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Solubilization of a [^{3}H]Cimetidine Binding Site from Rat Brain

A Clonidine-Sensitive H-2 Receptor Subtype?

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SUMMARY

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A binding site for [^{3}H]cimetidine was obtained from rat brain membranes solubilized with digitonin. The site displayed saturability, high affinity, and drug specificity for imidazole H-2 antagonists and was able to bind ligand at physiological temperatures. The regional distribution of binding sites paralleled that of neuronal histamine projections. Displacement of binding did not occur readily with H-2 agonists, and, although 2-guanidino-4-[2-(2-cyano-3-methyl-guanidino)ethyl-thiomethyl]thiazole (ICI 125,211, (a non-imidazole H-2 antagonist) displaced [^{3}H]cimetidine, it was less potent than imidazole antagonists. Micromolar concentrations of clonidine, a substance thought to stimulate a central imidazole H-2 receptor subtype, were able to displace [^{3}H]cimetidine binding, and chronic treatment of rats with clonidine *in vivo* resulted in down-regulation of the sites. These data suggest that the solubilized [^{3}H]cimetidine binding site is associated with the putative clonidine-sensitive H-2 receptor subtype.

The mammalian brain has been shown to contain effector systems involving both histaminergic H-1 and H-2 receptors (1, 2). Cellular events such as catecholamine release, phospholipid turnover, and glycogenolysis are linked specifically to histamine H-1 receptors (1–4), whereas histamine H-2 receptors mediate adenylate cyclase activation (1, 2). Radioligand receptor binding studies have used [^{3}H]mepyramine to evaluate the properties of H-1 receptors and to typify their ontogeny and transsynaptic regulation (2, 5–8); H-1 receptors also have been successfully solubilized (9). Recently [^{3}H]cimetidine, a histamine H-2 antagonist, has been used as a ligand to study the binding characteristics of crude membrane preparations of guinea pig and rat brain. In contrast to studies of the H-1 receptor, results obtained with cimetidine have been more complex (10–12). Although binding is observed readily at 4°, no specific binding is seen at physiological temperatures (11). Second, cimetidine binding in some studies of crude preparations of rat brain may display multiple components with differential sensitivities to copper (10–12). Because of these discrepancies, solubilized preparations of the receptors may offer a better opportunity to evaluate [^{3}H]cimetidine binding, as has been done successfully for H-1 receptors (9). In the current study, receptors from the rat brain have been solubilized with digitonin, and the resultant binding of [^{3}H]cimetidine has been evaluated.

Adult Sprague-Dawley rats (Zivic-Miller, Allison Park, Pa.) of either sex, weighing 150–200 g, were decapitated and the brains were homogenized (Polytron, 15 sec) in 5 volumes of ice-cold 0.05 M sodium-potassium-phosphate buffer (pH 7.45). Subsequent operations were carried out at 0–4° unless otherwise specified. The homogenate was centrifuged at 1,000 × *g* for 5 min to remove cell debris and the nuclear fraction, and the supernatant was centrifuged at 50,000 × *g* for 20 min. The pellet was resuspended in 5 volumes of the phosphate buffer and recentrifuged. The final pellet was resuspended in phosphate buffer (2.5 ml/g of original tissue, wet weight) containing 1% digitonin, using four or five up-down strokes in a smooth glass homogenizer fitted with a Teflon pestle. The preparation was then stirred for 30 min and centrifuged at 100,000 × *g* for 60 min. The resulting supernatant was stored at −20° until the binding studies were performed (usually the next day). The solubilized receptor preparation was found to be unstable after 2–3 days of storage. The protein content ranged from 1 to 2 mg/ml as estimated by the method of Lowry *et al.* (13).

To assess [^{3}H]cimetidine binding, 800 μl of the solubilized preparation were added to varying concentrations of [^{3}H]cimetidine (100 μl) with or without 100 μM cold cimetidine to assess nonspecific binding. The mixture was incubated at 37° for 30 min and was then placed on

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ice for 5 min. Subsequently, 250 μl of phosphate buffer containing 10% activated charcoal and 2.5% bovine serum albumin were added, thoroughly mixed for 2 min, and quickly centrifuged in a Beckman microfuge. An aliquot of the supernatant was counted for radioactivity in a Triton-based scintillation cocktail, and specific binding was determined as the difference between samples with and without unlabeled cimetidine. The 30-min incubation was sufficient to reach equilibrium binding values ±10%, and no decrease was observed even after 50 min of incubation. The effects of H-2 antagonists or agonists, of H-1 antagonists, of clonidine, or of Cu^{2+} were assessed by adding these agents to the incubation mixture.

This method for receptor solubilization and binding assay is similar to that described by Gavish *et al.* (9) for H-1 and γ-aminobutyric acid receptors. The concentration of unlabeled cimetidine used to assess nonspecific binding was taken from values reported by Kendall *et al.* (11) for crude rat brain membrane preparations.

N-[*methyl*-^{3}H]cimetidine (24–26 Ci/mmole) was a generous gift from Amersham Corporation (Arlington Heights, Ill.). Promethazine and triprolidine were obtained from Burroughs-Wellcome laboratories (Research Triangle Park, N. C.), and cimetidine, 4-methylhistamine, dimaprit, mepyramine, diphenhydramine, and metiamide were kindly donated by Smith Kline & French Laboratories (Philadelphia, Pa.). ICI 125,211 was kindly provided by Dr. S. J. Enna (University of Texas Medical School) and clonidine by Dr. J. N. Davis. (Duke University Medical School).

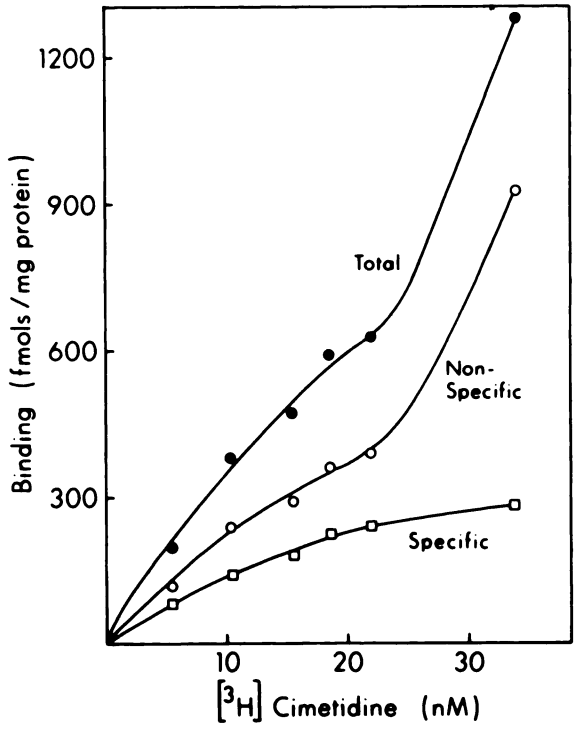

FIG. 1. *Saturation curve of [^{3}H]cimetidine binding in solubilized preparations from rat brain*

Specific binding (□) was determined as the difference between total binding (●) and nonspecific binding (○). Data represent means of triplicate determinations. Each experiment was performed three times in separate preparations, and standard errors were generally 5–10% of the value.

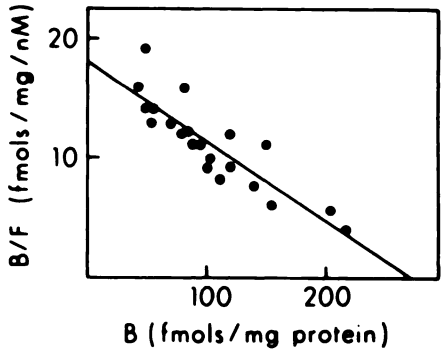

FIG. 2. *Scatchard analysis of specific [^{3}H]cimetidine binding (0.8–34 nM) in solubilized preparations from rat brain*

Data represent values obtained in four separate membrane preparations from 6–10 animals. Each point is the mean of triplicate assays. K_d = 15.7 ± 0.5 nM and B_{max} = 231 ± 6 fmoles/mg of protein.

The solubilized membrane preparation exhibited specific binding of [^{3}H]cimetidine at 37° which was saturated at approximately 30 nM of the ligand (Fig. 1). Nonspecific binding, determined in the presence of unlabeled cimetidine, was generally 40–50% of the total binding but increased sharply at high concentrations of cimetidine. Thus, unlike the crude membrane preparation (11), the solubilized preparation demonstrates binding at physiological temperatures.

Scatchard analysis revealed a single specific binding component with a K_d of 15.7 nM and a B_{max} of 276 fmoles/mg of protein (Fig. 2). The solubilized form of the receptor thus contains a single high-affinity site for the radioactive ligand compared with the relatively low affinity (K_d 400–2100 nM), and possibly a multiple component situation as reported in crude rat brain membrane preparations (11). In crude preparations, Cu^{2+} has been found to increase the capacity of the cimetidine binding site and to shift the specificity of binding (11). This property was lost upon solubilization, as copper had no effect on the binding characteristics of [^{3}H]cimetidine in the present study (Table 1). Several possibilities may explain these differences. First, the solubilized receptor may not be the same site as that present in the crude preparation; molecular heterogeneity of receptors for H-2 antagonists, including possibly separate entities, have been inferred from responses seen in tissue slices versus cell-free preparations (14). Alternatively, solubilization may remove a copper-sensitive regulatory component of the receptor.

Isolation of solubilized binding sites from different brain regions indicated basic similarities with the distri-

TABLE 1

Effect of Cu^{2+} on binding characteristics of [^{3}H]cimetidine in solubilized membrane preparation from rat brain

Data represent means ± standard error of three determinations in a preparation pooled from eight animals. Specific binding was determined at 12.1 nM [^{3}H]cimetidine, and the Scatchard analysis was performed as in Fig. 2.

Addition	Specific binding	K_d	Maximal binding
	fmoles/mg protein	*nM*	*fmoles/mg protein*
None	156 ± 2	13	231
50 μM Cu^{2+}	149 ± 6	13	231

TABLE 2

Regional distribution of [^{3}H]cimetidine binding to solubilized rat brain preparations

Data represent means ± standard error of four experiments from pooled tissues from four rats each. The [^{3}H]cimetidine concentration was 12.1 nM.

Region	Specific binding
	fmoles/mg protein
Hypothalamus	184 ± 6
Midbrain + thalamus	150 ± 13
Hippocampus	250 ± 14
Corpus striatum	64 ± 2
Medulla + pons	44 ± 6
Cerebellum	64 ± 6
Cortex	45 ± 7
Whole brain	164 ± 15

TABLE 3

Effect of clonidine administration in vivo on [^{3}H]cimetidine binding to solubilized preparations from rat brain regions

Data represent means ± standard error from preparation from the number of animals indicated in parentheses. The [^{3}H]cimetidine concentration was 12.1 nM.

Treatment	Specific binding		
	Hippocampus	Hypothalamus	Rest of brain
	fmoles/mg protein		
Control	175 ± 12 (6)	206 ± 10 (3)	100 ± 2 (6)
Clonidine (2.5 mg/kg s.c. for 9 days)	100 ± 3[a] (6)	144 ± 9[a] (3)	87 ± 2[a] (6)

[a] $p < 0.001$ versus controls.

bution of neuronal histamine (1, 2), i.e., large amounts in hypothalamus, midbrain + thalamus, and hippocampus, with a much smaller degree of binding in corpus striatum, cerebellum, and cerebral cortex (Table 2). The distribution stands in marked contrast to that seen with crude preparations, where [^{3}H]cimetidine binding sites were found to be uniformly distributed throughout the brain (15).

Displacement curves with several histamine H-2 drugs indicated that antagonists could displace [^{3}H]cimetidine binding, whereas agonists were much less effective (Fig. 3). Metiamide, a typical imidazole histamine H-2 antagonist, was fully efficacious in displacing [^{3}H]cimetidine and displayed a lower affinity than did cimetidine. 4-Methylhistamine, an H-2 agonist, was less potent, followed by histamine and dimaprit, another H-2 agonist. All of the H-1 antagonists (triprolidine, promethazine, mepyramine, diphenhydramine) were relatively ineffective, having IC_{50} values > 1 mM. The lower affinities of histamine H-2 agonists compared with H-2 antagonists for displacement of [^{3}H]cimetidine has been seen previously in broken cell preparations (15) and could reflect either separate agonist and antagonist forms of the receptor, or the existence of multiple recognition sites (16). In this regard, Karppanen (17) has summarized evidence that an "imidazole H-2 receptor subtype" may exist which is clonidine-sensitive and which is responsible for some of the histamine-like actions of clonidine in the central nervous system, such as H-2-like stimulation of adenylate cyclase (18). Clonidine was found to displace [^{3}H]cimetidine from the solubilized receptor (Fig. 3); furthermore, although ICI 125,211, a non-imidazole H-2 antihistamine, could displace [^{3}H]cimetidine, its potency was far lower than that seen for its antagonism of histaminergic effects in other tissues (19). These results suggested that the solubilized [^{3}H]cimetidine binding site could be the imadazole H-2 subtype. If this hypothesis were correct, down-regulation of [^{3}H]cimetidine binding should occur with repeated clonidine administration. Rats were given 0.9% NaCl solution (controls) or clonidine (2.5 mg/kg s.c.) daily for 9 days and killed 24 hr after the last injection. [^{3}H]Cimetidine binding to solubilized receptors indicated decreases in specific binding of approximately 40% in hippocampus and hypothalamus and 13% in the rest of the brain (Table 3). This reduction did not reflect simply residual clonidine in the preparation, as no inhibition was obtained if brain homogenates from untreated animals were exposed to clonidine (2.5 μg/g of brain) prior to receptor isolation (data not shown). It is

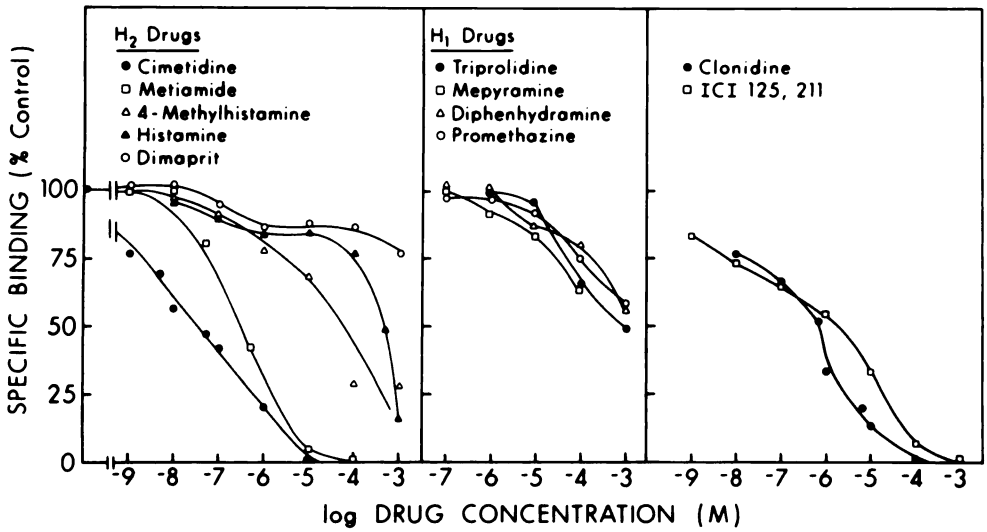

FIG. 3. *Inhibition of specific [^{3}H]cimetidine binding in rat brain solubilized preparations by imidazole type H-2 antagonists and agonists (left), by H-1 antagonists (center), and by clonidine or ICI 125,211 (right)*

Data represent displacement curves generated from several different preparations.

therefore likely that the solubilized [^{3}H]cimetidine binding site represents the clonidine-sensitive H-2 subtype.

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