0026-895X/87/010100-09$02.00/0
Copyright © by The American Society for Pharmacology and Experimental Therapeutics
All rights of reproduction in any form reserved.
MOLECULAR PHARMACOLOGY, 32:100-108

MOL PHARM

Muscarinic Receptor Heterogeneity in Rat Central Nervous System. II. Brain Receptors Labeled by [^{3}H]Oxotremorine-M Correspond to Heterogeneous M2 Receptors with Very High Affinity for Agonists

MICHEL GILLARD, MAGALI WAELBROECK, and JEAN CHRISTOPHE

Department of Biochemistry and Nutrition, Medical School, Université Libre de Bruxelles, B-1000 Brussels, Belgium (M.G., M.W., J.C.), and UCB Research Laboratories, Chemin Le Foriest, B-1420 Braine l'Alleud, Belgium (M.G.)

Received December 15, 1986; Accepted April 14, 1987

SUMMARY

We compared the binding characteristics of muscarinic receptors labeled by [^{3}H]oxotremorine-M ([^{3}H]oxo-M) in homogenates of brain cortex and heart from rat. In both tissues [^{3}H]oxo-M bound, with the same K_D (6.5 nM), to a fraction of the receptors labeled by [^{3}H]-*N*-methylscopolamine ([^{3}H]NMS). This [^{3}H]oxo-M receptor population represented, respectively, 15–20% and 35–40% of the total number of [^{3}H]NMS receptors in cortex and heart. The three unlabeled agonists oxotremorine, carbamylcholine, and pilocarpine, when tested in competition with [^{3}H]oxo-M, displayed a homogeneous super high affinity toward [^{3}H]oxo-M-labeled receptors, and were unable to discriminate between brain and heart receptors labeled by [^{3}H]oxo-M. By contrast, selective muscarinic antagonists showed some selectivity for either brain or heart [^{3}H]oxo-M-labeled receptors. We analyzed competition curves between [^{3}H]oxo-M and secoverine, pirenzepine, AF-DX 116, dicyclomine, or gallamine, assuming the existence of one or two receptor subclasses. Heart muscarinic receptors labeled by [^{3}H]oxo-M were homogeneous M2 receptors of the C type with very low affinity for pirenzepine (K_I = 400 nM). Brain [^{3}H]oxo-M-labeled receptors were heterogeneous receptors, with 30% (the B type) having a higher affinity for dicyclomine and a lower affinity for AF-DX 116 and gallamine than cardiac receptors, whereas the remaining 70% (the C type) showed "cardiac-like" binding properties. Both [^{3}H]oxo-M-labeled subtypes in cortex homogenates had a low affinity for pirenzepine, indicating that [^{3}H]oxo-M labeled only B and C (M2) receptors in this tissue. GTP inhibited completely [^{3}H]oxo-M binding in heart homogenates with an IC_{50} at 300 nM. In cortex homogenates, GTP showed the same potency, but its efficacy was much lower (with only 30% maximal inhibition). [^{3}H]oxo-M dissociation kinetics were monophasic in heart homogenates and biphasic in cortex homogenates. [^{3}H]oxo-M dissociation from both tissues was slowed by gallamine and *d*-tubocurarine and accelerated by GTP. We found no correlation between B versus C [^{3}H]oxo-M receptors, GTP-sensitive versus GTP-insensitive receptors, and rapidly versus slowly dissociating receptors, suggesting that [^{3}H]oxo-M labeled a large variety of muscarinic receptor-regulatory protein complexes, all having an SH affinity for agonists.

Many *in vitro* and *in vivo* studies support the hypothesis of muscarinic receptor heterogeneity (for review see Refs. 1 and 2). Two classifications have been proposed: 1) Birdsall *et al.* (3) suggested one based on agonist binding. Indeed, muscarinic agonists discriminate three states or types of receptors: SH, H, and L receptors with, respectively, super high (SH), high (H), and low (L) affinity for agonists (3); 2) Hammer *et al.* (4) demonstrated the existence of three receptor subtypes with different affinities for pirenzepine, the first selective antagonist available. These receptors were called A, B, and C receptors (5) with, respectively, high, intermediate, and low affinity for pirenzepine. The A, B, and C receptor subtypes are often reduced to two: the A subtype is then called "M1" and the B and C subtypes "M2." The relationship that could exist between agonist binding and pirenzepine binding classifications is still unclear.

Rat brain muscarinic receptors consist of a mixture of M1 and M2 receptors, based on pirenzepine binding studies (4, 5). Our previous work (6, 7) indicated that brain M2 receptors are heterogeneous so that three, rather than two, muscarinic receptor subtypes, corresponding to the A, B, and C receptor subtypes as defined by Hammer *et al.* (4) and Birdsall *et al.* (5) coexist in rat brain homogenates.

This research was aided by Grant 3.4571.85 from the Fonds de la Recherche Scientifique Médicale (Belgium).

ABBREVIATIONS: [^{3}H]PZ, [*N-methyl*-^{3}H]pirenzepine; [^{3}H]NMS, [*N-methyl*-^{3}H]scopolamine methylchloride; [^{3}H]oxo-M, [*methyl*-^{3}H]oxotremorine M acetate; AF-DX 116, [11-({2-[(diethylamino)-methyl]-1-piperidinyl} acetyl)-5,11-dihydro-6H-pyrido(2,3-*b*) (1,4)benzodiazepin-6-on]; 4-DAMP, 4-diphenylacetoxy-*N*-methyl-piperidine methbromide; I_{50}, concentration of unlabeled drug necessary to inhibit 50% of tracer binding; K_I, unlabeled drug dissociation constant; K_D, tracer dissociation constant; k_{on}, association rate constant; k_{off}, dissociation rate constant.

MOLECULAR PHARMACOLOGY
aspet
Downloaded from molpharm.aspetjournals.org at Universidade do Estado do Rio de Janeiro on December 5, 2012

In this work, we investigated the correlation between SH, H, and L agonist receptor subclasses and A, B, and C antagonist receptor subclasses. To achieve this aim, we: 1) analyzed agonist binding to A receptors (labeled by [^{3}H]PZ), B receptors (labeled with [^{3}H]NMS), and total receptors (A + B + C) (labeled by [^{3}H]NMS); and 2) examined the binding of selective antagonists to SH receptors labeled by [^{3}H]oxo-M, an agonist. Since these results indicated that most (but not all) receptors in the SH state belonged to the C "cardiac-like" receptor subclass, we compared [^{3}H]oxo-M binding and association and dissociation kinetics in heart and brain homogenates.

Materials and Methods

[^{3}H]NMS (74 Ci/mmol) and [^{3}H]oxo-M (72 Ci/mmol) were purchased from the Radiochemical Centre (Amersham, Bucks, England). [^{3}H]PZ (84 Ci/mmol) was obtained from New England Nuclear (Dreieich, FRG).

The following drugs were generous gifts: pirenzepine (gastrozepin) from Dr. R. Hammer (Institute di Angeli, Milano, Italy) and dicyclomine chloride from Merrel Dow (Brussels, Belgium). Dr. A. Giachetti (Institute di Angeli, Milano, Italy) helped us to obtain AF-DX 116 from Dr. K. Thomae GmbH (Biberach, FRG). Atropine sulfate, carbamylcholine chloride, gallamine triethiodide, and *d*-tubocurarine chloride were from Sigma Chemical Co. (St. Louis, MO). Pilocarpine was purchased from Janssen Chemica (Beerse, Belgium).

Tissue Preparation

Male Wistar albino rats (200–300 g) were sacrificed by decapitation, and the brain cortex and the whole heart were rapidly removed. Cortices were homogenized (5%, w/v) in an ice-cold 20 mM Tris-HCl (pH 7.5) buffer containing 250 mM sucrose and then stored until use in liquid nitrogen. Hearts were first rinsed in an isotonic solution (0.9% NaCl) and thereafter were homogenized in 2–3 ml of the same buffer as cortex, using an Ultraturrax homogenizer at maximal speed for 5 sec. Cardiac homogenates were filtered through two layers of medical gauze and were further diluted to a final concentration of 15% (w/v) and stored in liquid nitrogen until use.

Binding Experiments

Incubation conditions were adapted for each tracer or tissue to: 1) allow tracer binding to reach equilibrium, 2) keep the proportion of bound tracer below 20% of the total radioactivity offered, and 3) use a tracer concentration below or close to its K_D value for the receptors investigated.

Nonspecific binding was defined as binding observed in the presence of 1 μM atropine. In the system described below, nonspecific binding was essentially due to tracer binding to the filters. To reduce this binding, we presoaked all filters, for at least 2 hr, in 0.05% polyethyleneimine. After this pretreatment, the remaining nonspecific [^{3}H]oxo-M binding did not exceed 10–15% of total binding in brain cortex, and 20–25% in heart at the highest [^{3}H]oxo-M concentration used. It was below 10% of total binding when using [^{3}H]NMS (for both tissues) or [^{3}H]PZ (for cortex homogenates).

[^{3}H]oxo-M binding. Incubations were carried out at 25° for 10 min in 1 ml of 50 mM sodium phosphate buffer (pH 7.4) enriched with 2 mM $MgCl_2$, 1% bovine serum albumin, appropriate unlabeled drug concentrations, 2.7 nM [^{3}H]oxo-M, and 400 μg of protein from cortex or 600 μg of protein from heart homogenates. After this incubation, 2 ml of ice-cold 50 mM phosphate buffer (pH 7.4) were added to each sample, followed by rapid filtration through GF/C glass-fiber filters (Whatman, Maidstone, England). The filters were rinsed three times with 2 ml of the same buffer. After drying, the radioactivity was counted in a Toluol-Permablend cocktail.

[^{3}H]oxo-M saturation curves were obtained in cortex and heart homogenates using tracer concentrations varying from 0.2 to 15 nM.

[^{3}H]oxo-M dissociation kinetics were obtained as follows: after equilibrium was reached, tracer dissociation was induced by isotopic dilution with 1 μM atropine or 1 μM atropine plus the indicated concentration of the agent studied (GTP, gallamine, *d*-tubocurarine).

[^{3}H]NMS binding. We used the same experimental conditions as for [^{3}H]oxo-M except for the incubation period and protein concentration. With cortex homogenates, incubations were performed at 25° for 2 hr, using 40 μg of protein/ml, in the presence of 0.2 nM [^{3}H]NMS. When using heart homogenates, the incubation time was 15 min, the protein concentration was 120 μg/ml, and the [^{3}H]NMS concentration was 0.4 nM.

[^{3}H]NMS saturation curves were obtained in both tissues using a concentration range varying from 0.025 to 2 nM [^{3}H]NMS.

[^{3}H]PZ binding. Experiments with this tracer were performed only in cerebral cortex, under conditions similar to those used for [^{3}H]oxo-M. Protein concentration was 200 μg/ml, the incubation time was 1 hr, and the tracer concentration was 4 nM.

Protein Concentrations

Protein concentrations were determined according to the method of Lowry *et al.* (8) with bovine serum albumin as standard.

Data Analysis

Saturation curves were transformed according to the method of Scatchard (9) and analyzed by linear regression. Total amount of receptors (B_{max}) and dissociation constant (K_D) were given as mean ± standard deviation. Competition curves were analyzed by a computer-assisted curve-fitting procedure (10) as discussed in Results. Whenever possible, we used the Cheng and Prusoff equation (11) to calculate the unlabeled drug K_i value.

Results

Carbamylcholine binding to [^{3}H]oxo-M-, [^{3}H]NMS-, and [^{3}H]PZ-labeled receptors. [^{3}H]oxo-M may bind to SH and H receptors in brain (3). By contrast with the results of Birdsall *et al.* (3), agonist/[^{3}H]oxo-M competition curves obtained in heart and brain homogenates were superimposable with a Hill coefficient equal to 1.0 under our incubation conditions. [^{3}H]oxo-M labeled SH-like receptors only, as indicated by the very high carbamylcholine affinity in both tissues. This result suggested that [^{3}H]oxo-M dissociated from the H receptors during filtration.

By contrast, carbamylcholine/[^{3}H]NMS competition curves (in heart and brain) and carbamylcholine/[^{3}H]PZ competition curves (in brain) yielded Hill coefficients below 1.0, indicating that the agonist recognized two or more antagonist-labeled sites with different affinities. We therefore used a computer-assisted curve-fitting procedure to determine the proportion and affinity for carbamylcholine of each subclass.

As shown in Fig. 1, carbamylcholine inhibited [^{3}H]NMS binding at lower concentrations in rat heart (a pure C receptor population) as compared to rat cortex (a mixture of A, B, and C receptors). Analysis of the competition curves shown in Fig. 1 indicated that this result reflected a greater proportion of high affinity sites (SH or cardiac "high affinity" and H or cardiac "low affinity") in heart, rather than greater agonist affinity constants, in heart as compared to brain homogenates. The presence of L (cardiac "very low affinity") sites in heart was, in fact, not statistically significant in the absence of GTP.

We have previously demonstrated that [^{3}H]NMS recognizes three receptor types with different affinities in brain. We therefore attempted to measure separately the carbamylcholine interaction with each subtype. To analyze agonist binding to

MOL PHARM
MOLECULAR PHARMACOLOGY
aspet
Downloaded from molpharm.aspetjournals.org at Universidade do Estado do Rio de Janeiro on December 5, 2012

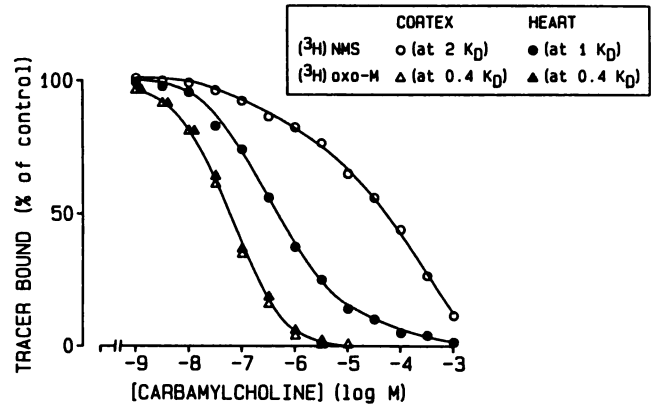

Fig. 1. Comparison of [^{3}H]NMS and [^{3}H]oxo-M-labeled receptors in cerebral cortex and heart homogenates from rat. Carbamylcholine competition for binding of 0.2 nM [^{3}H]NMS in cortex (○), 0.4 nM [^{3}H]NMS in rat heart (●), and 2.4 nM [^{3}H]oxo-M in cortex (Δ) and heart (▲) were obtained as explained under Materials and Methods. The results are representative of three to eight experiments performed in duplicate.

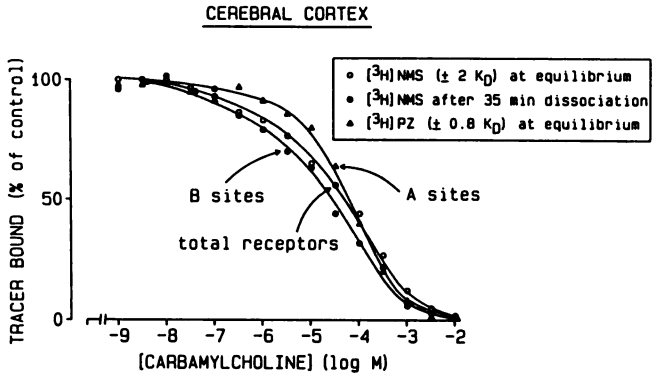

Fig. 2. Comparison of carbamylcholine binding to rat cerebral cortex A receptors, B receptors, and total receptors labeled at low [^{3}H]NMS concentration. ○, total receptors. Cerebral cortex homogenates were incubated in the presence of 0.2 nM [^{3}H]NMS and the indicated carbamylcholine concentrations, and duplicate samples were filtered at equilibrium. ●, B sites. Cerebral cortex homogenates were preincubated in the presence of 0.2 nM [^{3}H]NMS and the indicated carbamylcholine concentrations. After 35 min of isotopic dilution, [^{3}H]NMS had dissociated from more than 99% of C receptors, 97% of A receptors, and 50% of B receptors (see Refs. 6 and 7). Residual [^{3}H]NMS binding (to B receptors) was measured by filtration, as explained under Materials and Methods. ▲, A sites, Cerebral cortex homogenates were incubated in the presence of a low 5 nM [^{3}H]PZ concentration and the indicated carbamylcholine concentrations. Under these conditions, about 90% of the labeled receptors belonged to the A type. Representative of three to eight experiments performed in duplicate.

the A subtype, we used competition curves against a low [^{3}H]PZ concentration (Fig. 2). We were unable to detect any SH receptors when using [^{3}H]PZ as tracer. One-fifth of A receptors had H affinity for carbamylcholine, the other receptors having L affinity (Table 1). To analyze agonist binding to the dissociation-resistant B subtype, we used the isotopic dilution technique previously described (6, 7). As shown in Fig. 2, B receptors had a slightly higher than average affinity for carbamylcholine. Analysis of competition curves suggested that this was due to a rather large proportion of sites with SH or H affinity for carbamylcholine (Table 1). Taken together, A sites (42% of [^{3}H]NMS-labeled receptors) and B sites (44% of [^{3}H]NMS-labeled receptors) accounted for all [^{3}H]NMS binding to sites with L affinity for carbamylcholine, most of [^{3}H]NMS binding

TABLE 1

I_{50} values (in μM) and percentage of [^{3}H]NMS bound to muscarinic receptor subclasses SH, H, and L as determined from [^{3}H]NMS-carbamylcholine competition curves in rat heart and brain homogenates

Subclasses		SH	H	L	Total
Heart[a]	I_{50}[b]	0.08	2.0	60	0.5
	%	45	47	8	100
Brain cortex[c]					
Total sites	I_{50}	0.1	10	300	
	%	15	35	50	
B sites	I_{50}	0.1	10	300	2.5
	%	9	19	17	45
A sites	I_{50}	—	3	100	6.3
	%	0	8	34	42
A sites + B sites	%	9	27	51	87
(Total − (A + B) sites)	%	6	8	0	13

[a] The [^{3}H]NMS concentration used was 0.4 nM (0.8 K_D value in heart).
[b] Carbamylcholine K_i values, obtained by applying the Cheng and Prusoff equation (11) to data corresponding to a single receptor population, were as follows: C sites = 0.04/1.1/21.0 μM; B sites = 0.04/2.8/85 μM; A sites = −/1.7/56 μM. The differences in K_i values were not statistically significant.
[c] The [^{3}H]NMS concentration used (0.2 nM) was equal to 1.3 K_D for A sites, 2.5 K_D for B sites, and 0.4 K_D for C sites in brain. The [^{3}H]PZ concentration was 5 nM = 0.8 K_D value for A sites.

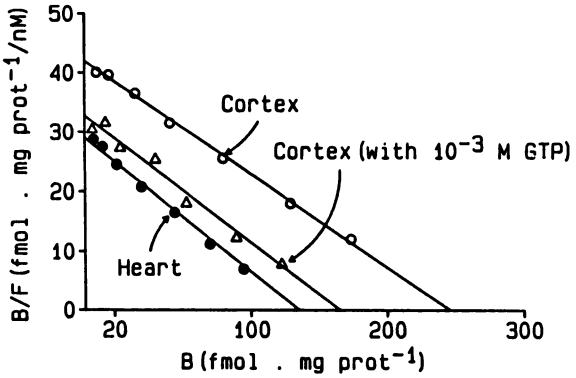

Fig. 3. [^{3}H]oxo-M Scatchard plots. Brain cortex (Δ, ○) and heart (●) homogenates were incubated in the presence of 0.2–15 nM [^{3}H]oxo-M, and in the absence (○, ●) or presence (Δ) of 10^{-3} M GTP. Specific binding was measured as explained under Materials and Methods. The results are represented according to the method of Scatchard (9). [^{3}H]oxo-M binding in heart was not detectable in the presence of GTP. Representative of three to five experiments in duplicate.

to sites with H affinity, and about half of [^{3}H]NMS binding to sites with SH affinity (see Table 1). This suggested that, as in rat heart, C receptors had SH or H affinity for carbamylcholine, with few or no receptors with L affinity.

[^{3}H]oxo-M saturation curves. A representative saturation curve is shown in Fig. 3. Typically, we obtained, at a 15 nM [^{3}H]oxo-M concentration, a total binding of about 5000 cpm per assay in cortex and 3000 cpm per assay in heart with a nonspecific binding of 500–600 cpm. In both tissues, [^{3}H]oxo-M bound with the same high affinity to a single population of receptors (Table 2). Receptors labeled by [^{3}H]oxo-M represented 15–20% only as opposed to 40–50% of the total amount of receptors determined by [^{3}H]NMS binding in, respectively, brain cortex and heart homogenates (Table 2).

Agonist competition curves for [^{3}H]oxo-M binding. To characterize receptors labeled by [^{3}H]oxo-M, we compared competition curves of carbachol for [^{3}H]oxo-M binding in both tissues (Fig. 1). We had previously shown that carbamylcholine

MOL PHARM
MOLECULAR PHARMACOLOGY
aspet
Downloaded from molpharm.aspetjournals.org at Universidade do Estado do Rio de Janeiro on December 5, 2012

TABLE 2

Comparison of [^{3}H]oxo-M binding and [^{3}H]NMS binding to rat heart and cortex receptors

	Cortex	Heart
[^{3}H]oxo-M		
K_D (nM)	6.7 ± 0.4	6.4 ± 1.0
B_{max} (fmol · mg prot^{-1})	218 ± 18	151 ± 14
[^{3}H]NMS		
K_D (nM)	0.15 ± 0.02	0.49 ± 0.03
B_{max} (fmol · mg prot^{-1})	1212 ± 65	353 ± 22
B_{max} [^{3}H]oxo-M/[^{3}H]NMS	18%	42%

distinguishes different receptor states when [^{3}H]NMS is used as tracer. By contrast, competition curves of carbachol for [^{3}H]oxo-M binding were superimposable in both tissues (Fig. 1). The Hill coefficients of these curves were close to 1.0, indicating that all [^{3}H]oxo-M-labeled receptors had the same affinity for carbachol (Table 3) (see also Ref. 3). The carbachol K_i value for these receptors was comparable to that calculated for SH receptors by analysis of [^{3}H]NMS competition curves (compare data shown in Tables 1 and 3).

Oxotremorine and pilocarpine (two muscarinic agonists) also displaced [^{3}H]oxo-M binding, as expected for drugs recognizing all receptors with the same affinity (Fig. 4). The K_i values for the three agonists studied were the same in cortex and heart homogenates (Fig. 4, Table 3), and compatible with values expected for SH receptors (3).

Antagonist competition curves. Analysis of ^{3}H-labeled antagonist binding suggested that SH receptors belong to types B and C. To confirm this hypothesis, we performed competition curves between [^{3}H]oxo-M and selective or nonselective muscarinic antagonists. Secoverine displayed no selectivity for receptors labeled by [^{3}H]oxo-M in the two tissues (Fig. 5). By contrast, pirenzepine had a 2.5-fold higher affinity for brain receptors (Table 3, Fig. 5). The best fit was obtained, in each case, with a one-site model, suggesting that [^{3}H]oxo-M-labeled receptors were more than 95% made of M2 (= B + C?) receptors. (An M1 population representing 5% or less of the total binding is undetectable in these experiments.)

Competition curves of AF-DX 116 (a cardioselective molecule) and dicyclomine (an A/B preferring drug) are shown in Figs. 6 and 7, and data analysis is given in Table 3. AF-DX 116 recognized 71% of brain SH receptors with a high affinity, similar to that for cardiac receptors (C sites). Low affinity receptors had an AF-DX 116 K_i value compatible with binding to A or B sites [see the accompanying paper (7)].

Dicyclomine bound with high affinity to 33% of brain receptors (with a K_i value typical of A/B sites); the 67% remaining receptors displayed a low affinity for dicyclomine, comparable to that found in cardiac tissue (C sites).

Since we were unable to detect [^{3}H]oxo-M-labeled sites with high affinity for pirenzepine (A sites), the above results indicated that SH sites could be subdivided into 65–70% C sites and 30–35% B sites. This was in full agreement with ^{3}H-labeled antagonist-carbamylcholine competition curves as: 1) we could not find any A receptors with SH affinity for carbamylcholine, and 2) C receptors were responsible for half of the tracer binding to SH sites at low [^{3}H]NMS concentrations (i.e., under conditions where C receptors were underrepresented when compared to B receptors).

Gallamine (gallamine triethiodide) competition curves for [^{3}H]oxo-M binding were comparable to AF-DX 116 competition curves: gallamine recognized 65% of cortex receptors with an affinity similar to its affinity for heart receptors, and 35% of cortex receptors with a 30-fold lower affinity. Gallamine behaves as a cardioselective allosteric antagonist on isolated organs (reviewed in Ref. 12). The above results suggested that this cardioselectivity partially reflected at least preferential inhibition of agonist binding to C as compared to B receptors.

Taken together, these results suggested that: 1) [^{3}H]oxo-M-labeled SH receptors were 65–70% C receptors and 30–35% B receptors, and 2) gallamine distinguished B and C receptors, being selective for C receptors.

Effects of GTP on [^{3}H]oxo-M binding. We examined the effects of GTP on [^{3}H]oxo-M binding in both tissues. As shown in Fig. 8, GTP completely inhibited [^{3}H]oxo-M binding in heart homogenates but reduced [^{3}H]oxo-M binding in cortex homogenates by only 30%. This guanine nucleotide was equipotent in both tissues with a half-maximal inhibitory concentration of about $3 \cdot 10^{-7}$ M.

GTP (10^{-3} M) addition to the cortex incubation medium

TABLE 3

Comparison of [^{3}H]oxo-M binding inhibition by three muscarinic agonists and five muscarinic antagonists, in rat cortex and heart homogenates

[^{3}H]oxo-M	Cortex		$R1$[b]	n_H[c]	Heart	
	K_{i1}[a]	K_{i2}[a]			K_i[a]	n_H[c]
			%			
Agonists						
Oxotremorine	7.1 ± 1.1		100	0.93	6.6 ± 0.8	1.02
Carbamylcholine	40 ± 5		100	0.91	42 ± 7	0.88
Pilocarpine	254 ± 35		100	0.88	330 ± 61	0.90
Antagonists						
Secoverine	10.1 ± 1.3		100	1.14	9.2 ± 1.3	1.12
Pirenzepine	201 ± 30[d]		100	0.90	495 ± 63	1.01
Dicyclomine[e]	3.5 ± 0.6	72 ± 11	33	0.79[e]	128 ± 28	0.99
AF-DX 116[e]	65.0 ± 5.6	702.6 ± 98.4	71	0.72[e]	76.9 ± 8.1	0.95
Gallamine[e]	90 ± 13	2797 ± 415	65	0.64[e]	124 ± 17	0.87

[a] K_i values, calculated using the Cheng and Prusoff equation (11) were expressed in nM ± standard deviation. The validity of this procedure was verified by comparing secoverine, carbamylcholine, AF-DX 116, and dicyclomine competition curves at 3 and 18 nM [^{3}H]oxo-M.

[b] Proportion of high affinity $R1$ receptors (percentage of total).

[c] Hill slope.

[d] Significantly lower value in cortex, when compared to heart ($p < 0.01$).

[e] Significantly ($p < 0.01$) better fitted by a two-site model in cortex.

MOL PHARM
MOLECULAR PHARMACOLOGY
aspet
Downloaded from molpharm.aspetjournals.org at Universidade do Estado do Rio de Janeiro on December 5, 2012

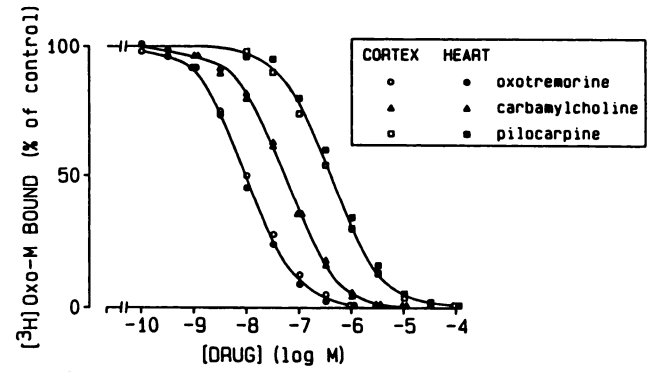

Fig. 4. [^{3}H]oxo-M competition curves in rat heart and cortex. Heart (●, ▲, ■) and cortex (○, △, □) homogenates were incubated in the presence of 2.4 nM [^{3}H]oxo-M and the indicated concentrations of oxotremorine (●, ○), carbamylcholine (▲, △), and pilocarpine (■, □). Specific binding was measured as explained under Materials and Methods. Representative of three to five experiments performed in duplicate.

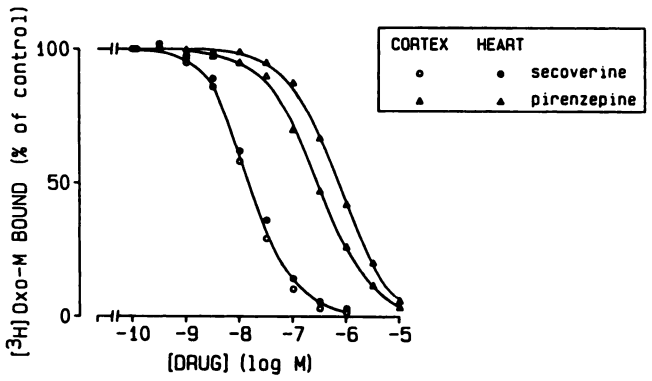

Fig. 5. [^{3}H]oxo-M/secoverine or pirenzepine competition curves in rat heart and cerebral cortex. Heart (●, ▲) and cortex (○, △) homogenates were incubated in the presence of 2.4 nM [^{3}H]oxo-M and the indicated concentrations of secoverine (●, ○) or pirenzepine (▲, △). Specific binding was measured as explained under Materials and Methods. Representative of three to five experiments performed in duplicate.

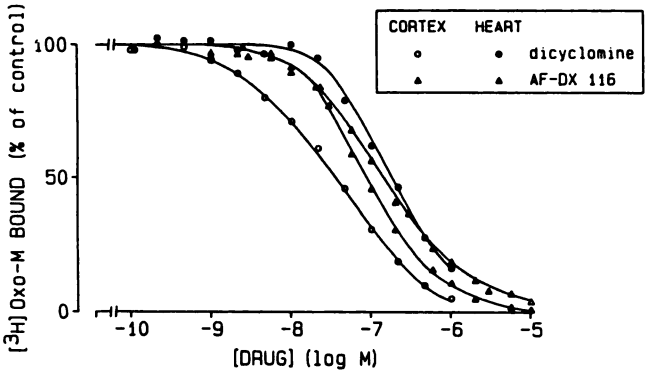

Fig. 6. [^{3}H]oxo-M/dicyclomine or AF-DX 116 competition curves in rat heart and cerebral cortex. Heart (●, ▲) and cortex (○, △) homogenates were incubated in the presence of 2.4 nM [^{3}H]oxo-M and the indicated concentrations of dicyclomine (●, ○) or AF-DX 116 (▲, △). Specific binding was measured as explained under Materials and Methods. Average of 8–10 experiments performed in duplicate.

slightly decreased [^{3}H]oxo-M binding. The affinity of [^{3}H]oxo-M (Fig. 2), and AF-DX 116 and dicyclomine (results not shown), as well as the proportions of high and low affinity receptors (for AF-DX 116 and dicyclomine) were not modified by GTP in rat brain. This suggested that [^{3}H]oxo-M binding

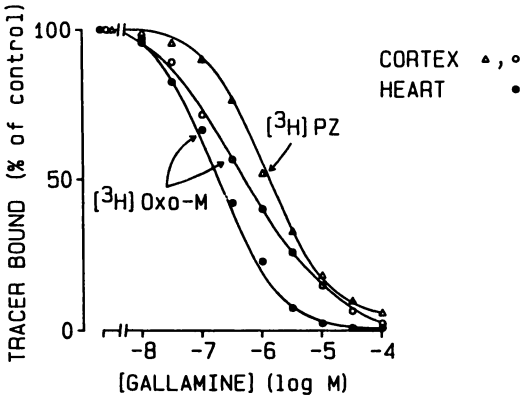

Fig. 7. [^{3}H]oxo-M/gallamine competition curves in rat heart and cerebral cortex. Heart (●) and cortex (○, △) homogenates were incubated in the presence of 2.4 nM [^{3}H]oxo-M (●, ○) or 4 nM [^{3}H]PZ (cortex only) (△) and the indicated concentrations of gallamine. Specific binding was measured as indicated under Materials and Methods. Average of five to eight experiments performed in duplicate.

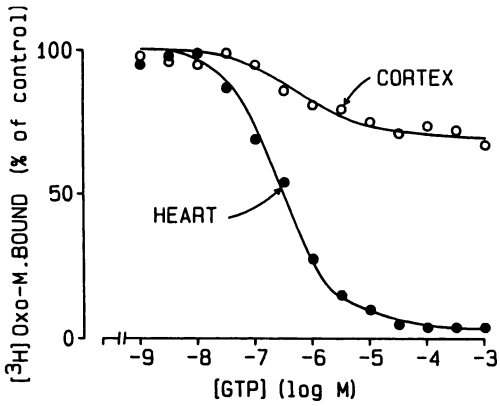

Fig. 8. Effects of GTP on [^{3}H]oxo-M binding. Heart (●) and cortex (○) homogenates were incubated in the presence of 2.4 nM [^{3}H]oxo-M and the indicated GTP concentrations. Specific binding was measured as explained under Materials and Methods. Representative of four experiments performed in duplicate.

to B and C receptors was equally GTP insensitive in cortex. In heart homogenates we did not observe any [^{3}H]oxo-M binding in the presence of 10^{-3} M GTP.

We considered the possibility that a factor present in the cortex preparation was responsible for reversibly or irreversibly destroying the GTP sensitivity of SH sites. However, co-incubation of cortex and heart homogenates gave a [^{3}H]oxo-M/GTP inhibition curve that was precisely that expected from [^{3}H]oxo-M binding in control unmixed homogenates (not shown).

Kinetic experiments. [^{3}H]oxo-M dissociated from heart muscarinic receptors with a k_{off} of 0.37 min^{-1}, corresponding to a half-life of about 2 min (Fig. 9). The analysis of association kinetics yielded a k_{on} value of $6.11 \cdot 10^7$ min$^{-1} \cdot$nM^{-1} (data not shown). The k_{off}/k_{on} ratio was 6.06 nM, in good agreement with the K_D value found in saturation experiments (Fig. 2).

[^{3}H]oxo-M kinetics were more complex in brain homogenates: as shown in Fig. 9, dissociation kinetics were curvilinear with the slow component representing about 20% of total binding. We calculated the expected [^{3}H]oxo-M association kinetics, using the dissociation rate constants found in Fig. 9 (2.3 min^{-1} and 0.15 min^{-1}), and assuming an overall K_D value of 6 nM.

MOL PHARM
MOLECULAR PHARMACOLOGY
aspet
Downloaded from molpharm.aspetjournals.org at Universidade do Estado do Rio de Janeiro on December 5, 2012

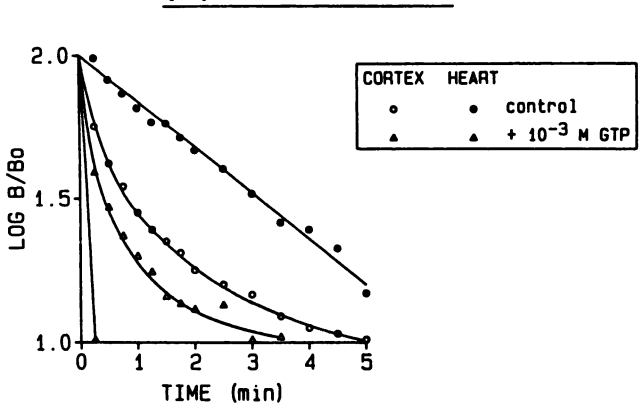

Fig. 9. Effects of GTP on [³H]oxo-M dissociation from cerebral cortex and heart receptors. Effect of GTP. Heart (●, ▲) and cortex (○, △) homogenates were preincubated for 15 min at 25° in the presence of 2.4 nM [³H]oxo-M. Dissociation was then induced by addition of 1 μM atropine (control: ○, ●) or 1 μM atropine and 10^{-3} M GTP (+GTP: △, ▲). Duplicate samples were filtered after the indicated time intervals. The results are presented on a semilog scale. Representative of 3–10 experperformed in duplicate.

Experimental results were in good quantitative agreement with those expected when assuming either the existence of two receptors with an equal affinity for [³H]oxo-M or [³H]oxo-M-induced receptor isomerization. It is sometimes possible to exclude one of these models by measuring association kinetics at various tracer concentrations (13). In the present case, theoretical calculations, assuming tracer concentrations up to 12 nM, indicated that the results expected from the two above models would be very similar, and could not be discriminated experimentally: due to the low proportion (20%) of "slow receptors," binding expected from both models would be within 3% of each other.

Previous results (discussed under Antagonist Competition Curves, Results) indicated that [³H]oxo-M was able to recognize two receptor subclasses (B and C receptors) with similar affinities. We therefore considered whether the C and B receptors corresponded, respectively, to "fast" and "slow" [³H]oxo-M binding. To test this hypothesis, we 1) preincubated brain homogenates for 10 min with AF-DX 116 or dicyclomine and added [³H]oxo-M 1 min only before filtration, and 2) preincubated brain homogenates for 10 min with [³H]oxo-M and either AF-DX 116 or dicyclomine, and then added 1 μM atropine 2 min before filtration.

Theoretical calculations indicated that—assuming two independent sites—[³H]oxo-M binding to the fast and slow sites varies from 80/20% at equilibrium to 94/6% in 1-min incubation experiments and 5/95% after 2 min dissociation. We therefore expected significant variations of the selective antagonist competition curves. However, AF-DX 116 and dicyclomine competition curves were biphasic, 30–35% of the [³H]oxo-M-labeled receptors having a low affinity for AF-DX 116 and a high affinity for dicyclomine in all three conditions.

This result indicated that the "fast" receptors (96% of the [³H]oxo-M pulse-labeled receptors) as well as the "slow" receptors (95% of the [³H]oxo-M dilution-resistant receptors) had heterogeneous B + C binding properties.

This was in marked contrast with [³H]NMS binding, where the fraction of [³H]NMS binding to sites with high affinity for AF-DX 116 or pirenzepine varied with the incubation conditions (6, 7)—indicating that [³H]NMS had different association and dissociation rate constants for A, B, and C receptors.

Effects of GTP on the dissociation kinetics of [³H]oxo-M. We induced tracer dissociation by simultaneous addition of atropine and 10^{-3} M GTP (Fig. 9). Fifteen sec after the addition of atropine-GTP, less than 10% of initial binding was observed in heart as compared to 91% when dissociation was induced by atropine alone. By contrast, in cerebral cortex, 40% of initial binding remained after atropine-GTP addition as compared to 56% when dissociation was induced by atropine alone.

The dissociation of [³H]oxo-M from brain muscarinic receptors was biphasic, in the presence as well as in the absence of GTP. [³H]oxo-M binding after atropine-GTP addition was, on an average, 20–30% lower than in control conditions at each time point. This suggested that GTP inhibited to the same extent [³H]oxo-M binding to "rapidly" and "slowly" dissociating receptors.

Effects of allosteric drugs on dissociation kinetics of [³H]oxo-M. Gallamine and *d*-tubocurarine are allosteric drugs capable of affecting dissociation kinetics of muscarinic drugs from their receptors. We therefore tested their effects on [³H]oxo-M dissociation rates from cortex and heart (Fig. 10). As expected, the two drugs decreased the dissociation rate of [³H]oxo-M in both tissues.

To determine the efficacy and potency of gallamine and *d*-tubocurarine in heart and cortex homogenates, we induced [³H]oxo-M dissociation with mixtures of 1 μM atropine and the indicated final drug concentrations (Fig. 11).

Gallamine dose-effect curves on [³H]oxo-M dissociation rates were superimposable in both tissues. At the highest concentration tested, gallamine decreased the dissociation rate by 50% (we were unable to determine its maximal effect).

d-Tubocurarine behaved differently in the two tissues, the concentration needed to decrease the tracer dissociation rate by 50% being 3-fold lower in heart homogenates. The maximal effect was apparently reached at 1 mM *d*-tubocurarine. At this concentration, the drug inhibited tracer dissociation by 85% in heart homogenate and 75% in brain homogenate. Both dose-effect curves could be analyzed assuming a single population of binding sites with EC_{50} values of 1.10^{-5} and $3.1\ 10^{-5}$ M, respectively, in heart and brain homogenates. The *d*-tubocurarine selectivity was, however, too small to exclude the hypothesis of a B/C, rather than brain/heart selectivity: the brain dose-effect curve was also compatible with EC_{50} values of 8.10^{-5} and 1.10^{-5} M, respectively, for B and C receptors.

Discussion

Muscarinic receptor heterogeneity has been demonstrated in many binding studies. In our experience, drugs inhibiting [³H]NMS binding to heterogeneous receptors (with Hill coefficients below 1.0) in the 50 mM sodium phosphate buffer fall into one of the three following categories.

1) Allosteric antagonists such as gallamine and d-tubocurarine. These molecules induce receptor heterogeneity by modifying muscarinic ligand association and dissociation rates (14, 15).

2) Selective antagonists such as pirenzepine (4), *4-DAMP* (Refs. 7 and 16; this work), *and AF-DX 116* (17). These molecules detect a receptor heterogeneity which cannot be explained by allosteric interactions with a nonmuscarinic site, or in cardiac membranes by interaction with GTP-binding protein(s).

MOL PHARM
MOLECULAR PHARMACOLOGY
aspet
Downloaded from molpharm.aspetjournals.org at Universidade do Estado do Rio de Janeiro on December 5, 2012

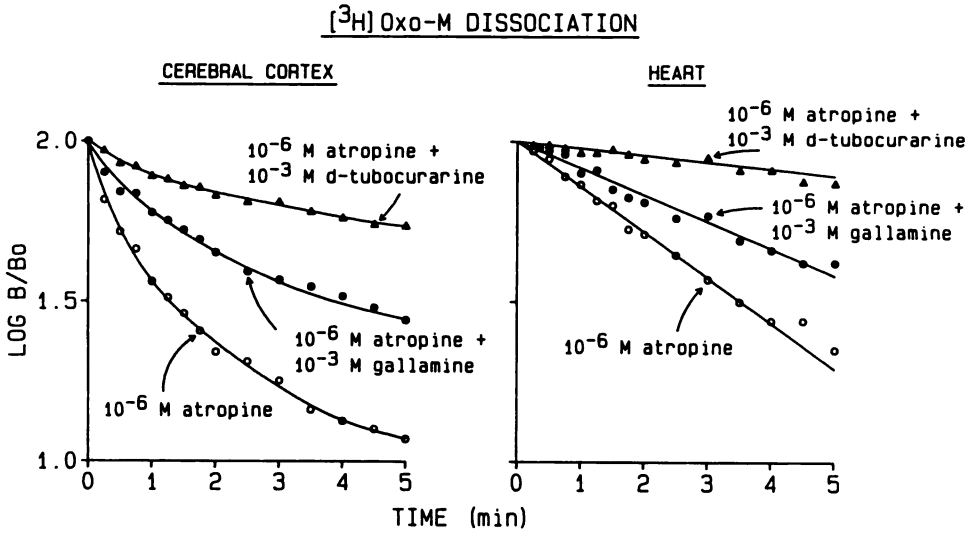

Fig. 10. Effects of the allosteric drugs gallamine and *d*-tubocurarine on [^{3}H]oxo-M dissociation from heart and brain receptors. Cortex (*left*) and heart (*right*) homogenates were preincubated for 15 min at 25° in the presence of 2.4 nM [^{3}H] oxo-M. Dissociation was then induced by addition of 1 μM atropine (control: ○), 1 μM atropine, and 1 mM gallamine (●), or 1 μM atropine and 1 mM *d*-tubocurarine (▲). Duplicate samples were filtered after the indicated time intervals. The results are presented on a semilog scale. Representative of 3–10 experiments performed in duplicate.

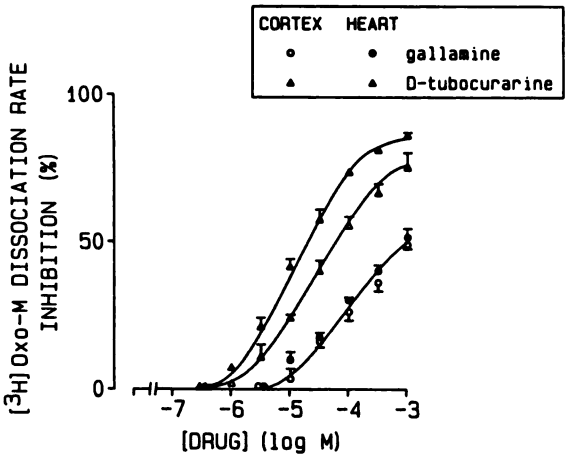

Fig. 11. Inhibition of the [^{3}H]oxo-M dissociation rate by gallamine and *d*-tubocurarine. Heart (●, ▲) and cortex (○, △) homogenates were preincubated for 15 min at 25°, followed by simultaneous addition of 1 μM atropine and the indicated *d*-tubocurarine (▲, △) or gallamine (●, ○) concentrations. Duplicate samples were filtered 2.5 min or 5 min after this addition to, respectively, cortex and heart homogenates in order to reach approximately the same amount of residual binding. The dissociation rate was estimated as log (B_{eq}) − log (B_t) where B_{eq} and B_t represent [^{3}H]oxo-M before and after dissociation, respectively. The 0% inhibition means there was no effect on the [^{3}H]oxo-M dissociation rate; 100% inhibition means total prevention of [^{3}H]oxo-M dissociation ($B_{eq} = B_t$). The degree of [^{3}H]oxo-M dissociation rate inhibition by gallamine and *d*-tubocurarine did not change with time (Fig. 10 and results not shown). Average ± standard deviation of three experiments performed in duplicate.

3) Agonists like carbachol or oxotremorine-M. These agonists are able to detect the existence of three receptors or receptor states, with super high (SH), high (H), and low (L) affinity (3). In cardiac membranes both high affinity states (SH-like and H-like) are converted to a lower (L-like) affinity state by GTP addition to the incubation medium (18).

As discussed in the accompanying paper (7), brain muscarinic receptors can be subdivided into three subclasses with different affinities for selective antagonists.

Three non-interconvertible agonist subclasses coexist in brain (3). It is generally admitted that, although[^{3}H]pirenzepine labels multiple agonist affinity states (H and L receptors) (19), ^{3}H-labeled agonists label SH receptors with low affinity for pirenzepine (20–22). It is also known that the M2 receptors labeled by ^{3}H-labeled agonists in rat brain and heart preparations have different binding properties (20–23). The agonist-binding properties of both brain and cardiac muscarinic receptors vary markedly, however, with the buffer composition. For example, Birdsall *et al.* (24) recently demonstrated that agonists detect SH receptors with high affinity for pirenzepine (A receptors) only if large $MgCl_2$ concentrations are added to the incubation medium, but have a very low affinity for A receptors if $MgCl_2$ is not included in the incubation buffer. We therefore decided to investigate agonist binding to each antagonist receptor subclass, hoping to find some correlation between the two classifications in the sodium phosphate/2 mM $MgCl_2$ buffer we generally use. We chose carbachol to investigate agonist binding to SH, H, and L receptors labeled by [^{3}H]NMS and [^{3}H] PZ, since this agonist is very selective (3) and allows more precise competition curve analysis. [^{3}H]oxo-M was used to investigate agonist and antagonist binding to receptors with SH affinity for agonists. SH receptors labeled by [^{3}H]oxo-M corresponded to SH receptors observed in [^{3}H]NMS binding studies, in terms of receptor concentration and of agonist and antagonist affinities, as discussed below.

Analysis of ^{3}H-labeled antagonist/carbachol competition curves and of [^{3}H]oxo-M/antagonist competition curves gave similar results: 1) we did not detect any A receptors in the SH state under our incubation conditions, and 2) B receptors were able to form high affinity (SH and H) states somewhat less efficiently than C receptors. C receptors were in fact responsible for 65–70% of [^{3}H]oxo-M binding to SH receptors (Figs. 5 and 6) and for about half of SH receptors labeled by a low [^{3}H] NMS concentration (Table 1).

[^{3}H]oxo-M labeled only SH receptors in heart and cortex homogenates, thereby allowing us to measure the affinity of agonists or antagonists for this receptor or receptor state (Figs. 1 and 2).

[^{3}H]oxo-M, oxotremorine, carbamylcholine, and pilocarpine, like the nonselective antagonist secoverine (25), showed the same affinity for cardiac and brain SH receptors, suggesting that they did not discriminate between brain (B)/, brain (C)/, and cardiac (C)/SH receptors. This contrasted with dicyclomine and AF-DX 116, which presented the same B/C selectivity as that found in [^{3}H]NMS binding studies.

MOL PHARM
MOLECULAR PHARMACOLOGY
aspet
Downloaded from molpharm.aspetjournals.org at Universidade do Estado do Rio de Janeiro on December 5, 2012

MOL PHARM

MOLECULAR PHARMACOLOGY

However, despite their similar affinities for agonists and selective antagonists, the binding properties of C/SH receptors in brain and heart were remarkably different for the following two reasons.

1) [³H]oxo-M association and dissociation kinetics were biphasic in cortex, in contrast with heart homogenates. We were unable to correlate the fast and slow binding phases with either B or C receptors (not shown) or with receptors sensitive or unsensitive to GTP (Fig. 9) (see below for discussion). It is possible, therefore, that [³H]oxo-M induced a slow receptor isomerization with a low affinity in cortex but not in heart.

None of the association or dissociation rate constants obtained in rat brain, assuming either isomerization or heterogeneous receptors, corresponded to the association/dissociation rate constants observed in heart. This suggested that a similar SH state (as judged by muscarinic drug affinity constants) could be reached by different reaction pathways (as judged by kinetic constants). The muscarinic ligands tested were unable to distinguish, at equilibrium, the rapidly dissociating brain SH state, the slowly dissociating brain SH state, and the cardiac SH state (with intermediary dissociation rate).

2) Cortex SH receptors were remarkably insensitive to GTP: 70–80% of cortex SH receptors were either able to maintain an SH conformation despite GTP binding to the associated regulatory protein or were able to reach an SH state without the intervention of a GTP-binding protein. The muscarinic ligands tested were unable to distinguish, at equilibrium, this SH state from the GTP-sensitive SH state observed in cardiac membranes.

Taken together, the above results suggest that the equilibrium conformation of SH muscarinic binding sites, recognized by muscarinic agonists and by nonselective and selective antagonists, was very similar in heart and cortex. C/SH receptors in both tissues had the same affinity for carbamylcholine, oxotremorine, and pilocarpine, on the one hand, and secoverine, dicyclomine, and AF-DX 116, on the other hand. However, the reaction pathway leading to [³H]oxo-M binding (reflected by association and dissociation kinetics) as well as the receptor interaction with effector protein(s) (reflected by GTP effects on agonist binding) was markedly different in heart and cortex homogenates.

We had previously shown that binding of allosteric drugs to their accessory sites on muscarinic receptors is exquisitely sensitive to receptor conformation. For example, the affinity of gallamine and *d*-tubocurarine for their accessory site on rat heart (C) receptors changed when these receptors were occupied by muscarinic ligands, in a tracer-dependent manner. Gallamine had a 33-fold higher and *d*-tubocurarine a 6-fold lower affinity for [³H]NMS-occupied receptors as compared to [³H] oxo-M-occupied receptors in rat heart (15). Hoping to better characterize the differences and similarities between cortex and heart C/SH conformation, we decided to compare the effects of gallamine and *d*-tubocurarine on [³H]oxo-M dissociation from these receptors. The results shown in Figs. 10 and 11 indicated that the conformation of the accessory site(s) of [³H] oxo-M-occupied cortex and cardiac SH receptors were very similar, if not identical (the small *d*-tubocurarine preference for cardiac [³H]oxo-M-labeled receptors could be due to a low C > B selectivity).

In conclusion: 1) we did not find any direct correlation between agonist and antagonist classes or states of muscarinic receptors in rat brain. There was an inverse correlation between average agonist affinities and pirenzepine affinities, but each antagonist-defined receptor class (A, B, C) recognized agonists with multiple affinities (SH, H, L).

2) The selective antagonists dicyclomine, AF-DX 116, and gallamine detected the B-C heterogeneity of [³H]oxo-M-labeled sites in cortex, and showed the same affinity for cortex and heart C/SH receptors. The nonselective antagonist secoverine and the four agonists tested (oxotremorine-M, oxotremorine, carbamylcholine, and pilocarpine) had the same affinity for cortex and heart SH receptors. Gallamine and *d*-tubocurarine recognized their accessory sites on brain and heart C/SH receptors occupied by [³H]oxo-M with very similar or identical affinity. These results suggested that the conformation of the C/SH binding subunit was very similar in heart and cortex.

3) By contrast, the reaction pathway leading to an identical or similar binding protein conformation was different in heart and cortex, as indicated by different kinetics and by different interactions with GTP-binding protein(s).

Acknowledgments

We thank Dr. A. Giachetti (Institute di Angeli, Milano, Italy) for helping us to obtain AF-DX 116 from Dr. K. Thomae GmbH (Biberach, FRG).

References

1. Birdsall, N. J. M., and E. C. Hulme. Muscarinic receptors subclasses. *Trends Pharmacol. Sci.* **4**:459–463 (1983).
2. Ehlert, F. J., W. R. Roeske, and H. I. Yamamura. Muscarinic cholinergic receptor heterogeneity. *Trends Neuro Sci.* **5**:336–339 (1982).
3. Birdsall, N. J. M., A. S. V. Burgen, and E. C. Hulme. The binding of agonists to brain muscarinic receptors. *Mol. Pharmacol.* **14**:723–736 (1978).
4. Hammer, R., C. P. Berrie, N. J. M. Birdsall, A. S. V. Burgen, and E. C. Hulme. Pirenzepine distinguishes between different subclasses of muscarinic receptors. *Nature (Lond.)* **283**:90–92 (1980).
5. Birdsall, N. J. M., A. S. V. Burgen, R. Hammer, E. C. Hulme, and J. Stockton. Pirenzepine—a ligand with original binding properties to muscarinic receptors. *Scand. J. Gastroenterol.* **15**(suppl. 66):1–4 (1980).
6. Waelbroeck, M., M. Gillard, P. Robberecht, and J. Christophe. Kinetic studies of [³H]-*N*-methylscopolamine binding to muscarinic receptors in the rat central nervous system: evidence for the existence of three classes of binding sites. *Mol. Pharmacol.* **30**:305–314 (1986).
7. Waelbroeck, M., M. Gillard, P. Robberecht, and J. Christophe. Muscarinic receptor heterogeneity in rat central nervous system. I. Binding of four selective antagonists to three muscarinic receptor subclasses: a comparison with M2 cardiac muscarinic receptors of the C type. *Mol. Pharmacol.* **32**:91–99 (1987).
8. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193**:265–275 (1951).
9. Scatchard, D. G. The attraction of proteins for small molecules and ions. *Ann. N. Y. Acad. Sci.* **51**:660–672 (1949).
10. Richardson, A., and A. L. Humrich. A microcomputer program for the analysis of radioligand binding curves and other dose-response data. *Trends Pharmacol. Sci.* **5**:47–49 (1984).
11. Cheng, Y., and W. H. Prusoff. Relationship between the inhibition constant (K_i) and the concentration of inhibitor which causes a 50 percent inhibition of an enzyme reaction. *Biochem. Pharmacol.* **22**:3099–3108 (1973).
12. Mitchelson, F. Heterogeneity in muscarinic receptors: evidence from pharmacologic studies with antagonists. *Trends Pharmacol. Sci.* **5**(suppl.): 12–16 (1984).
13. Tolkovsky, A. M. Etorphine binds to multiple opiate receptors of the caudate nucleus with equal affinity but with different kinetics. *Mol. Pharmacol.* **22**:648–656 (1982).
14. Stockton, J. M., N. J. M. Birdsall, A. S. V. Burgen, and E. C. Hulme. Modification of the binding properties of muscarinic receptors by gallamine. *Mol. Pharmacol.* **23**:551–557 (1983).
15. Waelbroeck, M., P. Robberecht, P. De Neef, and J. Christophe. Effects of *d*-tubocurarine on rat cardiac muscarinic receptors: a comparison with gallamine. *J. Recept. Res.*, in press.
16. Nilvebrant, L., and B. Sparf. Dicyclomine, benzhexol and oxybutynine distinguish between subclasses of muscarinic binding sites. *Eur. J. Pharmacol.* **123**:133–143 (1986).
17. Giraldo, E., R. Hammer, and H. Ladinsky. Binding profile in rat brain of compound AF-DX 116, a novel cardioselective muscarinic receptor antagonist of the competitive type. *Trends Pharmacol. Sci.* **7**(suppl.): 80 (1986).
18. Waelbroeck, M., P. Robberecht, P. Chatelain, and J. Christophe. Rat cardiac

Downloaded from molpharm.aspetjournals.org at Universidade do Estado do Rio de Janeiro on December 5, 2012

muscarinic receptors. I. Effects of guanine nucleotides on high- and low-affinity binding sites. *Mol. Pharmacol.* **21**:581–588 (1982).
19. Watson, M., H. I. Yamamura, and W. R. Roeske. [^{3}H]Pirenzepine and (−)-[^{3}H]quinuclidinyl benzylate binding to rat cerebral cortical and cardiac muscarinic cholinergic sites. I. Characterization and regulation of agonist binding to putative muscarinic subtypes. *J. Pharmacol. Exp. Ther.* **237**:411–418 (1986).
20. Bevan, P. [^{3}H]Oxotremorine-M binding to membranes prepared from rat brain and heart: evidence for subtypes of muscarinic receptors. *Eur. J. Pharmacol.* **101**:101–110 (1984).
21. Vickroy, T. W., W. R. Roeske, and H. I. Yamamura. Pharmacological differences between the high-affinity muscarinic agonist binding states of the rat heart and cerebral cortex labeled with (+)-[^{3}H]cismethyldioxolane. *J. Pharmacol. Exp. Ther.* **229**:747–755 (1984).
22. Potter, L. T., D. D. Flynn, H. E. Hanchett, D. L. Kalinoski, J. Luber-Narod, and D. C. Mash. Independent M_1 and M_2 receptors: ligands, autoradiography and functions. *Trends Pharmacol. Sci.* (suppl.) 22–31 (1983).
23. Vickroy, T. W., H. I. Yamamura, and W. R. Roeske. Differential regulation of high-affinity agonist binding to muscarinic sites in the rat heart, cerebellum, and cerebral cortex. *Biochem. Biophys. Res. Commun.* **116**:284–290 (1983).
24. Birdsall, N. J. M., E. C. Hulme, and J. M. Stockton. Muscarinic receptor heterogeneity. *Trends Pharmacol. Sci.* **5**(suppl.): 4–8 (1984).
25. Brunner, F., M. Waelbroeck, and J. Christophe. Secoverine is a nonselective muscarinic antagonist on rat heart and brain receptors. *Eur. J. Pharmacol.* **127**:17–25 (1986).

Send reprint requests to: Professor Jean Christophe, Department of Biochemistry and Nutrition, Medical School, Université Libre de Bruxelles, Boulevard de Waterloo 115, B-1000 Brussels, Belgium.

MOL PHARM
MOLECULAR PHARMACOLOGY
aspet
Downloaded from molpharm.aspetjournals.org at Universidade do Estado do Rio de Janeiro on December 5, 2012