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ACCELERATED COMMUNICATION

Novel Receptor Site Involved in Enhancement of Stimulus-Induced Acetylcholine, Dopamine, and Serotonin Release

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SUMMARY

The cognitive enhancer DuP 996 [3,3-bis(4-pyrindinylmethyl)-1-phenylindolin-2-one] and its structural analogs enhance the K^+-stimulated release of acetylcholine, dopamine, and serotonin in brain slices, without effect on basal release. A novel receptor site labeled by [^{3}H]DuP 996 has been identified. The [^{3}H]DuP 996 binding site has a K_d of 19 nM and a B_{max} of 102 fmol/mg of protein. Binding to this site is specific, saturable, reversible, and time, pH, and temperature dependent. Specific binding is decreased by treatment with trypsin and not affected by phospholipase C. Specific binding is inhibited by Ca^{2+} and increased by Mn^{2+} but not affected by Na^+, K^+, or Mg^{2+}. The [^{3}H]DuP 996 binding sites are heterogeneously distributed in brain, with striatum and hypothalamus having highest density and cerebellum lowest. The [^{3}H]DuP 996 binding site does not belong to any known class of receptor site, because [^{3}H]DuP 996 binding could not be displaced by a broad variety of standard pharmacological agents and neuropeptides. Physiological significance of this binding site is suggested by the excellent correlation between the binding affinity for this site and the potency to enhance K^+-stimulated release of acetylcholine for a series of DuP 996 analogs. Ligands for this receptor site may have therapeutic potential for the treatment of cognitive deficits and neurodegenerative diseases.

Recent findings suggest that the cerebral cholinergic system may be involved in the senile decline of cerebral function. Cortical ACh synthesis and release decline as a function of age in experimental animals (1–3). In Alzheimer's disease, there is a marked reduction in the number of cholinergic cell bodies in the nucleus basalis of Meynert, resulting in a decrease of choline acetyltransferase activity, acetylcholinesterase activity, and ACh synthesis in the cortical and hippocampal projection areas (4–7). In addition, the noradrenergic, the dopaminergic, and the serotonergic systems also appear to be deficient in a majority of patients with Alzheimer's disease (8, 9). Marked deficits in cognitive performance and cortical presynaptic cholinergic markers can be induced in experimental animals by lesioning of the nucleus basalis magnocellularis, which contains the cholinergic cell bodies that send their projections to the cortex (10–14). These cognitive deficits can be attenuated by cholinergic drugs (15, 16). Moreover, clinical trials with cholinesterase inhibitors such as physostigmine and tetrahydroaminoacridine have shown improvement in cognitive measures in patients with Alzheimer's disease (17–21).

A strategy for enhancement of neuronal function is to enhance endogenous stimulus-induced neurotransmitter release, which would result in an increase of the amount of neurotransmitters solely when their release is triggered by excitation of the neurons. Such action should result in an improvement of the signal-to-noise ratio during transmission, without overload toxicity that is typical of cholinesterase inhibitors and without the distortion of temporal patterns in neuronal transmission caused by direct receptor agonists. We have shown that DuP 996 enhances K^+-stimulated release of ACh, DA, and 5-HT, but not NE and GABA, in brain slices *in vitro* and enhances cortical ACh release *in vivo* (22, 23). L-Glutamate release is only slightly enhanced by DuP 996 (22, 23). DuP 996 has significant "memory-enhancing" effects in a variety of animal models of learning and memory. Specifically, DuP 996 has been shown to protect against hypoxia-induced passive avoidance deficit in rats (24, 25). In addition, DuP 996 is active in both the shock- and appetite-motivated procedures and has been shown to enhance performance levels when administered after training, as well as before training trials (24). In clinical studies, DuP 996 induced electroencephalographic changes, demonstrating significant central effects of vigilance-improving properties (26).

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ABBREVIATIONS: ACh, acetylcholine; DA, dopamine; NE, norepinephrine; 5-HT, 5-hydroxytryptamine (serotonin); KR, Krebs-Ringer; FRC, fractional rate constant; GABA, γ-aminobutyric acid.

The present paper describes a novel receptor site for DuP 996 and its analogs and presents the characterization of this receptor.

Materials and Methods

Membrane preparation. Male Sprague-Dawley rats (Charles River Breeding Laboratories, MA), at 200–250 g, were euthanized by decapitation. Rat brain membranes were prepared according to previously published procedures (27). Brain regions were dissected according to the method of Heffner *et al.* (28). Brain tissues were homogenized (20 sec) in 10 volumes (w/v) of ice-cold 0.32 M sucrose with a Brinkman Polytron. The homogenate was centrifuged at 920 × *g* for 10 min at 4°. The supernatant was centrifuged at 47,000 × *g* for 20 min. The resulting membrane pellet was resuspended in 10 volumes (original w/v) of 50 mM Tris·HCl (pH 7.4) and incubated at 37° for 30 min, to degrade and dissociate any bound endogenous ligands present. The membranes were then centrifuged at 47,000 × *g* for 20 min and resuspended in 50 mM Tris·HCl buffer. Protein concentrations were determined by the method of Bradford (29).

[^{3}H]DuP 996 binding. Aliquots of brain membranes (0.5–1 mg of protein) were incubated with [^{3}H]DuP 996 (41.5 Ci/mmol; custom synthesis by DuPont-NEN, Boston, MA) in 50 mM Tris·HCl buffer, pH 7.4, in a final volume of 1 ml, at room temperature for 45 min. The samples were rapidly filtered through Whatman GF/B glass filters (which had been previously soaked for 3 hr in 0.2% polyethylenimine), using a Brandel 24R cell harvester (Brandel, Gaithersburg, MD) attached to an Edwards 18 two-stage vacuum pump for very rapid filtration. The filters were washed two times, each time with 5 ml of ice-cold Tris·HCl buffer. Nonspecific binding was determined in the presence of 10 μM DuP 996. A concentration of 10 nM [^{3}H]DuP 996 was used in all studies except the saturation experiments. Radioactivity was determined by liquid scintillation counting. Binding data for saturation studies were analyzed using the LIGAND program (30). The binding rate constant k_1 was determined by the equation $k_1 = k'_1/[R]/[L]$, where k'_1 was determined experimentally, $[R] = B_{max} \times$ tissue, and $[L]$ was the ligand concentration. The rate constant k_{-1} was determined by the equation $k_{-1} = 0.693/t_{1/2}$.

ACh release assays. Superfusion neurotransmitter release assays were performed according to the method of Nickolson *et al.* (23). Male Wistar rats (Charles River) weighing 175–200 g were used. Rats were decapitated and brains were dissected immediately. Slices (0.3 mm thick) from the parietal cortex were prepared manually by using a recessed Lucite guide. Slices were subsequently cut into 0.25- × 0.25-mm squares with a McIlwain tissue chopper.

Cerebral cortical slices (about 100 mg of wet weight) were incubated in 10 ml of KR medium containing 116 mM NaCl, 3 mM KCl, 1.3 mM $CaCl_2$, 1.2 mM $MgCl_2$, 1.2 mM KH_2PO_4, 1.2 mM Na_2SO_4, 25 mM $NaHCO_3$, and 11 mM glucose, to which 10 μCi of [^{3}H]choline (specific activity, 80 Ci/mmol; DuPont-NEN) and 10 nmol of unlabeled choline had been added to give a final concentration of 1 μM. Incubation was carried out for 30 min at 37°, under a steady flow of 95% O_2/5% CO_2.

After a 15-min washout of nonspecifically bound radioactivity during superfusion, the collection of fractions of 4 min (1.2 ml) each was started. Hemicholinium-3 (10 μM) was added in the medium to prevent choline uptake. After 3 × 4 min, the KR medium was changed to a KR medium with 25 mM KCl (high-K^+-KR medium; S_1) for depolarization-induced stimulation of release. The high-K^+-KR medium was then substituted by vehicle- or DuP 996-containing low- or high-K^+-KR medium, and superfusion was continued for 3 × 4 min with low-K^+-KR medium, for 1 × 4 min with high-K^+-KR medium (S_2), and for 2 × 4 min with low-K^+-KR medium.

All superfusion fractions were collected in liquid scintillation counting vials. After superfusion, the slices were removed from the superfusion columns and extracted in 1.0 ml of 0.1 N HCl. To each superfusion fraction and extract, 12 ml of Liquiscint scintillation cocktail were then added, and samples were quantified by liquid scintillation counting.

The rate of release was expressed as FRC, which is equal to the dpm of the fraction/(the total dpm in the slices at the beginning of the fraction × the time of fraction). The increase in FRC for each column during stimulation ($FRCS_2$), measured in the presence of vehicle or drug, was divided by the corresponding $FRCS_1$, to yield the S_2/S_1 ratio. DuP 996 effects were evaluated by expressing this ratio for experimental columns (DuP 996 present during S_2) as a percentage of the ratio calculated for the concomitantly run control (no DuP 996 present during S_2).

Results

The structure of [^{3}H]DuP 996 is shown in Fig. 1.

[^{3}H]DuP 996 exhibited saturable specific binding to rat brain membranes. Specific binding represented 60–45% of total binding for the range of 1–32 nM [^{3}H]DuP 996, respectively. Analyses of [^{3}H]DuP 996 saturation studies using the LIGAND computer program best fit a one-site model and yielded a linear Scatchard plot (Fig. 2), with a K_d of 19 nM and a B_{max} of 102

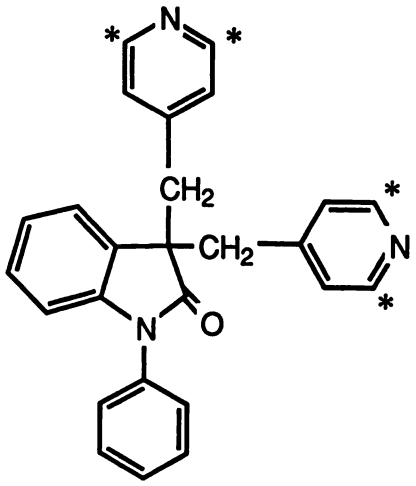

Fig. 1. The structure of [^{3}H]DuP 996. *, Positions of the tritium label.

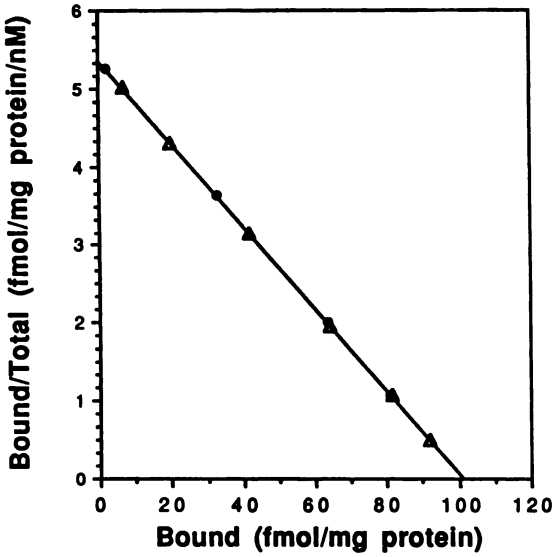

Fig. 2. Scatchard analysis of the equilibrium binding of [^{3}H]DuP 996 to rat brain membranes. Experiments were performed at 25°, with 60-min incubations. The concentration range of [^{3}H]DuP 996 was 1–185 nM. Data represent the combination of two experiments, with each *point* representing the average of six determinations. The data were analyzed by using the LIGAND computer program (30).

TABLE 1

Effects of buffer, cations, and anions on the specific binding of [^{3}H]DuP 996

Binding was performed in the presence of various buffer, cation, and anion concentrations. Controls represent binding in the presence of 50 mM Tris·HCl buffer. Each value represent the mean ± standard error of two or three experiments in duplicate.

Compound	Bound
mM	*% of control*
Tris·HCl	
5	108 ± 3
20	101 ± 4
NaCl, 200	99 ± 3
KCl, 200	97 ± 5
$CaCl_2$	
3	89 ± 14
5	76 ± 2[a]
6	62 ± 8[a]
12	59 ± 9[a]
25	64 ± 3[a]
50	55 ± 3[a]
$MgSO_4$	
10	103 ± 3
25	101 ± 6
50	89 ± 5
$MnCl_2$	
2	109 ± 23
3	91 ± 7
6	156 ± 13[a]
12	171 ± 5[a]

[a] $p < 0.05$.

fmol/mg of protein. The rates of [^{3}H]DuP 996 association and dissociation are shown in Fig. 3. [^{3}H]DuP 996 binding reached equilibrium by 40 min at 25°. The calculated rate constant for association (k_1) was 4.3×10^6 M^{-1} min^{-1}. The dissociation was initiated by the addition of 10 μM DuP 996. The dissociation $t_{1/2}$ of 10 min was determined in a semilogarithmic plot, and a k_{-1} of 0.0693 min^{-1} was calculated. The K_d calculated from the kinetic data using k_{-1}/k_1 was 16 nM, which is very similar to the K_d determined by equilibrium binding.

The optimal temperature for [^{3}H]DuP 996 binding was around 25° (Fig. 4A), and the optimal binding pH was around pH 7 (Fig. 4B). [^{3}H]DuP 996 binding exhibited a linear relationship with membrane protein concentration up to 1.4 mg of protein/assay (Fig. 4C). [^{3}H]DuP 996 binding was eliminated by incubation of brain membranes with trypsin but not with phospholipase C (Fig. 5). Varying the Tris buffer concentration from 5 to 50 mM had no significant effect on specific binding. The cation Ca^{2+} was found to significantly inhibit binding, by a maximum of 45%, whereas Mn^{2+} at 6 mM and above significantly increased specific binding (Table 1). Other cations, such as Na^+, K^+, and Mg^{2+}, had no effect. A wide range of pharmacological standards, including peptide and nonpeptide ligands for many receptors and ion channels, were not able to inhibit the binding of [^{3}H]DuP 996 (Table 2).

[^{3}H]DuP 996 binding was heterogeneously distributed over rat brain areas (Fig. 6). The [^{3}H]DuP 996 binding site density was highest in striatum and hypothalamus and lowest in cerebellum. [^{3}H]DuP 996 binding was also found in peripheral tissues such as the kidney and ileum.

The biological importance of this [^{3}H]DuP 996 binding site was studied by examining the relationship between binding affinity and potency of enhancing K^+-stimulated ACh release. After study of DuP 996 and 30 of its structural analogs, an excellent correlation was obtained between binding affinity for this site and potency to enhance K^+-stimulated ACh release (r = 0.96) (Fig. 7). The syntheses and structure-activity relationship of these DuP 996 structural analogs will be published elsewhere.

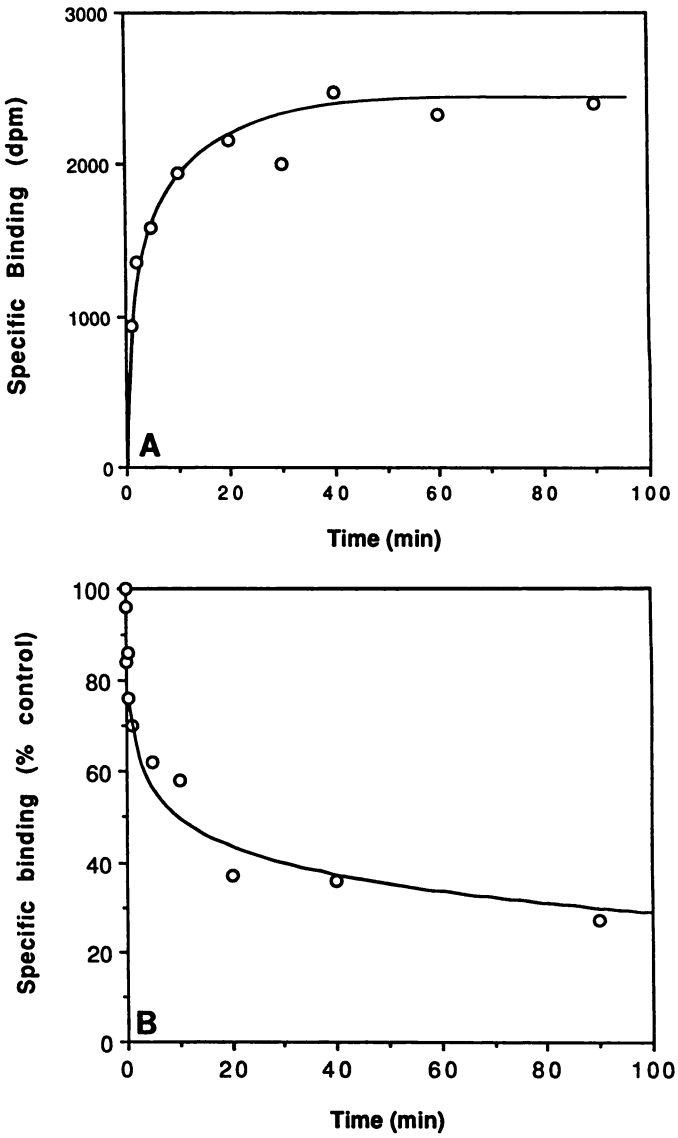

Fig. 3. Rates of association (A) and dissociation (B) for [^{3}H]DuP 996 (10 nM) binding to rat brain membranes. Experiments were performed at 25°. For the dissociation experiments, membranes were incubated with [^{3}H]DuP 996 for 45 min, and then 10 μM DuP 996 was added to initiate dissociation of bound [^{3}H]DuP 996. Each *point* represents the mean of three experiments in duplicate.

Discussion

DuP 996 is a cognitive enhancer currently in clinical trials for the treatment of Alzheimer's disease. DuP 996 was discovered for its unique pharmacological properties in enhancing K^+-stimulated release of ACh, DA, and 5-HT. DuP 996 has no effect on their basal release (22, 23). In order to understand the mechanism of action of DuP 996, we studied the interaction of [^{3}H]DuP 996 with brain membranes.

[^{3}H]DuP 996 binding is saturable and reversible. [^{3}H]DuP 996 binds to a single class of sites in rat brain membranes, with moderately high affinity. The B_{max} of this binding site is within

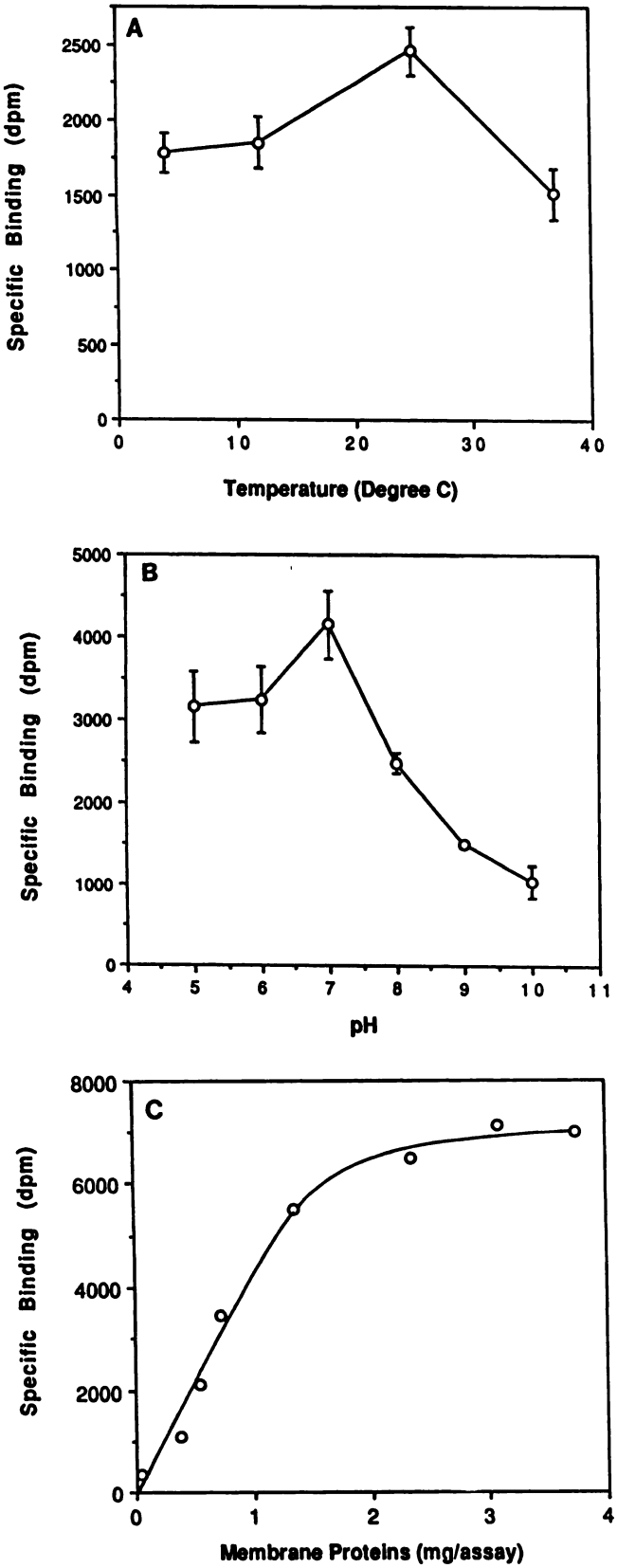

Fig. 4. Effects of temperature (A), pH (B), and membrane protein concentration (C) on the specific binding of 10 nM [^{3}H]DuP 996 to rat brain membranes. Experiments in A and C were performed with 50 mM Tris·HCl, pH 7.4, and experiments in B were performed with 100 mM Tris·HCl buffer. Each *point* represents two experiments in duplicate, and the *bars* represent standard errors of four determinations for each point.

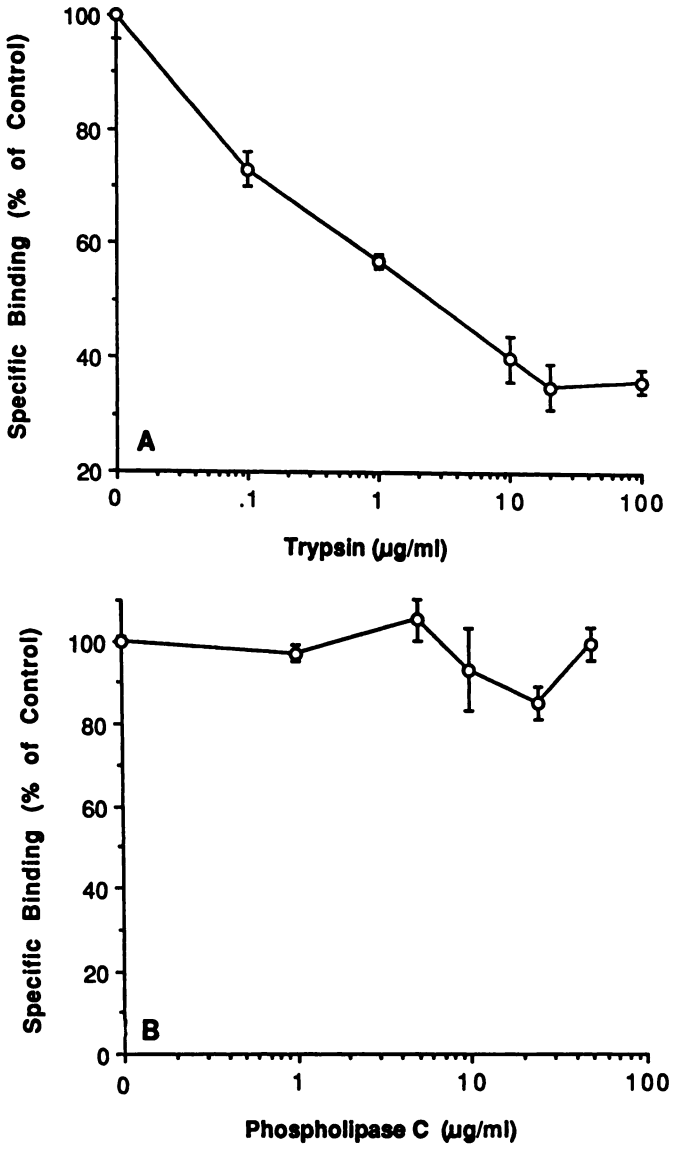

Fig. 5. Effects of incubation with trypsin (A) and phospholipase C (B) on the binding of [^{3}H]DuP 996 to rat brain membranes. Brain membranes were preincubated with either trypsin or phospholipase C for 10 min at 25°, and then 10 nM [^{3}H]DuP 996 was added and the membranes were incubated for an additional 45 min before filtration. Each *point* represents two experiments in duplicate, and the *bars* represent standard errors of four determinations for each point.

the range of many neurotransmitter receptors. The binding is concentration, time, temperature, pH, and membrane dependent. This binding site is sensitive to trypsin but not to phospholipase C, suggesting that the site is a protein. The [^{3}H]DuP 996 binding is selectively inhibited by Ca^{2+}, increased by Mn^{2+}, and not affected by Na^+, K^+, or Mg^{2+}. The significance of these effects is not known at this time. The [^{3}H]DuP 996 binding site exhibits heterogeneous regional distribution in rat brain, a property that is common for known neuronal receptors.

The [^{3}H]DuP 996 binding site has satisfied many biochemical requirements for the characterization of a receptor. However, the [^{3}H]DuP 996 binding site must be correlated with a biological function before it can be classified as a receptor. In this respect, it is important that the binding affinities of compounds for this site correlate very well with their potencies to enhance the release of ACh. Thus, the [^{3}H]DuP 996 binding site may

TABLE 2

Pharmacological standards that do not bind to the [^{3}H]DuP 996 binding site

All the pharmacological standards were tested up to a concentration of 10^{-4} M (K_i > 100,000 nM), with the exception of apamin, which was tested up to 10^{-5} M.

Adrenergic	Histaminergic	Peptidergic
NE	Cimetidine	Adrenocorticotropic hormone (1–24)
Isoproterenol	Pyrilamine	Angiotensin III
Yohimbine	Opioid	Bradykinin
Amino acid-related	Naloxone	Cholecystokinin
N-Methyl-D-aspartate	Purine	Cortisol-releasing factor
Strychnine	Adenosine	Luteinizing hormone-releasing hormone
Benzodiazepine	Serotoninergic	Neurokinin A
Diazepam	Mianserin	Neurokinin B
Cholinergic	Haloperidol	Neuropeptide Y
Atropine	Ritanserin	Substance P
Nicotine	σ	Thyrotropin-releasing hormone
Dopaminergic	(+)-SKF 10,047	Channel blockers
SCH 23390	Phencyclidine	Diltiazem
Amphetamine	Cognition-related	Verapamil
Haloperidol	Caffeine	Phencyclidine
GABAergic	Piracetam	Apamin
GABA	Tetrahydroaminoacridine	Veratidine
	Reuptake inhibitor	3,4-Aminopyridine
	Amitriptyline	

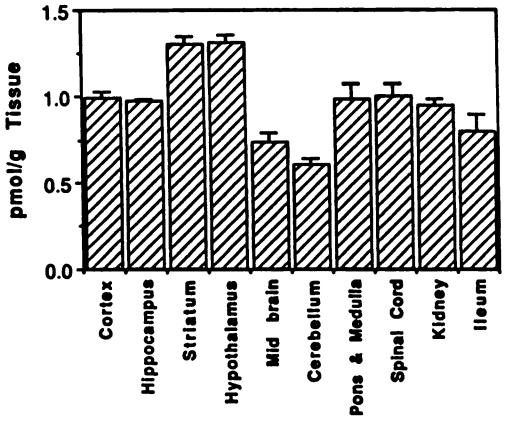

Fig. 6. Regional distribution of [^{3}H]DuP 996 binding in rat brain membranes. Brain regions were dissected according to the method of Heffner *et al.* (28). Each *bar* represent three experiments in duplicate, with standard errors indicated.

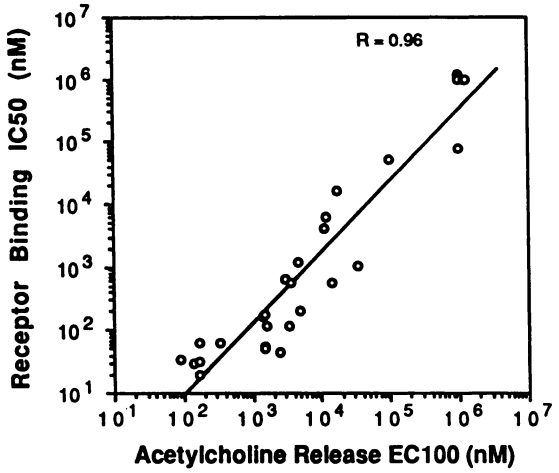

Fig. 7. Correlation of [^{3}H]DuP 996 receptor binding affinity of DuP 996 and its 30 structural analogs with the potency of these compounds to enhance K^+-stimulated ACh release by 100% (EC_{100}). ACh release assay was performed according to the method of Nickolson *et al.* (23).

be qualified as a receptor for the action of DuP 996. The characteristics of the DuP 996 receptor site are unique. A wide range of pharmacological standards for receptors and ion channels are inactive in displacing [^{3}H]DuP 996 from its binding site. Moreover, the property of releasing ACh, DA, or 5-HT, but not NE or GABA, upon depolarization, without affecting baseline release, is not shared by any known compound.

At this time, we cannot rule out the possibility that this receptor site could be part of an ion channel. However, calcium, sodium, and potassium channel ligands, such as diltiazem, verapamil, phencyclidine, veratidine, apamin, and 3,4-aminopyridine, do not bind to this receptor. In addition, these ion channel ligands do not have an effect similar to DuP 996 in enhancing neurotransmitter release.

In conclusion, the present paper describes a novel binding site, the physiological significance of which is related to enhancement of the stimulus-induced release of some but not all neurotransmitters.

Further studies on this site may lead to the discovery of novel compounds that may enhance neurotransmitter function in certain disease states, like Alzheimer's disease, and may yield new valuable tools for the study of the mechanism of neurotransmitter release.

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