Filters were placed in vials, 4 ml of xylene-Lumax (3:1) scintillation fluid was added, and radioactivity was counted after an 8-h equilibration period.

For peripheral benzodiazepine binding site studies, cerebral cortical and kidney membranes (350 μl) were incubated with 25 μl [^{3}H]PK 11195 (1.5 nM final concentration) in the absence (total binding) or presence of 10^{-10} to 5×10^{-5} M (final concentration) unlabeled PK 11195 or D,L-laudanosine. Peripheral benzodiazepine binding sites in the kidney were also assayed with 25 μl [^{3}H]Ro 5-4864 (2 nM final concentration) in the absence (total binding) or presence of 10^{-10} to 5×10^{-5} M (final concentration) unlabeled PK 11195 or D,L-laudanosine. The rest of the procedure was as described above for central benzodiazepine receptor binding assays, except that 10 μM unlabeled PK 11195 was used to determine nonspecific binding.

Muscarinic cholinergic receptors were determined using [^{3}H]QNB according to the method of Yamamura and Snyder.[12] Binding assay in a final volume of 1 ml Tris-HCl buffer, *p*H 7.4, contained 50 μl of [^{3}H]QNB (0.5 nM final concentration) and 850 μl cerebral cortical membranes in the absence (total binding) or presence of 10^{-10} to 5×10^{-5} M (final concentration) unlabeled atropine sulfate or D,L-laudanosine. Incubation for 60 min was performed at 25° C. The rest of the procedure was as described above for central benzodiazepine receptor assays, except that 10 μM unlabeled atropine sulfate was used to determine nonspecific binding.

Stock solutions of clonazepam, PK 11195, and D,L-laudanosine were prepared in alcohol, and the final concentration of alcohol in all the assays was 1%.

Results

Table 1 presents the potency of clonazepam to displace [^{3}H]FNZ from central benzodiazepine receptors, the potency of PK 11195 to displace [^{3}H]PK 11195 and [^{3}H]Ro 5-4864 from peripheral benzodiazepine binding sites, and also the potency of atropine sulfate to displace [^{3}H]QNB from muscarinic receptors. All these unlabeled ligands showed inhibition constant (K_I) values to their respective receptors in the nanomolar range.

The potency of D,L-laudanosine (10^{-10} to 5×10^{-5} M, final concentration) to displace [^{3}H]FNZ, [^{3}H]PK 11195, [^{3}H]Ro 5-4864, and [^{3}H]QNB from their respective receptors was also tested. The K_I values obtained from such displacement experiments were higher than 5×10^{-5} M (table 1), which means that this compound was inactive at the aforeexamined receptor sites.

Discussion

Atracurium is a competitive neuromuscular blocking agent that is independent of hepatic and renal pathways

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TABLE 1. Potency of Various Ligands to Bind to Central and Peripheral Benzodiazepine Receptors and to the Muscarinic Receptors

Ligand	Tissue	Displacer Compound	K_I (nM)
[^{3}H]FNZ	Cerebral cortex	Clonazepam	1.2
[^{3}H]FNZ	Cerebral cortex	D,L-laudanosine	>50,000
[^{3}H]PK 11195	Cerebral cortex	PK 11195	0.8
[^{3}H]PK 11195	Cerebral cortex	D,L-laudanosine	>50,000
[^{3}H]PK 11195	Kidney	PK 11195	1.8
[^{3}H]PK 11195	Kidney	D,L-laudanosine	>50,000
[^{3}H]Ro 5-4864	Kidney	PK 11195	3.8
[^{3}H]Ro 5-4864	Kidney	D,L-laudanosine	>50,000
[^{3}H]QNB	Cerebral cortex	Atropine sulfate	0.6
[^{3}H]QNB	Cerebral cortex	D,L-laudanosine	>50,000

Membrane preparations and binding experiments are described in "Materials and Methods." Specific binding was determined in the presence of 8–10 concentrations in triplicates of various unlabeled ligands to estimate IC_{50} values (concentration causing 50% inhibition of [^{3}H]ligand binding). The K_I values were calculated from the equation $K_I = IC_{50}/[(1 + F/K_D)]$, where F = [^{3}H]ligand concentration and K_D = equilibrium dissociation constant. Values are the mean of three separate experiments with less than 15% variability.

for terminating its action. At physiologic conditions a chemical reaction called Hofmann elimination occurs, and laudanosine plus a quaternary monoacrylate are formed. The quaternary monoacrylate is unstable in plasma and may also undergo a further breakdown by the same process of Hofmann elimination. Therefore, two molecules of laudanosine are potentially generated from one molecule of atracurium.[13]

High doses of laudanosine (15 $mg \cdot kg^{-1} \cdot h^{-1}$) given to lightly anesthetized dogs, yielding plasma concentration of 17 $\mu g/ml$ (5×10^{-5} M), have been reported to produce convulsive seizures.[10] In the present study we found that at a concentration of 5×10^{-5} M, D,L-laudanosine failed to displace [^{3}H]FNZ and [^{3}H]PK 11195 from central benzodiazepine receptors and peripheral benzodiazepine binding sites, respectively, in the cerebral cortex and [^{3}H]Ro 5-4864 and [^{3}H]PK 11195 from peripheral benzodiazepine binding sites in the kidney. The rationale for using two peripheral benzodiazepine ligands for the binding assays was based on a recent observation that an isoquinoline binding site and a benzodiazepine binding site are not identical in all species. For instance, in the calf only PK 11195 labels peripheral benzodiazepine binding sites with high affinity, whereas Ro 5-4864 binds with much lower potency; yet both compounds present similar affinities in the rat.[14] This could give rise to an assumption that there are two domains in one receptor site that could be affected differently by D,L-laudanosine.

Muscarinic antagonists are effective against convulsions produced by acetylcholine receptor agonists[11] and decrease the convulsive threshold for pentylenetetrazol in mice lesioned with kainic acid.[15] Therefore, we also tested whether D,L-laudanosine interacts with muscarinic receptor sites. We found that D,L-laudanosine failed to inhibit [^{3}H]QNB binding to the muscarinic cholinergic receptors in the cerebral cortex. These *in vitro* studies suggest that laudanosine-induced convulsions are not mediated *via* either the benzodiazepinergic or the central muscarinic receptor system.

The results from this study suggest that although diazepam suppresses laudanosine-induced seizures, its effect is probably not specific because even at a high concentration D,L-laudanosine failed to bind to central benzodiazepine receptors or to peripheral benzodiazepine binding sites. It is possible that not only diazepam, but also other drugs such as hydantoin or the general anesthetic barbiturates, which are potent anticonvulsants, may suppress laudanosine-induced seizures.

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