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Antagonist Binding Properties of Five Cloned Muscarinic Receptors Expressed in CHO-K1 Cells

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SUMMARY

A family of five cholinergic muscarinic receptor genes (m1, m2, m3, m4, and m5) has recently been identified and cloned. In order to investigate the pharmacological properties of the individual muscarinic receptors, we have transfected each of these genes into Chinese hamster ovary cells (CHO-K1) and have established stable cell lines expressing each receptor. In the present study we have examined the antagonist binding properties of each muscarinic receptor. Antagonists were chosen that had previously been proposed to be selective for muscarinic receptor subtypes and included pirenzepine, AF-DX 116, methoctramine, dicyclomine, hexohydrodifenidol, hexahydrosiladifenidol, hexocyclium, and silahexocyclium. m1, m2, and m3 receptors express binding properties similar to those expected of high affinity pirenzepine-type receptors of cerebral cortex ("M1"), low affinity pirenzepine-type receptors of atria ("M2 cardiac type"), and the intermediate affinity pirenzepine-type receptors found in exocrine glands ("M2 glandular type"), respectively. The M1/M2 schema cannot readily accommodate the binding properties of the m4 and m5 receptors. Pirenzepine, methoctramine, and hexahydrosiladifenidol were the most selective agents for the m1, m2, and m3 receptors, respectively. None of the antagonists used in this study were uniquely selective for either the m4 or m5 receptors. The diverse binding profiles of individual cloned receptors and the widespread distribution of m1–m4 mRNAs indicate that radioligand binding studies performed on primary tissues may actually be assessing the composite properties of a heterogeneous mixture of muscarinic receptor subtypes.

The pharmacology and binding properties of muscarinic receptors have been studied extensively for many years (for review see Refs. 1 and 2). A number of agents have been proposed to distinguish among muscarinic receptor subtypes. The most widely used classification system was based upon two selective antagonists, PZP and AF-DX 116. M1 receptors were characterized as expressing a high affinity toward PZP and an intermediate affinity toward AF-DX 116; conversely, M2 "cardiac type" have a low affinity toward PZP and high affinity toward AF-DX 116; M2 "glandular type" express a low affinity toward both PZP and AF-DX 116 (3–5). Further subdivisions of the M1 receptor have been suggested on the basis of functional studies using HHD and its silicon analogue, HHS (6, 7). Recent molecular cloning studies undertaken by ourselves (8, 9) and others (10–14) have demonstrated the existence of a family of five muscarinic receptor genes, all of which share the same proposed overall structure and a large degree of sequence identity. We have designated these receptors m1, m2, m3, m4, and m5 to signify the order in which the genes were cloned. m1 and m2 are rat homologues of the previously cloned porcine cortical and cardiac receptors, respectively (10–12), whereas m3, m4, and m5 represent novel receptors. The mRNAs encoding these receptors have also been mapped throughout the central (15, 16) and autonomic nervous systems[2] using *in situ* hybridization procedures. These studies demonstrated that most tissues express a heterogeneous mixture of receptor subtypes and, thus, are not well suited to characterization of individual receptors. In order to overcome this problem, we have inserted the coding sequences of the muscarinic receptor genes into the Okayama/Berg pCD mammalian expression vector and have used these constructs to transfect Chinese hamster ovary cells (CHO-K1) and select stably transformed cell lines. These cell lines provide an opportunity to assess the characteristics of individual muscarinic receptors expressed in homogeneous cell lines. The purpose of the present study was to use a range of antagonists to characterize the binding properties of m1, m2, m3, m4, and m5 receptors.

Materials and Methods

Preparation of plasmid DNA. The coding region of the m1 receptor is derived from a rat cDNA library whereas the coding sequences corresponding to the m2, m3, m4, and m5 receptors were

While this manuscript was in review, a manuscript appeared [Akiba *et al.*, *FEBS Lett.* **235**:257–261 (1988)] that described the affinities of PZP, AF-DX 116, and HHS for m1–m4 receptors expressed in oocytes. Their reported values are very close to our own.

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ABBREVIATIONS: PZP, pirenzepine; NMS, *N*-methylscopolamine; HHS, hexahydrosiladifenidol; HHD, hexahydrodifenidol.

derived from a human genomic library. The constructs containing the coding regions of the rat m1, human m2, human m3, human m4, and human m5 have been described previously (8, 9). m1 cDNA was isolated directly from a rat cerebral cortex pCD library and m2, m3, and m4 cDNAs were inserted into the Okayama/Berg pCD expression vector, whereas the m5 DNA was inserted into a modified version of the same vector, pCD-PS (9). Plasmid DNA was isolated and purified by two sequential density gradient centrifugations through CsCl, according to previously described procedures (17).

Cell culture. Chinese hamster ovary cells (CHO-K1) were used throughout these studies. Growth medium consisted of minimum Eagle's medium supplemented with 10% fetal calf serum (GIBCO, Grand Island, NY), 1000 units each of penicillin G and streptomycin, and 4 mM glutamine (M. A. Bioproducts, Walkersville, MD). Except for the overnight transfection (see below), cells were incubated in a humidified incubator at 37° and 5% CO_2. Selection medium consisted of growth medium containing 400 μg/ml concentrations of the neomycin analog G418 (GIBCO). Selection medium was changed every 3 days.

Transfection procedures. Cells were transfected using the modified calcium phosphate procedure described by Chen and Okayama (18). CHO-K1 cells were seeded into six-well plates at density of 10^5 cells/well. Twenty-four hours later, 4 μg of pCD vector containing a muscarinic receptor cDNA and 0.4 μg of pCDneo (18) were mixed with 200 ml of 0.1 M $CaCl_2$ and 200 μl of 2 × BBS, pH 6.89, at room temperature for 10 min before addition to one well containing 2 ml of growth medium. Cells were transfered to a humidified incubator at 35° and 3% CO_2 and incubated overnight. The following day, cells were washed and given fresh medium. Twenty-four hours later, cells were trypsinized and split at a ratio of 1:20 into four 10-cm plates. The next day, selection medium was added and selection continued for 2–3 weeks. At the end of selection, transfected cells were harvested and plated at limiting dilution into 96-well plates. Single colonies were selected, expanded, and assayed for [^{3}H]NMS binding capacity.

Membrane preparation. Transfected cells were grown to confluence, washed, and scraped into cold binding buffer and homogenized for 30 sec in a Tekmar Tissumizer (setting 50). Membranes were pelleted at 14,000 × g and rehomogenized, and the membrane protein was determined using a Bio-Rad protein assay dye reagent. Membrane concentration was adjusted to 1 mg of protein/ml and stored frozen at −80° before use. Membranes were also prepared from salivary glands of 200-g male Sprague-Dawley rats. Submaxillary glands were dissected free of sublingual glands before homogenization in a Tekmar tissumizer (2 min; setting 80). The membrane suspension was filtered through two layers of cheesecloth, pelleted, rehomogenized, and stored as described for the transfected cells.

Radioligand binding assays. All membranes, drugs, and radioligands were made up in binding buffer, which consisted of 25 mM sodium phosphate (pH 7.4) containing 5 mM magnesium chloride. Assays were conducted in 1 ml total volume. Final membrane protein concentrations were 30 μg/ml (m1), 70 μg/ml (m2), 4 μg/ml (m3), 7 μg/ml (m4), 3 μg/ml (m5), and 150 μg/ml (submaxillary gland). For direct binding assays, [^{3}H]NMS concentrations between 2.5 and 300 pM were used. For inhibition experiments, either 150 or 300 pM [^{3}H]NMS was used. Incubations were initiated by addition of [^{3}H]NMS and carried out at 22° for 3 hr. Displaceable binding was assessed in the presence of 1 μM atropine sulfate. Binding was terminated by filtration through a Brandel cell harvester onto Whatman GF/C filters. Membranes were washed three times with ice-cold binding buffer before being dried, transferred to 10 ml of scintillant (NEN Aquasol), and counted in an LKB 1217 Beta counter.

Data analysis. All data points were determined in duplicate. Points were plotted as the mean of at least three independent experiments. Data from direct binding experiments were fitted to the equation $a = \{B_{max} \cdot x^n/K_d\}/(1 + x^n/K_d)$ to derive the Hill number and fitted to $a = \{B_{max} \cdot x/K_d\}/(1 + x/K_d)$ in order to compute the equilibrium dissociation constant K_d and the binding capacity B_{max}; a is the amount of [^{3}H]NMS specifically bound, x is the concentration of [^{3}H]NMS, and n is the Hill number. Data were corrected for depletion of [^{3}H]NMS before analysis (19). Data from inhibition experiments were fitted to the equation %[^{3}H]NMS bound = $100 - [100x^n IC_{50}/(1 + x^n/IC_{50})]$ to obtain the Hill number and to the equation %[^{3}H]NMS bound = $100 - [100x/IC_{50}/(1 + x/IC_{50})]$ to obtain the IC_{50} inhibition constant. Equilibrium dissociation constant (K_i) were derived using the Cheng-Prusoff correction (20), $K_i = IC_{50}/(1+L/K_d)$, where L and K_d are the concentration and the equilibrium dissociation constant of [^{3}H]NMS, respectively. Data were analyzed by curvilinear regression with no weighting. Curves were computer generated using the program DA-TAPLOT (distributed by the National Technical Information Services) and run on a VAX II computer.

Drugs. PZP and AF-DX 116 were obtained from Karl Thomae, GmbH through the assistance of Dr R. Hammer (Boehringer Ingelheim); HHD, HHS, hexocyclium, and silahexocyclium were provided by Drs. Mutschler, Lambrecht, and Tacke (University of Frankfurt and Braunschweig); dicyclomine was obtained from Dr. E. Bohme of Merrell Dow (Cincinnati, OH); methoctramine was purchased from Research Biochemicals Inc., [^{3}H]NMS (73 Ci/mmol) was obtained from New England Nuclear (Boston, MA). Atropine sulfate was obtained from Sigma Chemical Co. (St. Louis, MO). pCDneo plasmids were donated by Dr. H. Okayama (NIMH, Bethesda, MD).

Results and Discussion

Saturation and inhibition curves and the binding parameters derived from the binding data are shown in Figs. 1, 2, and 3 and Tables 1 and 2.

[^{3}H]NMS, atropine, and HHD showed similar binding profiles. All three drugs expressed high affinities toward m1, m3, and m4 receptors, intermediate affinities toward the m5 receptor, and low affinities toward the m2 receptor, m1, m3, m4 > m5 > m2.

PZP had a high affinity only for the m1 receptor. Its affinity toward the m2 receptor was 57-fold less, whereas m3, m4, and m5 receptors had intermediate affinities, m1 > m3, m4, m5 > m2.

AF-DX 116 expressed a graded binding profile, with the highest affinity for the m2 receptor and the lowest affinity for the m5 receptor, m2 > m4 > m3 > m1 > m5.

HHS, hexocyclium, and silahexocyclium all showed high affinities for the m3 receptor and low affinities for the m2 receptor. This selectively was most marked for HHS (>4-fold), although hexocyclium and silahexocyclium showed higher affinities, m3 > m1, m4, m5 > m2.

Dicyclomine had a similar affinity for m1, m3, m4, and m5 receptors and a 17–24-fold lower affinity for the m2 receptor, m1, m3, m4, m5 > m2.

Methoctramine was the most selective agent for the m2 receptor. Its affinity for m2 receptors was 4-fold greater than that for the m1 receptor, 10–16-fold greater than that for the m4 and m5 receptors, and 33-fold greater than that for the m3 receptor, m2 > m1 > m4, m5 > m3.

[^{3}H]NMS bound to glandular muscarinic receptors with a K_d of 46 ± 26 pM and a Hill number of 0.92 ± 0.10 (data not shown). Of the antagonists tested, hexocyclium had the highest affinity toward the glandular receptor, hexocyclium > dicyclomine > HHS > Methoctramine > PZP > AF-DX 116 (Fig. 3; Table 2). The data obtained with the submaxillary gland were compared with the affinities of the same antagonists for the m1, m2, m3, m4, and m5 transformants. No significant difference was found between the affinities of these antagonists for the glandular receptor and the m3 receptor, whereas all other receptors showed significant differences with respect to

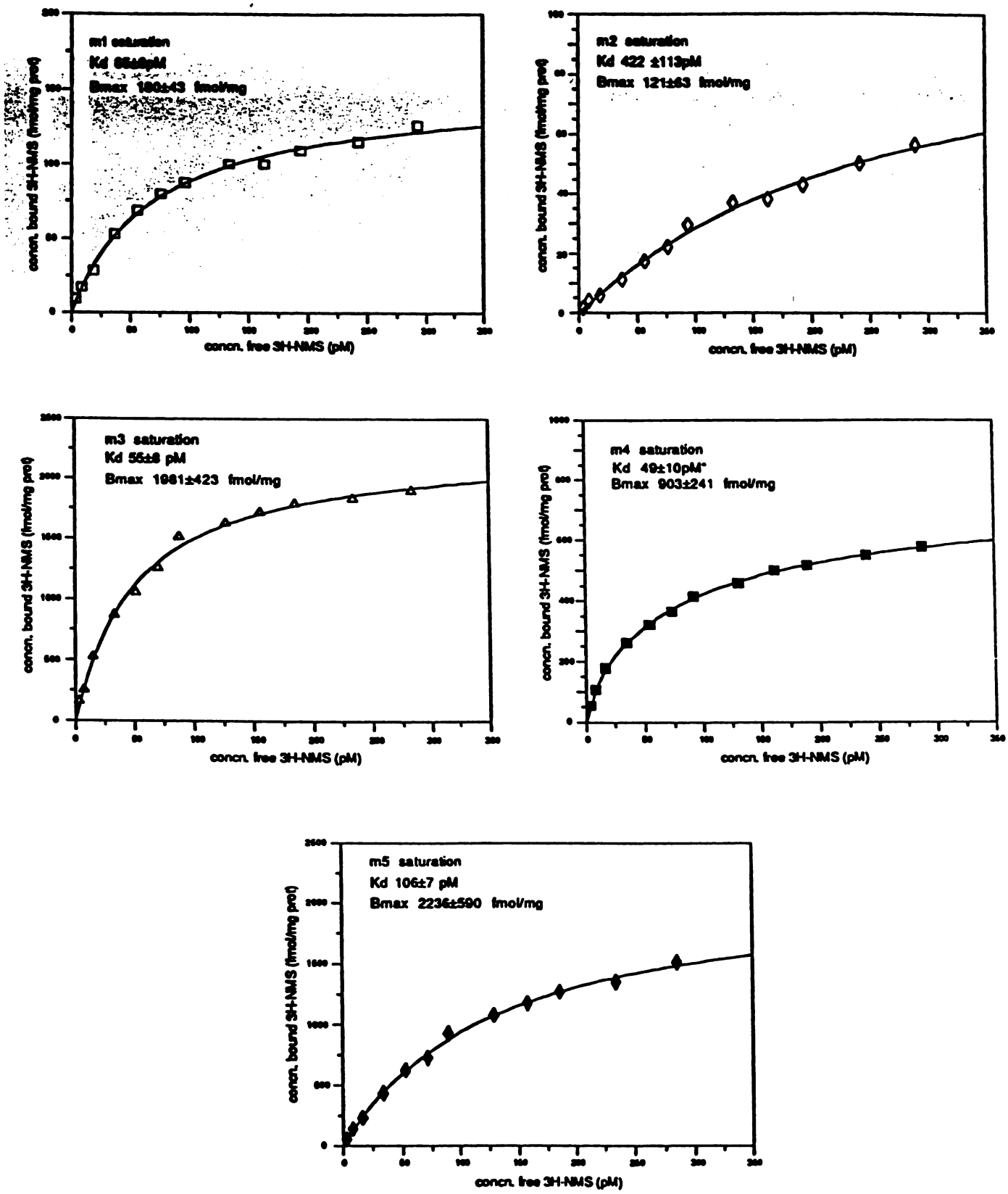

Fig. 1. [^{3}H]NMS saturation analysis of m1 (□), m2 (◇), me (△), m4 (■), and m5 (♦) transformants. Equilibrium dissociation constants (K_d; pM) and maximal binding capacities (B_{max}; fmol/mg of protein) of CHO-K1 cells stably transfected with m1, m2, m3, m4, and m5 DNA derived from [^{3}H]NMS saturation experiments (see Materials and Methods for experimental details) were generated using the equation $a=\{B_{max}\cdot x/K_d\}/(1+x/K_d)$. Untransfected CHO-K1 cells did not express any detectable binding (<2 fmol/mg of protein). Hill numbers were 0.98 ± 0.05 (m1); 1.00 ± 0.06 (m2); 1.01 ± 0.08 (m3); 0.75 ± 0.05 (m4); and 1.04 ± 0.14 (m5). Data represent the mean ± standard error of values derived from two to four experiments. *Hill numbers significantly different from unity ($p < 0.05$ in unpaired, one-tailed Student's t test).

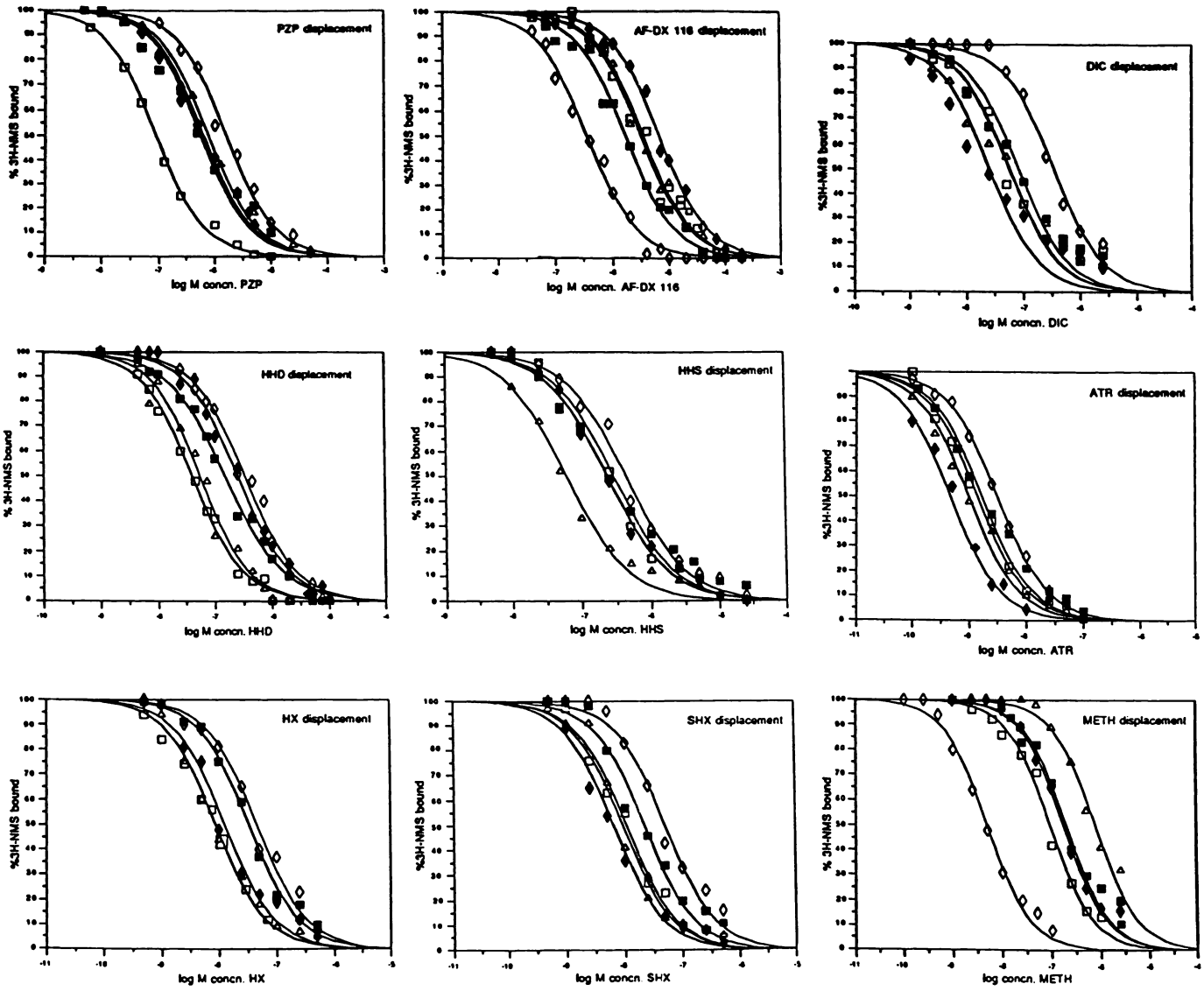

Fig. 2. Displacement of specific [^{3}H]NMS binding to m1 (□), m2 (◇), m3 (△), m4 (■), and m5 (♦) transformants. Concentration of [^{3}H]NMS was 150 pM for AF-DX 116 displacement studies and 300 pM for all other drugs. Specific binding was defined as that displaced by 1 mM atropine sulfate. *HX*, hexocyclium; *SHX*, silahexacyclium; *DIC*, dicyclomine; *METH*, methoctramine; *ATR*, atropine. All *points* represent the mean of values obtained from two to four individual experiments. *Curves* were generated to fit the equation: %[^{3}H]NMS bound = $100 - [\{100 \times /IC_{50}\}/(1 + x/IC_{50})]$.

at least one drug ($p < 0.05$ in an unpaired, double-tailed, Student t test).

The results of this study clearly demonstrate that muscarinic receptor genes transfected into mammalian fibroblasts express different antagonist binding profiles and, furthermore, that the currently used M1/M2 classification schemata do not adequately account for the range of binding characteristics expressed by m1, m2, m3, m4, and m5 receptors. Previous attempts to characterize the binding properties of muscarinic receptors have relied, for the most part, on cells or membranes prepared from tissues such as cerebral cortex, heart, ileum, and salivary glands as sources of muscarinic receptors. These studies suffer from the limitation that many of these tissues have been shown by *in situ* hybridization studies to express a mixture of muscarinic receptor genes (8, 15, 16). For instance, rat cerebral cortex expresses m1, m3, and m4 receptor genes. Although we have, as yet, no knowledge of the extent of receptor diversity expressed at the protein level, it seems likely that radioligand binding studies performed on cerebral cortex would, in fact, assess the composite binding properties of a heterogeneous mix of receptors. One of the purposes of this study was to compare the binding properties of cloned muscarinic receptors with the binding properties of muscarinic receptors expressed *in vivo*. Muscarinic receptors have been classified as M1 or M2 largely on the basis of their affinities for PZP and AF-DX 116. M1 receptors have a high affinity for PZP and an intermediate affinity for AF-DX 116; M2 cardiac type have a low affinity for PZP and a high affinity for AF-DX 116; M2 glandular type have an intermediate affinity for PZP and a low affinity for AF-DX 116 (3–5). Recent binding studies have characterized muscarinic receptors present in cerebral cortex, heart, and exocrine glands using a number of selective muscarinic antagonists including HHD, HHS, hexocyclium, silahexocyclium, dicyclomine, and methoctramine (5, 21–28). Comparison of inhibition binding constants obtained in the present study with those obtained in other recent studies is complicated

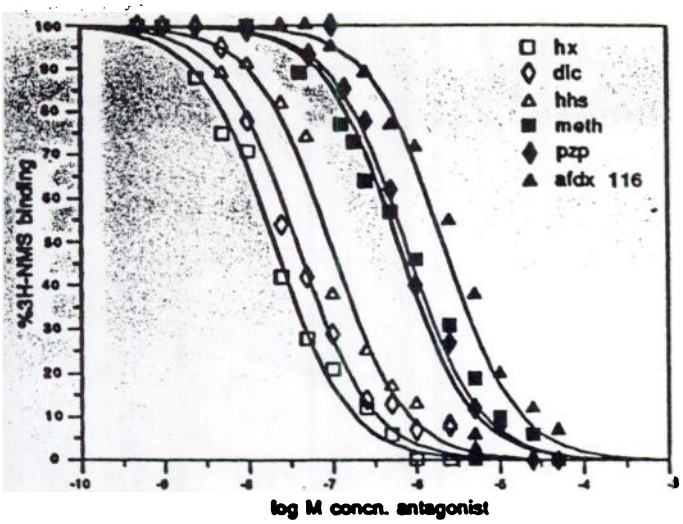

Fig. 3. [^{3}H]NMS displacement from rat submaxillary gland membranes. Membranes were prepared and displacement assays were conducted as described in Materials and Methods. Concentration of [^{3}H]NMS was 300 pM. Specific binding was defined as that displaced by 1 mM atropine sulfate. Plotted data represent the mean of two individual experiments. *Curves* were computer generated to fit the equation %[^{3}H]NMS bound = $100 - [\{100 \times /IC_{50}\}/(1 + x/IC_{50})]$. Abbreviations are as in Fig. 2.

because reported affinity values for a given antagonist often vary by up to an order of magnitude. Although the absolute affinity of a drug for its receptor may vary according to assay conditions, the relative difference in affinity of a drug towards different receptors is less variable and, thus, presents a more consistent criterion for comparison. A summary of these data is provided in Table 3.

Characterization of the m1 and m2 receptors. Identification of the m1 and m2 receptors as M1 and M2 receptors, respectively, was initially suggested by the observation that m1 and m2 receptors expressed the highest affinity for PZP and AF-DX 116, respectively. Furthermore, the 57-fold selectivity of PZP for the m1 receptor and the 7-fold selectivity of AF-DX 116 for the m2 receptor agree well with the relative difference in affinities reported for M1 cortical and M2 cardiac receptors (3, 29, 30). This conclusion was evidenced further by the 18-fold and 10-fold m1 selectivity displayed by HHD and hexocyclium and by the 10-fold m2 selectivity displayed by Methoctramine. Similar selectivities of 12-fold for HHD and hexocyclium towards cortical receptors and 7–16-fold selectivty of methoctramine toward cardiac receptors have been reported (25–27). Further corroboration is provided by the distribution of m1 mRNA and m2 mRNA. Northern blot analysis and *in situ* hybridization studies of muscarinic receptor mRNA distribution in the central nervous system (8, 15, 16) and autonomic nervous system[2] demonstrated a prevalence of m1 mRNA in cerebral cortex and m2 mRNA in atria, as expected for M1 and M2 cardiac receptors, respectively. However, caution is necessary in concluding that M1 receptors are a homogeneous class of receptors. M1 receptors are usually labeled using [^{3}H]PZP; because the difference in affinity between m1 and m4 receptors is only 5-fold, it is possible that the use of [^{3}H]PZP to label cerebral cortex muscrinic receptors will result in the labeling of both m1 and m4 receptors, depending upon the conditions of the assay. The difference in affinity between m1 and m4 receptors for the antagonists used in this study is less than 3-fold and, therefore, these agents may not adequately distinguish between them. Thus, it is possible that "M1" receptors are actually a mixture of m1 and m4 receptors.

Identification of the m3 receptor as the glandular type muscarinic receptor. The affinities of the m3, m4, and m5 receptors toward PZP and AF-DX 116 do not readily allow them to be classified as M1 or M2. The identification of the m3 receptor as the low affinity PZP-type receptor described in exocrine tissue (M2 glanduar type) was suggested by 1) the presence of m3 mRNA in pancreas (14) and salivary gland[2] and 2) the relatively large sizes of the protein encoded by the m3 cDNA (8, 14) and the glandular muscarinic receptor (31). Because there were few data available on the affinities of the antagonists used in this study toward the glandular receptor, a parallel study was undertaken to examine the affinities of these drugs for the muscarinic receptor of the rat submaxillary gland, using the same assay conditions as those used for the cloned receptors. The results of this experiment are shown in Fig. 3. The binding profile of the m3 receptor approximates the binding profile of the glandular receptor, the difference in inhibition constants for any particular drug being less than 2-fold. Comparison of the m3 and glandular receptor binding constants indicated that there was no significant difference between the two receptors ($p < 0.05$). The binding parameters of the m1, m2, m4, and m5 receptors differ significantly for at least one of the antagonists used in this study. Because the glandular receptor would be expected to express a low affinity toward AF-DX 116, we were initially concerned that the affinity of AF-DX 116 toward the m3 receptor was less than 5-fold its affinity for the m2 receptor. Most studies report a 20–35-fold difference in affinities between the cardiac and glandular receptor (5, 21, 24), although there are reports of much lower differences, namely 5-fold (27) and 12-fold (28). As can be seen from Table 2, under the assay conditions employed in the present study, AF-DX 116 expresses a similar intermediate affinity toward the glandular receptor and the m1 receptor. The selectivities of HHD, HHS, and hexocyclium agree well with the glandular receptor selectivity of these antagonists (24, 26, 28). Further evidence is also provided by the low affinity of methoctramine toward both the m3 and glandular receptor (23, 27, 28). The most useful agent for distinguishing the m3 receptor from other muscarinic receptors appears to be HHS, which expresses a 4–25-fold higher affinity for the m3 receptor than for the m1, m2, m4, and m5 receptors. Thus, the binding properties, localization, and larger size of the protein encoded by the m3 cDNA are entirely consistent with its identification as the glandular muscarinic receptor.

Characterization of m4 and m5 receptors. Neither the m4 nor m5 receptors express binding properties that readily allow them to be classified as M1 or M2, although it is possible that m4 receptors may have been classified as M1 receptors in previous studies (see above). Furthermore, none of the antagonists used in this study is uniquely selective for either the m4 or m5 receptors. Because the affinities of m1, m3, an m4 receptors towards either [^{3}H]NMS or [^{3}H]quinuclidinyl benzilate[3] are very similar and because m1, m3, and m4 mRNAs coexist in the cerebral cortex, it is likely that that binding experiments on membranes from cortex using these agents would label all three receptors. Use of low concentrations of

[2] N. J. Buckley, M. R. Brann, T. I. Bonner, and C. Stanford, manuscript in preparation.

TABLE 1

Binding parameters of m1, m2, m3, m4, and m5 receptors derived from [^{3}H]NMS displacement experiments shown in Fig. 2

IC_{50} represents the 50% inhibition value; *n* represents the Hill number; K_i represents the inhibition constant obtained using the Cheng-Prusoff equation and the [^{3}H]NMS K_D values from Fig. 1; numbers in parenthesis represent the number of individual experiments. All data represent the mean ± standard error of values obtained from the individual experiments. Abbreviations are as in Fig. 2.

		m1	m2	m3	m4	m5
PZP	K_i (nM)	16	906	180		
	IC_{50} (nM)	89 ± 16	1540 ± 90	1170 ± 150	561 ± 70	628 ± 70
	n	0.97 ± 0.17	1.07 ± 0.10	1.04 ± 0.16	0.75 ± 0.05*	0.80 ± 0.05*
		(2)	(3)	(3)	(3)	(4)
AF-DX	K_i (nM)	1300	186	838		2800
	IC_{50} (μM)	4.3 ± 1.2	0.36 ± 0.23	3.1 ± 0.4	1.8 ± 0.3	6.8 ± 0.9
	n	0.97 ± 0.11	0.96 ± 0.15	1.06 ± 0.10	0.79 ± 0.03*	0.92 ± 0.03
		(2)	(2)	(2)	(3)	(2)
HHD	K_i (nM)	11	200	16		83
	IC_{50} (nM)	37 ± 6	280 ± 105	61 ± 21	76 ± 16	199 ± 57
	n	0.98 ± 0.04	0.97 ± 0.06	1.03 ± 0.08	0.86 ± 0.04*	0.96 ± 0.05
		(4)	(4)	(4)	(3)	(3)
HHS	K_i (nM)	44	249	10		63
	IC_{50} (nM)	246 ± 28	424 ± 42	66 ± 34	298 ± 140	242 ± 53
	n	0.96 ± 0.08	1.16 ± 0.06	1.03 ± 0.06	0.72 ± 0.16*	1.00 ± 0.13
		(2)	(3)	(2)	(3)	(3)
HX	K_i (nM)	2.3	23	1.4	5.5	3.7
	IC_{50} (nM)	13 ± 4.3	40 ± 1	9 ± 0.7	39 ± 21	14 ± 6.2
	n	1.02 ± 0.09	0.95 ± 0.04	0.99 ± 0.18	1.03 ± 0.17	0.94 ± 0.20
		(2)	(2)	(3)	(3)	(2)
SHX	K_i (nM)	2.0	35	1.2	3.2	2.0
	IC_{50} (nM)	11 ± 1.0	59 ± 16	8 ± 2.3	23 ± 8	8 ± 2.1
	n	0.94 ± 0.08	1.04 ± 0.16	1.06 ± 0.04	0.92 ± 0.14	0.91 ± 0.08
		(3)	(3)	(3)	(3)	(2)
ATR	K_i (nM)	0.21	1.5	0.15		0.21
	IC_{50} (nM)	1.2 ± 0.34	2.6 ± 1.3	1.0 ± 0.1	2.1 ± 0.9	0.8 ± 0.3
	n	0.95 ± 0.07	0.93 ± 0.11	1.01 ± 0.08	0.79 ± 0.04*	0.92 ± 0.09
		(2)	(2)	(3)	(3)	(2)
METH	K_i (nM)	16	3.6	118		57
	IC_{50} (nM)	92 ± 31	6.1 ± 2.6	770 ± 66	260 ± 137	217 ± 109
	n	1.08 ± 0.12	0.98 ± 0.07	0.96 ± 0.10	0.75 ± 0.11*	1.03 ± 0.10
		(3)	(3)	(3)	(3)	(3)
DIC	K_i		244			
	IC_{50} (nM)	57 ± 32	415 ± 186	67 ± 27	97 ± 40	53 ± 7
	n	0.68 ± 0.11*	1.10 ± 0.30	0.66 ± 0.11*	0.76 ± 0.15*	0.64 ± 0.04*
		(3)	(4)	(3)	(3)	(4)

* Hill number differs significantly from unity ($p < 0.05$ in an unpaired, one-tailed Student's *t* test).

TABLE 2

Binding parameters of muscarinic receptor antagonists to membranes prepared from rat submaxillary gland generated from curves shown in Fig. 3

Membranes were prepared and displacement assays were conducted as described in Materials and Methods. IC_{50} represents the 50% inhibition value; *n* represents the Hill number; K_i represents the inhibition constant obtained using the Cheng-Prusoff equation. [^{3}H]NMS K_D value was 46 pM; numbers in parenthesis represent the number of individual experiments. All data represent the mean ± standard error of values obtained from two individual experiments. Abbreviations are as in Fig. 2.

	PZP	AF-DX 116	HHS	HX	METH	DIC
K_i (nM)	107	448	12.4	2.3	88	5.1
IC_{50} (nM)	801 ± 81	3366 ± 420	93 ± 16	17.1 ± 5.0	662 ± 140	38.2 ± 3.6
n	0.90 ± 0.11	0.91 ± 0.05	1.02 ± 0.11	0.90 ± 0.12	0.65 ± 0.06*	0.95 ± 0.04
	(2)	(2)	(2)	(2)	(2)	(2)

* Hill number differs significantly from unity ($p < 0.05$ in an unpaired, one-tailed Student's *t* test).

PZP would lead to a preferential labeling of m1 receptors, whereas [^{3}H]AF-DX 116 would be expected to label different proportions of m2, m4, and possibly m5 receptors. The affinity values of [^{3}H]NMS, atropine, PZP, and AF-DX 116 for the m4 receptor approximate those reported for NG-108 cells (32, 33), a cell line that expresses m4 receptor mRNA (13). Because we have so far been unable to detect m5 mRNA in any tissue or cell line examined, no comparison of the binding profile of the m5 receptor is possible. If such a source of m5 receptor is found, then we would predict that it would be characterized by a low affinity for AF-DX 116 and an intermediate affinity for PZP, HHD, HHS, and methoctramine, a profile unique for the m5

TABLE 3

Binding profile of muscarinic receptor antagonists for muscarinic receptors derived from cerebral cortex, exocrine glands, and heart and for cloned muscarinic receptors expressed in CHO-K1 cells

Drug abbreviations are as in Table 1. *CX*, cerebral cortex; *G*, exocrine glands; and *H*, heart.

Drug	Binding Profile	References
PZP	CX > G > H m1 > m3, m4, m5 > m2	3, 6, 25, 27, 28
AF-DX 116	H > CX > G m2 > m4 > m3 > m1 > m5	5, 22, 25, 27, 28
HHD	CX = G > H m1, m3, m4 > m5 > m2	6
HHS	G > H m3 > m1, m4, m5 > m2	25
HX	CX > G > H m3 > m1, m4, m5 > m2	7
DIC	G > H m1, m3, m4, m5 > m2	25, 27, 28
METH	H > CX > G m2 > m1 > m4, m5 > m3	23, 27, 28

receptor. The most diagnostic compound of those tested appears to be HHD, which expresses an affinity for the m5 receptor that is 4–7-fold lower than that for the m1, m3, and m4 receptors and almost 3-fold higher than that for the m2 receptor.

As can be seen from Table 2, a number of binding isotherms exhibited low Hill numbers. Most prominent were the displacement curves generated from the m4 inhibition data. Low Hill numbers are most often attributed to recognition by the antagonist of more than one receptor site or receptor conformation or to interaction of the antagonist with a second binding site on the receptor molecule causing a negative cooperative effect on binding to the first site. All these alternatives have been described for endogenous cardiac muscarinic receptors (2, 34, 35). However, an extensive binding analysis is necessary to distinguish among these models. We are currently investigating this question.

It is important to know whether the binding properties of muscarinic receptors vary as a function of the host cell into which the corresponding receptor gene is transfected. We have expressed m1, m2, m3, and m4 receptors in COS-7 (8), A9 L,[3] and CHO-K1 cells (present study) and have examined their affinities for PZP. In all cell lines, PZP has high, low, and intermediate affinities for m1, m2, and m3 receptors, respectively. However, previous studies have shown that m1 and m4 receptors both express high affinities for PZP when expressed in COS-7 or A9 L cells, in contrast to the intermediate affinity of the m4 receptor for PZP when expressed in CHO-K1 cells (present study). Another study by Peralta *et al.* (13) also showed little difference in PZP affinity between m1 and m4 receptors, although in this case the absolute affinity values were 10–30-fold lower than those reported in other studies. As stated earlier, the expressed affinity of a drug for a receptor can vary over a wide range, according to the assay conditions used. Because the affinities of PZP for cloned muscarinic receptors expressed in different host cells were measured under different assay conditions, the reported differences in affinity values may merely reflect these different assay conditions. However, it is also possible that antagonist binding properties may vary according to the cellular environment in which the receptor is placed. Further studies are necessary to resolve this issue.

The findings that m1, m2, m3, m4, and m5 receptors express different antagonist binding properties and that m1, m2, m3, and m4 mRNAs are also frequently coexpressed in the same regions of the nervous system (8, 15, 16), provide a rationale for understanding the heterogeneous binding curves that are frequently observed using antagonists to label receptors in central and peripheral tissues. This perspective may be further complicated because analysis of genomic blots indicates the possible existence of additional muscarinic receptor genes (8, 9). The use of stable transformed cell lines to characterize the binding properties of individual muscarinic receptors should permit identification of subtype-specific drugs while mutagenesis of muscarinic receptor genes may help in identifying amino acid residues that confer drug selectivity to each receptor subtype.

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