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INTERACTIONS OF D600 (METHOXYVERAPAMIL) AND LOCAL ANESTHETICS WITH RAT BRAIN α-ADRENERGIC AND MUSCARINIC RECEPTORS

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Abstract.—D600 (methoxyverapamil) was found to inhibit the specific binding assayed in rat brain homogenates of the antagonist agents [^{3}H]WB 4101 and [^{3}H]QNB to the α-adrenergic and muscarinic receptors respectively. The IC_{50} concentrations of D600 in standard binding experiments were 1.7×10^{-6} M and 1.4×10^{-5} M, with calculated K_I values of 0.98×10^{-6} M and 8.83×10^{-6} M. Scatchard analyses showed these inhibitions to be competitive. Lidocaine and tetracaine also inhibited radioligand binding to these receptors, with K_I values of 5.25×10^{-4} M and 4.85×10^{-5} M for the α-receptor and 8.2×10^{-5} M and 6.94×10^{-6} M for the muscarinic receptor; these inhibitions also appeared to be competitive. Increasing the Ca^{2+} concentration in the assays to 10 mM did not influence the effects of D600 or the anesthetics. Analyses of inhibitions of muscarinic receptor binding produced by D600 and lidocaine over a range of pH indicated that the inhibitory species of D600 is the uncharged form, whereas the charged form of lidocaine is inhibitory. Interactions of D600 and lidocaine with the agonist site on the muscarinic receptor were studied by measuring the effects of these agents on the displacement of [^{3}H]QNB by the muscarinic agonist carbachol. Comparison of these results with a theoretical model indicates that carbachol, [^{3}H]QNB, and D600 or lidocaine competitively displace one another at the same agonist site. The binding of labeled naloxone to the opiate receptor was also inhibited by D600, the IC_{50} being 4×10^{-6} M. These inhibitory effects of D600 and the local anesthetics on different receptors suggest that these agents may act by a common mechanism, namely by perturbing membrane structures. These results suggest caution in interpreting experiments in which D600 and verapamil are used analytically as Ca antagonists to assess the involvement of Ca in a biological system.

Verapamil and its methoxy derivative, D600, are members of a group of compounds which have been termed "calcium-antagonists", since at low concentrations they inhibit transmembrane calcium fluxes [1, 2]. Electrophysiological studies with mammalian cardiac fibers have demonstrated [3] that these agents at concentrations of 10^{-6} M block, selectively, the inward current carried by calcium and that this effect can be antagonized by increasing the extracellular calcium concentration. Experiments by Nayler and Krikler [4] which explore the cellular locus of action of verapamil have shown that at 10^{-6} M this drug interferes with the superficially located cardiac cell membrane storage sites for calcium, while Williamson *et al.* [5] have demonstrated, directly, interference by 10^{-6} M verapamil with ^{45}Ca binding to isolated cardiac sarcolemmal preparations. This inhibition of calcium binding at the cell membrane appears to account for the negative inotropic effect of these compounds in cardiac muscle, and for the peripheral and coronary vasodilation resulting from effects on smooth muscle.

Because of their effectiveness in inhibiting calcium influx into cardiac and smooth muscle, verapamil and D600 have been used in various laboratories as diagnostic tools in assessing the role of calcium in several processes, although often at drug concentrations greatly in excess of those which produce cardiac effects. Van der Kloot and Kita [6], for example, attempted to use verapamil and D600 at a concentration of 5×10^{-4} M as specific inhibitors of stimulated calcium influx in crustacean muscle, but concluded that the expected specificity was not attainable, since these agents depressed conductance changes underlying inward and outward movements of sodium and potassium. Also, Golenhofen and Hermstein [7] employed D600 in a study of vascular smooth muscle activation and concluded that two calcium-activation mechanisms exist, one of which was inhibited by D600 at concentrations of up to 2×10^{-5} M, but that nonspecific depression of activation occurred at much higher D600 concentrations and was due to a local anesthetic-type effect. Further, at concentrations which are orders of magnitude higher than those required for inhibition of calcium influx, verapamil has been shown to affect other systems; it has an ED_{50} of 3.7×10^{-4} M for local anesthesia in frog sciatic nerve [8], and at concentrations of up to 6×10^{-3} M verapamil affects excitation–contraction coupling in frog sartorius muscle [9]. Thus, depending on the concentrations used, verapamil and D600 are capable of producing several actions in addition to their Ca-antagonist effects. During an investigation of the influences of various agents on rat brain muscarinic and α-adrenergic receptors, we have found that D600 at concentrations of 10^{-6}–10^{-5} M interferes markedly with the specific binding of selected radioligands to

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the receptors. This report describes the interactions of D600 with these receptors and indicates that caution should be used when interpreting experiments in which D600 or verapamil is used analytically to establish the involvement of calcium in biological systems.

METHODS

Radioligand binding assays. α-Adrenergic receptor binding was determined in rat brain cortex preparations essentially as described by Greenberg *et al.* [10]. Cortex was homogenized in 20 vol. of ice-cold Tris–HCl buffer (pH 7.7 at 25°) with a Brinkmann Polytron PT 10 for 1 min at a setting of 5.2, then centrifuged at 37,000 for 10 min, resuspended in the Polytron, and again centrifuged. The pellet was resuspended in buffer at a concentration representing approximately 30 mg of original cortex per ml of buffer. The tritium-labeled α-antagonist WB 4101 with a specific activity of 25.4 Ci/mmole was purchased from the New England Nuclear Corp., Boston, MA. The standard binding assays were run in triplicate in disposable glass test tubes, and contained approximately 1.0 mg of cortex protein plus 0.25 nM [^{3}H]WB 4101 and 50 mM Tris–HCl at pH 7.7 in a total volume of 2 ml; parallel triplicate assays additionally contained 10^{-4} M *l*-norepinephrine. Incubations were carried out in a Dubnoff shaking incubator for 30 min at 25°, at which time the incubation mixtures were filtered rapidly through Whatman GFB glass fiber filters and rapidly washed with 15 ml of ice-cold buffer, using a Millipore filter manifold. The filters were placed in vials containing 13 ml of Beckman Ready-Solv EP, shaken mechanically for 30 min to disintegrate the filter, then counted in a liquid scintillation counter. Specific [3H]WB 4101 binding was defined as the difference in binding between incubations carried out in the presence and the absence of 10^{-4} M norepinephrine.

Muscarinic receptor binding was measured in rat brain striatal preparations essentially as described by Yamamura and Snyder [11]. In the standard assay approximately 100 μg of striatal homogenate protein were incubated for 45 min at 25° in a final volume containing 50 mM phosphate, pH 7.4, with the concentrations of [^{3}H]quinuclidinyl benzilate (QNB) of specific activity 16.4 Ci/mmole (Amersham Corp., Arlington Heights, IL) and D600 or local anesthetics indicated in Results; parallel experiments also contained 10^{-6} M atropine. Incubation mixtures were then filtered through GFB glass fiber filters which were rapidly washed with 10 ml of ice-cold buffer, transferred to counting vials, disintegrated and counted. Specific [3H]QNB binding was defined as the difference in binding between incubations carried out in the presence and the absence of 10^{-6} M atropine. In experiments investigating inhibitory activities of charged and uncharged forms of D600 and lidocaine on the muscarinic receptor, the pH of the incubation medium was controlled over the range pH 6.3 to 8.2 by substituting 100 mM Tris–maleate or *N*-Tris(hydropymethyl)methyl-2-aminoethane sulfonic acid (TES) buffers for the standard phosphate, as described in the legend of Table 2; the filters were washed at the same pH at which the incubation was conducted. In experiments exploring the interactions of D600 and lidocaine with the agonist binding site of the muscarinic receptor, [^{3}H]QNB binding was measured at a radioligand concentration of 0.8 nM in the presence of various concentrations of carbachol to establish displacement of the [^{3}H]QNB binding isotherm by this agonist. The percentage displacement of [^{3}H]QNB binding by 100 μM carbachol was then determined in the presence of various concentrations of D600 or lidocaine, as calculated for the two interaction models described below and in the Appendix. The dissociation constants of D600 and the local anesthetics for the receptors were calculated from the relationship,

$$K_I = \frac{IC_{50}}{\left(1 + \frac{\text{radioligand concentration}}{K_{\text{radioligand}}}\right)},$$

where IC_{50} is the concentration of D600 or local anesthetic producing 50 per cent inhibition of specific radioligand binding and $K_{\text{radioligand}}$ is the dissociation constant determined in separate experiments described in Results. Protein was determined by the method of Lowry *et al.* [12], using a bovine albumin standard. D600, α-isopropyl α(*N*-methyl-*N*-homoveratryl)-α-aminopropyl-3,4,5-trimethoxyphenylacetonitrile, was donated by the Knoll Pharmaceutical Co., Whippany, NJ. Tetracaine and lidocaine hydrochlorides were purchased from the Sigma Chemical Co., St. Louis, MO.

RESULTS

Effects on the brain α-receptor. When the specific binding of [^{3}H] WB 4101 to rat brain cortex preparations was assayed at various initial concentrations of [^{3}H] WB 4101, it was found that non-specific binding increased linearly with the [^{3}H] WB 4101 concentration, was non-saturable, and that specific binding reached a maximum at approximately 2 nM [^{3}H]WB 4101. The influence of D600 on specific binding of [^{3}H]WB 4101 was then studied, using [^{3}H]WB 4101 at a standard concentration of 0.25 nM. Since some effects of D600 in other systems have been ascribed to local anesthetic actions [6, 7], the effects of the local anesthetics lidocaine and tetracaine on α-receptor binding were also studied and compared with those of D600. Also, Fleisch and Titus [13] had shown that these local anesthetics altered contractile responses of isolated rat aortic and tracheal smooth muscles to norepinephrine and carbachol, and they had postulated that the anesthetics altered the affinity with which such agonists interact with their receptors. Thus, it was of interest here to establish directly any local anesthetic–receptor interactions by using the radioligand binding assays. Figure 1 shows the dose–response curves for the inhibition of specific binding of [^{3}H]WB 4101 to the α-receptor by D600, lidocaine and tetracaine with an IC_{50} of 1.7×10^{-6} M for D600 and for lidocaine and tetracaine of 9.1×10^{-4} M and 8.4×10^{-5} M respectively.

An analysis of the mechanisms of binding inhibition was made by assaying specific [^{3}H]WB 4101 binding at radioligand concentrations of 0.125, 0.25, 0.5 and

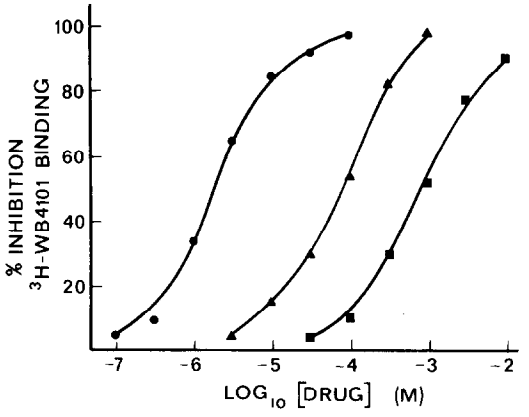

Fig. 1. Inhibitory effects of D600, lidocaine and tetracaine on specific binding of [3H]WB 4101 by the rat brain α-adrenergic receptor. Specific binding was determined as described in Methods at a radioligand concentration of 0.25 nM. Key: D600 (●); lidocaine (■); and tetracaine (▲). The curves represent the means of data from five separate experiments.

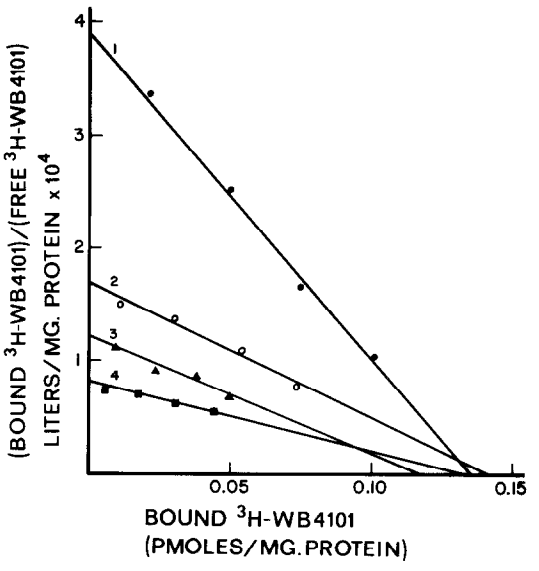

Fig. 2. Scatchard analyses of the inhibitions of specific binding of [^{3}H]WB 4101 by the α-adrenergic receptor produced by D600, lidocaine and tetracaine. Specific binding was determined at [^{3}H]WB 4101 concentrations of 0.125, 0.25, 0.5 and 1.0 nM in the absence of other drugs, curve 1, and in the presence of 1×10^{-6} M D600, curve 2; 1×10^{-3} M lidocaine, curve 3; and 1×10^{-4} M tetracaine, curve 4. All experiments were repeated 3–5 times, and the plots were drawn from the linear regression analyses of these data. Statistical analysis shows that the intercepts on the × axis are not significantly different.

1.0 nM in the presence and the absence of approximately IC_{50} concentrations of D600, lidocaine and tetracaine. The Scatchard analyses [14] of these data are shown in Fig. 2, in which the slopes represent $-1/K_d$, where the K_d is the apparent dissociation constant of the radioligand–receptor interaction and the intercepts on the abscissa represent the number of binding sites per unit protein. [^{3}H]WB 4101 binds, in the absence of inhibitors, to a single population of binding sites (Fig. 2, curve 1) with a K_d of 3.64×10^{-10} M, and there are 0.135 pmoles of [^{3}H]WB 4101 bound per mg of protein in the brain cortex preparations. It can also be seen that D600, lidocaine and tetracaine all increase the apparent K_d for [^{3}H]WB 4101 (i.e. decrease the affinity), but do not significantly alter the number of radioligand binding sites. Thus, all three drugs appear to act as reversible competitive inhibitors of [^{3}H]WB 4101 binding. Calculations using the equations described in Methods give a K_I value for D600 of 0.98×10^{-6} M and of 5.25×10^{-4} M and 4.85×10^{-5} M for lidocaine and tetracaine respectively (Table 1).

Effects on the brain muscarinic receptor. In order to differentiate further the actions of D600 and the local anesthetics, the effects of these three drugs on the muscarinic receptor were studied by measuring the specific binding of the muscarinic antagonist [^{3}H]-QNB to rat brain striatal homogenates. Figure 3 shows the inhibition of binding produced by various concentrations of these agents, assayed at a standard [^{3}H]QNB concentration of 0.2 nM. The IC_{50} concentrations obtained from these plots were: D600, 1.4×10^{-5} M; lidocaine, 1.3×10^{-4} M; and tetracaine, 1.1×10^{-5} M. Analyses of the mechanisms of inhibi-

Table 1. Comparison of effects of D600, lidocaine and tetracaine on rat brain α-adrenergic and muscarinic receptors

Drug	Receptor				
	α-Adrenergic		Muscarinic		
	IC_{50} (M)	K_I (M)	IC_{50} (M)	K_I (M)	$\frac{K_I\ \alpha\text{-adrenergic}}{K_I\ \text{muscarinic}}$
D600	1.7×10^{-6}	0.98×10^{-6}	1.4×10^{-5}	8.83×10^{-6}	0.11
Lidocaine	9.1×10^{-4}	5.25×10^{-4}	1.3×10^{-4}	8.2×10^{-5}	6.4
Tetracaine	8.4×10^{-5}	4.85×10^{-5}	1.1×10^{-5}	6.94×10^{-6}	6.98
$\frac{K_I\ \text{lidocaine}}{K_I\ \text{tetracaine}}$	10.82		11.81		

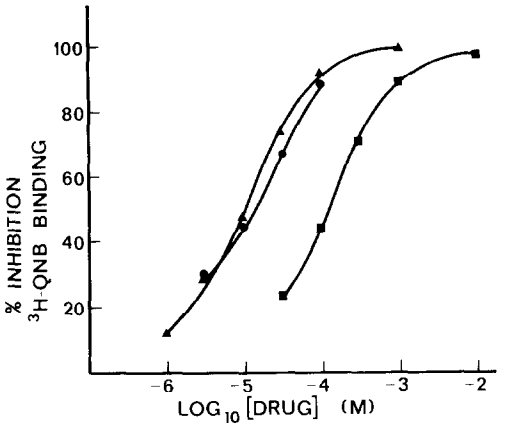

Fig. 3. Inhibitory effects of D600, lidocaine and tetracaine on specific binding of [^{3}H]QNB by the rat brain muscarinic receptor. Specific binding was determined as described in Methods at a radioligand concentration of 0.2 nM. Key: D600 (●); lidocaine (■); and tetracaine (▲). The curves represent the means of data from five separate experiments.

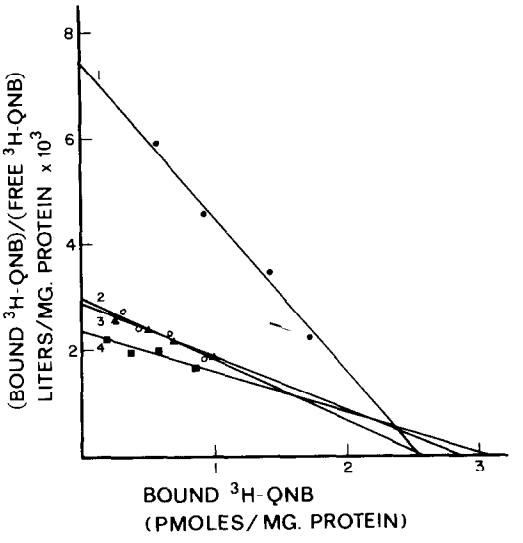

Fig 4. Scatchard analyses of the inhibitions of specific binding of [^{3}H]QNB by the muscarinic receptor produced by D600, lidocaine and tetracaine. Specific binding was determined at [3H]QNB concentrations of 0.1, 0.2, 0.4 and 0.8 nM in the absence of other drugs, curve 1, and in the presence of 1.4×10^{-5} M D600, curve 4; 1.3×10^{-4} M lidocaine, curve 2; 1.1×10^{-5} M tetracaine, curve 3. These experiments were repeated 3–5 times and the plots drawn from linear regression analyses of these data. Statistical analysis of these data indicates that the intercepts on the x axis are not significantly different.

tion were made by assaying binding at [^{3}H]QNB concentrations of 0.1, 0.2, 0.4 and 0.8 nM in the presence and the absence of approximately IC_{50} concentrations of D600, lidocaine and tetracaine. Scatchard plots of these data are shown in Fig. 4, where it can be seen that [^{3}H]QNB binding occurs at a single population of binding sites with a K_d of 3.41×10^{-10} M and that each of the drugs decreased the slope and, therefore, decreased the apparent affinity of the receptor for [^{3}H]QNB, with no significant change in the number of [^{3}H]QNB binding sites. Thus, D600, lidocaine and tetracaine all appear to act as reversible competitive inhibitors of the [^{3}H]QNB binding function of the muscarinic receptor. K_I values for these drugs were calculated using the IC_{50} data from Fig. 3 and the K_D for [^{3}H]QNB obtained in the control experiments of Fig. 4, as described above. The K_I for D600 was 8.83×10^{-6} M; for lidocaine, 8.2×10^{-5} M; and for tetracaine, 6.94×10^{-6} M. Comparison of these values with corresponding data from experiments with the α-receptor (Table 1) shows that the muscarinic receptor is about 9 times less sensitive than the α-receptor to D600, but is more sensitive than the α-receptor to both of the local anesthetics.

Aronstam *et al.* [15] have shown that the specific binding of [^{3}H]QNB to the rat brain muscarinic receptor has an unusually broad pH optimum, so that it is possible to study the effect of varying the pH

Table 2. Effect of pH on inhibition of radioligand binding to muscarinic receptor produced by D600 and lidocaine*

		D600 (2×10^{-5} M total)			Lidocaine (1.3×10^{-4} M)		
pH	Control binding (pmoles/mg)	Concentration, uncharged ($\times 10^{-5}$ M)	Inhibition observed (%)	Inhibition expected (%)	Concentration, charged ($\times 10^{-4}$ M)	Inhibition observed (%)	Inhibition expected (%)
6.3	0.370	0.67	39	40			
6.8	0.804	1.22	59	51	1.20	54	54
7.4	0.785	1.72	61	58	0.98	50	50
8.2	0.929	1.95	65	60	0.43	40	33

*Specific binding of [3H]QNB to rat striatal homogenates was measured as described in Methods at a radioligand concentration of 0.2 nM, but with the following buffers substituted for the standard phosphate. These buffers were all present at a final concentration of 100 mM; at pH 6.3 Tris-maleate was used, and at pH 6.8, 7.4 and 8.2, TES–HCl. The observed inhibitions were determined at constant total D600 and lidocaine concentrations at the different pH levels; the mean values from four separate experiments are shown. The expected inhibitions were obtained by calculation of the concentration of uncharged or charged forms of the drugs present at a given pH, taking the pK_a of D600 to be 6.6 and of lidocaine 7.9, as described in the text.

on the inhibition of binding to this receptor produced by lidocaine and D600 to determine whether the charged or uncharged forms of these drugs are the active species. Table 2 shows the results of these experiments. The control specific binding activity was more sensitive to a decrease in pH than Aronstam *et al.* [15] had found, with a marked decrease observed at pH 6.3. However, as the pH was increased, there was an increasing inhibition produced by a constant total D600 concentration of 2×10^{-5} M (from 39 per cent inhibition at pH 6.3 to 65 per cent inhibition at pH 8.2). If we assume that the pK_a of D600 is altered insignificantly by the additional methoxy group and is thus, 6.6, as for verapamil [9], then the concentration of uncharged D600 would be 0.67×10^{-5} M at pH 6.3 and 1.95×10^{-5} M at pH 8.2. With the assumption that only the uncharged form of D600 exerts inhibitory effects, the inhibitions to be expected from these concentrations were calculated from the dose–response curve in Fig. 3 and are shown in Table 2. The close similarities between the observed and the expected inhibitions seen in Table 2 strongly suggest that the uncharged form of D600 is the active species inhibiting [^{3}H]QNB binding to this receptor. Similar experiments were performed with lidocaine ($pK_a = 7.9$) at a constant total lidocaine concentration of 1.3×10^{-4} M. In contrast, decreasing inhibition was found as the pH was increased. Table 2 shows the comparison of the observed and expected inhibitions, as calculated for the charged species from the lidocaine dose–response curve of Fig. 2; the reasonably close correlation suggests that with lidocaine the inhibition is produced by the charged form of the local anesthetic.

Since the cholinergic antagonist QNB is known to interact with accessory sites adjacent to, and in addition to, the Ach binding region of the muscarinic receptor [15, 16], it is conceivable that D600 and the local anesthetics could displace [^{3}H]QNB binding by interacting with an accessory receptor region, while having no effect on the binding of agonists to the receptor. The interactions of D600 and lidocaine with the agonist site on the muscarinic receptor were, therefore, studied by measuring the effects of these agents on the displacement of [^{3}H]QNB by the muscarinic agonist carbachol. These experiments were designed to differentiate between two theoretical models, designated cases 1 and 2, the equations for which are presented in the Appendix. In case 1 it was assumed that [^{3}H]QNB, carbachol, and D600 or lidocaine all bind to the same site according to the first order mass action law described by equations 1–3. In case 2 it was assumed that carbachol and D600 or lidocaine bind to different sites, although both sites have regions in common with the antagonist binding site and hence both carbachol and the two drugs displace antagonist ([^{3}H]QNB) binding; equations describing these interactions are also shown in the Appendix.

Figure 5 shows the percentage inhibition of 0.8 nM [^{3}H]QNB binding produced by 100 μM carbachol in the presence of various concentrations of D600 and lidocaine. Maximum (100 per cent) binding was defined as the specific binding that occurred in the presence of both 0.8 nM [^{3}H]QNB and a given concentration of D600 or lidocaine, these being 1, 3, 10 and 20 times their respective K_I concentrations as determined above. It can be seen that the

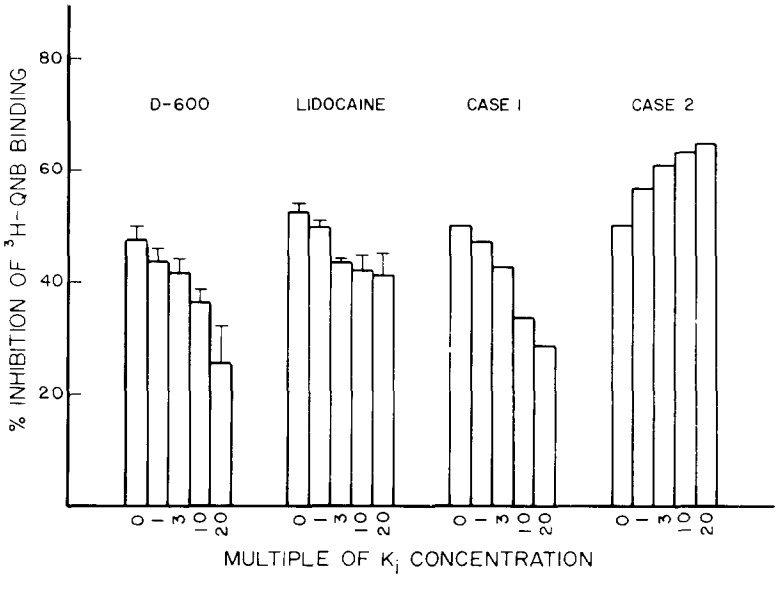

Fig. 5. Influence of D600 and lidocaine on the inhibition produced by carbachol of the specific binding of [^{3}H]QNB to the rat brain muscarinic α-receptor. The radioligand concentration was 0.8 nM. All experiments contained striatal homogenate, 0.8 nM [3H]QNB and 100 μM carbachol, together with D600 or lidocaine at concentrations 1, 3, 10, and 20 times their respective K_I concentrations. In these experiments, maximal binding was defined as specific binding occurring in the presence of [3H]QNB and the indicated concentration of D600 or lidocaine. Also shown are the theoretical inhibitions of specific binding predicted from the case 1 and 2 interaction models described in the text and the Appendix.

inhibition produced by carbachol in the presence of D600 was very similar to the theoretical displacement expected from an interaction described in case 1. These data, therefore, are consistent with the hypothesis that [^{3}H]QNB, carbachol and D600 all competitively displace one another at the same, agonist site. Lidocaine also decreased the inhibition of [^{3}H]QNB binding produced by carbachol, although the inhibition of carbachol binding produced by the high concentrations of lidocaine was not as great as predicted by the case 1 model.

DISCUSSION

A significant finding of the present study is that D600 at concentrations often used to produce "calcium antagonism", additionally affects brain muscarinic and α-receptors. From the data summarized in Table 1, it is clear that D600 has a significantly greater effect on the α-receptor than the muscarinic receptor ($K_I = 0.98 \times 10^{-6}$ vs 8.8×10^{-6} M) and, from Figs. 2 and 4, that the inhibition appears to be competitive in both cases. The nature of the inhibition suggests that D600 exerts its effects here either by interacting at the binding sites of the two receptors, or at other nearby sites which influence the radioligand binding sites in an allosteric manner. Since the binding sites of the two receptors must be quite different, in that they accept structurally dissimilar α-adrenergic or muscarinic agents, it would seem less likely that D600 interacts with the actual binding sites than by affecting these sites via adjacent allosteric loci. In considering the mechanisms of attachment of QNB and acetylcholine to the muscarinic receptor, both these molecules contain esteratic and cationic sites, but QNB additionally possesses aromatic groups capable of hydrophobic interaction with lipids and the hydrophobic areas of membranes [16]. Since the active species of D600 inhibiting this receptor is the uncharged form, as was found by Bondi [9], for the release of Ca in skeletal muscle, it is possible that D600 may be acting here by competing with QNB for hydrophobic areas of the receptor. The data of Fig. 5 show that D600 competes with carbachol and, therefore, acts also to influence the agonist binding site of the muscarinic receptor; thus, the binding of D600 must affect the binding of both agonists and antagonists to the receptor. Verapamil has high lipid affinity [17] and D600, with an additional methoxy group, would be similarly lipophilic and thereby expected to distribute well into membrane structures. Thus, the speculation could be made that the spectrum of effects produced by the Ca-antagonists, ranging from inhibition of Ca influx at low concentrations to the receptor effects described here and to local anesthetic effects at much higher concentrations, may result from a continuum of perturbations of membrane structure associated with increasing drug concentration. Although no studies have been reported on membrane perturbation by Ca-antagonists, we have shown recently that receptor binding functions are inhibited by those concentrations of alkanols and halothane which also increase membrane fluidity.*

*A. S. Fairhurst and P. Liston, manuscript submitted for publication.

While the present study does not identify the mechanisms underlying receptor inhibition, it does establish that D600, at concentrations employed by some workers to implicate Ca in various processes, additionally inhibits receptor binding activity, so that receptor-D600 interactions must be considered when interpreting the results of such experiments. From these findings it may be anticipated also that other types of receptors will be found to be sensitive to D600, and we have preliminary data indicating that the rat brain opiate receptor is also inhibited, with an IC_{50} of approximately 4×10^{-6} M for D600. However, it would seem that inhibition of the muscarinic receptor plays little part in the inhibition of Ca influx in smooth muscle produced by D600, since this latter inhibition occurs at D600 concentrations $\leqslant 10^{-7}$ M and also in tissues stimulated by K^+ wherein the muscarinic receptor is bypassed [18, 19].

Although some effects of high concentrations of D600 have been ascribed to local anesthetic effects [6, 7], and indeed verapamil with an ED_{50} of 3.7×10^{-4} M is 1.6 times more potent than procaine in blocking conduction in frog sciatic nerve preparations [8], the receptor inhibition data presented here show distinct differences between D600 and lidocaine or tetracaine. First, the inhibitory form of lidocaine acting on the muscarinic receptor appears to be the charged species, whereas for D600 it is the uncharged species, indicating that the interaction of this local anesthetic with some membrane component influencing radioligand binding is different from that of D600. A cationic group is a well known requirement of cholinergic ligands so that the observation that the protonated form of lidocaine is the active species also suggests that lidocaine produces a competitive inhibition of [^{3}H]QNB binding. However, this interaction may be more complex since deviations from competitive inhibition were observed at high concentrations of lidocaine (Fig. 5). Local anesthetics produce a wide variety of effects on biological membranes, although the molecular events underlying these effects are largely unknown. These drugs have been shown to compete with Ca in the lobster neuron [20], so that interaction with a Ca-binding site on the muscarinic and α-receptors was considered as a possible mechanism of action here. However, the IC_{50} for the local anesthetics for both receptors was not changed by the addition of 10 mM Ca to the incubation media; the IC_{50} for D600 for the receptors was similarly unchanged by 10 mM Ca.

A second difference found here between the actions of local anesthetics and D600 on the receptors is that, in contrast to the relative sensitivities of the receptors to D600, the muscarinic receptor is more sensitive to these local anesthetics than is the α-receptor. The ratio of K_I lidocaine to K_I tetracaine is very similar for both receptors (Table 1), suggesting that these anesthetics bind to a common site associated in some manner with each receptor, but that the influence of such an anesthetic binding site on the radioligand site is different for the muscarinic and α-receptors. Since the ED_{50} for lidocaine in blocking conduction in the frog sciatic nerve is 3.8×10^{-3} M and for tetracaine acting on the giant squid axon is 1×10^{-4} M [21], it is clear from Table 1 that inhibition of receptor binding occurs at similar or lower con-

centrations than produce classical local anesthetic effects. Papahadjopoulos *et al.* [22] have suggested that the effects of local anesthetics on membranes may be mediated via membrane fluidity changes associated with phospholipid interactions, so that it is conceivable that local anesthetic effects and the inhibition of receptor binding functions produced by D600, lidocaine and tetracaine may have, as a common basis, the perturbation of membrane structure. However, the molecular events underlying any such perturbations must be different because the active species of the D600 and local anesthetic molecules affecting the receptors are different.

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APPENDIX

Equations are derived below which describe the binding of a radiolabeled antagonist in the presence of a non-labeled agonist and another non-labeled ligand. These equations are based on two different theoretical models designated as case 1 and case 2, and establish the theoretical basis necessary for determining whether a ligand interacts with the agonist binding region of the muscarinic receptor.

Case 1. Radiolabeled antagonist and the other two non-labeled ligands all bind to the same site according to the law of mass action as described by equations 1–3.

$$(A) + (R) \rightleftharpoons (AR) \qquad \frac{(A)(R)}{(AR)} = K_A \tag{1}$$

$$(B) + (R) \rightleftharpoons (BR) \qquad \frac{(B)(R)}{(BR)} = K_B \tag{2}$$

$$(C) + (R) \rightleftharpoons (CR) \qquad \frac{(C)(R)}{(CR)} = K_C \tag{3}$$

$$(R_T) = (R) + (RA) + (RB) + (RC), \tag{4}$$

where (R) is the concentration of free receptors, (R_T) is the total receptor concentration, (A) is the concentration of radiolabeled antagonist, (B) is the concentration of non-labeled agonist, and (C) is the concentration of a non-labeled ligand. (RA), (RB) and (RC) are the concentrations of receptor complexes and K_A, K_B and K_C are dissociation constants. The solution to this system of equations is given below and is a special case of the general solution given by Feldman [23].

$$f_{BC} = \frac{(AR)}{(R_T)} = \frac{(A)}{(A) + K_A(1 + (B)/K_B + (C)/K_C)}, \tag{5}$$

where f_{BC} represents the fractional occupancy of A in the presence of B and C. When B is equal to its IC_{50} concentration ($B = B_{50}$), then:

$$f_{BC} = \tfrac{1}{2} f_C, \tag{6}$$

where f_C is the fractional occupancy of A in the presence of C. It follows from equation 6 that:

$$\frac{(A)}{(A) + K_A(1 + (B_{50})/K_B + (C)/K_C)} = \tfrac{1}{2}\frac{(A)}{(A) + K_A(1 + (C)/K_C)}. \tag{7}$$

By rearrangement:

$$B_{50} = K_B(1 + (A)/K_A + (C)/K_C). \tag{8}$$

Equation 8 illustrates that, if all three ligands bind to the same site, the IC_{50} of B for half maximal displacement of A will increase with increasing concentrations of another ligand C. Thus, if the binding of A is expressed as a percentage of the maximum amount of binding that occurs in the presence of A and C only, then the percentage inhibition of binding produced by the agonist B will decrease with increasing concentrations of C.

Case 2. Non-labeled agonist and another non-labeled ligand bind to different sites; however, both sites have regions in common with the antagonist binding site and hence both ligands displace antagonist binding. All ligands bind according to the law of mass action as described by equations 1–3. In addition, the following equations apply:

$$(B) + (CR) \rightleftharpoons (BCR) \qquad \frac{(B)(CR)}{(BCR)} = K_B \tag{9}$$

$$(C) + (BR) \rightleftharpoons (BCR) \qquad \frac{(C)(BR)}{(BCR)} = K_C \tag{10}$$

$$R_T = (R) + (AR) + (BR) + (CR) + (BCR), \tag{11}$$

where (BCR) represents the tertiary receptor complex of B and C. Since B and C bind at distinct non-interacting sites on the same receptor, equations 9 and 10 imply:

$$\frac{(B)(R)}{(BR)} = \frac{(B)(CR)}{(BCR)} = K_B \tag{12}$$

$$\frac{(C)(R)}{(CR)} = \frac{(C)(BR)}{(BCR)} = K_C. \tag{13}$$

The analytical solution to equations 1–3 and 9–13 is:

$$f_{BC} = \frac{(AR)}{(R_T)} = \frac{(A)}{(A) + K_A(1 + (B)/K_B + (C)/K_C + (B)(C)/K_BK_C)} \quad (14)$$

At the IC_{50} concentration of $B(B = B_{50})$:

$$f_{BC} = \tfrac{1}{2}f_C \quad (15)$$

$$\frac{(A)}{(A) + K_A(1 + (B_{50})/K_B + (C)/K_C + (B_{50})(C)/K_BK_C)} = \tfrac{1}{2}\frac{(A)}{(A) + K_A(1 + (C)/K_C)} . \quad (16)$$

By rearrangement:

$$B_{50} = K_B\frac{(1 + (A)/K_A + (C)/K_C)}{1 + (C)/K_C} . \quad (17)$$

Two extreme conditions of equation 17 are worthwhile to consider. In the absence of C, equation 17 reduces to:

$$B_{50} = K_B(1 + (A)/K_A).$$

However, as the concentration of C becomes large such that $(C)/K_C \gg (A)/K_A$, then equation 17 reduces to:

$$B_{50} = K_B .$$

According to the conditions of case 2, as the concentration of C increases, the displacement binding isotherm of B shifts to the left and asymptotically approaches the true binding isotherm of B. Consequently, the per cent inhibition of radiolabeled ligand binding produced by B will increase with increasing concentrations of C. These results are exactly opposite to the consequences of case 1.