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Sialic Acid Residues as Catalysts for M_2-Muscarinic Agonist-Receptor Interactions

EL BDAOUI HADDAD, YVES LANDRY, and JEAN PIERRE GIES

Laboratoire de Neuroimmunopharmacologie, Université Louis Pasteur-Strasbourg I, BP 24, 67401 Illkirch Cedex, France

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SUMMARY

The role of sialic acid residues in the interactions of muscarinic agonists with the cardiac M_2 muscarinic receptor was investigated by competitive binding experiments using the lipophilic radioligand (−)-[*benzilic*-4,4-^{3}H]quinuclidinyl benzilate ([^{3}H]QNB) and the hydrophilic ligand [*N-methyl*-^{3}H]scopolamine methyl chloride ([^{3}H]NMS). Direct labeling of the agonist binding sites was performed with the radiolabeled agonist [*methyl*-^{3}H]oxotremorine M acetate ([^{3}H]oxo-M). Neuraminidase decreased the affinity of the M_2-selective agonist carbamylcholine in competitive binding experiments performed with [^{3}H]QNB and [^{3}H]NMS. The binding of the M_1-selective agonist (4hydroxy-2-butynyl)trimethylammonium chloride *m*-chlorocarbanilate (McN-A-343), of the M_1-selective antagonist pirenzepine, and of the M_2-selective antagonist 11-({2-[(diethylamino)methyl]-1 piperidinyl}acetyl)-5,11-dihydro-6*H*-pyrido(2,3*b*)(1,4)benzodiazepin-6-on (AF-DX-116) were not affected by neuraminidase. Neuraminidase did not modify the binding parameters of ^{3}H-antagonists but reduced the number of agonist binding sites revealed by [^{3}H]oxo-M. The removal of sialic acid decreased the half-life of the receptor-agonist complex. The present results suggest that removal of sialic acid reduces the formation of super-high affinity agonist-receptor complexes. Sialic acid may catalyze macroscopic binding by enhancing accumulation of the agonist at the membrane surface.

Complementary DNAs for five different muscarinic acetylcholine receptors (m1, m2, m3, m4, and m5) have been isolated from rat and human genomes and expressed in mammalian cells (1–4). The various binding states of agonists observed in tissues may be related to these distinct gene products, to posttranslational modifications of receptor proteins, or to specific conformational states imposed upon the receptor proteins by membrane lipids and coupling proteins.

The muscarinic receptors m1, m2, m3, and m4 differ in the number and spacing of the potential *N*-glycosylation sites. Two sites were noted near the amino terminus of the m1 muscarinic receptor, three in the m2, five in the m3, and three in the m4 receptors (5). The glycoprotein nature of the solubilized muscarinic receptor from bovine cerebral cortex (M_1) has been demonstrated (6). The solubilized muscarinic acetylcholine receptor from porcine atria (M_2) has sialoglycoprotein characteristics, inasmuch as it interacts specifically with lectin affinity resins (7). Further studies have revealed the presence of 21 sialic acid residues (8).

We reported that the removal of sialic acid residues by neuraminidase treatment reduced the affinity of muscarinic agonists for cerebellum, heart, and lung muscarinic receptors but not for striatum muscarinic receptors (9). In order to characterize the role of sialic acid residues in the interactions of muscarinic agonists with the M_2 "cardiac-like" muscarinic receptor, we compared the affinity of selective agonists and antagonists for the receptor labeled with either [^{3}H]QNB or [^{3}H]NMS in the absence or presence of neuraminidase. We also examined the effects of sialic acid removal on the direct binding of agonists by labeling the agonist binding sites with [^{3}H]oxo-M. Our results indicate that membrane surface sialic acids are necessary to enhance direct agonist binding.

Experimental Procedures

Materials. [^{3}H]QNB (33.1 Ci/mmol), [^{3}H]NMS (73.8 Ci/mmol), and [^{3}H]oxo-M (82 Ci/mmol) were obtained from the Radiochemical Center (New England Nuclear Boston, MA). Neuraminidase (type V, from *Clostridium perfringens*) (EC 3.2.1.18), carbamylcholine chloride (carbachol), pilocarpine hydrochloride, oxotremorine sesquifumarate, atropine sulfate, bacitracin, and the protease inhibitor PMSF were purchased from Sigma Chemical Co. (St. Louis, MO). McN-A-343 was from Research Biochemicals (Natick, MA). Pirenzepine dihydrochloride and AF-DX 116 base were gifts from Boehringer-Ingelheim (Ingelheim, FRG). All drugs were prepared in the assay buffer with the exception of AF-DX 116, which was dissolved in 1 N HCl and diluted

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ABBREVIATIONS: [^{3}H]QNB, (−)-[*benzilic*-4,4-^{3}H]quinuclidinyl benzilate; [^{3}H]NMS, [*N-methyl*-^{3}H]scopolamine methyl chloride; [^{3}H]oxo-M, [*methyl*-^{3}H]oxotremorine M acetate; AF-DX 116, 11-({2-[(diethylamino)methyl]-1 piperidinyl}acetyl)-5,11-dihydro-6*H*-pyrido(2,3*b*)(1,4)benzodiazepin-6-on; McN-A-343, (4 hydroxy-2-butynyl)trimethylammonium chloride *m*-chlorocarbanilate.

with buffer, and PMSF, which was dissolved in isopropylic acid and diluted with buffer.

Tissue preparation for binding experiments. Male Wistar rats weighing 200 to 300 g (Iffa-Credo, L'Arbresle, France) were sacrificed by decapitation and the hearts were removed. Whole hearts were dissected free of connective tissues, rinsed, blotted dry, and weighed. Membrane preparation was performed at 0–4°, as described previously (10). Briefly, tissues were minced and homogenized in 0.25 M ice-cold sucrose. After centrifugation at 2500 × *g* for 10 min at 4°, the pellets were suspended, homogenized, and resedimented at the same speed. The two supernatant fluids were pooled and diluted with 60 volumes of 50 mM Na_2HPO_4-KH_2-PO_4 buffer, pH 7.4. The mixtures were then centrifuged at 33,000 × *g* for 30 min at 4°. The resulting pellets were resuspended (2 mg of protein/ml) in buffer (145 mM NaCl, 2 mM $MgCl_2$, 10 mM NaH_2PO_4, pH 7.4) before use in radioligand binding assays.

Protein concentrations. Protein concentrations were determined according to the method of Spector (11), using bovine γ-globulin as the standard.

[^{3}H]QNB binding. [^{3}H]QNB binding was carried out with 1 ml of membrane suspension containing 0.2 nM [^{3}H]QNB, various concentrations of unlabeled drugs, and appropriate amounts of membrane preparation (45–60 μg of protein/assay). Incubations were performed at 37° for 1 hr, with or without neuraminidase. Under these conditions, the steady state for specific binding (in the absence or presence of neuraminidase) and the linear relationship between specific [^{3}H]QNB binding and the amount of membrane proteins added were verified. The density of binding sites (B_{max}) and the equilibrium dissociation constant (K_D) for the radioligand were determined by saturation experiments that used increasing concentrations of radioligand (5–2000 pM) and that were analyzed with Scatchard plots. Under these incubation conditions, equilibrium was reached for all concentrations of radioligand used (data not shown). Bound [^{3}H]QNB was harvested by rapid vacuum filtration through GF/B glass fiber filters (Whatman, Inc, Clifton, NJ). Filters were rinsed twice with 5 ml of ice-cold phosphate buffer and placed in vials with 7 ml of scintillation cocktail (Ready-Solv HP; Beckman Instruments, Inc, Fullerton, CA). A vigorous 20-min shaking, followed by overnight extraction, was required for quantitative desorption of the filter-bound radioactivity (12), which was then measured in a liquid scintillation counter with a 45% efficiency. Specific [^{3}H]QNB binding was defined as the difference between total binding and binding in the presence of 10^{-6} M atropine. Nonspecific binding was less than 5% and mostly due to [^{3}H]QNB binding to filters.

[^{3}H]NMS binding. We used the same experimental conditions as for [^{3}H]QNB binding. Incubations were performed at 37° for 1 hr, with or without neuraminidase, in the presence of 0.2 nM [^{3}H]NMS and 45–60 μg of protein/assay. [^{3}H]NMS saturation curves were obtained using a concentration range varying from 10 to 2000 pM. Equilibrium was reached for all concentrations of radioligand used under these incubation conditions (data not shown).

[^{3}H]Oxo-M binding. Incubations were carried out at 25° for 50 min in 1 ml of membrane suspension containing 0.5 nM [^{3}H]oxo-M and 80–120 μg of protein. [^{3}H]Oxo-M saturation curves were performed using a concentration range varying from 0.2 to 6 nM. To reduce nonspecific binding, essentially due to tracer binding to the filters, we presoaked all filters in 0.05% polyethyleneimine. After this pretreatment, the remaining nonspecific [^{3}H]oxo-M binding did not exceed 20–25% of total binding.

Kinetics of [^{3}H]oxo-M binding. Rat heart membranes were preincubated [in the presence of the protease inhibitors (184 μg/ml, bacitracin and 0.1 mM PMSF)] for 60 min at 25°, in the absence or presence of neuraminidase (3 units/mg of protein). After the preincubation period, membranes were washed by a centrifugation at 33,000 × *g* for 30 min. The resulting pellets were resuspended in buffer and membrane suspensions were used for kinetic experiments. Kinetic experiments were carried out at 25° in a total volume of 1 ml of membrane suspension containing 0.37 nM [^{3}H]oxo-M and 180–250 μg of protein, in the absence or presence of 1 μM atropine. [^{3}H]Oxo-M dissociation kinetics were obtained as follows: after equilibrium had been reached, tracer dissociation was induced by isotopic dilution with 1 μM atropine. Specific binding was determined at appropriate time intervals.

Neuraminidase treatment. Membranes were suspended, with or without neuraminidase, in the presence of the competitive ligand and one of the three tracers for 1 hr at 37° ([^{3}H]QNB and [^{3}H]NMS) or for 50 min at 25° ([^{3}H]oxo-M). The lack of interference of the enzyme with ligand binding (using the three radiolabeled ligands) was verified by adding denatured neuraminidase (100° for 30 min) to membrane preparations. The active enzyme did not modify the association rate of the two radiolabeled antagonists. The effects of neuraminidase on muscarinic receptor binding properties were irreversible. The irreversibility was tested by treating homogenates with neuraminidase at 37° at pH 7.4 for 1 hr, washing it away, and then measuring binding properties as in our earlier study (9). In view of the lack of neuraminidase effects on the equilibrium of [^{3}H]QNB and [^{3}H]NMS binding and the irreversible effects of neuraminidase on muscarinic binding, we included the enzyme directly in the incubation medium. The commercial enzyme contains, according to the manufacture, small amounts of proteases with activity 1300 times lower than that of neuraminidase. Control experiments in the presence of protease inhibitors indicated that this contamination was negligible, so all effects are due to sialic acid release. Sialic acid released by the treatment was measured with the fluorometric thiobarbituric acid method described by Hammond and Papermaster (13).

Data analysis. Experimental data for the saturation and inhibition studies were analyzed, as described earlier (14), using the nonlinear regression analysis described by Munson and Rodbard (15) (LIGAND program; Elsevier-Biosoft, Cambridge, UK). The precision of fit to a one- or two-site model was determined with an F test ($p < 0.01$), by comparing the residual sum of squares for fitting data to a one- or two-site model. Data were weighted with the reciprocal of the variance. To facilitate comparisons between ligands and between tissues, IC_{50} values were converted to K_i values, called $K_{0.5}$ as suggested by Weiland and Molinoff (16), using the Cheng and Prusoff equation (17). K_i was independent of the concentration of radioligand, unlike IC_{50} as shown previously (18). The kinetic parameters of [^{3}H]oxo-M binding were calculated as follow: the first-order rate constant for dissociation (k_{-1}) was calculated from the slope of the line generated by a plot of $\ln (B_t/B_e)$ versus t, where B_t and B_e are the amount of [^{3}H]oxo-M bound at time t and at equilibrium, respectively. The association rate constant (k_1) was calculated by assuming pseudo-first-order kinetics, which can be described by the relationship $\ln (B_e/B_e - B_t) = k_{obs}\, t$; the slope of the line generated by a plot of $\ln (B_e/B_e - B_t)$ versus t is equal to the rate constant (k_{obs}). The second-order rate constant for association (k_1) was obtained using the relationship $k_1 = (k_{obs} - k_{-1})/[L]$, where k_{-1} is the first-order rate constant for dissociation of [^{3}H]oxo-M, determined as described below, and $[L]$ is the concentration of [^{3}H]oxo-M used.

Results

Sialic acid removal from heart membranes. In order to correlate the neuraminidase effects observed in binding studies, we measured the amount of sialic acid released by the enzyme, using the fluorometric technique described above. This release followed an exponential time course (Fig. 1). Sialic acid release was maximal when 1 unit of neuraminidase was incubated for 1 hr at 37°, pH 7.4, with the membranes (Fig. 1, *inset*). Then we used the optimal enzymatic conditions by incubating rat heart membranes for 1 hr with 3 units of neuraminidase/mg of protein. After 60 min, 3 units of neuraminidase released 6.22 ± 0.32 nmol/mg of protein. This represented 55–60% of the total sialic acid content. Similar results were obtained at 25° by Nagatomo *et al.* (19).

Saturation experiments. When [^{3}H]QNB and [^{3}H]NMS

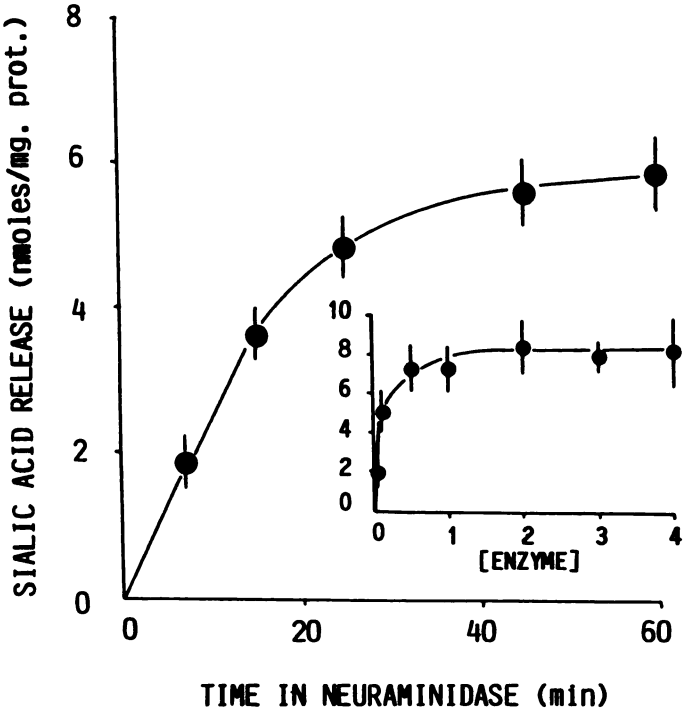

Fig. 1. Time course of sialic acid release from heart membranes by neuraminidase. Cardiac membranes (600 μg of protein/assay) were incubated with 3 units of neuraminidase/mg of protein at 37° for different times. *Inset* release of sialic acid after 1 hr of incubation with different doses of neuraminidase. The effect was maximal with 3 units/mg of protein. Data are from three experiments performed in duplicate.

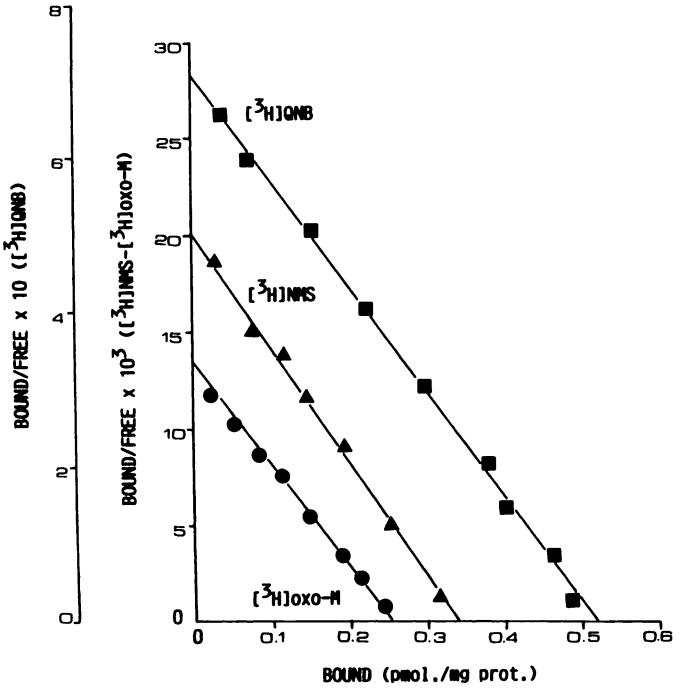

Fig. 2. Scatchard analysis of the binding of three radiolabeled muscarinic ligands to heart membranes. Data represent four ([^{3}H]NMS) or six ([^{3}H]QNB and [^{3}H]oxo-M) experiments with each radioligand. The Hill coefficients for each of the three curves was 1.

TABLE 1

Radioligand binding characteristics in rat heart membranes

The K_D and B_{max} were determined by nonlinear regression analysis with the computerized method LIGAND, as described previously (14). Total receptors (B_{max}) and dissociation constants (K_D) are given as means ± standard errors from four to six independent experiments performed in duplicate.

	K_D		B_{max}	
	Control	Treated	Control	Treated
	pM		*fmol/mg of protein*	
[^{3}H]QNB	38.1 ± 0.3	36.9 ± 3.1	585 ± 6	558 ± 27
[^{3}H]NMS	915 ± 53	900 ± 57	443 ± 56	406 ± 45
[^{3}H]Oxo-M	1590 ± 130	1577 ± 135	238 ± 18	137 ± 17

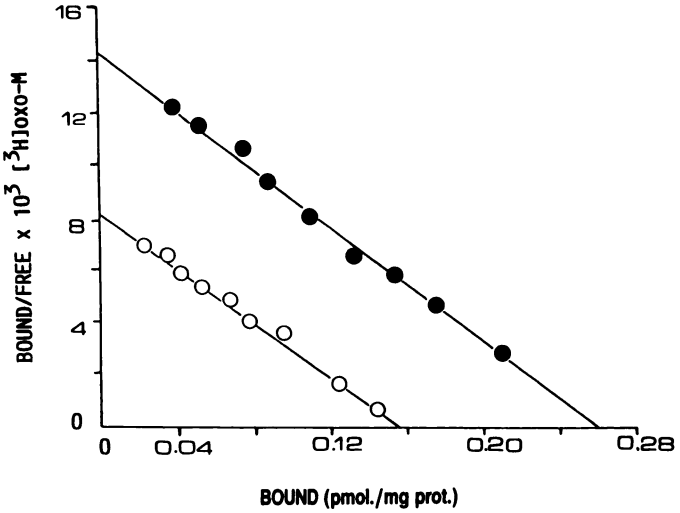

Fig. 3. Scatchard plots of specific [^{3}H]oxo-M binding in the absence (●) or the presence (○) of neuraminidase (3 units/mg of protein). The total number of agonist binding sites measured with [^{3}H]oxo-M was 238 and 137 fmol/mg of protein in the absence and presence of neuraminidase, respectively. Data are from five experiments performed in duplicate.

were used to label muscarinic acetylcholine receptors in rat heart homogenates, specific binding of both ligands demonstrated saturability. Scatchard analysis of the saturation isotherms indicated that both ligands bound to a homogeneous receptor population (Fig. 2). The affinity of [^{3}H]QNB was 24 times higher than that of [^{3}H]NMS. Moreover, as reported by Lee and El-Fakahany (20), [^{3}H]NMS labeled only 76% of the receptors identified by [^{3}H]QNB (Table 1). [^{3}H]Oxo-M bound with a higher dissociation constant (K_D = 1.59 nM) to rat heart membranes. There was no evidence of heterogeneity among agonist binding sites (Fig. 2). Receptors labeled by [^{3}H]oxo-M represented 41% of the receptors determined by [^{3}H]QNB binding. Neuraminidase had no effect on the K_D and B_{max} of the two radiolabeled antagonists. The effect of neuraminidase on agonist interaction at muscarinic agonist binding sites was investigated by generating saturation binding isotherms with the radiolabeled agonist [^{3}H]oxo-M. Surprisingly, neuraminidase decreased the number of binding sites without affecting the affinity of this agonist (Table 1, Fig. 3). The same effect was obtained when the membranes were pretreated with neuraminidase (in the presence of protease inhibitors), as was performed for the kinetic studies. This effect could be attributed to a release of sialic acid by neuraminidase, because the denatured enzyme (100° for 30 min) did not affect the saturation profile of [^{3}H]oxo-M and because the protease contamination was taken as being negligible.

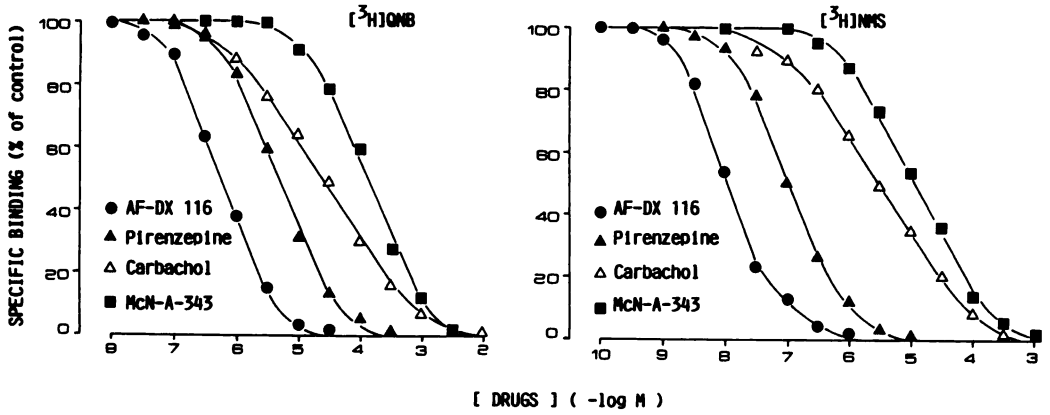

Fig. 4. Inhibition of [^{3}H]QNB and [^{3}H]NMS binding by muscarinic agonists and antagonists. Experimental conditions were as described in Experimental Procedures. Values are means from three independent experiments performed in duplicate.

TABLE 2

Inhibition of [^{3}H]QNB and [^{3}H]NMS binding in rat heart membranes by selective muscarinic ligands

$K_{0.5}$ values were determined from competitive binding curves (illustrated in Fig. 4). Hill slope factors, calculated from the competitive binding curves, are shown in parentheses. Data are given as means ± standard errors of three to five independent experiments performed in duplicate.

	$K_{0.5}$			
	[^{3}H]QNB		[^{3}H]NMS	
	Control	Treated	Control	Treated
	μM			
Carbachol	4.95 ± 0.25 (0.59)[a]	18.94 ± 1.41 (0.47)[a]	2.41 ± 0.15 (0.64)[a]	5.43 ± 0.73 (0.59)[a]
K_H[b]	0.47 ± 0.18 (42%)[c]	0.92 ± 0.19 (41%)[c]	0.27 ± 0.08 (44%)[c]	1.21 ± 0.45 (45%)[c]
K_L[b]	22.34 ± 3.04 (58%)[d]	71.82 ± 11.28 (59%)[d]	11.03 ± 3.62 (56%)[d]	35.03 ± 9.62 (55%)[d]
McN-A-343	18.38 ± 0.17 (0.89)	21.52 ± 2.36 (0.92)	12.32 ± 5.28 (0.91)	13.75 ± 6.72 (0.89)
AF-DX-116	0.109 ± 0.007 (1.03)	0.110 ± 0.009 (1.02)	0.006 ± 0.001 (1.11)	0.006 ± 0.001 (1.06)
Pirenzepine	0.776 ± 0.003 (1.05)	0.895 ± 0.038 (1.11)	0.078 ± 0.005 (1.15)	0.074 ± 0.004 (1.10)

[a] The two-site analysis fit is significantly better ($p < 0.01$) than that for the one-site model.
[b] K_H and K_L are the equilibrium dissociation constants for the high and low affinity sites according to a two-site model.
[c] High affinity receptors as a percentage of the total receptor population ($B_H \times 100/[B_H + B_L]$).
[d] Low affinity receptors as a percentage of the total receptor population ($B_L \times 100/[B_H + B_L]$).

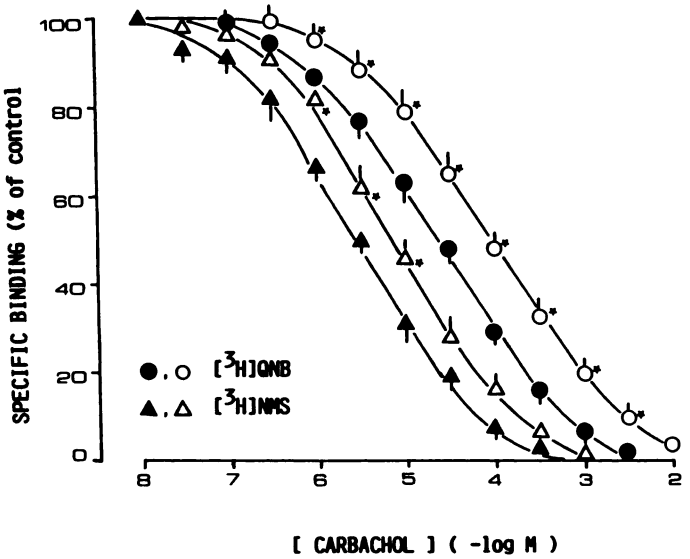

Fig. 5. Inhibition of [^{3}H]QNB and [^{3}H]NMS binding by carbachol in the absence (*closed symbols*) or the presence (*open symbols*) of neuraminidase (3 units/mg of protein). Data are from four experiments carried out in duplicate.

[^{3}H]QNB and [^{3}H]NMS binding. Selective muscarinic agonists and antagonists were tested for their ability to compete with [^{3}H]QNB (0.2 nM) or [^{3}H]NMS (0.2 nM) for specific binding sites in heart homogenates, to investigate whether neuraminidase affected the binding of these drugs to the muscarinic M_2 receptor. The M_1-selective agonist McN-A-343, the M_1-selective antagonist pirenzepine, and the M_2-selective antagonist AF-DX 116 displayed specific binding to [^{3}H]QNB- and [^{3}H]NMS-labeled sites according to the law of mass action, with steep slopes, giving Hill coefficients not significantly different from unity. However, the M_2-selective agonist carbachol (21, 22) inhibited specific [^{3}H]QNB and [^{3}H]NMS binding with Hill coefficients significantly less than unity. The competition curves for control membranes are shown in Fig. 4 and the $K_{0.5}$ values in Table 2. The $K_{0.5}$ values given for the AFDX116/[^{3}H]NMS and the pirenzepine/[^{3}H]NMS competition curves are much lower than those calculated by Hammer and co-workers (23, 24) but are in accord with those obtained by Korc *et al.* (25).

TABLE 3

Inhibition of [^{3}H]QNB binding in rat heart membranes (in the absence or presence of neuraminidase) by carbachol, in the presence of protease inhibitors

Data are given as means ± standard errors of four independent experiments performed in duplicate

	$K_{0.5}$	K_H[a]	K_L[a]
		μM	
Control	2.96 ± 0.64	0.83 ± 0.48 (43%)[b]	12.90 ± 2.73 (57%)[c]
Treated	9.04 ± 0.41	0.91 ± 0.24 (47%)[b]	65.06 ± 8.04 (53%)[c]

[a] K_H and K_L are the equilibrium dissociation constants for the high and low affinity sites according to a two-site model.
[b] High affinity receptors as a percentage of the total receptor population ($B_H \times 100/[B_H + B_L]$).
[c] Low affinity receptors as a percentage of the total receptor population ($B_L \times 100/[B_H + B_L]$).

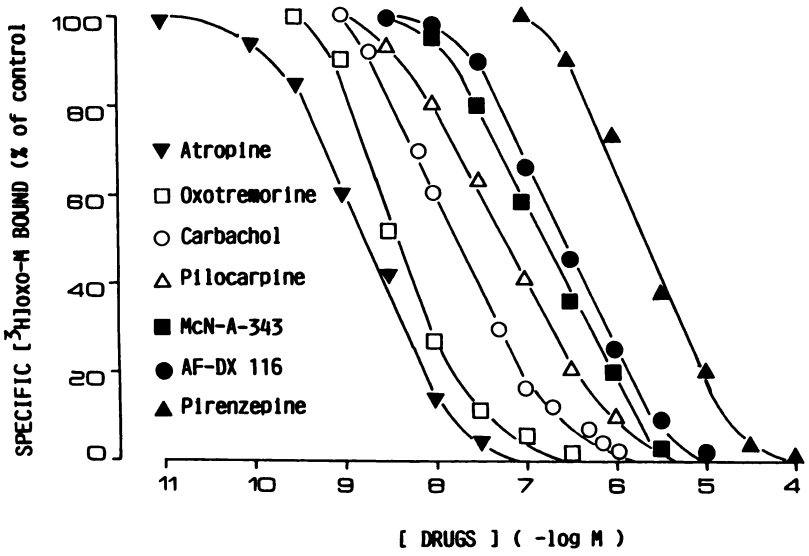

Fig. 6. Inhibition of [^{3}H]oxo-M binding by muscarinic agonists and antagonists. Experimental conditions were as described in Experimental Procedures. Values are means of five independent experiments performed in triplicate.

TABLE 4

Inhibition of [^{3}H]oxo-M binding in rat heart membranes by selected muscarinic ligands

$K_{0.5}$ values were determined from competitive binding curves (illustrated in Fig. 6). Data are given as means ± standard errors of three independent experiments performed in duplicate. Hill slope factors are shown in parentheses.

	$K_{0.5}$			
	Control		Treated	
	nM			
Oxotremorine	2.63 ± 0.20	(1.09)	3.85 ± 0.12	(1.4)
Carbachol	10.02 ± 1.75	(0.85)	9.71 ± 0.29	(0.88)
Pilocarpine	70.20 ± 1.94	(1.02)	86.04 ± 2.95	(1)
McN-A-343	210 ± 100	(0.88)	250 ± 170	(1.06)
Atropine	1.54 ± 0.14	(0.86)	0.95 ± 0.04	(0.88)
AF-DX 116	250 ± 70	(0.91)	238 ± 55	(0.86)
Pirenzepine	1680 ± 120	(1.18)	ND[a]	

[a]ND not determined.

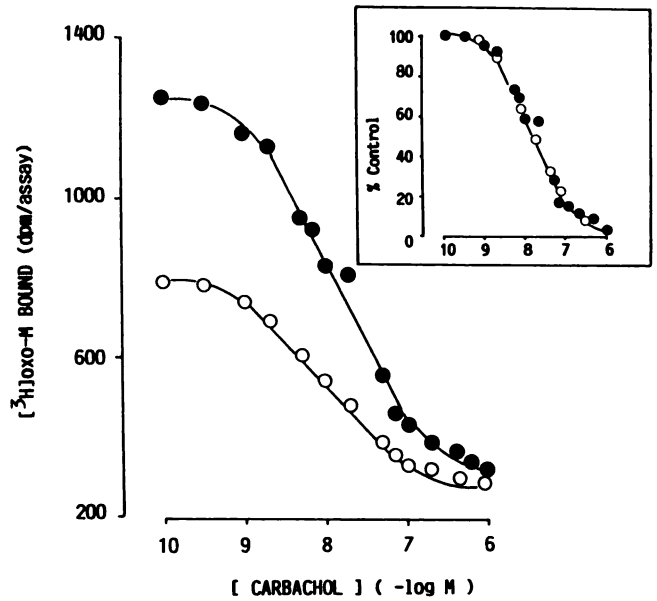

Fig. 7. Inhibition of [^{3}H]oxo-M binding by carbachol in the absence (●) or the presence (○) of neuraminidase (3 units/mg of protein). Data are from three experiments carried out in duplicate.

Neuraminidase affected only carbamylcholine binding to [^{3}H]QNB- and [^{3}H]NMS-labeled receptors. The binding of the M_1-selective agonist McN-A-343 and the M_1- (pirenzepine) and M_2- (AF-DX 116) selective antagonists was not affected by neuraminidase (Table 2). Neuraminidase reduced the affinity for carbamylcholine without affecting the biphasic profile of the competitive curves obtained with the two tracers (Fig. 5). The neuraminidase-induced shift in the carbachol competition curves was much more pronounced for the displacing of [^{3}H]QNB than of [^{3}H]NMS.

Because of the potential problem of proteolysis in the heart membranes, protease inhibitors [bacitracin (184 μg/ml) and 0.1 mM (PMSF)] were included in the buffer used for membrane preparation and for binding assays. The effects of neuraminidase obtained using carbachol against [^{3}H]QNB binding were similar to those obtained without these proteases inhibitors in the assay buffer (Table 3).

[^{3}H]Oxo-M binding. To characterize receptors labeled by [^{3}H]oxo-M, we compared the competitive binding curves of agonists and antagonists (Fig. 6). All the competitive binding curves had Hill coefficients equal to 1.0. We showed previously that carbamylcholine distinguishes different receptor states when [^{3}H]QNB or [^{3}H]NMS are used as tracers. In contrast, the Hill coefficients for competitive binding curves of carbachol for [^{3}H]oxo-M binding were close to 1, indicating that all [^{3}H]oxo-M-labeled receptors had the same affinity for carbachol (Table 4) (see also ref. 26). The carbachol K_i values for these receptors were comparable in the experiments performed with or without neuraminidase; only the number of binding sites was lower (Fig. 7).

Kinetics of [^{3}H]oxo-M binding. [^{3}H]Oxo-M binding reached steady state within 15 min of incubation, at the concentration used (0.37 nM) (Fig. 8). The association rate constant (k_1) was 1.49 ± 0.24 × 10^8 min^{-1} M^{-1} and 1.62 ± 0.17 × 10^8 min^{-1} M^{-1} for control and neuraminidase-pretreated membranes, respectively.

[^{3}H]Oxo-M dissociated from rat heart membranes with rate constants (k_{-1}) of 0.20 ± 0.01 min^{-1} for control and 0.47 ± 0.05 min^{-1} for neuraminidase-pretreated membranes (Fig. 8 *inset*), corresponding to a half-life of the complex of 3.36 ± 0.28 min

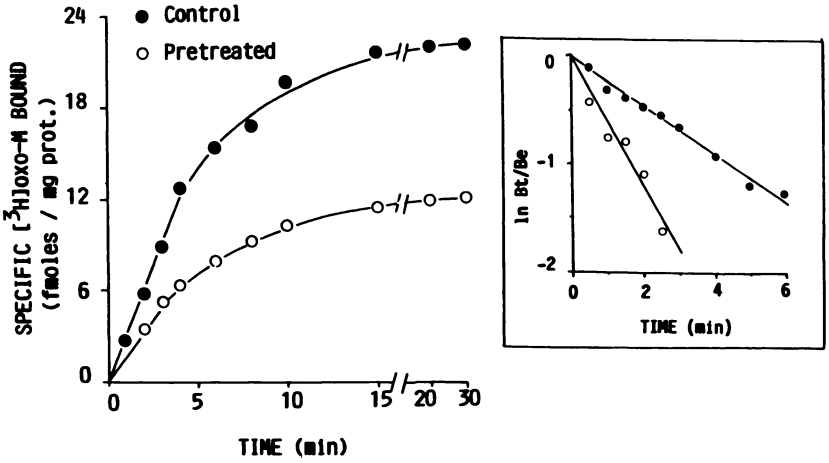

Fig. 8. Kinetics of [^{3}H]oxo-M binding to rat heart membranes. Washed rat heart membranes, which were pretreated with or without neuraminidase (3 units/mg of protein), were incubated with 0.37 nM [^{3}H]oxo-M at 25°. *Inset*, semilogarithmic plot of the dissociation kinetics data. The association and dissociation rate constants were calculated as indicated in Data analysis. The data shown are the average of duplicate determinations at each point and are representative of three similar experiments.

and 1.61 ± 0.18 min for control and neuraminidase-pretreated membranes, respectively.

The control kinetic parameters agree closely with those reported by Waelbroeck *et al.* (27). The kinetically determined dissociation constant (k_{-1}/k_1) for control (1.62 ± 0.22 nM) and neuraminidase-pretreated membranes (2.28 ± 0.29 nM) was similar to the K_D calculated under equilibrium binding conditions (1.59 nM; Table 1).

Discussion

The binding properties of the labeled antagonists [^{3}H]QNB and [^{3}H]NMS and of the labeled agonist [^{3}H]oxo-M to rat heart membranes were compared, in the absence and presence of neuraminidase.

Treatment of rat heart membranes with neuraminidase hydrolyzes 50% of the sialic acid residues with kinetics (Fig. 1) similar to those reported in heart cells (28). Sialic acid residues of the membrane surface are linked to glycoproteins and gangliosides (29). Neuraminidase treatment affected the binding of the M_2-selective agonist carbachol selectively by displacing the [^{3}H]QNB or [^{3}H]NMS competitive binding curves to the right, without changing the proportion of high and low affinity binding components (Table 2, Fig. 5). An irreversible decrease in the affinity was observed, as described in Experimental Procedures. Neuraminidase did not affect the binding profiles of the M_1-selective agonist McNA343 or of the M_1- and M_2-selective antagonists pirenzepine and AF-DX 116. The K_D and B_{max} of the two radiolabeled antagonists were not affected. The decrease in affinity noted for carbachol could not be attributed to a decrease in the affinity of the tracers ([^{3}H]QNB and [^{3}H]NMS) or to a decrease in the population of the high affinity binding component of carbachol, which remained constant after neuraminidase treatment (42–45%; Table 2), as revealed by competitive binding experiments with [^{3}H]QNB or [^{3}H]NMS. Moreover, this neuraminidase effect could not be attributed to protease activity, because the shift was also observed in the presence of protease inhibitors (Table 3).

It is important to understand the nature of the membrane sites labeled by [^{3}H]oxo-M under the present assay conditions. As shown originally by Birdsall *et al.* (26), muscarinic agonists apparently bind to multiple forms or states of a single muscarinic receptor drug binding site. These states for carbamylcholine, which were originally referred to as super-high, high, and low affinity agonist states, appear to exist in various proportions within most tissues. Despite this multiplicity of agonist binding states, under the assay conditions described here, [^{3}H]oxo-M appears to label a single high affinity state (K_D = 1.59 nM) in the myocardium. The dissociation constant (K_D) of this [^{3}H]oxo-M binding state is similar to the indirectly predicted values for the super-high affinity agonist binding state (26). Therefore, throughout this manuscript, we have referred to the [^{3}H]oxo-M binding state as a "high affinity agonist state," which is obviously the highest affinity state detectable for [^{3}H]oxo-M under these assay conditions. The possibility cannot, however, be excluded that a second population of binding sites may exist with lower affinity (high affinity), as reported by Bevan (30). [^{3}H]QNB and [^{3}H]NMS binding revealed high affinity agonist binding states. The affinity of carbachol for these binding sites was lower than that for the [^{3}H]oxo-M binding sites, suggesting that these sites may be different. Neuraminidase decreased the number of sites labeled by [^{3}H]oxo-M (super-high affinity sites), whereas [^{3}H]QNB- and [^{3}H]NMS-labeled muscarinic receptors were unaffected. Neuraminidase affected the B_{max} but apparently not the K_D (Fig. 3). The dissociation rate constant (k_{-1}) for [^{3}H]oxo-M is increased after neuraminidase treatment from 0.20 ± 0.01 min^{-1} to 0.47 ± 0.05 min^{-1} (Fig. 8).

In competition studies with [^{3}H]QNB or [^{3}H]NMS, only the high and low affinity agonist binding sites were revealed; the neuraminidase effect was only observed by a shift of the carbachol competition curve to the right and not by a decrease of the super-high affinity agonist binding sites, because these were not revealed by the competition experiments.

The finding that neuraminidase inhibits [^{3}H]oxo-M binding without affecting the total number of receptors measured with [^{3}H]QNB or [^{3}H]NMS agrees with reports that GTP (27) or *N*-ethylmaleimide (31) reduces high affinity agonist binding to muscarinic receptors. Our results show that the removal of sialic acid reduces the formation of the super-high affinity agonist-receptor complex (i.e., the B_{max} of [^{3}H]oxo-M decreases), but the K_D of the remaining super-high affinity sites is not affected. Approximately 60% of the [^{3}H]oxo-M binding activity remains after neuraminidase treatment. We suggest that sialic acid is necessary for super-high affinity agonist binding. One explanation for these data might be that the charged agonists (carbachol, [^{3}H]oxo-M) bind to sialic acid by ionic interactions between the positively charged quaternary ammonium of the agonists and the negatively charged carboxyl

group of sialic acid. Neuraminidase releases sialic acid and thus, alters the agonist-sialic acid interaction, which in turn reduces agonist binding. Several lines of investigation demonstrate that neuraminidase digestion, which specifically releases sialic acid, alters selectively the serotonin receptor in isolated smooth muscles (32), increases insulin binding (33), enhances the accessibility of β-adrenergic receptors (34), and decreases the number of α-adrenergic and muscarinic receptors (35). The loss of agonist binding upon sialic acid removal could be due to loss of sialic acid either from the muscarinic receptor or from some other component of the membrane surface. Sialic acid might catalyze macroscopic binding by enhancing agonist interaction at the membrane surface. As Sargent and Schwyzer (36) proposed for peptide ligands, the importance of the membrane as an antenna for and modifier of ligand-receptor interactions has to be considered. Our present studies revealed that sialic acid contributes to the formation of the highest affinity agonist binding sites but did not determine whether sialic acid residues, in the vicinity of or on the muscarinic receptor, are important for muscarinic receptor function. Further studies are underway to elucidate the physiological significance of sialic acid residues in modulating muscarinic agonist effects.

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Send reprint requests to: Jean Pierre Gies, Laboratoire de Neuroimmunopharmacologie, Université Louis Pasteur-Strasbourg I, BP 24, 67401 Illkirch Cedex, France.