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Structural and Stereochemical Requirements for Muscarinic Receptor Binding

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SUMMARY

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The ability of stereoisomers of muscarinic agonists and antagonists to inhibit specific [^{3}H](DL)3-quinuclidinyl benzilate binding to receptors in rat brain and guinea pig ileum longitudinal muscle is compared to their ability to stimulate or inhibit contraction of the ileum longitudinal muscle. The close correlations observed with receptor antagonists and weak agonists emphasize the equivalence of muscarinic receptors identified by biochemical and physiological techniques. The correlations with the most biologically potent agonists were poor, suggesting high intrinsic activity and large spare receptor populations for these compounds. Muscarinic receptors display stereoselectivity with the enantiomers of certain strong agonists in their production of ileum muscle contractions but not in their ability to inhibit [^{3}H](DL)3-quinuclidinyl benzilate binding. Although minor differences are observed between binding stereoselectivity in the muscle and brain tissues, the results suggest a similar structural geometry in the two receptors.

INTRODUCTION

Determination of the equivalence of biochemically identified muscarinic binding sites with physiologically responsive sites is difficult in the central nervous system. Suggestions of equivalence have come from studies correlating central binding properties and peripheral pharmacologic responses (1-4). Direct correlations of central binding potency with central functions such as postsynaptic electrophysiological responses are difficult; however, correlations with central activity as evidenced by behavioral disturbances have been found (3).

The stereoselectivity of muscarinic interactions is of interest because of the insight such information can yield into the geometry of binding sites and the relationship between agonist and antagonist sites (5). Stereospecific ligand binding has aided the biochemical identification of neurotransmitter receptors and can serve as a probe for the structural integrity of receptor binding sites (6). In this study the enantiomers of a number of muscarinic agonists and antagonists are compared in their ability to block the binding of [^{3}H](DL)3-quinuclidinyl benzilate (QNB)[1] to the muscarinic re-

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[1] The abbreviations used are: QNB, [^{3}H](DL)3-quinuclidinyl benzilate; BSA, bovine serum albumin; DFP, diisopropyl fluorophosphate.

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ceptors in rat brain and guinea pig ileum and their ability to stimulate or inhibit ileum longitudinal muscle contraction.

MATERIALS AND METHODS

Microsomal membranes were prepared from the telencephalon of adult Wistar rats or from the longitudinal muscle of guinea pig ileum by homogenization in 10 volumes of 50 mM sodium phosphate, pH 7.4. The supernatant after a 1,000 × *g* centrifugation for 5 min was spun at 40,000 × *g* for 20 min. The neural membrane pellet was suspended in buffer and used without further treatment. The muscle membrane pellet was suspended in a minimal volume of deionized, glass-distilled water, lyophilized and stored at −20° until use. Protein concentrations were routinely determined from UV absorbance measurements in 2% SDS (7) and compared to determinations on identically prepared membrane fractions by the method of Lowry *et al.* (8) using BSA as a standard.

Muscarinic receptor binding was measured by the specific binding of [^{3}H](DL)3-quinuclidinyl benzilate (New England Nuclear, 29.4 Ci/mmole), by a modification of the method of Yamamura and Snyder (9), as previously described (10). Neural (25–50 μg protein) or muscle (75–150 μg protein) membranes were added to incubation media containing various concentrations of QNB in the presence and absence of a single concentration of the drug being evaluated, in a final volume of 5 ml. The concentration of binding sites was kept below 10^{-11} M, and the concentration of free QNB was adjusted by the amount that was bound. Incubation time was 90 min at 20°. Bound QNB was separated from free ligand by filtration with suction on Whatman GFB glass fiber filters. After washing once with 7 ml buffer, the radioactivity content of the filters was determined by scintillation counting. Unlabeled scopolamine (10^{-6} M) was included in a duplicate set of incubation media to determine nonspecific binding. Nonspecific binding rose from 5 to 65% of the total binding as the concentration of QNB was increased from 50 to 4,000 pM. Nonspecific binding was generally 10–20% higher in ileum muscle compared to brain membranes. For determinations of the receptor affinities of acetyl-β-methylcholines, membrane preparations (5 mg protein/ml) were pretreated with 10^{-4} M diisopropyl fluorophosphate (DFP) for 10 min at room temperature. The concentration of DFP was reduced to 2 μM by dilution before binding was assayed. This treatment did not affect receptor concentration or QNB affinity in control preparations.

Receptor concentrations and dissociation and inhibition constants were determined from parameters of double reciprocal plots of the binding data as revealed by linear regression analyses.

The (+) and (−) enantiomers of hyoscyamine were resolved by the method of Werner and Miltenberger (11). The optical isomers of acetyl-β-methylcholine were prepared by the method of Major and Bonnett (12).

RESULTS AND DISCUSSION

Double reciprocal plots of QNB binding to neural and ileal membranes in the absence and presence of a number of muscarinic ligands are depicted in Fig. 1. The inhibition of QNB binding to receptors in both tissues was competitive with all drugs tested, maximal binding not varying significantly from control levels. The dissociation constant (K_d) for QNB binding to brain receptors was $1.28 \pm 0.21 \times 10^{-10}$ M, and the receptor density was 1.7 ± 0.2 pmoles/mg membrane protein (n = 10). With longitudinal muscle receptors the K_d was 1.97 ± 0.12 × 10^{-10} M, and maximal binding was 0.68 ± 0.10 pmoles/mg membrane protein (n = 8).

The pK_i values (− log inhibition constant in M) of QNB binding for the various drugs are presented in Table 1 along with previously determined (13, 14) pA_2 and pD_2 values[2] for their pharmacological actions in guinea pig ileum. The correlation between the pK_i values and the pA_2 values for antagonism of acetylcholine-induced contrac-

[2] pA_2 is the negative logarithm of the antagonist concentration in M that requires a doubling of the dose of the agonist to compensate for the action of the antagonist. pD_2 is the negative logarithm of the agonist concentration in M at which the drug gives 50% effect.

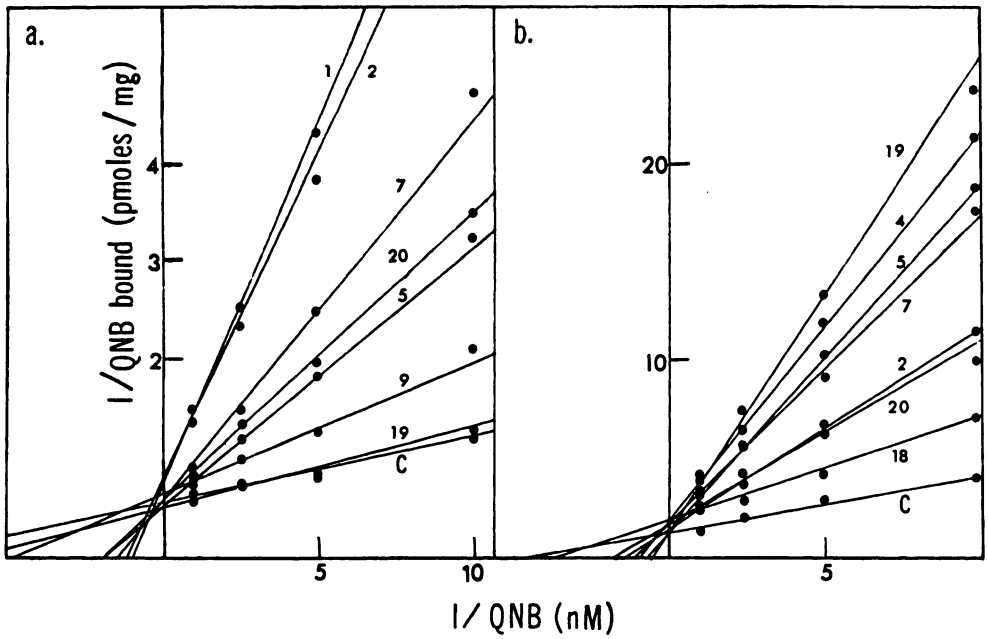


FIG. 1. *Double reciprocal plots of QNB binding to (a) neural and (b) longitudinal muscle receptors in the absence (c) and presence of various cholinergic ligands (identified in Table 1)*

Data points representing binding at high concentrations of QNB (2 and 4 nM) are omitted for clarity. The lines are drawn from least squares analyses of all the data. Each point is the average of triplicate determinations of binding performed in a single experiment. The drugs and concentration used were: *1* and *2*, 10^{-7} M; *4* and *5*, 10^{-8} M; *7*, 10^{-6} M; *9*, 10^{-5} M; *18* and *20*, 10^{-9} M; *19*, 10^{-9} M with brain receptors and 10^{-7} M with ileum receptors.

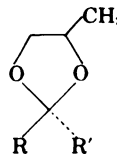
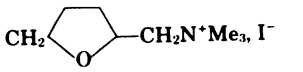
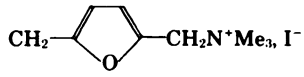
tion of guinea pig ileum longitudinal muscles is presented in Fig. 2a; the corresponding information for the agonists is shown in Fig. 2b. The pK_i values of receptor antagonists with brain receptors are in close agreement with those of the guinea pig ileum. There is a consistent discrepancy between inhibition constants for antagonism of QNB binding and muscle contraction, muscarinic affinity being 3.5 ± 1.3-fold greater in the binding studies. It is possible that these systematic differences are related to the use of different temperatures in the binding (20°) and physiological (37°) experiments or are experimental artifacts inherent in the design of the binding or pharmacological studies.

With the exception of S(L) acetyl-β-methylcholine (*17*), there is also fairly close agreement between the pK_i values for agonists of inhibition of QNB binding to neural and muscle receptors. There are, however, several major differences between the muscarinic activity of agonists measured by binding versus muscle contraction studies. With weak agonists, i.e., those agonists which produce half maximal muscle contraction only at concentrations above 1 μM, there is a good correlation between binding and pharmacological potencies. On the other hand, strong agonists ($pD_2 > 6.8$) inhibit very little QNB binding when present at their pD_2 concentrations. The differences in patterns of muscarinic activity between the weak and strong agonists is illustrated in Fig. 2b; the tenuous relationship between affinity for the QNB binding site and efficiency in producing smooth muscle contractions with strong agonists is in contrast to the good relationship with weak agonists.

The present results indicate that when present in the concentrations at which they produce half maximal muscle contractions, the strong agonists occupy only between 0.5 and 5.3% of the QNB binding sites. The only exception is drug *17* which occupies 22.3% of the binding sites at its pD_2 concen-

TABLE 1

Antagonism of QNB binding to muscarinic receptors in rat brain and guinea pig ileum by various cholinergic ligands

#	Drug		Affinity constants			
			Brain receptors	Guinea pig longitudinal muscle		
			$pK_i \pm SD$[a]	$pK_i \pm SD$[a]	$pA_2 \pm SE$ (n)[b]	$pD_2 \pm SE$ (n)[b]
						
	R	R′				
1	C_6H_5	C_6H_5 (RS)	8.08 ± 0.15	7.95 ± 0.10	7.66 ± 0.04 (6)	
2	C_6H_5[c]	C_6H_5	7.94 ± 0.12	7.87 ± 0.09	7.55 ± 0.03 (6)	
3	C_6H_5[d]	C_6H_5	7.99 ± 0.09	8.08 ± 0.14	7.62 ± 0.03 (6)	
4	C_6H_{11}	C_6H_5 (2S, 4S)	8.68 ± 0.06	8.89 ± 0.22	8.03 ± 0.03 (7)	
5	C_6H_{11}	C_6H_5 (2R, 4R)	8.45 ± 0.01	8.24 ± 0.08	7.73 ± 0.02 (3)	
6	$CH_3(CH_2)_3$	$CH_3(CH_2)_3$	7.20 ± 0.01	7.19 ± 0.13	6.59 ± 0.03 (4)	
7	$CH_3(CH_2)_2$	$CH_3(CH_2)_2$	6.67 ± 0.03	6.62 ± 0.21	6.00 ± 0.04 (3)	
8	CH_3	CH_3	5.03 ± 0.03	5.03 ± 0.04		4.87 ± 0.03 (4)
9	C_2H_5	C_2H_5	4.98 ± 0.01	4.78 ± 0.02		4.89 ± 0.02 (4)
10	CH_3	H	5.28 ± 0.02	5.60 ± 0.02		7.61 ± 0.02 (19)
11	H	CH_3	5.26 ± 0.08	5.66 ± 0.05		7.14 ± 0.05[e]
12	CCl_3	H	5.42 ± 0.10	5.45 ± 0.03		5.80 ± 0.08[e]
13	H	CCl_3	5.28 ± 0.09	5.21 ± 0.14		5.30 ± 0.13[e]
14			5.21 ± 0.05	5.50 ± 0.13		6.84 ± 0.08[e]
15			6.35 ± 0.07	6.02 ± 0.09		7.64 ± 0.08[e]
16	R(D) acetyl-β-methylcholine		4.23 ± 0.08	4.25 ± 0.06		4.22 ± 0.06[e]
17	S(L) acetyl-β-methylcholine		5.99 ± 0.10	6.70 ± 0.13		7.24 ± 0.04[e]
18	RS(±) hyoscyamine		9.27 ± 0.27	9.42 ± 0.13	9.10 ± 0.06	
19	R(+) hyoscyamine		7.99 ± 0.10	8.05 ± 0.08	7.80 ± 0.06	
20	S(−) hyoscyamine		9.52 ± 0.01	9.56 ± 0.21	9.30 ± 0.05	

[a] The binding affinities were determined from binding curves (n = 2–5) performed separately on different tissue preparations in the presence of the same concentration of each drug. Each point of each curve was the average of three independent binding determinations. The values and variations listed are from linear regression analyses of double reciprocal binding plots of the grouped data. Inhibition constants from the separate curves generally varied less than 20%.

[b] From Chang *et al.* (13) and Chang and Triggle (14).

[c] Derived from L-glycerol-1-tosylate.

[d] Derived from D-glycerol-1-tosylate.

[e] From Triggle (unpublished results, n ≥ 3).

tration. The weak agonists (*8, 9, 12, 13* and *16*) occupy approximately 50% of the binding sites at their pD_2 concentrations. The weak agonists have low intrinsic activity; apparently their ability to stimulate muscle contraction is directly proportional to their occupancy of binding sites with maximal receptor occupancy necessary for maximal stimulation. These findings suggest that the strong agonists possess high intrinsic effi-

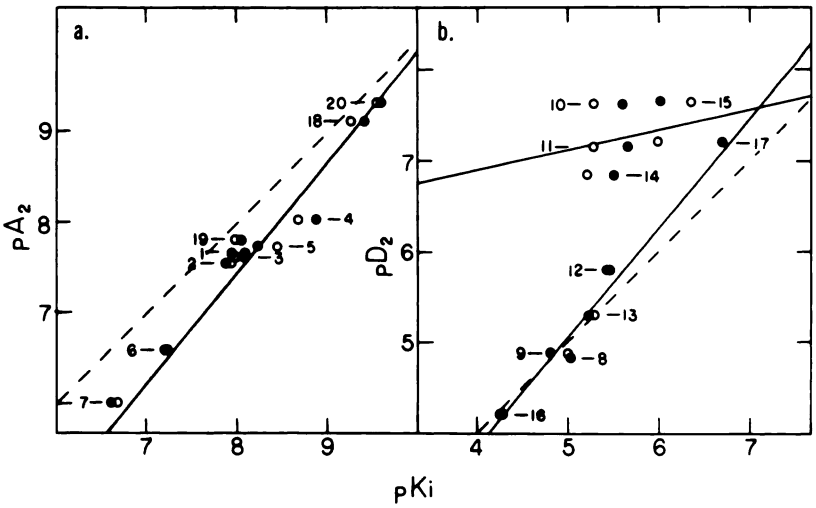


FIG. 2. *Correlation between pK_i values for inhibition of QNB binding to the muscarinic receptor of rat brain* (○) *and guinea pig ileum longitudinal muscle* (●) *by the muscarinic ligands identified in Table 1 and their physiological potency (solid lines)*

The dashed lines indicate the relationship of identity, $pK_i = pA_2$ or pD_2.

a) Correlation between pK_i values and pA_2 values for inhibition of ileum muscle contraction by muscarinic antagonists. Linear regression analyses indicate slopes of 1.1 ± 0.1 ($r = 0.98$) between brain binding and muscle contraction inhibition data, 1.1 ± 0.2 ($r = 0.98$) between muscle binding and contraction data and 1.1 ± 0.2 ($r = 0.98$) for the combined binding information and pA_2 values.

b) Correlation between pK_i values and pD_2 values for stimulation of longitudinal muscle contraction by muscarinic agonists. Linear regression analyses indicate close correlations between pD_2 values and pK_i values for inhibition of QNB binding to brain, ileum and combined brain and ileum receptors ($r = 0.95$, 0.96 and 0.95, respectively) with slopes of 1.2 ± 0.2 in each case. The corresponding correlation for the strong agonists (*10, 11, 14, 15* and *17*) is much less obvious ($r = 0.34$) and the slope of the line (0.21 ± 0.05) deviates significantly from that of the line depicting the realtionship $pK_i = pD_2$.

cacy and large spare receptor populations (15, 16). The relationship between pD_2 and the difference between pK_i and pD_2 values for the dioxolane stereoisomers and furan compounds is linear with both brain and ileum receptors (Fig. 3). This is possibly suggestive of a flexible coupling between the muscarinic binding site and the ion channel varying from essentially 1:1 to 1:100 for the most potent members of the series. Or it may be that these potent agonists open channels for longer durations. It is also possible that the strong agonists are involved in some unanticipated interactions with smooth muscle unrelated to receptor mechanisms during the initiation of biological responses, or that the binding sites labeled by QNB do not identify all the physiologically relevant sites of interaction for these ligands.

The preceding calculations of agonist receptor occupancy were made using the K_i as the measure of binding affinity and assuming that agonist binding follows the mass action formalism. This assumption appears to be incorrect; muscarinic receptors exist in at least two populations which differ with respect to their affinity for receptor agonists, but not antagonists (4). The inhibition of QNB binding by the various agonists was determined at agonist concentrations close to their K_i values. Since previous reports indicated that approximately one-third of forebrain receptors exist in the highest agonist affinity state, the agonists would be expected to occupy both high and low agonist affinity receptors at their K_i concentrations (4, 10, 17). Therefore the measures of agonist binding affinity provided in this report should not be considered a complete account of agonist binding properties.

Structure activity relationships. The 1,3-dioxolane nucleus is the basic structure of

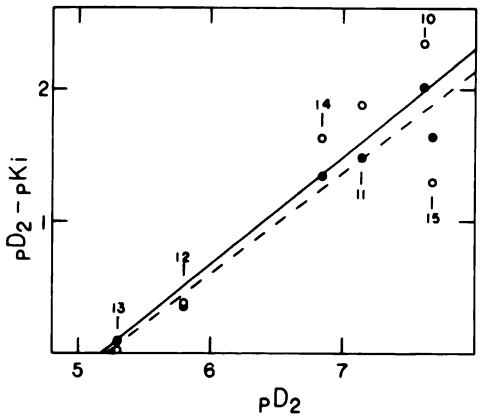


FIG. 3. *Relationship between the difference between biological and binding potencies ($pD_2 - pK_i$) and biological activity (pD_2) for a series of dioxolane and furan agonists with guinea pig ileum* (●) *and brain* (○) *muscarinic receptors*

With ileum receptors, the data fit the line $pD_2 - pK_i = 0.76\,pD_2 - 4.01$, with a correlation coefficient of 0.98. The corresponding relationship with brain receptors is $pD_2 - pK_i = 0.82\,pD_2 - 4.27$, with a correlation coefficient of 0.89. The lines represent the best fit to the data points as revealed by linear regression for the brain (solid line) and ileum (dashed line) receptors.

a large, well-characterized series of muscarinic agonists and antagonists which has proven especially useful in elucidating the stereochemical requirements for muscarinic action (2, 13, 14, 18–24). The relative lack of stereoselectivity in muscarinic antagonism of intestinal muscle contraction that is found with the stereoisomers of 2,2-diphenyl (*2, 3*) and the *trans* 2-phenyl-2-cyclohexyl (*4, 5*) derivatives of 1,3-dioxolane-4-dimethylaminoethyl methiodide is also evident in both CNS and longitudinal muscle receptor binding properties. The somewhat greater activity of compound *4* compared to *5* is in agreement with a previous study in which the absolute configuration at the acetal carbon (C_2) was found to be the most important factor governing activity of both *cis* and *trans* enantiomers of dioxolane antagonists (S > R) (24). The absolute configuration at C_4 (*2 versus 3*) has less influence on binding and physiological activities. The configuration at C_4, however, was reported by another group to have a greater influence on the inhibition of QNB binding by *cis*-2-methyl dioxolanes (*10*) and the mixed agonist-antagonist diisopropyl dioxolanes (2) than we observe with *2* and *3*.

The geometrical relationship between the C_2 and C_4 substituents (as in *10* and *11* and *12* and *13*) has little effect on binding affinity, in contrast to the 300% and 50% greater activities of the *cis* isomers (*10* and *12*, respectively) in eliciting muscle contraction. The lack of stereoselectivity for *10* and *11* in receptor binding compared to muscle stimulation suggests that these compounds produce their biological effects through interactions at a site(s) which differs in structure from that of the QNB-identified muscarinic binding site(s). The strict chemical requirements for high biological activity is apparent from a comparison of the trichloromethyl dioxolanes (*12, 13*) and the closely related methyldioxolanes (*10, 11*). While their binding potencies are identical, *12* and *13* possess only one-hundredth the agonist activity of *10* and *11*.

In contrast to the relative lack of stereoselectivity in receptor binding by *2* and *3* and *4* and *5*, the (−) isomer of atropine (*20*) has 35-fold higher binding activity at central and peripheral receptors than (+) hyoscyamine (*19*), in excellent agreement with its greater pharmacologic activity in guinea pig ileum. (Previous reports indicate a greater stereospecific index for the hyoscyamine isomers (25); our results may indicate an incomplete resolution of the isomers.) These results are consistent with previous findings in which stereoselectivity in muscarinic antagonists is less obvious when the center of asymmetry is located in the ester's imino alcohol moiety as opposed to the acidic portion (22–28). This is not unexpected, since the binding of muscarinic antagonists is dominated by the interactions of the large hydrophobic moieties located in the acidic portions of cholinergic esters (29, 30). The close correlation of antagonists, but not agonists, with certain physicochemical characteristics such as hydrophobicity (13), is probably a reflection of this situation.

Compared to the D(−) enantiomer (*16*), L(+) β-methyl acetylcholine (*17*) has 60- and 250-fold more activity in inhibiting

QNB binding in the brain and ileum, respectively. The reason for the difference in binding affinity with brain *versus* ileum receptors is unclear; however, the degree of stereoselectivity in brain receptor binding is less than that of ileum with the enantiomers of all compounds tested, except atropine.

The correlation between the physiologically- and biochemically-determined affinity constants for a number of muscarinic antagonists and weak agonists underscores the reliability of the QNB binding assay, as applied in its present form, for the identification of muscarinic acetylcholine receptors in subcellular preparations. The similar drug stereoselectivity of peripheral and central muscarinic acetylcholine receptors suggests similar structural geometries in the two receptors.

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