0026-895X/86/02096-08$02.00/0

MOLECULAR PHARMACOLOGY, 30:96-103

Effects of *In Vivo* and *In Vitro* Treatments with *N*-Ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline on Putative Muscarinic Receptor Subtypes in Rat Brain

ANDREW B. NORMAN and IAN CREESE[1]

Department of Neurosciences, School of Medicine, University of California, San Diego, La Jolla, California 92093

Received January 19, 1986; Accepted May 6, 1986

SUMMARY

N-Ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline (EEDQ) was found to irreversibly decrease the B_{max} of [^{3}H](−)-quinuclidinyl-benzilate [(−)QNB] binding in rat brain following *in vivo* administration or by incubation of tissue homogenates with EEDQ *in vitro*. A greater reduction in the B_{max} of [^{3}H](−)QNB binding was observed in the hippocampus and cortex than in the brainstem following *in vivo* or *in vitro* treatment with EEDQ. Competition of pirenzepine for [^{3}H](−)QNB binding was best described by computer-derived models assuming two binding sites in all brain regions. However, following EEDQ treatment there was a rightward shift in the pirenzepine competition curves for the remaining [^{3}H](−)QNB-binding sites in all brain regions. Computer analysis of the pirenzepine competition curves indicated that this was due to a selective decrease in the number of [^{3}H](−)QNB-binding sites having high affinity for pirenzepine. Although the binding of [^{3}H](−)QNB to the site having lower affinity for pirenzepine was apparently unaltered, the affinity of pirenzepine for this binding site was significantly lowered following both *in vivo* and *in vitro* treatment with EEDQ. Thus, EEDQ differentially modifies muscarinic receptor-binding sites having high and low affinity for pirenzepine. The reduction in the B_{max} of [^{3}H](−)QNB binding and the rightward shift in the pirenzepine competition curve elicited by EEDQ both *in vivo* and *in vitro* could be prevented by co-administration of reversible muscarinic antagonists, thereby demonstrating that EEDQ interacts at the ligand recognition site of muscarinic receptors. These data suggest that the putative muscarinic receptor subtypes discriminated by pirenzepine may represent differences in the accessibility of pirenzepine and EEDQ to a homogeneous population of [^{3}H](−)QNB-binding sites or, alternatively, that these muscarinic receptor-binding sites discriminated by pirenzepine and EEDQ represent structurally distinct molecular entities.

From physiological (1) and receptor binding (2–6) studies, the novel muscarinic receptor antagonist pirenzepine has been reported to distinguish two putative subtypes of muscarinic receptors in brain and peripheral tissues. These muscarinic receptor subtypes are proposed to have identical affinities for classical muscarinic antagonists such as QNB, scopolamine, and atropine, in spite of their differential affinities for pirenzepine. The site having high affinity for, and pharmacological sensitivity to, pirenzepine has been designated the M_1 muscarinic receptor, and the site having lower affinity for pirenzepine has been designated the M_2 muscarinic receptor. Radioligand binding studies have demonstrated a marked regional variation in the ratio of these high and low affinity pirenzepine-binding sites in the rat brain. The cortex and hippocampus are reported to contain mainly high affinity pirenzepine, M_1, binding sites, whereas the brainstem has a greater proportion of low affinity pirenzepine, M_2, binding sites (4, 5). There is some controversy, however, surrounding this classification. Thus, there are reports that, in peripheral tissues, pirenzepine is equipotent for all muscarinic receptors (7). Furthermore, solubilized receptors from brain tissue may have interconvertible states of [^{3}H](−)QNB-binding sites having high and low affinity for pirenzepine (Ref. 8, but see Refs. 9 and 10). It is therefore unclear at present whether these two muscarinic receptor subtypes discriminated by pirenzepine represent distinct molecular entities or two conformational states of the same receptor protein. The lack of other selective pharmacological agents for these putative muscarinic subtypes has severely limited the further delineation of their pharmacological and biochemical characteristics.

EEDQ has been reported to act as an irreversible antagonist at various monoaminergic receptors in peripheral tissues and in brain. EEDQ irreversibly blocks binding to α-adrenergic receptors in peripheral tissues (11) and blocks binding to D_1

This work was supported by a research grant from the Alzheimer's Disease and Related Disorders Association, Inc., and the Alzheimer's Center Grant AG05131.

[1] Recipient of Research Scientist Development Award MH00316.

ABBREVIATIONS: QNB, quinuclidinylbenzilate; EEDQ, *N*-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline; EDTA, ethylenediaminetetraacetate; ACh, acetylcholine.

and D_2 dopamine receptors (12) and S_2 serotonergic receptors (13) in brain following peripheral administration. EEDQ has also been reported to act as an irreversible antagonist of muscarinic receptors in peripheral tissues *in vitro* (14).

We report here that EEDQ acts as an irreversible antagonist of [^{3}H](−)QNB binding to rat brain muscarinic receptors *in vivo* and *in vitro* and, furthermore, demonstrate that EEDQ differentially modifies [^{3}H](−)QNB-binding sites having high and low affinity for pirenzepine.

Experimental Procedures

Materials. [^{3}H](−)QNB (specific activity 30 Ci/mmol) was purchased from Amersham, Deerfield, IL. Atropine sulfate and scopolamine hydrobromide were purchased from Sigma Chemical Co., St. Louis, MO. EEDQ was purchased from Aldrich Chemical Co. Milwaukee, WI. Pirenzepine dihydrochloride was generously donated by Dr. D. Jenden, University of California, Los Angeles, and Boehringer Ingelheim, Ridgefield, CT.

***In Vivo* EEDQ treatments.** Male Sprague-Dawley rats (Simonsen Laboratories, Gilroy, CA; 160–200 g) were used in all experiments. For investigation of the *in vivo* effects of EEDQ, the compound was dissolved in ethanol/water (1:1, v/v) and rats were injected intraperitoneally with EEDQ (10–20 mg/kg) or vehicle with a volume of 1 ml/kg. Rats were decapitated 3 hr later and the brain was rapidly removed into ice-cold saline. Individual brain regions were dissected on an ice-cold glass Petri dish, placed in plastic vials, frozen in liquid nitrogen, and stored at −70° until use. Tissue from individual control and EEDQ-treated rats was homogenized by a Tekmar Tissumizer (setting 7, 10 sec) in 50 volumes of cold 50 mM Tris-HCl buffer (pH 7.7 at 25°), washed twice by centrifugation (48,000 × *g*, 10 min), and resuspended in fresh buffer. The final resuspension was into assay buffer consisting of 50 mM Tris-HCl containing 5 mM $MgSO_4$ and 0.5 mM EDTA (pH 7.7 at 25°).

***In vivo* receptor protection.** In these experiments rats were subcutaneously administered scopolamine (0.7 mg/kg) dissolved in saline) or vehicle 30 min before injection of EEDQ (20 mg/kg i.p.). The rats were then decapitated 3 hr after administration of EEDQ, the cerebral cortex was removed, and the B_{max} and K_D of [^{3}H](−)QNB binding were determined in saturation experiments.

***In Vitro* EEDQ treatments.** Cortex, hippocampus, and brainstem from untreated male Sprague-Dawley rats (180–200 g) were individually homogenized in 50 volumes of cold 50 mM Tris-HCl buffer (pH 7.7 at 25°) and centrifuged at 48,000 × *g* for 10 min. The pellets were then resuspended to a concentration of 10 mg original wet weight tissue in warm Tris-HCl buffer and divided into two (for pirenzepine competition experiments) or eight (for time course and EEDQ concentration experiments) 12-ml aliquots in plastic centrifuge tubes. The tubes were then placed in a water bath at 37° and 120 μl of EEDQ (10^{-1}–10^{-4} M) in an ethanol/water vehicle were added to give a final concentration of EEDQ of 10^{-3}–10^{-6} M. Vehicle alone was added to one of the aliquots of each tissue which served as the control. Following incubation for 20 or 50 min, 12 ml of ice-cold Tris-HCl buffer were rapidly added to all tubes, and the tissues were washed three times by centrifugation and resuspended into fresh buffer. The final resuspension was into assay buffer.

***In vitro* receptor protection.** In these experiments cerebral cortex was homogenized and washed once by centrifugation and then divided into aliquots as described above. Aliquots were incubated at 37° for 20 min in the presence and absence of 1 μM atropine in the presence and absence of 100 μM EEDQ. Tissue was then washed as described above and the B_{max} and K_D of [^{3}H](−)QNB binding and pirenzepine competition for [^{3}H](−)QNB binding were determined as described below.

Binding assays. All [^{3}H](−)QNB binding assays were conducted in a total assay volume of 5 ml for 90 min at 25°. Pirenzepine competition curves were generated using three intermediate concentrations per log unit in the presence of 0.2 or 0.4 nM [^{3}H](−)QNB, using 1 mg original wet weight of tissue per tube for cortex and hippocampus and 2 mg original wet weight of tissue per tube for brainstem. Under these conditions greater than 95% of added [^{3}H](−)QNB remained free. [^{3}H](−)QNB saturation assays used 0.5 mg of tissue per tube for cortex and hippocampus and 1 mg of tissue per tube for brainstem with a concentration range of 0.005–0.5 nM [^{3}H](−)QNB. Although a low tissue concentration was used in saturation studies, at the lower concentrations of [^{3}H](−)QNB up to 35% of added [^{3}H](−)QNB was bound to the tissues in the absence of atropine, thus significantly reducing the free concentration of [^{3}H](−)QNB. Direct measurement of the free concentration of [^{3}H](−)QNB were made in aliquots of supernatant by centrifugation of a replicate. These measurements demonstrated that greater than 95% of [^{3}H](−)QNB added to the tube could be accounted for by specifically bound and free [^{3}H](−)QNB. Thus, there was very little [^{3}H](−)QNB nonspecifically bound to either the tissue or the walls of the glass test tubes used. Atropine (1 μM) was used to define nonspecific binding in all experiments. Assays were terminated by rapid filtration over Whatman GF/C filters and washed three times with 5 ml of cold Tris-HCl buffer. Filters were placed in plastic minivials and 4 ml of Cytoscint (Westchem, San Diego, CA) were added. Radioactivity trapped on the filters was measured using a Beckman LS 7500 liquid scintillation counter at an efficiency of 52%.

Data analysis. The computer analyses employed the weighted, nonlinear least squares curve-fitting program LIGAND (15, 16) which uses a general model for complex ligand-receptor binding systems (17). The exact treatment of experimental data has been described previously in detail (16). Briefly, competition curves were first analyzed according to a four-parameter logistic equation (18), and the slope factor (pseudo-Hill coefficient, designated n_H) was derived. The curves were then analyzed according to a model for the binding of the radioligand and competing drug to one, two, or more binding sites. Deviation of the observed points from the predicted values were weighted according to the reciprocal of the predicted variance (19). Testing for statistical difference between models was obtained by comparing their residual variances of fits to the data by a partial *F* test (20). A model for two binding sites was retained only when it fitted the data significantly better than a model for a single binding site ($p < 0.05$, partial *F* test). For these analyses, nonspecific binding of [^{3}H](−)QNB was constrained to experimentally determined values. In some experiments, computer-derived parameter estimates from different curves were tested for significance of difference. The control curves were first analyzed allowing the parameter estimates to reach their optimal values. The affinities of pirenzepine for the two putative muscarinic binding sites in EEDQ-treated tissue were then allowed to reach their optimal values, and these values were then constrained to those values found in control tissue. The effect of this constraint on the goodness of fit was tested and the parameters were considered statistically indistinguishable if the constraining process did not significantly worsen the fit. Saturation curves were analyzed by Scatchard analysis (21). In some experiments, statistical significance was determined using a two-tailed Student's *t* test for comparison of means.

Results

Following intraperitoneal injection of EEDQ, rats displayed marked catalepsy and some of the rats displayed convulsions. As shown in Fig. 1, Scatchard analysis of [^{3}H](−)QNB saturation data demonstrated a single homogeneous population of [^{3}H](−)QNB-binding sites in rat cerebral cortex from both control and EEDQ-treated rats. As shown in Table 1, there was a significant reduction in the B_{max} of [^{3}H](−)QNB binding to cortex, hippocampus, and brainstem 3 hr after injection of EEDQ. However, the reduction in the B_{max} of [^{3}H](−)QNB binding in the EEDQ-treated compared to control rats was significantly greater in the hippocampus and cortex (approximately 56% and 53% reduction, respectively) than in the brain-

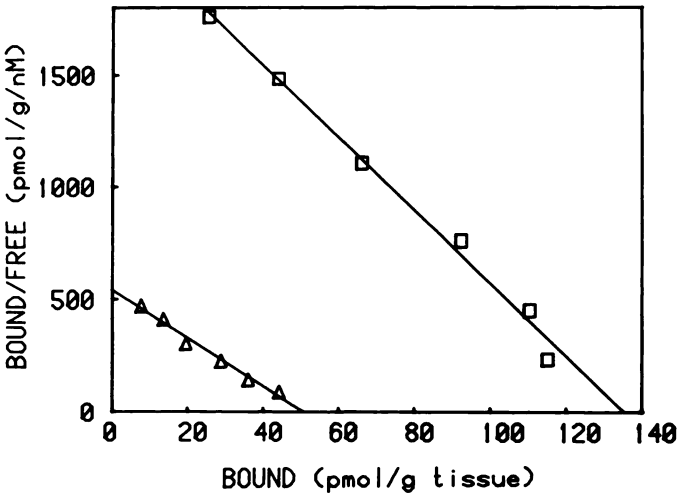

Fig. 1. Effect of *in vivo* EEDQ administration on the specific binding of [^{3}H](−)QNB to homogenates of rat cerebral cortex. Rats were injected intraperitoneally with EEDQ (20 mg/kg) (△) or vehicle (□) and decapitated 3 hr later. [^{3}H](−)QNB saturation assays were conducted as described in Experimental Procedures using a concentration range of 0.005—0.5 nM [^{3}H](−)QNB. A 1 μM concentration of atropine defined nonspecific binding. The B_{max} and K_D values of this representative experiment were, respectively, 136.5 pmol/g of tissue and 61 pM for control, and 50.6 pmol/g of tissue and 93 pM for tissue from EEDQ-treated rats. This represents a 63% decrease in the specific binding of [^{3}H](−)QNB.

stem (34% reduction) ($p < 0.01$). The reductions in the B_{max} of [^{3}H](−)QNB binding could not be reversed by repeated washing of the tissues.

Similar to the effects produced by *in vivo* treatment with EEDQ, Scatchard analysis of [^{3}H](−)QNB saturation data demonstrated that incubating tissue homogenates for 20 min *in vitro* with 100 μM EEDQ produced a significant reduction in the B_{max} of [^{3}H](−)QNB binding with no significant effect on the K_D of [^{3}H](−)QNB in all brain regions assayed (Table 2). The reduction in the B_{max} of [^{3}H](−)QNB binding was greater in the hippocampus and cortex (approximately 69% and 59% reductions, respectively) compared to the reductions obtained in the brainstem (approximately 36%) ($p < 0.01$).

As shown in Fig. 2, there was a concentration-dependent, irreversible reduction in the specific binding of 0.5 nM [^{3}H](−)QNB following incubation of homogenates of hippocampus and brainstem (and cortex; data not shown) with EEDQ *in vitro*. EEDQ was significantly more effective at reducing [^{3}H](−)QNB binding to homogenates of hippocampus than to brainstem. The apparent EC_{50} of EEDQ for the inhibition of [^{3}H](−)QNB binding under the conditions employed in these experiments was approximately 3×10^{-4} M in the brainstem and 9×10^{-5} M in the hippocampus (and the cortex; data not shown). Nonspecific binding of [^{3}H](−)QNB was not significantly altered by any concentration of EEDQ, being 200 ± 30 cpm in control tissue and 190 ± 28 cpm at the highest concentration of EEDQ (3 mM) employed.

As shown in Fig. 3, there was a time-dependent reduction in the specific binding of 0.5 nM [^{3}H](−)QNB produced by 100 μM EEDQ in homogenates of hippocampus and brainstem (and cortex; data not shown). However, the irreversible reduction of [^{3}H](−)QNB binding was slower in brainstem and less complete compared to the effects observed in the hippocampus (or cortex; data not shown).

Pirenzepine has previously been reported to discriminate multiple muscarinic receptor-binding sites labeled by [^{3}H](−)QNB (3, 6). Similarly, in the present experiments, pirenzepine competition for [^{3}H](−)QNB binding was best described by a computer-derived model assuming two binding sites in all brain regions assayed (see *control curve*, Fig. 4). In rat cerebral cortex, EEDQ treatment, *in vivo*, produced a 67% decrease in specific [^{3}H](−)QNB binding and shifted pirenzepine competition curves over to the right and made them less steep (Fig. 4). Computer-assisted analysis of pirenzepine competition curves following EEDQ treatment demonstrated that the proportion of sites having low affinity for pirenzepine was increased in tissue from EEDQ-treated rats (Table 3). As also shown in Table 3, a similar shift to the right in the pirenzepine competition curve for [^{3}H](−)QNB binding was found in hippocampus and brainstem.

Computer-assisted analysis of pirenzepine competition curves demonstrated a significant increase ($p < 0.001$, two-tailed t test) in the *proportion* of [^{3}H](−)QNB-binding sites having low affinity for pirenzepine in all three brain regions. This demonstrated that there was a greater reduction in the *absolute* number of [^{3}H](−)QNB-binding sites having high affinity for pirenzepine. The LIGAND program was used to calculate the concentration of the high and low affinity pirenzepine binding sites. As seen in Table 3, EEDQ-treatment produced a significant loss of [^{3}H](−)QNB-binding sites having high affinity for pirenzepine in hippocampus, cortex and brainstem, respectively. In contrast, there was no significant loss of [^{3}H](−)QNB-binding sites having low affinity for pirenzepine. When the affinity of pirenzepine for its high affinity binding

TABLE 1

Effect of *in vivo* administration of EEDQ on [^{3}H](−)QNB binding in rat brain

Rats were injected intraperitoneally with EEDQ (20 mg/kg) or vehicle and decapitated 3 hr later. [^{3}H](−)QNB saturation assays used 0.5 mg of tissue per replicate for hippocampus and cortex and 1 mg of tissue per replicate for brainstem and a concentration range of 0.005–0.5 nM [^{3}H](−)QNB. Nonspecific binding was defined by 1 μM atropine in all experiments. B_{max} and K_D values represent the mean ± standard error from the number of rats shown in parentheses.

	Control		EEDQ treated		Percentage of decrease from mean control B_{max}
	B_{max}	K_D	B_{max}	K_D	
	pmol g/tissue	pM	pmol g/tissue	pM	
Hippocampus ($n = 6$)	128.5 ± 5.6	48.6 ± 5.0	56.4 ± 8.8*	61 ± 8.4	56
Cortex ($n = 14$)	146.4 ± 5.5	74.9 ± 6.7	67.8 ± 4.6*	97.2 ± 7.6	53
Brainstem ($n = 6$)	38.1 ± 1.1	182.4 ± 11.5	25.1 ± 2.5*	127.5 ± 16.1	34

* Significantly different from control B_{max} ($p < 0.01$, two-tailed t test).

TABLE 2

Effect of *in vitro* EEDQ treatment on [^{3}H](−)QNB binding in rat brain

Tissue homogenates (10 mg/ml) were divided into two aliquots and incubated in the absence and presence of 100 μM EEDQ for 20 min at 37°, and the tissues were then washed three times to remove any remaining EEDQ. [^{3}H](−)QNB saturation assays used 0.5 mg of tissue per replicate for hippocampus and cortex and 1 mg of tissue per replicate for brainstem, and a concentration range of 0.005–0.5 nM [^{3}H](−)QNB. Nonspecific binding was defined by 1 μM atropine in all experiments. Values represent the mean ± standard error from the number of experiments shown in parentheses.

	Control		EEDQ treated		Mean percentage decrease from control B_{max}
	B_{max}	K_D	B_{max}	K_D	
	pmol g/tissue	*pM*	*pmol g/tissue*	*pM*	
Hippocampus (n = 4)	72.2 ± 3.7	45.8 ± 2.3	28.0 ± 2.7[a]	58.2 ± 14.8	61 ± 2
Cortex (n = 6)	91.9 ± 7.5	54.1 ± 3.4	37.2 ± 4.2[a]	60.0 ± 3.2	59 ± 4
Brainstem (n = 3)	27.5 ± 2.3	94.6 ± 18.1	17.2 ± 0.9[a]	94.5 ± 11.4	36 ± 5

[a] Significantly different from control B_{max} ($p < 0.01$, two-tailed t test).

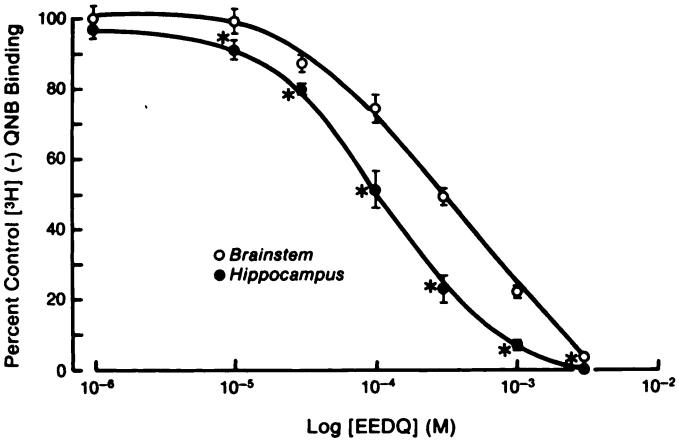

Fig. 2. Effect of increasing concentrations of EEDQ *in vitro* on the subsequent specific binding of [^{3}H](−)QNB to homogenates of rat hippocampus and brainstem. Membrane homogenates were divided into eight identical aliquots and incubated with seven different concentrations of EEDQ. One aliquot of each tissue was incubated with vehicle only and served as the control. All aliquots were incubated for 20 min at 37° and then washed three times and assayed for [^{3}H](−)QNB binding using a single concentration of [^{3}H](−)QNB (0.5 nM). Nonspecific binding was defined by 1 μM atropine. The apparent EC_{50} of EEDQ under these conditions was 9×10^{-5} M for homogenates of hippocampus and 3×10^{-4} M for homogenates of brainstem. *Points* represent the mean and standard error from four separate experiments. *, significantly different from values observed in brainstem, $p < 0.01$, two-tailed t test.

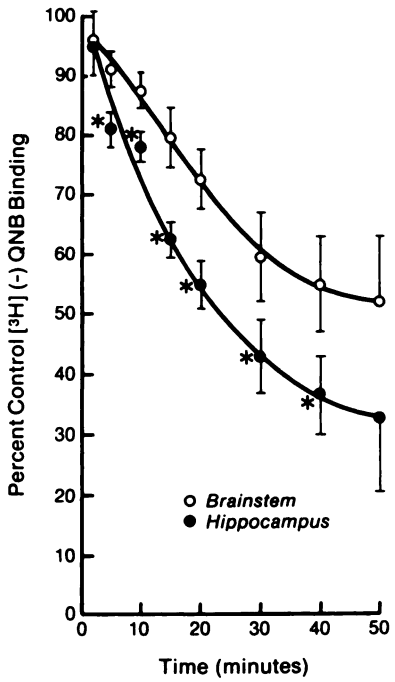

Fig. 3. Time-dependent EEDQ-induced reduction in specific [^{3}H](−)QNB binding to homogenates of hippocampus and brainstem *in vitro*. Membrane homogenates were divided into eight identical aliquots and incubated for 50 min at 37°. EEDQ (100 μM) was added to individual aliquots at various times prior to termination of the incubation. Incubation was terminated by dilution with cold buffer, and the tissues were washed three times by centrifugation and resuspended in fresh cold buffer. One aliquot of each tissue was not incubated with EEDQ and served as the control. The tissue in each aliquot was assayed for [^{3}H](−)QNB binding using a single concentration of [^{3}H](−)QNB (0.5 nM). Nonspecific binding was defined by 1 μM atropine. There was no significant difference in nonspecific binding between the control and EEDQ-treated tissue at any time point. *Points* represent the mean and standard error from five separate experiments. *, significantly different from values observed in brainstem, $p < 0.01$, two-tailed t test.

sites in tissues from EEDQ-treated rats was constrained to values found in tissues from control rats, the fit to the data points was not significantly different, thereby demonstrating that these values were statistically indistinguishable. However, when the affinity of pirenzepine for its low affinity binding sites in hippocampus and cortex was constrained to values found in tissues from control rats, the fit to the data points was significantly impaired ($p < 0.05$, partial F test), demonstrating that these values were significantly different. The affinity of pirenzepine for its low affinity binding site is approximately 6-, 5-, and 2-fold lower in hippocampus, cortex, and brainstem, respectively, in tissue from EEDQ-treated rats compared to control rats. This effect on the affinity of pirenzepine for its lower affinity binding site can be clearly observed in Fig. 4, where [^{3}H](−)QNB binding is completely inhibited at 3×10^{-6} M pirenzepine in cerebral cortex from control rats, whereas in cortex from EEDQ-treated rats there is still approximately 20% of the specific binding of [^{3}H](−)QNB remaining at this concentration of pirenzepine.

The classical muscarinic receptor antagonist (−)-scopolamine is generally believed to have identical affinity for all putative muscarinic receptor subtypes. In order to determine whether a supposedly non-subtype-selective antagonist was also affected by EEDQ, we examined its competiton for [^{3}H](−)QNB-binding sites in cortex in tissue from control and EEDQ-treated rats. Interestingly, as shown in Table 4, (−)-scopolamine also fits significantly better to a model assuming two binding sites in both control and EEDQ-treated tissue,

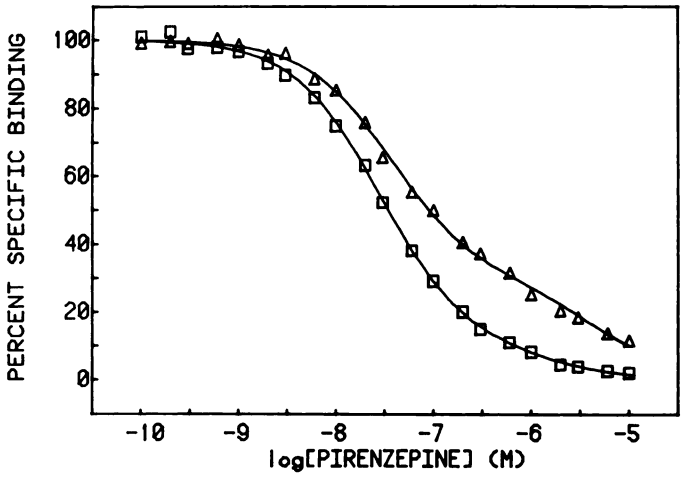

Fig. 4. Competition of pirenzepine for [^{3}H](−)QNB binding to membranes from rat cerebral cortex from control (□) and EEDQ-treated (△) rats. Rats were administered EEDQ (20 mg/kg) or vehicle intraperitoneally and were decapitated 3 hr later. In cerebral cortex the total and nonspecific binding values of [^{3}H](−)QNB (0.24 nM) in the representative experiment shown were 3937 cpm and 140 cpm, respectively, in control and 1386 cpm and 149 cpm, respectively, in tissue from EEDQ-treated rats, representing a 67% loss of specific [^{3}H](−)QNB binding. Each tube contained 1 mg original wet weight of tissue. Pirenzepine competition curves were analyzed by the computer program LIGAND. Both curves were best described by a computer-derived model assuming two binding sites: control: K_H = 5.4 nM, K_L = 157 nM, %R_L = 10%; EEDQ-treated: K_H = 7.3 nM, K_L = 912 nM, and %R_L = 29.8%.

although the difference in affinity for two [^{3}H](−)QNB-binding sites in control tissue was not as great as that observed with pirenzepine, and the proportion of lower affinity sites was therefore more difficult to establish accurately. Also of interest was the observation that, in tissue from EEDQ-treated rats, the competition of (−)-scopolamine was more shallow and the affinity of this low affinity component was lower than that observed in control tissue. The proportion of [^{3}H](−)QNB-binding sites having lower affinity for (−)-scopolamine in EEDQ-treated tissue was similar to the proportion of [^{3}H](−)QNB-binding sites having lower affinity for pirenzepine in EEDQ-treated tissue.

As shown in Table 5, the apparent selectivity of EEDQ for reducing the B_{max} of the high affinity pirenzepine-binding sites, which was observed *in vivo*, is maintained when homogenates of the brain regions are incubated with EEDQ *in vitro*. Again, a shift to the right in the pirenzepine competition curves was observed in all brain regions for the remaining [^{3}H](−)QNB-binding sites and there was an increase in the proportion of [^{3}H](−)QNB-binding sites having lower affinity for pirenzepine.

A potential explanation for the lack of effect of EEDQ on putative M_2 muscarinic cholinergic receptors is that they are "protected" by bound endogenous ACh, putative M_2 muscarinic receptors having higher affinity for agonists than putative M_1 muscarinic receptors (22). One set of experiments was designed to test whether residual endogenous ACh might account for the apparent lack of effect of EEDQ on [^{3}H](−)QNB binding to the site having low affinity for pirenzepine. Cerebral cortex was homogenized in 50 volumes of Tris-HCl buffer and centrifuged for 10 min at 48,000 × *g*. The tissue homogenate was resuspended to a concentration of 10 mg/ml in warm Tris-HCl buffer and incubated at 37° for 20 min, then diluted with buffer and washed twice by centrifugation and resuspended in fresh buffer. A wash procedure less rigorous than this has previously been shown to remove essentially all endogenous ACh (23). The homogenate was resuspended to 10 mg of tissue/ml, divided into two 10-ml aliquots, and incubated for 20 min at 37° in the presence or absence of 100 μM EEDQ. The tissues were then washed a further three times by centrifugation with the final resuspension into assay buffer (50 mM Tris-HCl containing 5 mM $MgSO_4$ and 0.5 mM EDTA). Pirenzepine competition for 0.4 nM [^{3}H](−)QNB binding was assayed as described above. EEDQ produced a 65% loss of specific [^{3}H](−)QNB binding and pirenzepine competition curves were again shifted to the right in EEDQ-treated compared to control tissue. Computer-assisted analysis demonstrated a significant increase in the proportion of [^{3}H](−)QNB-binding sites having lower affinity for pirenzepine. Thus, in tissue extensively washed and subjected to two 37° incubation steps [a much more rigorous wash procedure than has previously been demonstrated to remove endogenous ACh (23)], the selectivity of EEDQ for irreversibly reducing the binding of [^{3}H](−)QNB to the muscarinic binding site having high affinity for pirenzepine is still apparent.

As shown in Fig. 5, there was a protection of the binding of [^{3}H](−)QNB when rats were injected intraperitoneally with

TABLE 3

Computer-assisted analysis of pirenzepine competition for [^{3}H](−)QNB binding to brain areas from control and EEDQ-treated rats

Rats were treated intraperitoneally with EEDQ (20 mg/kg) or vehicle and decapitated 3 hr later. Specific [^{3}H](−)QNB binding to hippocampus and cortex was to 1 mg of tissue and specific binding to brainstem was to 2 mg of tissue. Pirenzepine competition curves were analyzed using the computer program LIGAND. K_H and K_L correspond to the affinity of pirenzepine for the putative M_1 and M_2 muscarinic receptors, respectively. R_L corresponds to the proportion of the total population of [^{3}H](−)QNB-binding sites having low affinity for pirenzepine. Values represent the mean ± standard error from the number of rats shown in parentheses.

	Specific [^{3}H](−)QNB binding	K_H	K_L	R_L	No. of putative M_1 binding sites	No. of putative M_2 binding sites
	cpm		*nM*	%	*pmol/g tissue*	
Hippocampus						
Control (n = 5)	3610 ± 175	5.5 ± 1.2	128.0 ± 41.3	8.4 ± 1.4	121.3 ± 6.4	10.9 ± 1.4
EEDQ treated (n = 5)	1468 ± 245[a]	6.1 ± 1.4	765.6 ± 237[a]	21.5 ± 3.8[a]	41.0 ± 9.1[a]	10.3 ± 0.6
Cortex						
Control (n = 8)	4001 ± 245	6.0 ± 0.5	121.4 ± 12.2	12.2 ± 1.1	137.1 ± 10.7	17.6 ± 1.2
EEDQ treated (n = 8)	1775 ± 214[a]	7.4 ± 0.5	576.1 ± 85.6[a]	25.6 ± 2.7[a]	50.6 ± 8.8[a]	15.3 ± 1.0
Brainstem						
Control (n = 5)	1305 ± 78	15.1 ± 2.4	344.5 ± 34.0	57.4 ± 2.8	11.4 ± 1.0	15.4 ± 1.4
EEDQ treated (n = 5)	1035 ± 62[a]	15.2 ± 1.5	607.4 ± 84.6[a]	71.0 ± 1.5[a]	5.7 ± 0.8[a]	15.0 ± 0.9

[a] Significantly different from control values ($p < 0.01$, two-tailed t test).

TABLE 4

Computer-assisted analysis of (−)-scopolamine competition for [^{3}H](−)QNB binding to cerebral cortex from control and EEDQ-treated rats

Rats were injected intraperitoneally with EEDQ (20 mg/kg) or vehicle and decapitated 3 hr later. Specific binding of [^{3}H](−)QNB was to 1 mg of tissue. Competition curves were analyzed using the computer program LIGAND. Nonspecific binding was defined by 1 μM atropine. Values represent the mean ± standard error from five separate experiments.

	K_H	K_L	R_L
	nM		%
Control	0.16 ± 0.02	0.8 ± 0.1	7.2 ± 0.9
EEDQ treated	0.15 ± 0.02	1.3 ± 0.2[a]	33.2 ± 2.5[b]

[a] Significantly different from control values ($p < 0.05$, two-tailed t test).
[b] Significantly different from control values ($p < 0.005$, two-tailed t test).

scopolamine (0.7 mg/kg) prior to injection with EEDQ, or when homogenates of cortex were incubated with atropine *in vitro* in the presence of EEDQ. Pretreatment of tissue with the reversible muscarinic antagonist, scopolamine, or atropine had no significant effect on the binding of [^{3}H](−)QNB. Furthermore, as shown in Fig. 6, when [^{3}H](−)QNB binding was protected from the irreversible modification produced by EEDQ *in vitro* by co-incubation with atropine, the shift to the right in the pirenzepine competition curve for [^{3}H](−)QNB binding was also prevented.

Discussion

Inhibition of [^{3}H](−)QNB binding elicited by EEDQ following both *in vivo* and *in vitro* treatment is clearly irreversible as repeated washes produced no increase in the B_{max} of [^{3}H](−)QNB. Furthermore, there was only a small change in the K_D of [^{3}H](−)QNB binding following the EEDQ treatment, whereas a simple competitive inhibition by EEDQ would be expected to result in a large change in the K_D with no change in the B_{max}.

The differential loss of [^{3}H](−)QNB binding between various brain regions following *in vivo* administration of EEDQ might have been accounted for by a differential distribution of EEDQ between these various regions. We therefore examined the effect of EEDQ on [^{3}H](−)QNB binding to these individual brain regions *in vitro* and found that the tissue specificity of EEDQ for reducing [^{3}H](−)QNB binding was maintained. The greater sensitivity to the loss of [^{3}H](−)QNB binding produced by EEDQ in the hippocampus and cortex compared to the brainstem correlates with the respective ratios of high to low affinity pirenzepine-binding sites in these tissues. In the hippocampus and cortex, which contain a majority of [^{3}H](−)QNB-binding sites having high affinity for pirenzepine (putative M_1 muscarinic receptors), a larger proportion of [^{3}H](−)QNB binding is lost following treatment with EEDQ compared with the loss of [^{3}H](−)QNB binding in the brainstem which contains a majority of [^{3}H](−)QNB-binding sites having low affinity for pirenzepine. Therefore, these data may suggest that the binding of [^{3}H](−)QNB to putative M_1 muscarinic receptors appears to be more susceptible to irreversible antagonism by EEDQ than is the binding of [^{3}H](−)QNB to putative M_2 muscarinic receptors.

It has been hypothesized that muscarinic receptors discriminated by pirenzepine might also possess differential affinities for agonists (22). Indeed, M_1 and M_2 muscarinic receptors have been defined on the basis of agonist affinities (24, 25) with agonists having been proposed to have higher affinity for M_2 muscarinic receptors than for M_1 muscarinic receptors (24, 25). Thus, a potential explanation for the selectivity of EEDQ for reducing the binding of [^{3}H](−)QNB to M_1 muscarinic receptors following *in vivo* administration might be that endogenous ACh is more tightly bound to and protects the M_2 muscarinic receptors from modification by EEDQ. This hypothesis is unlikely, however. When *homogenates* of cerebral cortex were subjected to an extensive wash procedure more than sufficient to remove endogenous ACh (23), prior to incubation with EEDQ, the selectivity of EEDQ for reducing the binding of [^{3}H](−)QNB to M_1 muscarinic receptors was still evident.

The mechanism of action of EEDQ is thought to involve the activation of peptide carboxyl groups forming a highly reactive mixed carbonic anhydride (26). In a receptor protein this reactive group may then interact with suitable nucleophilic groups, such as adjacent α-amino groups producing an irreversible covalent crosslinking of peptide chains within the receptor (26). It is therefore possible that the selectivity of EEDQ for putative M_1 and M_2 muscarinic receptors may be due to differences in the accessibility of peptide carboxyl groups on these two receptors to EEDQ. M_1 muscarinic receptors would therefore have carboxyl groups which are more readily accessible for interaction with EEDQ than does the M_2 muscarinic receptor.

TABLE 5

Effect of *in vitro* EEDQ treatment on pirenzepine competition for [^{3}H](−)QNB binding in rat brain

Tissue homogenates were divided into two equal aliquots and incubated at 37° for 20 min in the presence and absence of 100 μM EEDQ, and the tissues were then washed three times to remove any residual EEDQ. Pirenzepine competition curves were analyzed using the computer program LIGAND. K_H and K_L correspond to the affinity of pirenzepine for the putative M_1 and M_2 muscarinic receptors, respectively. R_L corresponds to the proportion of the total population of [^{3}H](−)QNB-binding sites having low affinity for pirenzepine. Values represent the mean ± standard error from the number of separate experiments shown in parentheses.

	Specific [^{3}H](−)QNB binding	K_H	K_L	R_L	No. of putative M_1 binding sites	No. of putative M_2 binding sites
	cpm	nM		%	pmol/g tissue	
Hippocampus (n = 4)						
Control	2406 ± 321	5.4 ± 1.3	227.4 ± 54.6	7.7 ± 2.1	99.1 ± 11.0	10.3 ± 1.4
EEDQ treated	1296 ± 129[a]	6.4 ± 1.6	270.4 ± 119.8	16.0 ± 0.5[a]	44.1 ± 3.3[a]	8.4 ± 1.5
Cortex (n = 5)						
Control	2875 ± 125	5.2 ± 1.0	97.3 ± 24.5	14.3 ± 2.0	95.2 ± 9.4	16.0 ± 2.7
EEDQ treated	1396 ± 105[a]	6.2 ± 1.2	241.3 ± 86.5[a]	23.5 ± 2.3[a]	42.0 ± 1.3[a]	15.8 ± 3.6
Brainstem (n = 4)						
Control	1378 ± 125	12.4 ± 3.9	70.1 ± 24.7	66.6 ± 2.9	8.9 ± 1.3	15.4 ± 1.8
EEDQ treated	1062 ± 81[a]	10.3 ± 2.4	151.2 ± 45.5[a]	73.9 ± 2.7[a]	4.9 ± 1.4[a]	13.4 ± 1.5

[a] Significantly different from control values ($p < 0.01$, two-tailed t test).

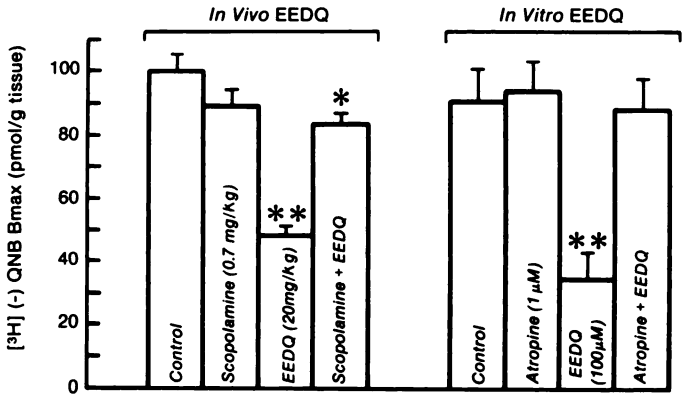

Fig. 5. Protection by muscarinic antagonists against the *in vitro* and *in vivo* reduction of specific [^{3}H](−)QNB binding in rat cerebral cortex produced by EEDQ. *In vivo EEDQ:* Rats were injected subcutaneously with scopolamine (0.7 mg/kg) or vehicle 20 min before intraperitoneal injection with EEDQ (20 mg/kg) or vehicle and decapitated 3 hr later. *In vitro EEDQ:* Homogenates of rat cerebral cortex were washed once, divided into equal aliquots, and incubated for 20 min at 37° with atropine (1 μM), EEDQ (100 μM), or atropine together with EEDQ. One aliquot received neither drug and served as the control. For all experiments B_{max} values were determined by Scatchard analysis of [^{3}H](−)QNB saturation data. Nonspecific binding was determined by 1 μM atropine. *, significantly different from control values, $p < 0.05$; **, $p < 0.001$, two-tailed *t* test.

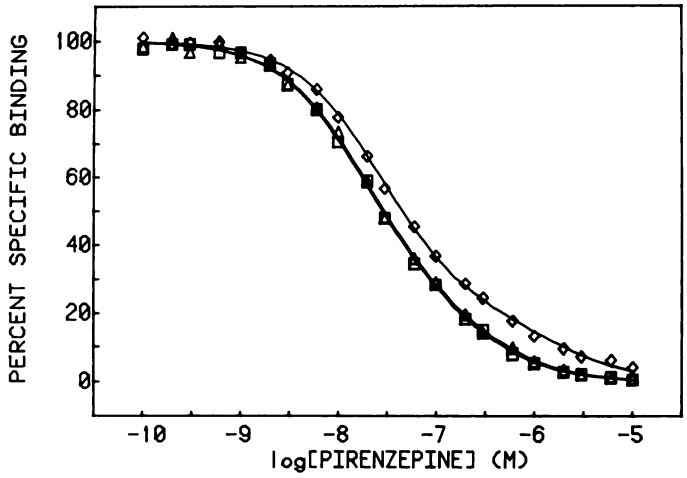

Fig. 6. Pirenzepine competition for [^{3}H](−)QNB binding in EEDQ-treated and atropine-protected rat cerebral cortex. Homogenates of rat cerebral cortex were washed, divided into three equal aliquots, and incubated for 20 min at 37° with: 100 μM EEDQ (◇), 1 μM atropine and 100 μM EEDQ (△), neither drug (control) (□), or atropine alone (curve not shown). All curves were best described by a computer-derived model assuming two binding sites: control: K_H = 4.6 nM, K_L = 150.5 nM, $\%R_L$ = 13%; EEDQ and atropine: K_H = 5.5 nM, K_L = 170.1 nM, $\%R_L$ = 12.5%; EEDQ-treated: K_H = 6.4 nM, K_L = 359.7 nM, $\%R_L$ = 20%.

Alternatively, the availability of suitable nucleophilic groups with which the activated carboxyl group(s) may react may be different in the putative M_1 and M_2 muscarinic receptors. Thus, the M_2 muscarinic receptor may not have suitable nucleophilic groups which are easily accessible to activated carboxyl groups, thereby reducing the probability of an activated carboxyl group interacting to form a covalent linkage with a nucleophile. The slower time course of inactivation of [^{3}H](−)QNB binding in the brainstem which contains a larger proportion of putative M_2 muscarinic receptors might be taken to indicate that nucleophilic groups are indeed less accessible to the activated carboxyl groups which may regenerate to stable carboxyl groups before covalent linkage occurs to an internal nucleophile.

It is also possible that the selectivity of EEDQ for M_1 and M_2 muscarinic receptors may be due to differences in the accessibility of these two receptors to EEDQ. It has been reported that muscarinic receptors differing in agonist affinity are located differentially on intracellular and extracellular membranes (27). It is possible that the intracellular receptors, which might be postulated to have low affinity for pirenzepine, are inaccessible to EEDQ. Indeed, although EEDQ is very lipophilic, it is not known whether it is able to cross neuronal membranes during the time that EEDQ is in an active form. EEDQ has been shown to be active for a relatively short period following *in vivo* administration (28). This laboratory has previously reported that EEDQ, when administered *in vivo*, does not functionally modify adenylate cyclase or the stimulatory guanine nucleotide-regulatory subunit (N_s), although both are modified following *in vitro* EEDQ treatment of membranes, suggesting that these moieties are inaccessible to EEDQ following *in vivo* administration (29). However, this possibility is unlikely to explain the selectivity of EEDQ for putative M_1 muscarinic receptors *in vivo* since, when *homogenates* of tissues were incubated with EEDQ, the selectivity of EEDQ for reducing [^{3}H](−)QNB binding to putative M_1 muscarinic receptors was still evident. [^{3}H](−)QNB binding to homogenates of brainstem, which have a greater proportion of low affinity pirenzepine-binding sites, was less susceptible to EEDQ in both a time- and a concentration-dependent fashion than was [^{3}H](−)QNB binding to hippocampus and cortex, which contain predominantly high affinity pirenzepine-binding sites. Furthermore, in homogenates of individual tissues, the binding of [^{3}H](−)QNB to M_1 muscarinic receptors was still more susceptible to irreversible modification by EEDQ than was the binding of [^{3}H](−)QNB to M_2 muscarinic receptors. Although the inaccessibility of intracellular receptors may not account for EEDQ selectivity *in vivo*, the possibility cannot be discounted that the microenvironment of the receptor, for example, a lipid annulus surrounding the receptor or sugar residues, may be different for the two receptors, thus impeding access of EEDQ.

A further observation does suggest that the putative M_2 muscarinic receptor is accessible to EEDQ, however. That we observed a significant shift in the affinity of pirenzepine for the putative M_2 muscarinic receptor, with no detectable change in the affinity of [^{3}H](−)QNB for these binding sites following EEDQ treatment, suggests that EEDQ may indeed modify these [^{3}H](−)QNB-binding sites. However, the modification of the M_2 muscarinic receptor would be different from that which occurs at the M_1 muscarinic receptor in that [^{3}H](−)QNB binding is abolished at the M_1 but not at the M_2 muscarinic receptor, whereas the M_2 muscarinic receptor is also modified such that pirenzepine no longer binds in the same fashion. Furthermore, the affinity of (−)-scopolamine for a population of [^{3}H](−)QNB-binding sites was also altered following EEDQ treatment. The proportion of remaining [^{3}H](−)QNB-binding sites having lower affinity for (−)-scopolamine was similar to the proportion of lower affinity pirenzepine-binding sites. This observation might suggest that these are the same binding sites which were modified by EEDQ. It is important to note that, due to the shift in affinity of pirenzepine for a population of [^{3}H](−)QNB-binding sites, it is not possible to definitively

conclude at this time that the modified lower affinity site, in fact, corresponds exclusively to a modified putative M_2 receptor.

That protection of [^{3}H](−)QNB binding is possible with muscarinic receptor antagonists both *in vivo* and *in vitro* suggests that EEDQ modifies the receptors at the ligand recognition site, rather than via an allosteric modification of the recognition site produced by a modification of a remote carboxyl group(s). That protection with scopolamine also blocks the change in pirenzepine competition for [^{3}H](−)QNB binding indicates that the change in affinity of pirenzepine by EEDQ is also restricted to modification of the ligand recognition site. Thus, if EEDQ also modifies the muscarinic receptor at remote sites, this would not appear to affect the conformation of the ligand recognition site. Whether possible interactions of EEDQ at other sites on the receptor remote from the ligand recognition site modifies coupling of the receptor to effector moieties remains to be elucidated.

It is possible that the differential accessibility of the putative muscarinic receptor subtypes discriminated by pirenzepine to EEDQ might explain the apparent selectivity of this compound. Although it still remains to be conclusively demonstrated that [^{3}H](−)QNB-binding sites having lower affinity for pirenzepine and (−)-scopolamine in EEDQ-treated tissues do in fact correspond to the putative M_2 muscarinic receptor, that differential modification by EEDQ of two distinct [^{3}H](−)QNB-binding sites does occur suggests that there are differences in the carboxyl groups and/or availability of internal nucleophilic groups between the ligand recognition sites of these two putative receptor subtypes. This would represent either different conformational states of the same molecular entity (differences in tertiary or quaternary structure of the receptor protein) or discrete molecular entities (different primary structures of the receptor).

Acknowledgments

We would like to thank Andrew Chen and Lawrence Farhat for excellent technical assistance, Dr. Joan Heller-Brown for helpful discussion, and Paula Martin for manuscript preparation.

References

1. Hammer, R., and A. Giachetti. Muscarinic receptor subtypes. M1 and M2 biochemical and functional characterization. *Life Sci.* **31**:2991–2998 (1982).
2. Hammer, R., C. P. Berrie, N. J. M. Birdsall, A. S. V. Burgen, and E. C. Hulme. Pirenzepine distinguishes between different subclasses of muscarinic receptors. *Nature (Lond.)* **283**:90–92 (1980).
3. Watson, M., W. R. Roeske, and H. I. Yamamura. [^{3}H]Pirenzepine selectively identifies a high affinity population of muscarinic cholinergic receptors in the rat cerebral cortex. *Life Sci.* **31**:2019–2023 (1982).
4. Watson, M., H. I. Yamamura, and W. R. Roeske. A unique regulatory profile and regional distribution of [^{3}H]pirenzepine binding in rat provide evidence for distinct M_1 and M_2 muscarinic receptor subtypes. *Life Sci.* **32**:3001–3011 (1983).
5. Wamsley, J. K., D. R. Gehlert, W. R. Roeske, and H. I. Yamamura. Muscarinic antagonist binding site heterogeneity as evidenced by autoradiography after direct labeling with [^{3}H]-QNB and [^{3}H]-pirenzepine. *Life Sci.* **34**:1395–1402 (1984).
6. Luthin, G. R., and B. B. Wolfe. Comparison of [^{3}H]pirenzepine and [^{3}H] quinuclidinylbenzilate binding to muscarinic cholinergic receptors in rat brain. *J. Pharmacol. Exp. Ther.* **228**:648–655 (1984).
7. Laduron, P. M., J. E. Leysen, and H. Gorissen. Muscarinic receptor: multiple sites or unitary concept? *Arch. Int. Pharmacodyn.* **249**:319–321 (1981).
8. Roeske, W. R., and J. C. Venter. The differential loss of [^{3}H]pirenzepine *vs* [^{3}H](−)quinuclidinylbenzilate binding to soluble rat brain muscarinic receptors indicates that pirenzepine binds to an allosteric state of the muscarinic receptor. *Biochem. Biophys. Res. Commun.* **118**:950–957 (1984).
9. Berrie, C. P., N. J. M. Birdsall, E. C. Hulme, M. K. Stockton, and J. M. Stockton. Solubilization and characterization of high and low affinity pirenzepine binding sites from rat cerebral cortex. *Br. J. Pharmacol.* **85**:697–703 (1985).
10. Luthin, G. R., and B. B. Wolfe. Characterization of [^{3}H]pirenzepine binding to muscarinic cholinergic receptors solubilized from rat brain. *J. Pharmacol. Exp. Ther.* **234**:37–44 (1985).
11. Belleau, B., R. Martel, G. Lacasse, M. Menard, N. L. Weinberg, and Y. G. Perron. *N*-Carboxylic acid esters of 1,2 and 1,4-dihydroquinolines. A new class of irreversible inactivators of the catecholamine alpha receptors and potent central nervous system depressants. *J. Am. Chem. Soc.* **90**:823–824 (1968).
12. Hamblin, M. W., and I. Creese. Behavioral and radioligand binding evidence for irreversible dopamine receptor blockade by *N*-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline. *Life Sci* **32**:2247–2255 (1983).
13. Battaglia, G., A. B. Norman, P. L. Newton, and I. Creese. *In vitro* and *in vivo* irreversible blockade of cortical S_2 serotonin receptors by *N*-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline: a technique for investigating S_2 serotonin receptor recovery. *J. Neurochem.* **46**:589–593 (1986).
14. Chang, K-J., J. F. Moran, and D. J. Triggle. Mechanism of cholinergic antagonism by *N*-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline (EEDQ). *Pharmacol. Res. Commun.* **2**:63–66 (1970).
15. Munson, P. J., and D. Rodbard. LIGAND: A versatile computerized approach for characterization of all ligand binding systems. *Anal. Biochem.* **107**:220–239 (1980).
16. DeLean, A., J. M. Stadel, and R. J. Lefkowitz. A ternary complex model explains the agonist-specific binding properties of the cyclase-coupled beta-adrenergic receptor. *J. Biol. Chem.* **255**:7108–7117.
17. Feldman, H. A. Mathematical theory of complex ligand-binding systems at equilibrium: some methods for parameter fitting. *Anal. Biochem.* **48**:317–338 (1972).
18. DeLean, A., P. J. Munson, and D. Rodbard. Simultaneous analysis of families of sigmoidal curves: application to bioassay, radioligand assay, and physiological dose-response curves. *Am. J. Physiol.* **235**:E97–E102 (1978).
19. Rodbard, D., R. H. Lenox, H. L. Wray, and D. Ramseth. Statistical characterization of the random errors in the radioimmunoassay dose-response. *Clin. Chem.* **22**:350–358 (1976).
20. Rodbard, D. Statistical quality control and routine data processing for radioimmunoassays and immunoradiometric assays. *Clin. Chem.* **20**:1255–1270 (1974).
21. Scatchard, G. The attractions of proteins for small molecules and ions. *Ann. N. Y. Acad. Sci.* **51**:660–672 (1949).
22. Birdsall, N. J. M., and E. C. Hulme. Muscarinic receptor subclasses. *Trends Pharmacol. Sci.* **4**:459–463 (1983).
23. Morrow, A. L., R. Loy, and I. Creese. Alteration of nicotinic cholinergic agonist binding sites in hippocampus after fimbria transection. *Brain Res.* **334**:309–314 (1985).
24. Flynn, D. D., and L. T. Potter. Different effects of *N*-ethylmaleimide on M1 and M2 muscarine receptors in rat brain. *Proc. Natl. Acad. Sci. USA* **82**:580–583 (1985).
25. Mash, D. C., D. D. Flynn, and L. T. Potter. Loss of M2 muscarine receptors in the cerebral cortex in Alzheimer's Disease and experimental cholinergic denervation. *Science (Wash. D. C.)* **228**:1115–1117 (1985).
26. Belleau, B., V. DiTullio, and D. Godin. The mechanism of irreversible blockade by *N*-carbethoxydihydroquinolines—model studies with typical serine hydrolases. *Biochem. Pharmacol.* **18**:1039–1044 (1969).
27. Zarbin, M. A., J. K. Wamsley, and M. J. Kuhar. Axonal transport of muscarinic cholinergic receptors in rat vagus nerve: high and low affinity agonist receptors move in opposite directions and differ in nucleotide sensitivity. *J. Neurosci.* **2**:934–941 (1982).
28. Leff, S. E., R. Gariano, and I. Creese. Dopamine receptor turnover rates in rat striatum are age-dependent. *Proc. Natl. Acad. Sci. USA* **81**:3910–3914 (1984).
29. Hess, E. J., G. Battaglia, A. B. Norman, and I. Creese. *In vivo* EEDQ specificity for D-1 dopamine receptor blockade: lack of effect on Ns or the catalytic subunit of adenylate cyclase. *Soc. Neurosci. Abstr.* **11**:313 (1985).

Send reprint requests to: Dr. Andrew B. Norman, Department of Neurosciences, M-008, School of Medicine, University of California, San Diego, La Jolla, CA 92093.