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Cooperativity Pattern in the Interaction of the Antiestrogen Drug Clomiphene with the Muscarinic Receptors

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SUMMARY

The possible interaction between the muscarinic receptors and the antiestrogenic drug clomiphene citrate was investigated in competition experiments using the highly specific tritiated antagonist *N*-methyl-4-piperidyl benzilate (4-NMPB) in various regions of rat brain. It was found that clomiphene can displace muscarinic antagonists from their receptor-ligand complexes. Binding analyses as well as determinations of the kinetics of dissociation of [^{3}H]4-NMPB-receptor complexes indicate the binding of more than one molecule of clomiphene in a positively cooperative pattern. These findings suggest that the nonsteroidal antiestrogenic drugs, e.g., clomiphene, might exert their effects not only through the specific estrogen receptor but also in conjunction with the muscarinic system.

INTRODUCTION

Nonsteroidal synthetic compounds such as clomiphene citrate, defined chemically as 1[-*p*-(β-diethylaminoethoxy)phenyl]1,2-diphenyl-2-chloroethylene, are widely used as ovulation inducers in humans and animals (see ref. 1 and references therein). These antiestrogenic compounds are thought to act at the hypothalamic-pituitary level by competing with endogenous 17β-estradiol for estrogen-receptor binding sites, although the mechanism of action is not yet clear.

From previous studies on the structure-activity relationships and quantum chemical calculations for muscarinic antagonists (2, 3), it became evident that clomiphene citrate might incorporate an acetylcholine-like molecular arrangement, especially in the region of the "cationic head." Its possible interaction with the muscarinic receptors was therefore investigated in competition experiments using the highly specific, tritiated antagonist 4-NMPB[2] (4, 5) in homogenates of various regions of rat brain.

MATERIALS AND METHODS

Materials

[^{3}H]4-NMPB, 69.7 Ci/mmole, was prepared by catalytic tritium exchange as described elsewhere (6). Its purity was >97%. Clomiphene citrate (Ikaclomine) was a gift from Dr. B. Weiner, of Teva Ltd. (Jerusalem, Israel). Its purity, determined by HPLC, was found to be >96%. Cisclomiphene (zuclomiphene, Isomer A; RMI 16,312) and transclomiphene (enclomiphene, Isomer B; RMI 16,289) were gifts of Dr. W. L. Albrecht of Merrell National Laboratories, Division of Richardson-Merrell Inc. (Cincinnati, Ohio). Tamoxifene was a gift from Dr. A. Eshkol, Sheba Medical Center (Tel-Hashomer, Israel). All other compounds were of the best grade available.

Methods

Adult male and female rats of the CD strain were obtained from Levinstein's Farm (Yokneam, Israel) and maintained in an air-conditioned room at 24 ± 2° for 14 hr (5 a.m.–7 p.m.) under fluorescent illumination and in darkness for 10 hr. Food from Assia Maabarot Ltd. (Tel-Aviv, Israel) and water were supplied ad libitum. The rats were then 3–4 months old and weighed 190–250 g. They were decapitated (between 10 a.m. and noon) and their brains were rapidly removed. The median hypothalamus [containing the median eminence and the ventromedial, the dorsomedial, and the arcuate hypothalamic nuclei (17–20 mg/animal)], the medulla-pons, the cortex, and the caudate putamen were dissected out in a cold room after identification with the aid of a stereotaxic atlas (7).

HPLC

HPLC analysis was carried out on the Waters Associates instrument Model 440 equipped with an absorbance detector (280 nM) and differential refractometer R401, with an analytical column (Whatman) (Partisil p × s 10/25 ODS) with a flow rate of 0.3 ml/min, using methanol as the solvent.

Binding Assay

Brain regions were homogenized, as described in detail previously (5), to yield a 3% homogenate (w/v). Homogenates prepared from the medulla-pons (three rats), cortex (one rat), caudate putamen (two rats), and the me-

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[2] The abbreviations used are: 4-NMPB, *N*-methyl-4-piperidyl benzilate; HPLC, high-pressure liquid chromatography; CPK, CPK atomic models (Ealing Corporation, Cambridge, Mass.).

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dian hypothalamus (five rats) were used for binding assays as follows: 50 μl of tissue preparation were incubated at 25° in 2 ml of modified Krebs-Henseleit solution (25 mM Tris-HCl, 118 mM NaCl, 4.69 mM KCl, 1.9 mM $CaCl_2$, 0.54 mM $MgCl_2$, 1.0 mM NaH_2PO_4, 11.1 mM glucose), pH 7.3, containing varying amounts of the labeled ligand. After 30 min of incubation, ice-cold Krebs solution (3 ml) was added and the contents were passed rapidly by suction through a glass filter (GF/C, Whatman, 25-mm diameter). The filters were washed three times in 3 ml of ice-cold Krebs solution. All procedures were completed within less than 10 sec. Binding assays were performed in triplicate, together with triplicate control samples containing 5×10^{-5} M unlabeled atropine. The filters were placed in vials containing 4 ml of scintillation liquid (Hydro-Luma, Lumac Systems, Inc., Titusville, Fla.) and were maintained at 25° for 30 min; the radioactivity was then measured by liquid scintillation spectrometry (Packard Prias Model PL) with a counting efficiency of 40–45%. Protein was determined by the method of Lowry *et al.* (8), using bovine serum albumin as a standard.

Specific binding was defined as the total binding minus the nonspecific binding, i.e., binding in the presence of 5×10^{-5} M unlabeled atropine. Direct binding studies, competition experiments, and kinetic experiments were carried out as described in detail in previous reports (5, 9).

Data Analysis

The competition curves were analyzed by assuming the following model in which the labeled ligand (An) and unlabeled ligand clomiphene (C) compete for the same binding site on the receptor (R); in addition, we assumed a possible ternary complex of the two ligands and the receptor, as depicted in Scheme 1; theoretical binding curves were fitted to the experimental data points using the nonlinear least-squares regression computer program BMDPAR,[3] revision date November 1979.

$$\begin{array}{ccc} R + An & \overset{K_1}{\rightleftharpoons} & RAn \\ + & & + \\ C & & C \\ {\scriptstyle K_2'}\ \Downarrow\Uparrow & & \Downarrow\Uparrow\ {\scriptstyle K_2'} \\ RC + An & \overset{K_1}{\rightleftharpoons} & RCAn \\ + & & \\ C & & \\ {\scriptstyle K_2''}\ \Downarrow\Uparrow & & \\ RC_2 & & \end{array}$$

SCHEME 1

$$K_1 = \frac{[R][An]}{[RAn]}$$

$$K_2' = \frac{[R][C]}{[RC]}$$

$$K_2'' = \frac{[RC][C]}{[RC_2]}$$

[3] Developed at the Health Science Computing Facility of the University of California at Los Angeles. The facility is sponsored by National Institutes of Health Research Resources Grant RR-3.

$[R]$, $[An]$, and $[C]$ are the concentrations of free receptor, antagonist, and clomiphene, respectively. $[RAn]$, $[RC]$, and $[RC_2]$ are the concentrations of the complexes of the receptor with the antagonist, with one molecule of clomiphene and with two molecules of clomiphene, respectively. The total concentration of the receptor is given by

$$[R_T] = [R] + [RAn] + [RC] + [RC_2] + [RAnC]$$

The free receptor concentration will thus be given by

$$[R] = \frac{[R_T]}{1 + \frac{[An]}{K_1} + \frac{[C]}{K_2'} + \frac{[An][C]}{K_2'K_1} + \frac{[C]^2}{K_2'K_2''}}$$

With these equations we can express the concentration of the bound antagonist $[RAn + RAnC]$ as a function of the dissociation constants and the concentrations of the two ligands, as follows:

$$[RAn] + [RAnC] = \frac{[R_T]}{1 + \frac{[An]}{K_1} + \frac{[C]}{K_2'} + \frac{[An][C]}{K_2'K_1} + \frac{C^2}{K_2'K_2''}} \left(\frac{(An)}{K_1} + \frac{(An)(C)}{K_2'K_1}\right)$$

Double reciprocal. Simple rearrangement of the above yields the following:

$$\frac{[R_T]}{[RAn] + (RAnC)} = \frac{\left[\frac{K_1}{[An]}\right]\left[1 + \frac{[C]}{K_2'} + \frac{[C]^2}{K_2''K_2'}\right]}{1 + \frac{[C]}{K_2'}} + 1 \qquad (1)$$

From Eq. 1 it can be seen that plotting of $[R_T]/([RAn] + [RAnC])$ versus $1/[An]$ should yield straight lines intersecting at 1.0 on the ordinate with a slope (α) such that

$$\alpha = K_1 + \frac{K_1[C]^2}{K_2''(K_2' + [C])}$$

This equation predicts that the behavior of α will vary as a function of clomiphene concentration. At low concentrations of clomiphene,

$$\alpha = K_1 + \frac{K_1[C]^2}{K_2''K_2'}$$

and the line will be exponential and will intersect the ordinate at K_1. Moreover, replotting of α as a function of $[C]^2$ will yield a straight line at low concentrations of C, with an intercept of K_1 and a slope of $K_1/(K_2'K_2'')$. At high concentrations of clomiphene plotting of α as a function of C should be linear, since then

$$\alpha = K_1 + \frac{K_1[C]}{K_2''}$$

and the slope of this linear portion of the curve is given by K_1/K_2''.

Dixon plot. Rearrangement of Eq. 1 yields

$$\frac{[R_T]}{[RAn]+[RCAn]} = 1 + \frac{1}{([An]/K_1)} + \frac{[C]^2/(K_2''K_2')}{([An]/K_1)(1+[C]/K_2')} \quad (2)$$

At low concentrations of C, i.e., $C \ll K_2'$,

$$\frac{[R_T]}{[RAn]+[RCAn]} = 1 + \frac{K_1}{[An]} + \frac{K_1}{(An)K_2''K_2'}[C]^2 \quad (3)$$

Plotting of $(R_T)/([RAn]+[RCAn])$ as a function of $[C]^2$ at various concentrations of antagonist (An) should yield straight lines. Replotting the slopes of these lines $(K_1/[An]K_2''K_2')$ as a function of $1/[An]$ should give a straight line intersecting the origin and having a slope α'' such that

$$\alpha'' = \frac{K_1}{K_2''K_2'}$$

RESULTS

The possible interaction of clomiphene (a mixture of two geometrical isomers) with the muscarinic receptor was tested by means of competition experiments with [^{3}H]4-NMPB (2.5 nM). At 2.5 nM [^{3}H]4-NMPB, there is approximately 85% occupancy of the sites. Figure 1 shows the results of competition experiments using homogenates from cortex, medulla-pons and median hypothalamus. Clomiphene did indeed inhibit the high-affinity binding of [^{3}H]4-NMPB, as well as that of ^{3}H-labeled *N*-methyl scopolamine (data not shown), thus indicating that clomiphene can act as an anticholinergic drug. The apparent K_D values determined from these experiments were in the range of 10^{-6} M (see below). Preliminary experiments (as described in detail in ref. 4) indicated that binding of clomiphene to the [^{3}H]4-NMPB sites was reversible and that reassociation could be repeated upon addition of [^{3}H]4-NMPB to dissociated receptor-clomiphene complex. Competition experiments were carried out in several brain regions with different concentrations of [^{3}H]4-NMPB in the presence of different concentrations of clomiphene. It had previously been found (5, 9) that binding of 0.5–16 nM [^{3}H]4-NMPB at 25° reaches equilibrium within less than 30 min. The results for the median hypothalamus, for example, are presented in Fig. 2, which shows that the value for maximal binding of [^{3}H]4-NMPB to median hypothalamus preparation is the same in both the absence and presence of unlabeled clomiphene. Similar results were obtained in other brain regions. When the data shown in Fig. 2A are replotted in a double reciprocal form (Fig. 3) according to Eq. 1 (see

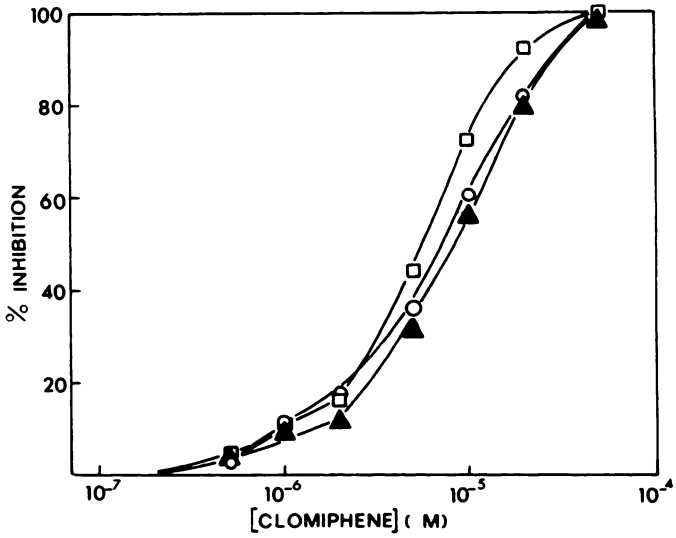

FIG. 1. *Binding of 2.5 nM [^{3}H]4-NMPB to homogenates of various brain regions (25°) in the presence of various concentrations of clomiphene citrate*

▲——▲, Cortex; □——□, medulla-pons; ○——○, median hypothalamus.

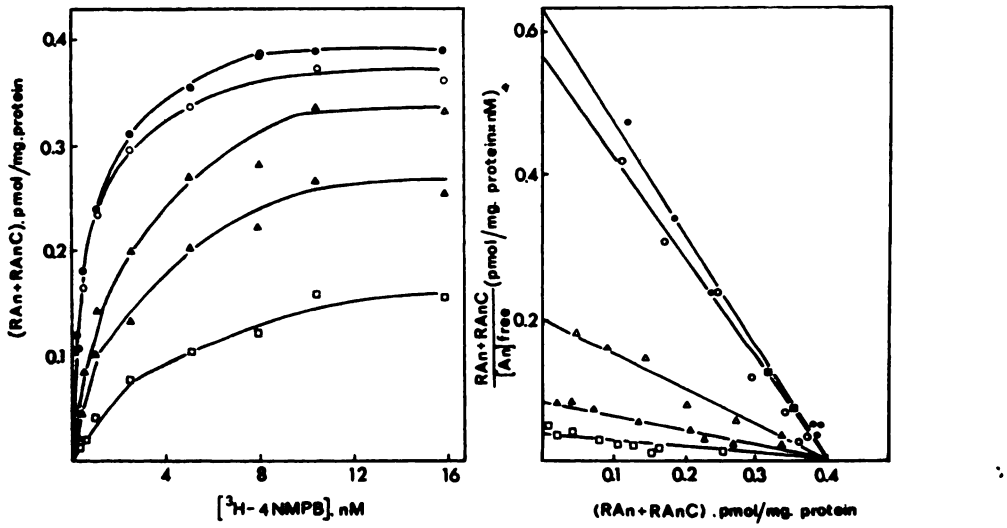

FIG. 2. *Specific binding of [^{3}H]4-NMPB as a function of its various concentrations in the presence of clomiphene citrate*

RAn + RAnC = bound [^{3}H]4-NMPB. Tissue homogenates (0.05 ml) of median hypothalamus of female rats were incubated with varying concentrations of [^{3}H]4-NMPB for 30 min at 25° in 2 ml of modified Krebs solution (pH 7.3) containing various concentrations of clomiphene citrate: ●——●, without added clomiphene citrate; ○——○, 1 μM; Δ——Δ, 5 μM; ▲——▲, 10 μM; □——□, 20 μM. Each point is the mean of triplicate samples whose standard error was less than 5%. The concentration of receptor binding sites was 95 pM.

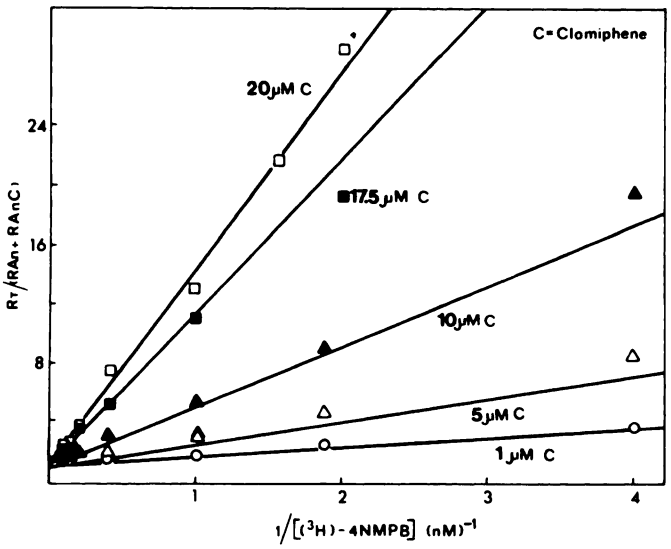

FIG. 3. *Double-reciprocal plots of data presented in Fig. 2*

The plot for 20 μM clomiphene citrate was obtained from data of separate experiments. R_T = maximal binding capacity of [^{3}H]4-NMPB; $RAn + RAnC$ = bound [^{3}H]4-NMPB. As indicated, each plot represents a different concentration of clomiphene citrate. The *solid line* is the best fit of the data obtained by computer analysis using nonlinear regression curve fitting.

Materials and Methods), the intersection of lines of 1.0 on the ordinate indicates that clomiphene displaces all of the specifically bound [^{3}H]4-NMPB in a competitive manner. A replot of the slopes of the lines depicted in Fig. 3 as a function of clomiphene concentration yields a parabolic curve (see Fig. 7), indicating a multiple binding of clomiphene to the muscarinic receptor. This phenomenon was not region-specific, since similar behavior was observed in the other brain regions under investigation.

Competition experiments (2 nM [^{3}H]4-NMPB and various clomiphene concentrations) were carried out in the medulla-pons and median hypothalamus homogenates in the presence of 100 μM GTP (10 min at 37°). The binding properties of [^{3}H]4-NMPB were essentially the same in the absence and presence of GTP.

The possible chemical alteration of clomiphene during the course of competition experiments was tested by HPLC. Aliquots were chromatographed and found to behave like the authentic compound.

Similar competition experiments performed with pure cisclomiphene and transclomiphene (10) indicated that there was no stereoselectivity; i.e., the K_D values determined for the two isomers were found to be very similar to each other and to that of the mixture of the geometrical isomers.

The rate of dissociation of [^{3}H]4-NMPB from its complex with the receptor in each of the separate brain regions was determined. Homogenates were incubated to equilibrium for 2 min at 25° in the presence of 5 nM [^{3}H]4-NMPB (5, 9). Under these conditions equilibrium is reached within 2 min (5, 9). Dissociation was initiated by adding either 50 μM unlabeled 4-NMPB or 50 μM clomiphene, and the samples were filtered immediately (zero time) and at various time intervals. The first-order plots for the dissociation of [^{3}H]4-NMPB from the various preparations deviate from linearity. This phenomenon was observed previously by us and is discussed at length elsewhere (4, 5, 9). We calculated the half-lives for the dissociation as depicted in Table 1. As shown in all regions investigated, dissociation was accelerated in the presence of clomiphene.

DISCUSSION

The results presented here clearly indicate that (a) clomiphene can displace muscarinic antagonists from their receptor-ligand complexes; (b) this phenomenon occurs in various brain regions; and (c) the binding patterns are indicative of binding of more than one molecule of clomiphene.

Competition between two compounds on the same biological site is believed to indicate that a common molecular structure exists. Thus, the antimuscarinic character of clomiphene might result from its acetylcholine-like molecular arrangement (see ref. 3 and references therein). The molecular structure of the benactyzine crystal (11) is shown in Fig. 4. This drug, which is an analogue of 4-NMPB, is an amino ester of benzilic acid, and is known to have antimuscarinic activity (12). In order to investigate the possibility that clomiphene mimics the spatial arrangement of benactyzine, we examined both CPK and Dreiding molecular models (Fig. 4, *left*). Fig. 4 (*right*) shows the relatively high degree of spatial correspondence evident in the models of these two drugs. The common molecular core present in the two compounds (*stippled circles*) constitutes the particular arrangement which is essential in order for interaction with the acetylcholine receptor to take place. This type of molecular arrangement was previously shown (2, 3, 13) to be shared by both muscarinic agonists and antagonists, thus supporting an hypothesis of their binding to a common site. The *upper region* in Fig. 4 (*right*) represents the molecular structural elements which are not commonly included in acetylcholine-like arrangements; the possibility suggests itself that this difference might account for another site of binding, and that this site might be responsible for the cooperativity discussed below.

The results of the competition experiments indicated

TABLE 1

Half-lives of dissociation of [^{3}H]4-NMPB from the receptor-ligand complex

Homogenates were incubated to equilibrium (2 min 25°) with 5 nM [^{3}H]4-NMPB. Dissociation was initiated by adding unlabeled 4-NMPB or clomiphene, and the samples were filtered immediately (zero time) and at various time intervals. ([^{3}H]4-NMPB$_{bound}$)$_{t=0}$ in picomoles per milligram of protein: 0.35 for median hypothalamus, 0.52 for posterior hypothalamus, 0.39 for preoptic area, and 0.29 for medulla-pons.

Region	$t_{1/2}$ in the presence of	
	50 μM 4-NMPB[a]	50 μM clomiphene[a]
	min	*min*
Median hypothalamus	5	3
Posterior hypothalamus	6	4
Preoptic area	3.5	2.5
Medulla-pons	5	0.25

[a] The mean of at least three experiments whose standard error was less than 5%.

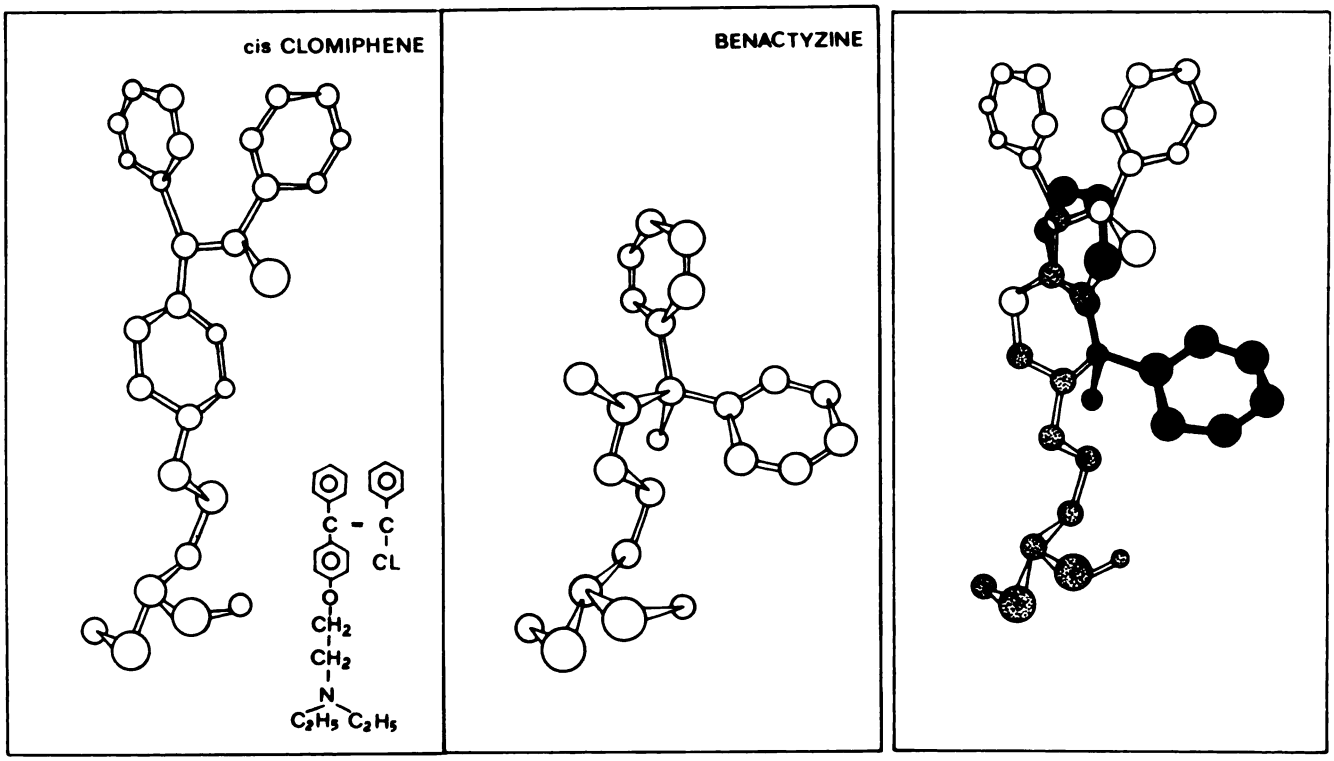

FIG. 4. *Left, allowed conformation of cisclomiphene as verified by CPK models; center, molecular model of benactyzine as found in the crystal structure (○) (11); right, superposition of cisclomiphene and benactyzine molecule (●)*

that more than one molecule of clomiphene binds to the receptor, and the data shown in Figs. 1 and 2 were therefore replotted according to Dixon (Fig. 5A) and Hill (Fig. 6). The curvature of the Dixon plots, as well as the slopes of Hill plots calculated at half-saturation as discussed by Dahlquist (14) (1.7 for the medulla-pons; 1.3 for the cortex, and 1.2 for the hypothalamus) further supported the notion that more than one molecule binds to the muscarinic receptor. The simplest model that can accommodate these results is given in Scheme 1. For the sake of simplicity, we assume in this model that the two isomers of the receptor-4-NMPB complex (4, 5) behave similarly. As predicted from this model (see Eq. 1), the plot of α (slope of the line) as a function of [*C*] (clomiphene concentration) (Fig. 7, *left*) should yield a curve composed of two parts (see Methods, double reciprocal), the lower exponential and the upper linear, and this was indeed observed. The slope of the linear part is given by

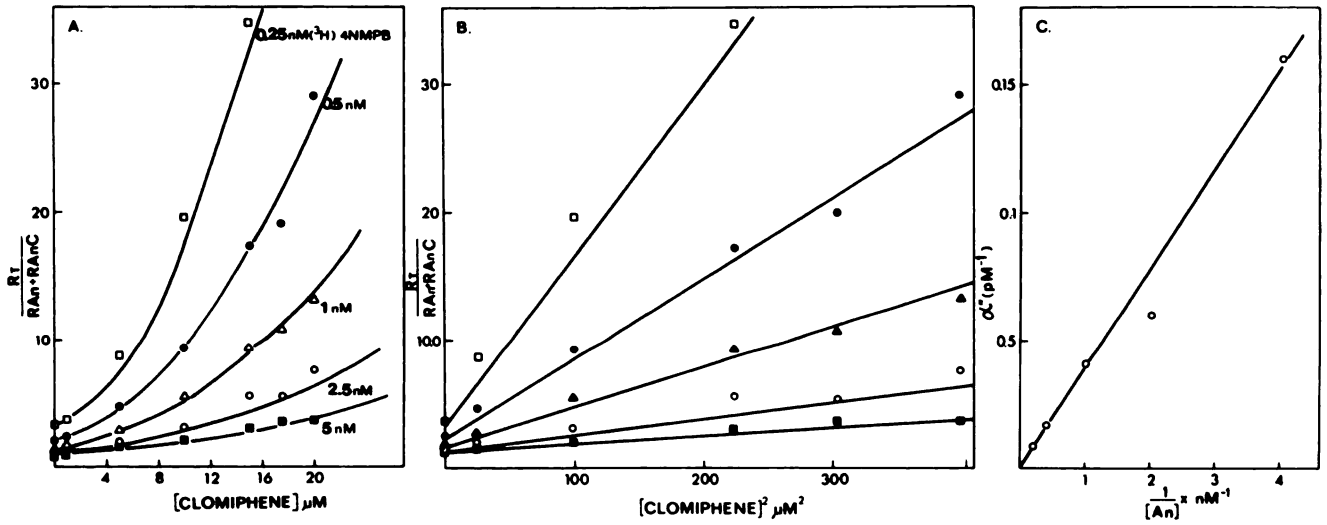

FIG. 5. *Dixon plots at various concentrations of [³H]4-NMPB*

Data are taken from Fig. 2 (as well as for additional concentrations of [³H]4-NMPB), where R_T = maximal binding capacity of [³H]4-NMPB and [*RAn* + *RAnC*] = bound [³H]4-NMPB as a function of (A) the concentration of clomiphene citrate, (B) the square of the concentration of clomiphene citrate, and (C) the slopes (α'') of the linear part of B as a function of 1/[³H]4-NMPB free concentration. The *solid line* is the best fit of the data obtained by computer analysis using nonlinear regression curve-fitting.

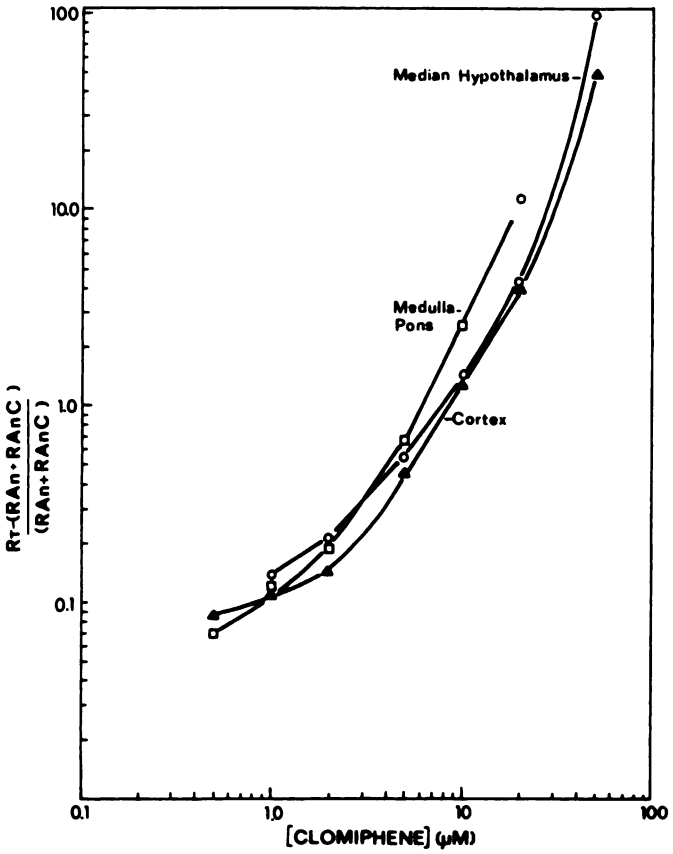

FIG. 6. *Hill plots of data given in Fig. 1*

K_1/K_2''. Moreover, in plotting α as a function of $[C]^2$ (Fig. 7, *right*), the exponential part in Fig. 7 (*left*) should now be linear, as indeed shown. The curved lines obtained in Dixon plots (Fig. 5A) can be linearized at low clomiphene concentrations by replotting the data as a function of $[C]^2$ (Fig. 5B) (see Materials and Methods, Dixon plots, Eq. 2). The slopes of these lines replotted as a function of $1/[An]$ are shown in Fig. 5C. Note the good agreement between the theoretical curves and the experimental data.

Analyses of these curves according to Eqs. 2 and 3 (see Materials and Methods) yields a K_D value of 6×10^{-10} M for 4-NMPB; as expected, this K_D value is in excellent agreement with that determined from direct binding of that ligand to the muscarinic receptors in the median hypothalamus (9). The value of the dissociation constant for the first clomiphene molecule (K_2') is $7.1 \pm 2.1 \times 10^{-5}$ M, whereas that for the second molecule (K_2'') is $2.1 \pm 0.25 \times 10^{-7}$ M.

As shown in Table 2, the measured values for the [^{3}H]4-NMPB binding constant are in agreement with those calculated using the nonlinear regression procedure, thus lending further support to the validity of the model presented in Scheme 1.

An alternative to the model presented in Scheme 1 is to exclude the ternary complex from this model. In such a case one would expect a linear curve when plotting the α value as a function of $[C]^2$ (slope = $K_1[C]^2/K_2'K_2''$). As shown in Fig. 7 (*right*), this is not the case.

The fact that clomiphene possesses muscarinic properties is not unexpected, since several of its reported side effects (15, 16), i.e., vasomotor flushes, abdominal-pelvic discomfort, nausea and/or vomiting, visual disturbances,

TABLE 2

Apparent binding constant for 4-NMPB measured from data given in Fig. 2 (right) and values calculated from the model given in Scheme 1

Values from the model in Scheme 1 were calculated by the nonlinear least-squares regression procedure.

Clomiphene concentrations	Binding constant measured	Binding constant calculated
μM	nM^{-1}	nM^{-1}
0	1.66 ± 0.07	1.66
1	1.41 ± 0.15	1.51
5	0.50 ± 0.07	0.60
10	0.21 ± 0.05	0.24
17.5	0.08 ± 0.02	0.08

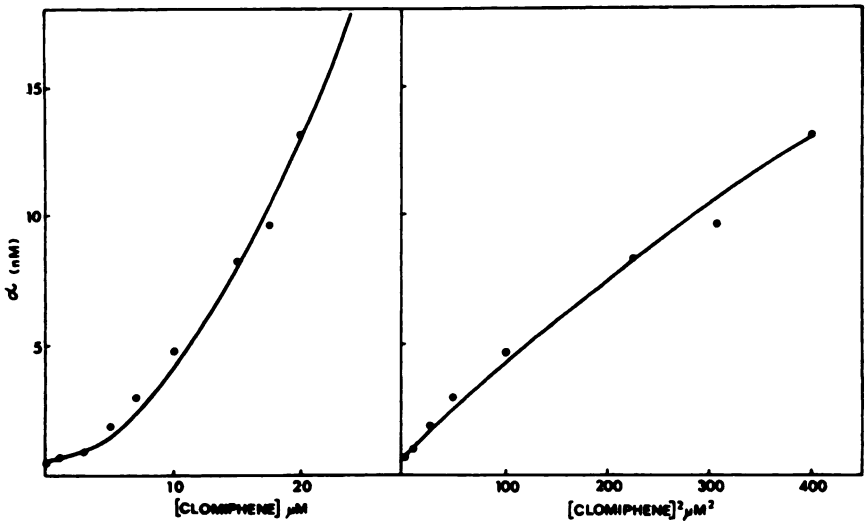

FIG. 7. *The slopes (α) of double reciprocal plots shown in Fig. 2, as a function of the concentration of clomiphene citrate (left) and the square of the concentration of clomiphene citrate (right)*

The *solid line* is the best fit of the data obtained by computer analysis using nonlinear regression curve-fitting.

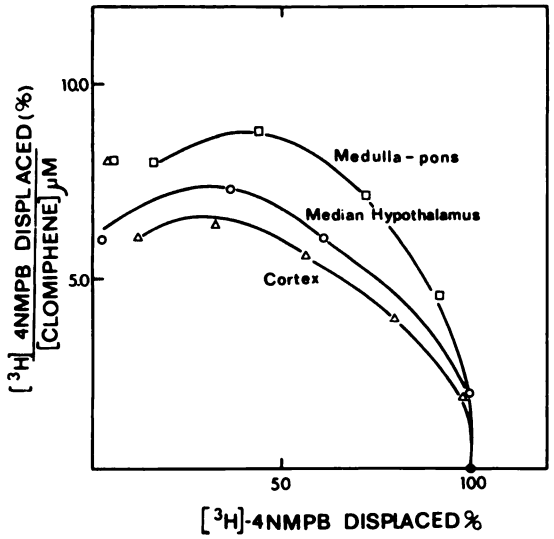

FIG. 8. *Scatchard analysis of the displacement of [^{3}H]4-NMPB by various concentrations of clomiphene citrate in various brain regions*

and memory impairment are most likely due to its anticholinergic-antimuscarinic properties. The fact that the K_D value for clomiphene binding is much lower than those of known muscarinic antagonists (5, 17), and is in fact closer to the values obtained for agonists, suggests that the drug might be acting as a cholinergic agonist or partial agonist rather than as an antagonist. However, in the guinea pig ileum system, clomiphene inhibits acetylcholine-induced contractions,[4] indicating that, at least in these peripheral receptors, clomiphene behaves as an antagonist. The possibility was considered that clomiphene behaves as an antagonist peripherally and as an agonist or partial agonist centrally. However, a recent case report (16) describing memory impairment as a result of high levels of clomiphene in a female patient tends to support an assumption of central antagonistic activity by this drug.

Experiments using widely varying receptor concentrations (up to 20-fold) yielded similar results. Nonspecific adsorption of clomiphene is thus unlikely to account for the deviation in the linearity of, e.g., Dixon plots. However, the fact that the gradient of the Hill plots appears to increase with increasing clomiphene concentrations suggests that nonspecific interaction cannot be dismissed altogether. The use of radiolabeled clomiphene currently being synthesized in our laboratory will help to clarify further the possibility of nonspecific anchoring of clomiphene to, e.g., lipid domains, as well as to elucidate its mode of action in both antagonists and agonists.

The model presented in Scheme 1 favors an assumption of the existence of positive cooperativity. This suggestion is strongly supported by (a) a difference of 2–3 orders of magnitude between the K_D values of the two clomiphene molecules, (b) the Scatchard plot (Fig. 8), and (c) the accelerated dissociation of the [^{3}H]4-NMPB-receptor complex observed in the presence of clomiphene (Table 1).

From other receptor systems, one might consider the possibility that the observed "positive cooperativity" is due to dimerization of clomiphene. In such a case one would expect a linear curve when plotting the α value as a function of [C]^2. As shown in Fig. 7 this is not the case. Furthermore, it is quite clear from the dissociation experiments that two-site interaction might offer a reasonable explanation for the observed acceleration. We therefore suggest that the first molecule of clomiphene binds to the muscarinic receptor at a site distal to the "native" antagonist site, leading to conformational changes such that, when the second clomiphene molecule enters the 4-NMPB zone of binding, the ternary complex formed (Scheme 1) has a much higher K_{off} and hence the dissociation of [^{3}H]4-NMPB is facilitated. It is therefore conceivable that the effect on the muscarinic receptors could be brought about through the agency of the first molecule of clomiphene in binding to another macromolecule, hence effecting a modulation of the muscarinic system in conjunction with another neuronal pathway (18).

[4] S. Avissar and M. Sokolovsky, unpublished data.

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