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Quantitation of Tolerance Development After Chronic Oxotremorine Treatment

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MARKS, M. J., L. D. ARTMAN AND A. C. COLLINS. *Quantitation of tolerance development after chronic oxotremorine treatment.* PHARMACOL BIOCHEM BEHAV. 19(1) 103–113, 1983.—A new procedure was developed to quantitate the tolerance which develops as mice are chronically infused with the muscarinic agonist, oxotremorine. Cumulative dose-response curves were constructed for the effects of oxotremorine on body temperature and rotarod performance by administering sequential injections to individual animals. These dose-response curves compare favorably to those constructed by injecting individual animals with one of several doses. The sequential injection technique was used to assess the magnitude of tolerance development to oxotremorine. A linear relationship between oxotremorine infusion rate (dose) and magnitude of change of the ED_{50} value for impairment of rotarod performance was observed, with animals receiving an infusion rate of 1.0 mg/kg/hr showing a 24-fold increase in ED_{50}. Dose-response curves for tolerant animals were parallel to those constructed for naive animals. The oxotremorine dose required to decrease body temperature to 35°C ($ED_{35°}$) was 80-fold greater than control in the group treated with 1.0 mg/kg/hr. The dose-response curves for tolerant animals were not parallel to those seen in naive animals. Time courses of recovery from a challenge dose of oxtoremorine suggest little change in metabolism occurred during chronic infusion. Chronic oxotremorine infusion resulted in a decrease in the total number of QNB binding sites. Both high- and low-affinity sites were reduced in number. Since no change in K_I for the muscarinic agonist, carbamylcholine, was observed, it seems unlikely that a change occurs in the affinity of the muscarinic receptor for agonists. Significant change in receptor number was detected only in animals that received higher doses of oxotremorine. Chronic oxotremorine treatment had no effect on choline uptake by synaptosomes prepared from any of five brain regions.

Oxotremorine Cholinergic Muscarinic receptors Choline uptake Tolerance

TOLERANCE development frequently accompanies chronic drug treatment. Several laboratories have reported the development of tolerance to the muscarinic cholinergic agonists tremorine [7,17] and oxotremorine [3, 16, 19, 20]. The early reports dealing with tolerance to muscarinic agonists were primarily phenomenological in nature. Recent investigations, however, have attempted to quantify tolerance more accurately and have, in addition, attempted to provide a biochemical explanation. Maayani *et al.* [19] noted that oxotremorine induces salivation, tremor, and hypothermia in mice, as well as impairing performance on a rotarod. Chronic oxotremorine injection (once daily for 10–14 days) resulted in a parallel shift to the right of the dose-response curves for each of these effects. The shifts in the dose-response curves appeared to be dose related in that a greater shift was seen after chronic treatment with higher oxotremorine doses. This tolerance was not accompanied by a change in the number or affinity of muscarinic receptors as measured in whole brain homogenates.

Numerous studies [9, 10, 14, 24, 29] have demonstrated that chronic inhibition of acetylcholinesterase (AChE) results in a reduction in muscarinic binding sites. Most of these studies suggested the reduction in muscarinic receptor number may account for tolerance to cholinesterase inhibitors, but only Uchida *et al.* [29] and Ehlert *et al.* [10] directly tested the correlation between tolerance and altered receptor number. These investigators noted that chronic inhibition of AChE with diisopropylfluorophosphate (DFP) resulted in an increase in the ED_{50} for oxotremorine-induced contractions of ileum. This tolerance was accompanied by a decrease in the number of muscarinic receptors [10,29] or in the affinity of those receptors for agonists [10].

Thus, tolerance to cholinergic agonists clearly develops, and alterations in receptors also seem to occur. However, it is not clear whether the receptor number changes readily explain the tolerance. The fact that Maayani *et al.* [19] achieved tolerance to oxotremorine without altering the number or affinity of muscarinic receptors suggests that tolerance to oxotremorine may not develop in the same way as does tolerance to AChE inhibitors. However, Ben-Barak *et al.* [3] have found that injection of oxotremorine twice daily did result in down regulation of muscarinic receptor in rats. Marks *et al.* [20] and Taylor *et al.* [28] have both administered cholinergic agonists by methods allowing greater exposure of the animals to the drugs. Taylor *et al.* [28] performed their studies by injecting carbamylcholine into the intrathecal space, thereby exposing the spinal cord to the agonist. Chronic exposure resulted in tolerance to the effects

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of carbamylcholine in a tail-flick test and also resulted in a substantial decrease in the number of muscarinic binding sites in the spinal cord. The decrease followed first-order kinetics over the 25-hr time period tested. In our earlier study [20], varying doses of oxotremorine were chronically infused into mice via an indwelling intravenous catheter [2], a procedure that facilitates the continual administration of drugs, such as oxotremorine, which have a short half-life. Chronic oxotremorine treatment resulted in tolerance to the rotarod-impairing and hypothermia-inducing effects of this drug. A dose-related reduction in muscarinic receptors in six brain regions was observed, but virtually complete tolerance to the challenge dose of oxotremorine was seen at the lowest chronic infusion doses, i.e., tolerance was observed before receptor number was noticeably altered.

The studies of Maayani *et al.* [19] and our initial study [20] may not have provided a totally reliable test of the hypothesis that tolerance to oxotremorine is due to altered receptor number. These studies failed to assess the effect of chronic oxotremorine on affinity of the muscarinic receptor for agonists. Birdsall *et al.* [4] detected super high-, high-, and low-affinity binding sites for muscarinic agonists, and found that the proportion of those sites differs among tissues. Ehlert *et al.* [9,10] observed that chronic DFP treatment alters high- but not low-affinity agonist binding sites. It therefore seemed possible that an effect of oxotremorine on the high-affinity muscarinic agonist site may not have been detected in our initial study.

Our previous study [20] also examined the effect of chronic oxotremorine treatment on the activity of the enzymes acetylcholinesterase and choline acetyltransferase. These enzymes were unaffected by chronic oxotremorine treatment. Another component of the cholinergic system, which may be affected by chronic treatment with oxotremorine and thereby contribute to the development of tolerance, is high-affinity choline uptake [30]. This uptake system appears to be coupled to the synthesis of the acetylcholine pool released by depolarization of nerve cell membranes [6,21]. Additionally, the high affinity uptake of choline *in vitro* can be affected by treatment with drugs that alter the turnover of acetylcholine [1, 22, 26]. Oxotremorine, which decreases the turnover of acetylcholine, decreases the rate of choline uptake. Since acute administration of oxotremorine affects choline uptake, changes in this process after chronic treatment may occur.

The experiments reported here were designed to assess the effect of chronic oxotremorine infusion on both the affinity and number of all muscarinic receptors, as well as the inhibition of these receptors by muscarinic agonists. The effect of chronic oxotremorine treatment on choline uptake was also determined. In addition, tolerance to oxotremorine was better quantified by constructing oxotremorine dose-response curves for individual animals. This technical advance allowed a better estimate of any possible correlation between tolerance development and receptor or other neurochemical alterations.

METHOD

Animals

Female C3H/2Ibg mice, bred in our colony, were used in these experiments. Before surgery at 70±10 days of age, the animals were housed on aspen shavings in metal cages (20×30×10 cm) with food (Wayne Sterilizable Lab Blox) and water available ad lib. The animal colony was maintained at 23±1.0° and had a 12-hr light cycle (lights on from 7:00 a.m. to 7:00 p.m.). After surgery, the animals were housed singly in the infusion chambers under identical environmental conditions.

Chronic Drug Infusion

Oxotremorine was continuously infused into the mice through a cannula of silastic tubing inserted in the right jugular vein according to the method of Barr *et al.* [2] under pentobarbital (60 mg/kg)/chloral hydrate (125 mg/kg) anesthesia.

Two days after surgery, the cannula was attached to thermoplastic tubing which was connected to a 1-ml syringe mounted on a Harvard infusion pump. Sterile saline (35 μl/hr) was administered for 2 days, after which drug infusion was begun. The five infusion rates were 0.1, 0.2, 0.3, 0.6, and 1.0 mg/kg/hr. The 0.1 mg/kg/hr group was treated with this dose for 7 days. The remaining groups were initially treated with 0.1 mg/kg/hr for 1 day, and the dose was subsequently increased by 0.1 mg/kg/hr daily until the final dosage was achieved. All animals in each group were maintained at their final infusion rate for 6 days.

Tolerance Test

Both control and treated animals were trained on the rotarod (Ugo Basile Co., Milan, Italy) before the effects of acute administration of oxotremorine were measured. Training was initiated at 10 rpm, the slowest rotation speed. When a mouse could remain on the device for 100 sec, rotation speed was increased successively to 12 rpm, 14 rpm, and 16 rpm. Training was complete in 1–2 hr. A trained animal was able to remain on the device for 100 sec when tested at 10-min intervals thereafter.

The acute effects of oxotremorine on rotarod behavior and body temperature were assessed in two groups of trained mice. In one group, each mouse received a single acute IP injection of one of five oxotremorine concentrations. Each mouse in the other group received six successive injections of the drug at various concentrations.

Those animals receiving a single injection were tested as follows: Upon completion of training, the animal was placed on the rotarod at a rotational speed of 16 rpm. Rotation continued for 100 sec or until the animal fell from the device, in which case the time was noted. After completion of the rotarod test, body temperature was measured using a Digitec 5810 rectal probe (YSI, Yellow Springs, OH) inserted 2.5 cm. The mouse was then given an IP oxotremorine injection of either 0.02, 0.04, 0.08, 0.12, or 0.20 mg/kg in saline. Fifteen minutes after injection, the animal was again tested for rotarod performance and body temperature.

Animals in the successive injection group were trained and tested for rotarod performance and body temperature as described above. Each animal then received an IP injection of oxotremorine at a dose of 0.02 mg/kg, and rotarod performance and body temperature were measured 15 min after injection. A second injection of 0.02 mg/kg was then administered (cumulative amount of drug was 0.04 mg/kg), and rotarod performance and body temperature were measured 15 min later. Injections were continued at 15-min intervals using the following doses: 0.04, 0.04, 0.08, and 0.20 mg/kg (corresponding cumulative doses were 0.08, 0.12, 0.20, and 0.40 mg/kg). Measures of rotarod performance and body temperature were obtained 15 min after each injection.

All chronically treated animals were trained on the rotarod before they were placed in the drug infusion cham-

bers. Animals in groups infused with five different drug doses were retrained on the rotarod after removal from the infusion chambers at the end of the treatment period. Beginning 60 to 90 min after removal from the chambers, animals received acute oxotremorine injections at 15-min intervals according to the following schedule: For final infusion rates of 0.1 mg/kg/hr and 0.2 mg/kg/hr, the individual acute injections were 0.1, 0.1, 0.2, 0.2, and 0.2 mg/kg (cumulative doses of 0.1, 0.2, 0.4, 0.6, and 0.8 mg/kg, respectively); for a final infusion rate of 0.3 mg/kg/hr, cumulative doses were 0.2, 0.4, 0.6, 1.0, and 2.0 mg/kg; for 0.6 mg/kg/hr, cumulative doses were 0.5, 1.0, 2.0, 3.0, and 4.0 mg/kg; for 1.0 mg/kg/hr, cumulative doses were 1.0, 2.0, 3.0, 5.0, and 8.0 mg/kg. Rotarod performance and body temperature were measured 15 min after each injection. Upward adjustment of oxotremorine dose permitted measurement of the effects of acute oxotremorine and comparison of these effects among treatment groups.

Data were fitted to a line by the method of least squares using either rotarod score or temperature as the dependent variable and log cumulative oxotremorine dose as the independent variable. Slopes and 50% confidence limits of these slopes were calculated to allow comparison of the lines generated for mice receiving each of the drug infusion rates. In addition, the lines were tested for parallelism. To test the effect of acute oxotremorine on rotarod behavior and temperature, the ED_{50} (acute cumulative dose giving a rotarod time of 50 sec) and $ED_{35°}$ (acute cumulative dose giving a body temperature of 35°) were calculated from the parameters of the lines.

Neurochemical Correlates

Separate groups of animals were used for assessment of QNB binding and quantitation of tolerance. Six days after the final oxotremorine infusion rate was achieved, an animal was removed from the infusion apparatus. Two hours later the animal was challenged with a single dose of oxotremorine to test for tolerance. This dose was 0.4 mg/kg for animals receiving a final infusion rate of 0.2 mg/kg/hr, and 1.0 mg/kg for animals receiving final infusion rates of 0.6 mg/kg/hr or 1.0 mg/kg/hr. Body temperature was measured 20 min after the acute injection. Mice were then returned to the chronic infusion apparatus and oxotremorine infusion was continued. The day after the tolerance test, animals were again removed from the infusion apparatus, and 2 hr later were killed by cervical dislocation. The brains were removed and dissected. Homogenates (4% w/v) of cerebral cortex, hindbrain (pons-medulla), hippocampus, corpus striatum, and midbrain (tissue remaining in the midbrain region after removal of hypothalamus, hippocampus, and striatum) were made in 50 mM potassium phosphate buffer (pH 7.4). Cerebellum and hypothalamus were discarded owing to the low level of QNB binding found in our initial study [20] and small size of the region, respectively.

Binding assays, a modification of the method of Yamamura and Snyder [31], were conducted as follows: Homogenate was pipetted into 10 ml of 20 mM potassium phosphate buffer (pH 7.4). Final protein amounts per tube were approximately 100 μg for cortex, 150 μg for hindbrain, 100 μg for midbrain, 80 μg for hippocampus, and 50 μg for striatum. The tubes were warmed to 37°, [^{3}H]-L-QNB (New England Nuclear, 40.2 Ci/mmol) was added, and the samples were mixed and incubated for 45 min at 37°. After the incubation, the particulate protein and bound QNB were collected by filtration onto Whatman GFA filters, and the filters were washed three times with 5 ml of ice-cold buffer. Results obtained using GFA filters under low vacuum were the same as those obtained using the finer mesh filters GFC and GFF (i.e., for cortex, GFA: $K_D = 17.2 \pm 2.2$ pM, $B_{max} = 1.67$ pmol/mg protein; GFC: $K_D = 17.9 \pm 3.1$ pM, $B_{max} = 1.64 \pm 0.10$ pmol/mg protein; GFF: $K_D = 16.0 \pm 1.7$ pM, $B_{max} = 1.54 \pm 0.09$ pmol/mg protein; all results mean±S.E.M., n=4). Filters were placed in Nalgene filmware scintillation bags (Nalge Co.), 3 ml of scintillation cocktail (toluene, 1.36 liter; Triton-X 100, 0.9 liter; 2,5-diphenoxazole, 10.6 g) were added, the bags were sealed, and the filters were mechanically crushed. Samples were counted at 20% efficiency using a Beckman Model 7000 liquid scintillation counter. Blanks determined by including either 1×10^{-4} M oxotremorine or 1×10^{-6} M atropine, or by omitting homogenate were identical. The blank most commonly used was that obtained by omitting protein from the assay.

The effects of oxotremorine on QNB binding were determined in brain regions from control animals and from animals that received the 1.0 mg/kg/hr oxotremorine infusion rate. The effects of carbamylcholine on QNB binding were determined in control mice and in those receiving final oxotremorine infusion rates of 0.2, 0.6, and 1.0 mg/kg/hr. Both muscarinic agonists were added prior to addition of [^{3}H]-L-QNB.

A single QNB concentration was used in a given experiment, and concentrations were relatively constant from one experiment to another. The average QNB concentrations ±S.E.M. were 79.2±2.0 pM for the carbamylcholine experiments and 76.2±3.6 pM for experiments using oxotremorine. The amount of QNB bound in the absence of added inhibitor represented 10.7±0.3% of the added ligand.

Since displacement of QNB by agonists appears to be biphasic [4], the displacement curves for both oxotremorine and carbamylcholine were fitted to the following two-site model:

$$\text{Total QNB bound} = \frac{QNB_1}{1 + I/K_1} + \frac{QNB_2}{1 + I/K_2}$$

where QNB_1 and QNB_2 are the amount of ligand bound in the absence of inhibitor to sites with the apparent inhibition constants K_1 and K_2 for oxotremorine or carbamylcholine, and I is the inhibitor concentration. Data were fitted to the two-site model using a method of least squares. The K_1 and K_2 in this equation are not the actual inhibition constants but are a function of QNB concentration, the K_D of QNB, and the actual K_I for inhibitor:

$$K_{1 \text{ or } 2} = \frac{[QNB]}{K_I\,(1 + [QNB]/K_D)}.$$

Since K_D and [QNB] were known within a given experiment, K_I values were calculated:

$$K_I = K/(1 + [QNB]/K_D).$$

In these assays whole, unwashed homogenate was used. The possibility that results obtained using this tissue preparation may differ from those obtained using a washed particulate fraction was assessed. Displacement of [^{3}H]-L-QNB by carbamylcholine was determined in both whole homogenate and washed particulate fraction obtained by four cycles of homogenization and centrifugation (15 min at 20000 rpm, Sorvall RC 2B). While the total binding of QNB to the washed pellets had, as expected, higher binding owing to removal of

soluble protein, the proportion of the two affinity states for carbamylcholine and the K_I for the agonist were unchanged. This result was the same for both control mice and those infused with 1.0 mg/kg/hr oxotremorine. The results obtained for cortex for control animals were: whole homogenate, QNB bound=1.30±0.08 pmol/mg protein, fraction high affinity=0.40±0.03, $K_I=0.7\pm0.3\times10^{-7}$ M, fraction low affinity=0.60±0.03, $K_I=2.0\pm0.8\times10^{-5}$ M; washed particulate fraction, QNB bound=1.68±0.05 pmol/mg protein, fraction high affinity=0.43±0.04, $K_I=1.5\pm0.4\times10^{-7}$ M, fraction low affinity=0.57±0.04, $K_I=3.0\pm0.9\times10^{-5}$ M. Similarly, the results obtained for cortex of mice infused with 1.0 mg/kg/hr oxotremorine were: whole homogenate, QNB bound =0.84±0.04 pmol/mg protein, fraction high affinity =0.42±0.03, $K_I=2.2\pm0.8\times10^{-7}$ M, fraction low affinity=0.58±0.03, $K_I=3.6\pm1.2\times10^{-5}$ M; washed particulate fraction, QNB bound=1.68±0.32 pmol/mg protein, fraction high affinity=0.38±0.04, $K_I=1.7\pm0.6\times10^{-7}$ M, fraction low affinity=0.62±0.04, $K_I=2.9\pm0.9\times10^{-5}$ M. Likewise, the displacement of QNB binding by carbamylcholine in midbrain, hindbrain, hippocampus, and striatum in both control and oxotremorine-infused mice was unaffected by repeated washing of the particulate fraction (data not shown).

Choline uptake measurements were made using a crude synaptosomal preparation as outlined by Gray and Whittaker [15]. Tissue from the five brain areas were homogenized in 0.32 M sucrose. After a centrifugation (10 min, 1475 × g) to separate the nuclei and cell debris, the crude synaptosomal pellet was collected by centrifugation at 22000 × g for 20 min. The resulting pellet was resuspended in 0.32 M sucrose and kept on ice for use in the choline experiments.

Choline uptake measurements were made at 37°. The composition of the uptake buffer was: NaCl, 118 mM; KCl, 4.7 mM; $MgSO_4$, 1.4 mM; $CaCl_2$, 1.3 mM; glucose, 20 mM; HEPES, 15.8 mM; pH 7.4. Synaptosomal suspensions (200 μl) were added to 700 μl of uptake buffer and incubated for 10 min at 37°. After the 10-min incubation, 100 μl of uptake buffer containing [^{3}H]-choline was added, and the samples were mixed and incubated for 2 min. [^{3}H]-choline concentration was 0.1 μM. After the 2-min incubation, the samples were poured onto GFA filters under gentle vacuum and washed three times with 3 ml volumes of ice-cold 154 mM NaCl. High-affinity uptake was determined as the difference between uptake in the absence or presence of 1×10^{-6} M hemicholinium-3. While only one concentration of [^{3}H]-choline was used to measure uptake in midbrain, hindbrain, hippocampus, and striatum, four concentrations were used for cortex samples to provide an estimate of the K_m for uptake.

Protein was measured by using the method of Lowry *et al.* [18] with bovine serum albumin as the standard.

Statistical Analyses

Dose-response curves were analyzed by least squares linear regression using data obtained from individual animals. Slopes and ED_{50} or $ED_{35°}$ values were derived from parameters of the lines. An estimate of the biological half-life of oxotremorine was obtained by fitting the data obtained for the return to normothermia and unimpaired rotarod performance to a linear function of time. Binding data were analyzed by one-way analysis of variance followed by Tukey's B post hoc test. Means were considered different if $p<0.05$.

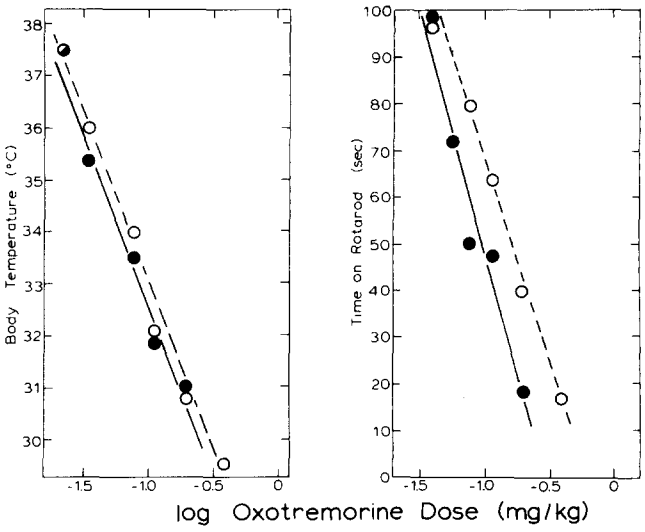

FIG. 1. Comparison of dose-response curves for oxotremorine effects on body temperature and rotarod performance. Body temperature (left panel) and rotarod performance (right panel) were determined in mice that received a single dose of oxotremorine (●) or in those receiving successive doses of the drug (○). Each point is the mean of 8–16 measurements.

RESULTS

The comparison between the effects of oxotremorine administered as a single acute dose or as a series of injections is shown in Fig. 1. The method of obtaining a specified drug dose had no effect on the log dose-response curves for oxotremorine-induced hypothermia. These curves are coincidental; that is, they have the same slope and the same $ED_{35°}$ (0.05 mg/kg). When impairment of rotarod performance was examined, a slightly different result was obtained. No significant differences in the slopes of the log dose-response curves were observed, but the curve for the group receiving sequential injection was shifted slightly to the right of that for the animals that received only a single oxotremorine injection. The corresponding ED_{50} values were 0.17 mg/kg and 0.10 mg/kg. Nevertheless, the close correspondence of results obtained using the two acute administration procedures suggests that sequential administration of oxotremorine is useful in the quantitative assessment of tolerance to this drug through the construction of dose-response curves for individual animals.

The effects of increasing dosages during chronic treatment were measured in groups of mice which received oxotremorine at one of five infusion rates (from 0.1 to 1.0 mg/kg/hr). The tests were conducted 6 days after the final drug concentration had been achieved, and concentrations of the acute injections were adjusted to provide measurable effects. Figure 2 illustrates the data obtained for body temperature. As the infusion rate of oxotremorine was increased, the log dose-response curve shifted to the right and a higher acute dose of drug was required to elicit the same temperature decrease. At each drug infusion rate, the lines for the test animals are significantly different from control ($p<0.01$). A similar shift to the right of the dose-response curves was observed for rotarod performance (see Fig. 3).

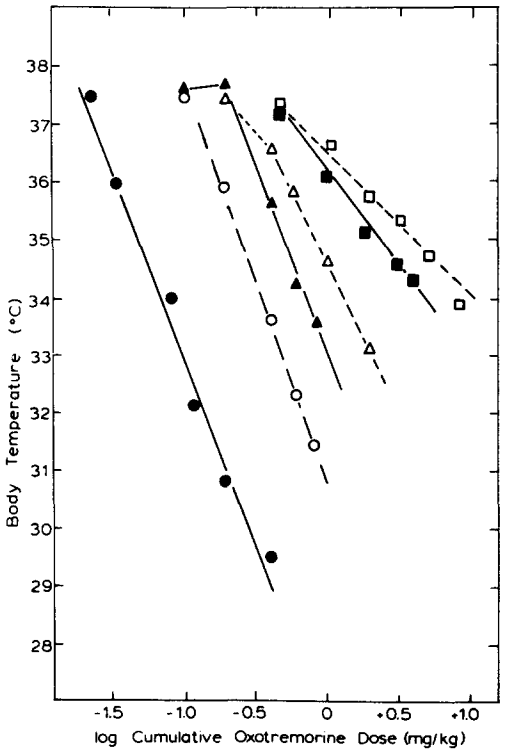

FIG. 2. Log dose-response curves for oxotremorine effects on body temperature. Chronically infused mice were tested for the hypothermic effects of oxotremorine using the successive injection technique. The oxotremorine infusion rates were: 0.0 (control, ●), 0.1 (○), 0.2 (▲), 0.3 (△), 0.6 (■), and 1.0 (□) mg/kg/hr. Each point is the mean for six animals; the lines were determined by the method of least squares.

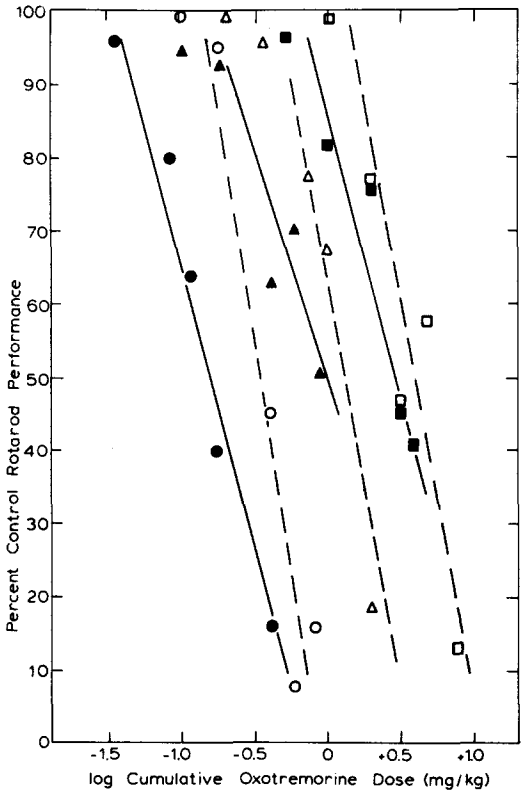

FIG. 3. Log dose-response curves for oxotremorine effects on rotarod performance. Chronically infused mice were tested for the rotarod debilitating effects of oxotremorine using the successive injection technique. The oxotremorine infusion rates were: 0.0 (control, (●), 0.1 (○), 0.2 (▲), 0.3 (△), 0.6 (■), and 1.0 □) mg/kg/hr. Each point is the mean for six animals; the lines were determined by the method of least squares.

None of the lines for infused animals coincides with that for controls ($p<0.02$).

The effect of oxotremorine on the parameters of these lines is displayed in Fig. 4. A dose-related decrease in slope for the effect of oxotremorine on body temperature was observed as infusion rate increased (see Fig. 4A). At the rates of 0.6 and 1.0 mg/kg/hr these slopes are significantly less than the slope of the line for the control group ($p<0.01$). Quantitation of the shift to the right of the dose-response curves is shown in Fig. 4C. The $ED_{35°}$ increased markedly as the infusion rate was raised. The 4-fold shift in $ED_{35°}$ observed for the 0.1 mg/kg/hr rate of infusion increased to 80-fold for the 1.0 mg/kg/hr rate. The drug-treated groups all had significantly greater ($p<0.01$) $ED_{35°}$than the control group. However, the change in slope of the dose-response curves caused at least part of the upward concavity of the $ED_{35°}$ curve as a function of infusion rate. Therefore, it must be recognized that the $ED_{35°}$ results shown in Fig. 4C are a function of the slopes of the dose-response curves, not merely a measure of the shift to the right of the curves.

The effects of increasing oxotremorine infusion rates on the development of tolerance to the debilitating effects of oxotremorine on rotarod performance are less complex. As shown in Fig. 4B, chronic infusion had no effect on the slopes of the dose-response curves for rotarod performance. The ED_{50} increased as a linear function of infusion rate (see Fig. 4D). The ED_{50} for the 0.1 mg/kg/hr group was approximately twice the control value, while the ED_{50} for the 1.0 mg/kg/hr group was about 25-fold higher than control.

We did not measure oxotremorine metabolism directly, but the biological half-life of the drug was estimated in control animals and in animals infused with oxotremorine at the 1.0 mg/kg/hr rate. Challenge doses of the drug (0.2 mg/kg and 5.0 mg/kg, respectively) were chosen to provide similar biological effects. The time courses for oxotremorine effect on impairment of rotarod behavior and on depression of body temperature are shown in Fig. 5. The shapes of the curves for control and treated animals are comparable, but not identical. Since the log dose-response curves for both biological effects were linear, both rotarod performance and body temperature should be proportional to log oxotremorine concentration injected. Assuming that the rate limiting step for reversal of drug effects in both groups was drug metabolism, an estimate of metabolism was made from the rising phase of the curves. For rotarod and temperature effects, respectively, the estimates of drug half-life made from these calculations were 30 and 22 min for control mice, and 43 and 13 min for treated mice.

Since oxotremorine is a muscarinic agonist, tolerance to the drug may result from an alteration in the amount of mus-

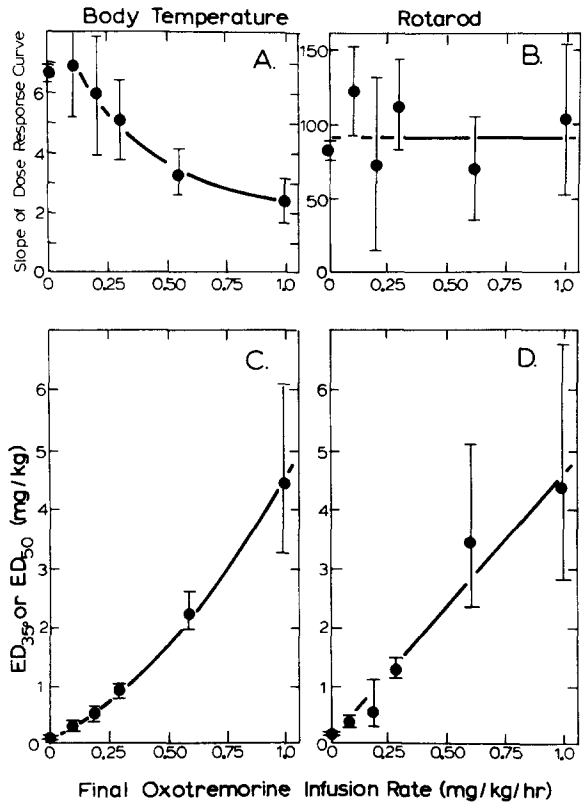

FIG. 4. Effect of oxotremorine infusion on slopes and $ED_{35°}/ED_{50°}$ for oxotremorine effects. Slopes±50% confidence limits were determined from the lines in Figs. 3 and 4 and are plotted as a function of oxotremorine infusion rate for body temperature (A) and rotarod performance (B). The $ED_{35°}$ for hypothermia (C) and the ED_{50} for rotarod effects (D) of chronic oxotremorine and the 50% confidence limits were calculated from the lines in Figs. 3 and 4.

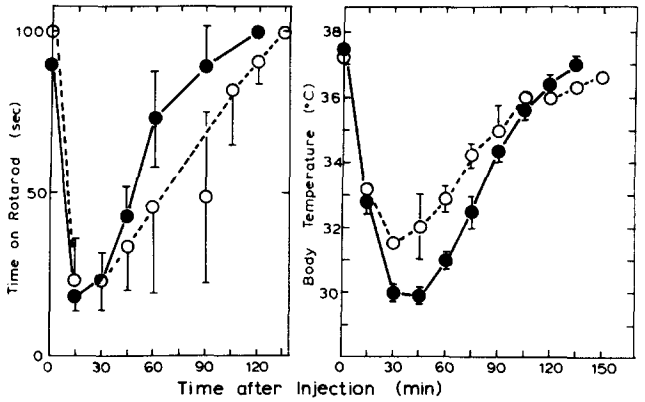

FIG. 5. Time course of oxotremorine effects on naive and oxotremorine-infused mice. Control (●, n=8) or oxotremorine-infused (○, n=3, final rate 1.0 mg/kg/hr) were challenged with acute doses of oxotremorine of 0.2 mg/kg and 5.0 mg/kg, respectively. Rotarod performance (left panel) and body temperature (right panel) were subsequently measured at the times indicated. Points represent mean±S.E.M.

TABLE 1

EFFECT OF OXOTREMORINE INFUSION IN QNB BINDING*

Brain region	Final oxotremorine infusion rate (mg/kg/hr)	K_D (pM)	B_{max} (pmol/mg)
Cortex	0.0	17.8 ± 2.5	1.804 ± 0.110
	1.0	23.8 ± 2.4	1.366 ± 0.080†
Midbrain	0.0	16.4 ± 1.9	0.952 ± 0.048
	1.0	20.8 ± 2.2	0.616 ± 0.051†
Hindbrain	0.0	17.3 ± 1.5	0.540 ± 0.044
	1.0	15.4 ± 1.3	0.290 ± 0.024†
Hippocampus	0.0	14.5 ± 2.3	1.527 ± 0.130
	1.0	23.5 ± 3.7	1.128 ± 0.073†
Striatum	0.0	16.7 ± 1.4	3.588 ± 0.483
	1.0	17.9 ± 2.1	2.263 ± 0.178

*Means ± S.E.M. for maximum QNB binding and K_D were determined for six control and six oxotremorine-infused mice (1.0 mg/kg hr). Binding is expressed as pmol/mg protein.

†Significantly different from control ($p<0.05$).

carinic receptor or in the affinity of this receptor for its agonists. Since both high- and low-affinity agonist binding sites appear to exist, a change in the affinity of an agonist for either of these sites may occur as well. To test these possibilities, the number of muscarinic receptors of each type and their affinities for agonists were measured using the muscarinic antagonist, [^{3}H]-L-QNB.

The K_D and B_{max} for QNB binding in five brain regions of either control mice or mice infused with oxotremorine (1.0 mg/kg/hr) are summarized in Table 1. Infusion had no effect on the K_D for QNB in any of the brain regions, but a significant decrease in the maximal binding was observed in the cortex, midbrain, hindbrain, and hippocampus of the drug-treated animals. The decrease in striatum almost achieved significance.

While the data in Table 1 demonstrate that oxotremorine infusion decreases total QNB binding, they do not allow the analysis of the relative proportion of high-affinity and low-affinity forms of the receptor. Information about the proportion of these forms was obtained by constructing log dose-response curves for displacement of QNB by oxotremorine and carbamylcholine. The QNB displacement curves for these two agonists in cerebral cortical homogenates from control and oxotremorine-infused mice (1.0 mg/kg/hr) are shown in Fig. 6. These data have been fitted to a two-site model as proposed by Birdsall *et al.* [4]. It is interesting to observe that QNB binding was lower in drug-treated animals than in controls, but the shapes of the curves are similar for the two groups. The similarity in shape indicates that the K_I (and therefore the K_D) for the agonists is unchanged by oxotremorine infusion.

To determine whether the K_I for oxotremorine or the ratio of high-affinity to low-affinity agonist binding sites were altered by drug treatment, these values were calculated in five brain regions (see Table 2). Total binding was significantly decreased in all regions except striatum. This decrease was not restricted to either the high- or low-affinity binding sites, since the percentage of high-affinity receptors was unchanged by infusion. No change in K_I was observed

TABLE 2

EFFECT OF OXOTREMORINE INFUSION ON OXOTREMORINE INHIBITION OF QNB BINDING*

Brain region	Infusion rate (mg/kg/hr)	Total QNB bound (pmol/mg)	High affinity		Low affinity		% High affinity
			Bound (pmol/mg)	K_I ($\times 10^8$ M)	Bound (pmol/mg)	K_I ($\times 10^7$ M)	
Cortex	0.0	1.51 ± 0.09	0.52 ± 0.05	3.6 ± 1.0	0.99 ± 0.07	9.7 ± 1.4	34.5 ± 2.3
	1.0	1.06 ± 0.11†	0.35 ± 0.04†	1.4 ± 0.2†	0.71 ± 0.06†	8.7 ± 0.7	32.9 ± 2.3
Midbrain	0.0	0.80 ± 0.05	0.31 ± 0.04	3.1 ± 0.4	0.49 ± 0.04	6.5 ± 0.6	38.7 ± 4.5
	1.0	0.47 ± 0.06†	0.15 ± 0.03†	1.9 ± 0.2†	0.32 ± 0.02†	6.3 ± 0.6	30.4 ± 2.7
Hindbrain	0.0	0.45 ± 0.04	0.32 ± 0.03	3.0 ± 0.3	0.13 ± 0.01	6.7 ± 0.7	70.5 ± 1.4
	1.0	0.24 ± 0.02†	0.16 ± 0.02†	5.5 ± 0.6†	0.08 ± 0.01†	11.1 ± 3.7	66.3 ± 3.6
Hippocampus	0.0	1.33 ± 0.10	0.25 ± 0.03	1.3 ± 0.6	1.08 ± 0.10	8.5 ± 0.6	19.4 ± 2.5
	1.0	0.86 ± 0.07†	0.20 ± 0.01	2.4 ± 0.9	0.66 ± 0.05†	9.0 ± 0.4	23.8 ± 1.4
Striatum	0.0	2.87 ± 0.52	0.81 ± 0.11	4.9 ± 1.2	2.06 ± 0.05	8.2 ± 1.0	30.6 ± 4.6
	1.0	1.71 ± 0.17	0.66 ± 0.08	16.6 ± 3.6†	1.05 ± 0.07	9.1 ± 0.6	37.8 ± 2.3

*The mean ± S.E.M. of total QNB binding, the amount calculated to be high- and low-affinity components of this binding, and their corresponding K_I's for oxotremorine were calculated by fitting dose-response curves similar to those in Fig. 6A to the two-site model. There were six mice in each group. Average QNB concentration was 76.2 pM. Binding is expressed as pmol/mg protein.

†Significantly different from control ($p<0.05$).

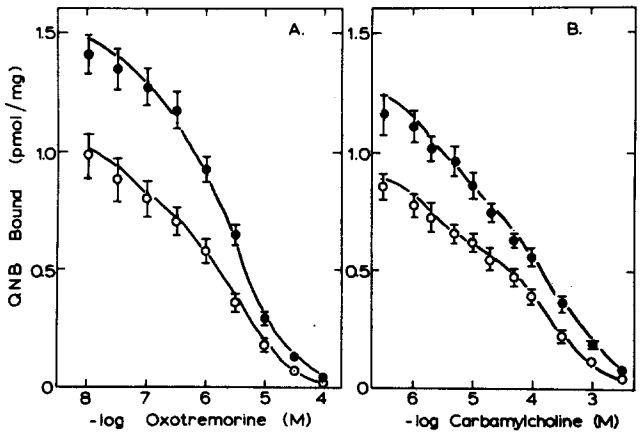

FIG. 6. Inhibition of QNB binding by agonists in control and chronically infused mice. The effects of oxotremorine (A) or carbamylcholine (B) on QNB binding were determined in control (●) or oxotremorine-infused (○, 1.0 mg/kg/hr) animals. Points represent mean±S.E.M. of six determinations. The data have been fitted to a two-site model as described in the text.

for the low-affinity form, nor was the K_I for oxotremorine at the high-affinity sites markedly affected. It should be noted that resolution of the K_I's for oxotremorine and amount of QNB bound by the high- and low-affinity sites is difficult owing to the relatively small differences in the K_I for this agonist:

$$\frac{K_I\text{low}}{K_I\text{high}} = 20\text{–}30.$$

Because of this problem in the analysis of oxotremorine displacement of QNB, carbamylcholine was employed more extensively to determine the proportions of agonist binding sites. Brain regions from control mice and mice infused with 0.2, 0.6, and 1.0 mg/kg/hr oxotremorine were analyzed for carbamylcholine displacement of QNB binding. Three drug-treated groups were examined to determine if a change in the ratio of high- to low-affinity sites might occur at one infusion rate but not at others. The parameters of the displacement curves are summarized in Tables 3 and 4. Total binding, as well as high- and low-affinity binding, decreased in each of the regions in a dose-dependent manner. In all regions examined, the total binding was significantly lower than control in the 1.0 mg/kg/hr group. In cortex and hindbrain, total binding was lower in the 0.6 mg/kg/hr group as well. However, the percentage of high-affinity agonist binding was unchanged by any treatment in any region. That is, there was no difference between the oxotremorine-induced decrease in QNB binding at the high- and low-affinity sites. This observation confirms the results that were found when oxotremorine was used as the inhibitor (see Table 2). The percentage of high-affinity sites measured using carbamylcholine as the inhibitor was slightly higher in each brain region than was the percentage in the oxotremorine experiment. No significant change in the K_I for carbamylcholine at either the high- or low-affinity sites occurred in any brain region at any infusion rate.

The uptake of [^{3}H]-choline by crude synaptosomal fractions prepared from five brain regions of mice infused with various concentrations of oxotremorine was measured. The results of these experiments are shown in Fig. 7. Oxotremorine infusion had no significant effect on choline uptake in any brain region. These measurements were made using a single concentration of choline and, therefore, do not provide information about the kinetics of the uptake process. It is unlikely that oxotremorine treatment altered both the K_m and V_{max} in such a manner that the uptake at a single substrate concentration was unchanged. Chronic treatment had no significant effect on the K_m for choline uptake in cortex: K_m(μM)±S.D.-control=0.4±0.2; 0.1 mg/kg/hr=0.7±0.4; 0.2 mg/kg/hr=0.4±0.2; 0.3 mg/kg/hr=0.4±0.3; 0.6 mg/kg/hr=0.3±0.2; and 1.0 mg/kg/

TABLE 3

EFFECT OF OXOTREMORINE INFUSION ON CARBAMYLCHOLINE INHIBITION OF QNB BINDING*

Brain region	Oxotremorine infusion rate (mg/kg/hr)	Total QNB bound (pmol/mg)	High affinity (pmol/mg)	Low affinity (pmol/mg)	% High affinity
Cortex	0.0	1.30 ± 0.09	0.55 ± 0.05	0.75 ± 0.04	41.5 ± 2.2
	0.2	1.15 ± 0.15	0.45 ± 0.05	0.70 ± 0.03	38.9 ± 2.7
	0.6	1.04 ± 0.05†	0.35 ± 0.02†	0.68 ± 0.03	33.8 ± 1.3
	1.0	0.93 ± 0.08†	0.34 ± 0.05†	0.59 ± 0.05	36.3 ± 3.3
Midbrain	0.0	0.76 ± 0.06	0.36 ± 0.03	0.40 ± 0.04	47.7 ± 3.1
	0.2	0.69 ± 0.06	0.35 ± 0.03	0.35 ± 0.03	49.0 ± 1.5
	0.6	0.58 ± 0.06	0.28 ± 0.04	0.30 ± 0.03	48.2 ± 3.5
	1.0	0.53 ± 0.03†	0.23 ± 0.03†	0.30 ± 0.02	43.3 ± 4.2
Hindbrain	0.0	0.38 ± 0.04	0.28 ± 0.03	0.10 ± 0.02	74.2 ± 3.3
	0.2	0.38 ± 0.03	0.26 ± 0.02	0.12 ± 0.02	70.4 ± 3.0
	0.6	0.26 ± 0.01†	0.17 ± 0.01†	0.09 ± 0.01	64.2 ± 3.7
	1.0	0.22 ± 0.01†	0.14 ± 0.01†	0.08 ± 0.01†	63.4 ± 3.3
Hippocampus	0.0	1.10 ± 0.13	0.31 ± 0.05	0.79 ± 0.23	27.9 ± 2.9
	0.2	1.12 ± 0.12	0.34 ± 0.05	0.79 ± 0.07	29.4 ± 1.6
	0.6	0.88 ± 0.08	0.25 ± 0.03	0.63 ± 0.06	28.7 ± 2.8
	1.0	0.64 ± 0.10†	0.24 ± 0.04	0.40 ± 0.06†	37.2 ± 3.1
Striatum	0.0	2.31 ± 0.27	0.99 ± 0.08	1.32 ± 0.20	43.9 ± 2.2
	0.2	2.16 ± 0.13	0.91 ± 0.05	1.25 ± 0.09	42.5 ± 1.7
	0.6	1.97 ± 0.14	0.83 ± 0.08	1.14 ± 0.10	42.3 ± 3.2
	1.0	1.56 ± 0.05†	0.61 ± 0.18†	0.94 ± 0.08†	39.0 ± 5.0

*The mean ± S.E.M. of total QNB binding and the amount calculated to be high- or low-affinity components of this binding were determined by fitting dose-response curves similar to those in Fig. 6B to the two-site model. Data were collected from six animals at each infusion rate. Average QNB concentration was 79.2 pM. Binding is expressed as pmol/mg protein.

†Significantly different from control ($p<0.05$).

hr=0.3±0.3. Cortex was used in these measurements because it was the brain region in which K_m determinations could be made using tissue from a single treated animal.

DISCUSSION

Our initial study concerning the effects of chronic oxotremorine infusion [20] provided clear evidence of the development of tolerance to oxotremorine. However, the method used to evaluate tolerance in that study did not permit a quantitative analysis of tolerance development. Data obtained with the sequential injection method in the present study suggest that the tolerance which develops during chronic oxotremorine infusion is dose dependent.

Maayani *et al.* [19] reported a 2- to 5-fold change in ED_{50} for the effects of oxotremorine on body temperature, salivation, tremor, and rotarod performance when daily injections of oxotremorine were administered for 10 to 14 days. The greater tolerance seen in our study probably is due, at least in part, to the higher dose of oxotremorine which was infused. In addition, it may be that some tolerance was lost between injections in the Maayani *et al.* study. Any acquired tolerance presumably would not have been lost in our studies because the animals were continuously exposed to oxotremorine.

Maayani *et al.* [19] also reported that degree of oxotremorine tolerance development in their study was dose dependent. This finding is supported by the results of our experiments. The shifts in the dose-response curves for rotarod impairment were parallel (see Figs. 2 and 4B), whereas nonparallel shifts of the dose-response curves were seen for the hypothermia-producing effects of oxotremorine (see Figs. 3 and 4A). These observations may suggest differing mechanisms of tolerance development for the two effects of oxotremorine.

It was previously demonstrated the chronic oxotremorine infusion had no effect on acetylcholinesterase activity and little effect on choline acetyltransferase activity. Likewise, the present study shows that chronic oxotremoreine treatment did not alter high-affinity choline uptake in any brain region tested. This uptake process, however, is affected by acute oxotremorine injection [1, 22, 26]. It appears, therefore, that acute alterations induced in a biochemical system by drug treatment will not necessarily be reflected in alterations of this system by chronic treatment with the same drug.

Our earlier study [20] demonstrated that chronic oxotremorine treatment elicits a dose-dependent decrease in the number of QNB binding sites in every brain region examined except cerebellum, and similar results were obtained in the present experiments. No changes in the affinity of muscarinic receptors for QNB were detected in either study. Chronic infusion of 0.1, 0.2, or 0.3 oxotremorine doses in our earlier study did not produce significant alterations in the number or affinity of QNB binding sites in any examined brain region except hypothalamus. The present study detected clear-cut tolerance (up to 10-fold) to oxotremorine's actions in animals treated in an identical fashion. These results substantiate our earlier conclusion that early stages of

TABLE 4

EFFECT OF OXOTREMORINE INFUSION ON K_I FOR CARBAMYLCHOLINE INHIBITION OF QNB BINDING*

Brain region	Oxotremorine infusion rate (mg/kg/hr)	K_I(HA) ($\times 10^7$ M)	K_I(LA) ($\times 10^5$ M)
Cortex	0.0	5.5 ± 1.0	5.7 ± 0.8
	0.2	4.8 ± 0.5	4.8 ± 0.4
	0.6	4.7 ± 0.6	4.3 ± 0.1
	1.0	4.3 ± 1.2	3.9 ± 0.2
Midbrain	0.0	3.1 ± 0.6	2.4 ± 0.6
	0.2	3.7 ± 0.8	3.1 ± 0.4
	0.6	5.1 ± 0.6	3.5 ± 0.6
	1.0	4.9 ± 0.7	3.4 ± 0.2
Hindbrain	0.0	3.4 ± 0.5	1.5 ± 0.3
	0.2	4.9 ± 1.0	2.2 ± 0.5
	0.6	8.4 ± 1.1	2.1 ± 0.4
	1.0	5.9 ± 1.4	1.9 ± 0.3
Hippocampus	0.0	5.3 ± 1.3	4.2 ± 0.4
	0.2	3.5 ± 0.6	3.9 ± 0.6
	0.6	5.4 ± 0.4	4.6 ± 0.6
	1.0	3.0 ± 0.8	3.2 ± 0.8
Striatum	0.0	5.1 ± 0.7	3.9 ± 0.9
	0.2	4.7 ± 1.3	3.8 ± 0.5
	0.6	4.2 ± 1.1	3.3 ± 0.2
	1.0	4.1 ± 1.1	3.7 ± 1.1

*K_I values ± S.E.M. for carbamylcholine were calculated for the high-affinity (HA) and low-affinity (LA) sites by fitting inhibition curves to the two-site model. Data were collected on the same six animals per group as those used in the experiment reported in Table 3. Infusion rate did not significantly affect either high- or low-affinity K_I values within brain regions.

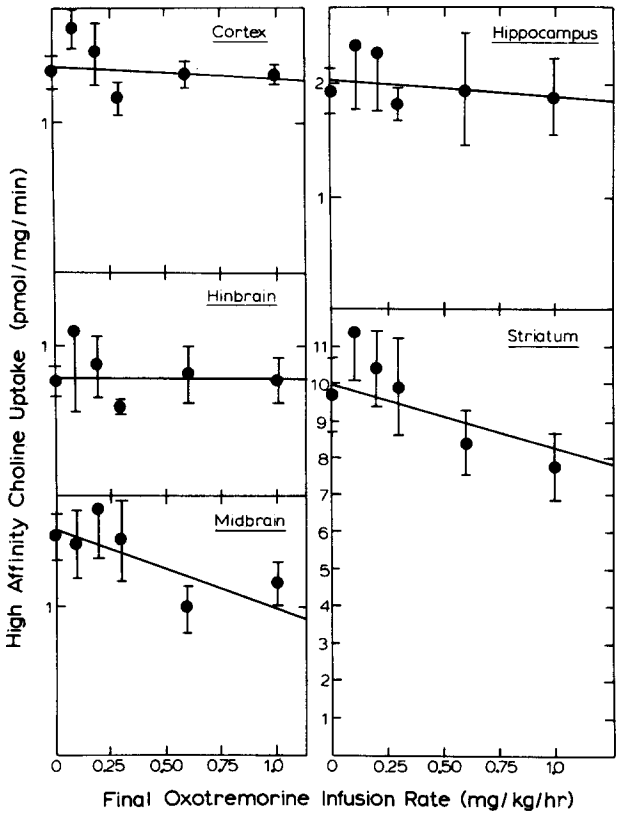

FIG. 7. Effect of oxotremorine on high-affinity choline uptake. High-affinity choline uptake was measured in synaptosomes prepared from each of five brain regions. Uptake was measured using 0.1 μM [^{3}H]-choline. Points represent mean±S.E.M. of 6–13 determinations.

the development of tolerance to oxotremorine are not explainable on the basis of a measurable alteration in receptor number or affinity.

The present results indicate no differential effect of chronic oxotremorine treatment on high-affinity and low-affinity muscarinic agonist binding sites. Since carbamylcholine appeared to differentiate the two forms of binding site better than did oxotremorine, dose-response curves for the effect of chronic oxotremorine on high- and low-affinity binding were constructed using carbamylcholine as the probe. The data presented in Table 3 demonstrate that chronic oxotremorine treatment decreases the total number of QNB binding sites in what appears to be a dose-related fashion. Both high- and low-affinity forms are affected, and the ratio of the two forms is unaltered. While regional differences in the percentage of high-affinity QNB binding sites were detected (e.g., 40% of total binding sites in cortex were high-affinity, whereas approximately 70% were high-affinity in hindbrain), in no region was the ratio of high- to low-affinity sites changed by chronic oxotremorine infusion. Similarly, chronic treatment did not appear to alter the K_I value for carbamylcholine inhibition of QNB binding. Taken together, these data strongly suggest that tolerance to oxotremorine, at least in its early stages, is not due to an altered number or ratio of muscarinic receptor subtypes, although the tolerance accompanying higher doses of oxotremorine may be due in part to reduced receptor number.

Little attention has been devoted to the question of whether chronic cholinergic activation alters the ratio, number, or affinity of high- and low-affinity binding sites for muscarinic agonists. Ehlert *et al.* [9,10] have examined the effect of chronic DFP treatment on agonist inhibition of QNB binding. In both rat striatum [9] and ileum [10] DFP treatment decreased total QNB binding but had no effect on the percentage of high-affinity agonist binding sites. These observations are in agreement with those presented here. While chronic oxotremorine infusion had no effect on the K_I for carbamylcholine and inconsistent effects on the K_I for oxotremorine at the high-affinity sites in our study, DFP treatment in the Ehlert *et al.* [9,10] studies appeared to increase the K_I for acetylcholine and oxotremorine at the high-affinity sites in striatum and to increase the K_I for acetylcholine and oxotremorine, but not for carbamylcholine, at the high-affinity sites in ileum. Treatment, tissue, and/or species differences may explain the minor dissimilarities between our data and those obtained by Ehlert *et al.* [9,10].

In general, the observation of binding sites with different affinity for ligands may result from the presence of several distinct receptor molecules, from differential coupling of a single receptor molecule to other proteins, or from the existence of several conformational states of a receptor with dif-

ferent affinities for the ligand. The heterogeneity of agonist binding to muscarinic receptors probably results from either coupling or conformational differences, rather than from the existence of structurally distinct receptors [5, 8, 12]. Whatever the molecular basis of the heterogeneity of agonist binding to muscarinic receptors, chronic oxotremorine treatment had no effect on the relative amount of high- and low-affinity agonist binding sites. Displacement of QNB binding by agonists does not allow a reliable estimate of the super-high-affinity site. Measurement of these sites using labeled agonists [4,11] will be required to determine if the super-high-affinity agonist sites are differentially affected.

It should be noted that the decreased number of QNB binding sites detected in this and our previous study is not likely to be due to an inhibition of QNB binding by residual oxotremorine [20]. In our initial study, we noted that neither injection of large oxotremorine doses shortly before sacrifice nor *in vitro* incubation of homogenates with oxotremorine altered QNB binding. Apparently, any residual oxotremorine is washed out during tissue homogenization and preparation. Any oxotremorine which remained in the tissue would have served to overestimate receptor loss [13].

Although it is not yet clear which brain areas control the behavioral phenomena used to test for tolerance development, the absence of detectable receptor changes after low doses of oxotremorine indicates that an explanation other than altered receptor number or affinity may be needed to explain the early stages of tolerance development. Enhanced drug metabolism explains tolerance to many agents. Although we have not directly assessed metabolism in our studies, the observation that hypothermia induced by oxotremorine returns to normothermia at similar rates in both naive and tolerant animals suggests similar metabolic rates. Another possible explanation for tolerance development may be altered receptor coupling. Su *et al.* [27] have reported that chronic stimulation with beta-adrenergic agonists results in uncoupling of the beta-adrenergic receptor from adenylate cyclase before the occurrence of changes in the number of beta-adrenergic receptors. Similarly, Richelson [23] has observed the rapid development of a decreased responsiveness of neuroblastoma cells to muscarinic agonists, as measured by cGMP synthesis. This decreased responsiveness occurs more quickly than the decrease in receptor number detected by Shifrin and Klein [25] in the same cell line. The results of these experiments and our studies suggest that the role of altered receptor coupling in tolerance to muscarinic agonists merits further investigation.

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