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TEMPERATURE-DEPENDENCE AND HETEROGENEITY OF MUSCARINIC AGONIST AND ANTAGONIST BINDING*

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Abstract—The binding parameters of muscarinic agonists and antagonists in rat central (brain, cerebellum and striatum) and peripheral (heart and lung) tissues were determined at 2° and 37° from competitive binding experiments with (^{3}H)-quinuclidinylbenzylate (QNB). Muscarinic ligands binding affinities for cerebellum, heart and lung were tightly correlated. These tissues were also characterized by their low concentration of muscarinic receptors with high affinity for agonists. In contrast, brain and striatum contained a higher concentration of muscarinic receptors with a lower affinity for agonists. The affinity of QNB was lower at 2° whereas that of other ligands was higher. The temperature-dependent shifts of competition curves differed from tissue to tissue and from compound to compound. The shifts were highest with gallamine and carbachol. The binding isotherms of muscarinic ligands were tentatively studied with a two-site binding model. The percentage of high- and low-affinity binding components of agonists differed with the compound. Guanine nucleotides and the temperature increase lowered agonist affinity without changing the proportions of the high- and low-affinity binding components. These results corroborate that the binding heterogeneity of muscarinic ligands does not depend only on the presence of two distinct receptors. Neither guanine nucleotides nor temperature changes allow conversion between the different putative conformational states of the muscarinic receptors.

High- and low-affinity binding sites for muscarinic agonists have been suggested by binding experiments [1, 2]. Study of pirenzepine [3], showing high (M_1) and low (M_2) affinity binding sites, seems to counter the homogeneity of muscarinic antagonist binding [4]. Brain and heart membranes have been reported potentially as models for M_1 and M_2 sites, respectively [5]. A correlation between high and low pirenzepine sites and low and high affinity agonist-binding sites has been suggested [6, 7], but this has not been validated. Indeed, the question remains open of whether binding selectively reflects the existence of different receptors or the involvement of conformational modifications in one receptor.

Modified conformational states might be induced by drug interactions with the receptor [8, 9], and might occur through an allosteric mechanism, involving a secondary binding site on the receptor molecule, as proposed for gallamine [10–12] and pancuronium [12]. The association of the receptor with different effectors might also lead to an apparent heterogeneity of ligand binding in one tissue and to different affinity levels for various tissues. GTP-dependent coupling factors might play a role in the conformation of the receptor [13–16]. Guanine nucleotide have been thought to induce a conversion of high affinity agonist-binding sites into low affinity ones [13, 14. 17], whereas other studies have not lead to such conclusions [18, 19]. These discrepancies might be related to the different incubation temperatures used [14]. Various temperature-dependent modifications in muscarinic ligand binding have been reported [20–23], but no extensive study has compared the effects of temperature changes on the binding of muscarinic antagonists and agonists and on their modulation by guanine nucleotides. We, however, did so, considering the binding heterogeneity of muscarinic ligands might differ with temperature.

MATERIALS AND METHODS

Membrane preparation. Male Wistar rats (200–250 g) were killed by decapitation and brains (whole brain minus cerebellum) were rapidly removed. Alternatively, the striatum and the cerebellum were taken separately. Whole heart and lungs were dissected free of connective and vascular tissue, rinsed, blotted dry and weighed. All membrane preparations were performed at 0°–4° according to the same procedure.

The fresh tissue was homogenized in 10 vol. (ml/g of tissue wet weight) of 0.25 M ice-cold sucrose with a Duall (brain) or an Ultra-Turrax (heart and lung) homogenizer. After low-speed centrifugation at 2500 g (r_{max}) for 10 min, the pellet was again homogenized in 10 vol. of 0.25 M sucrose and resedimented. The supernatants resulting from the two previous centrifugations were pooled and diluted with Na_2HPO_4–KH_2PO_4 buffer (pH = 7.4) to give 60 vol. of 50 mM phosphate buffer. The mixture was centrifuged at 33,000 g (r_{max}) for 30 min. The pellet was carefully homogenized in 5 vol. of 0.25 M sucrose and kept on ice prior to appropriate dilutions

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in 50 mM phosphate buffer (pH = 7.4) for binding assays.

Muscarinic receptor labelling. (^{3}H)-QNB binding was carried out in 2 ml (final vol.) of 50 mM phosphate buffer (pH = 7.4) containing 0.1 nM (^{3}H)-QNB, various concentrations of unlabelled drugs and appropriate amounts of membrane preparation (μg protein/assay: striatum, 20–30; brain, 35–45; heart, 70–90; cerebellum, 90–110; lung, 160–200). Incubations were performed at 2° for 22 hr at 37° for 50 min. Under both conditions, the steady state for specific binding and the linear relationship between specific (^{3}H)-QNB binding and the amount of added membrane proteins were verified.

Bound (^{3}H)-QNB was harvested by rapid vacuum filtration through GF/B glass fibre filters (Whatman). Filters were rinsed twice with 7 ml of ice-cold phosphate buffer and placed in vials with 7 ml of scintillation cocktail (Ready-Solv HP, Beckman). A vigorous 20 min shaking followed by overnight extraction were required for quantitative desorption of the filter-bound radioactivity [24] which was then counted in a liquid scintillation spectrometer with an efficiency of 45%. Specific (^{3}H)-QNB binding was defined as the difference between total binding and binding in the presence of 10^{-6} M atropine. The non-specific binding level was very low and mostly due to (^{3}H)-QNB binding to filters.

Binding data analysis. Competitive binding curves were analyzed in two ways by non-linear least-squares regression. First, an iterative programme originally described by McPherson [25] yielded binding isotherm of the competing ligand for the whole population of muscarinic sites in terms of IC_{50} value and of slope factor (p). This method minimized the sum of squares of the errors between the data points and the estimated curve. Values for p of less than 1 provided means for assessing the deviation from a simple mass action relationship for the binding. Such data were analyzed according to a two-site binding model [26]. The ligand was assumed to interact with two independent binding sites simultaneously obeying simply the law of mass action. This two-site binding model enabled us to calculate best-fit parameters for each population of sites, including the percentage of each site and the $IC_{50} \pm SD$, as described by Barlow [26]. K_i values were determined from IC_{50} values with the method of Cheng and Prusoff [27], assuming competitive interactions.

$$K_i = \frac{IC_{50}}{1 + (^3\text{H})\text{-QNB concentration}/K_D \text{ of } (^3\text{H})\text{-QNB}}$$

K_D for (^{3}H)-QNB was determined at 2° and 37° by Scatchard analysis. Prior checks were made [9] that IC_{50} of drugs (atropine and carbachol) increased linearly at 2° and 37° with increasing concentration of (^{3}H)-QNB (20–200 pM). In all cases, IC_{50} of drugs did increase linearly with increasing concentrations of radioligand while the corresponding K_i-values calculated with the Cheng and Prusoff equation were independent of the concentration of (^{3}H)-QNB used.

Protein concentrations were determined according to Spector [28], using bovine gamma-globulin as a standard.

Compounds. (−)-(3-^{3}H)-quinuclidinyl benzilate [(^{3}H)-QNB, 33 Ci/mmole] was purchased from New England Nuclear Corporation (Boston, MA). The following compounds were obtained from Sigma Chemical Company (St. Louis, MO): acetylcholine, acetyl-β-methyl choline, carbamylcholine, and choline chloride; scopolamine and pilocarpine hydrochloride; atropine sulphate; oxotremorine sesquifumarate; gallamine triethiodide and hexamethonium bromide. Trimethyl-ammonium hydrochloride, phenyltrimethylammonium chloride and tetramethylammonium iodide were from Ega-Chimie (Steinheim/Albuch, F.R.G.). Pirenzepine dihydrochloride was a gift from Boehringer-Ingelheim (Reims, France) and isopropamide iodide was from Delalande (Courbevoie, France). All other chemicals were of the highest purity available.

RESULTS

Tissue heterogeneity in muscarinic agonist and antagonist binding

The binding characteristics of muscarinic receptors were examined in different rat tissues (Fig. 1). The atropine binding curves fitted a simple Langmuir isotherm and the binding constants were similar in the different tissues (Table 1). The carbachol competitive binding curves were similar in peripheral tissues (heart and lung) and in the cerebellum, with K_i-values 20 times lower than those determined in whole brain or striatal membranes. In the latter, the carbachol binding curves did not fit a simple Langmuir isotherm and were shifted to high concentrations of carbachol. This heterogeneity in carbachol binding could not be attributed to variation in the binding dissociation constants of the radioligand

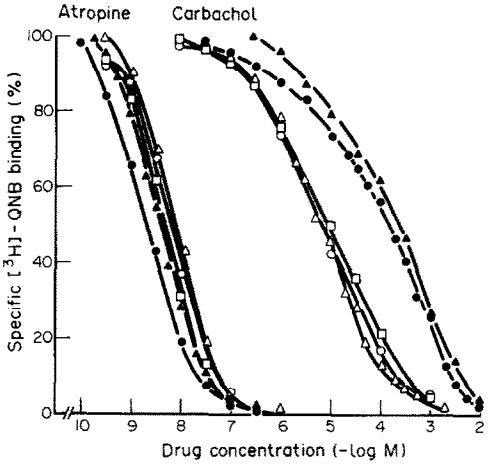

Fig. 1. Tissue dependence of the affinities of muscarinic receptor sites for atropine and carbachol. Membrane preparations from different tissues (striatum: ▲, brain: ●, lung: □, cerebellum: ○ and heart: △) and drug displacement experiments (atropine: left; carbachol: right) were performed under identical conditions (see Materials and Methods). Binding assays were run at 37° with 0.1 nM (^{3}H)-QNB. The values shown are means of at least three independent experiments performed in duplicate, and the corresponding K_i are given in Table 1.

Table 1. Comparative study of tissue enrichment and binding affinity parameters of muscarinic (^{3}H)-QNB binding sites

Tissues	(^{3}H)-QNB binding K_D (pM)	(^{3}H)-QNB binding B_{max} (pmol./g tissue)	Atropine $K_i \pm$ SD (nM)	Carbachol $K_i \pm$ SD (μM)
Striatum	26.7 ± 2.1	93.7	0.813 ± 0.030	34.265 ± 2.130
Brain	24.7 ± 1.0	58.5	0.408 ± 0.024	20.439 ± 1.230
Cerebellum	18.6 ± 3.0	4,37	0.930 ± 0.152	0.947 ± 0.160
Heart	25.5 ± 3.5	1.91	1.411 ± 0.160	1.308 ± 0.174
Lung	20.9 ± 1.9	0.24	0.780 ± 0.080	1.490 ± 0.170

Membrane preparations from different tissues were incubated at 37° with increasing concentrations of (^{3}H)-QNB (3–300 pM) as described under Materials and Methods. At least three independent saturation experiments enabled mean values (± SD) to be estimated for the (^{3}H)-QNB binding parameters (K_D and B_{max} values). IC_{50} were determined by non-linear regression analysis of the competitive binding curves and K_i calculated as described under Materials and Methods.

used (Table 1). Saturation experiments gave linear Scatchard plots (data not shown) over the range of (^{3}H)-QNB concentration studies (3–300 pM) which enabled K_D-values to be calculated. These affinities were similar in the different tissues, whereas B_{max} revealed large variations in the concentration of QNB-binding sites (Table 1).

Binding profiles of muscarinic sites at 37°

Data obtained with atropine and carbachol were extended to other muscarinic agonists and antagonists belonging to different chemical classes (Table 2). The affinities of the classical antagonists atropine, scopolamine, isopropamide and pirenzepine where higher in brain than in heart. In contrast, the apparent affinity of gallamine was higher in heart. The slope factors of the binding isotherms of classical antagonists were close to the unit in both tissues but were lower than 1 for pirenzepine in brain and for gallamine in brain and heart. The binding isotherms of these compounds are shown on Fig. 2.

Agonists displayed a nearly 1-(pilocarpine) to 16-times (carbachol) higher affinity for heart binding sites than for brain ones. Slope factors were lower than 1 in both tissues. Figure 3, left panel, shows that the similar affinities observed above for atropine and carbachol in heart, lung and cerebellum could be extended to other ligands with a variance coefficient of correlation close to zero. In contrast, comparing the affinities of muscarinic ligands in heart and brain (Fig. 3, right panel) reveals wide dispersion of data points around the correlation line. However, the sequence of affinity values was similar in both tissues.

Temperature dependence of muscarinic agonist and antagonists binding

The inhibition of (^{3}H)-QNB binding in brain and heart membrane was reinvestigated at 2° whereas the above data were obtained at 37°. Lowering the incubation temperature resulted in a reversible increase in the equilibrium dissociation constant (K_D) for (^{3}H)-QNB without affecting the binding site density (B_{max}). The temperature-dependent change in the affinity of (^{3}H)-QNB was reversible and prior incubation at 37° or 0° had no effect on the affinity determined in a subsequent incubation at a different temperature. Decreasing the temperature to 2° decreased the affinity of QNB and increased the affinity of the other ligands (Table 2). The affinity of classical antagonists and of pirenzepine was slightly higher at 2°, whereas that of gallamine was much enhanced, leading to a high ratio between K_i at 37° and at 2° (Table 2). These temperature-dependent shifts are illustrated for pirenzepine and gallamine in Fig. 2.

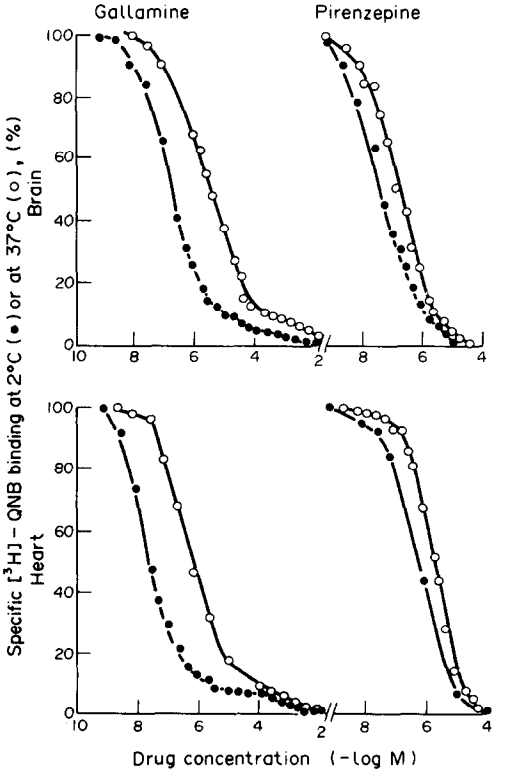

Fig. 2. Specific (^{3}H)-QNB binding to heart and brain membranes determined at 2° and 37° in the presence of various concentrations of gallamine and pirenzepine. Corresponding K_i are listed in Table 2.

Table 2. Comparison of K_i for binding isotherms of muscarinic ligands in rat brain and heart membranes at 37° and 2°

Compound	$K_i \pm$ SD (nM) at 37° Heart	at 37° Brain	at 2° Heart	at 2° Brain	$K_i37°/K_i2°$ Heart	$K_i37°/K_i2°$ Brain
Antagonists						
QNB	0.025 ± 0.003	0.025 ± 0.001	0.078 ± 0.002	0.086 ± 0.001	0.32	0.29
Atropine (ATROP)	1.41 ± 0.16	0.40 ± 0.024	0.795 ± 0.033	0.196 ± 0.007	1.77	2.04
Scopolamine (SCOPO)	2.16 ± 0.29	0.42 ± 0.02	0.76 ± 0.03	0.124 ± 0.004	2.84	3.39
Isopropamide (ISOPR)	3.49 ± 0.49	1.04 ± 0.06	1.11 ± 0.05	0.55 ± 0.01	3.14	1.89
Pirenzepine (PIRENZ)	397 ± 59	39.5 ± 2.2	341 ± 18	26.3 ± 0.5	1.16	1.50
Gallamine (GALLA)	210 ± 30	930 ± 60	17.6 ± 0.7	103 ± 3	11.93	9.03
Agonists						
Oxotremorine (OXO)	120 ± 20	490 ± 30	14.3 ± 0.6	81 ± 2	8.39	6.05
Acetylcholine (ACH)*	750 ± 110	4360 ± 300	220 ± 14	1211 ± 47	3.41	3.60
Carbachol (CARBA)	1310 ± 170	20440 ± 1230	161 ± 7	2823 ± 82	8.14	7.24
Acetyl-β-methylcholine (META)*	2060 ± 300	13990 ± 620	633 ± 34	1539 ± 61	3.25	9.09
Pilocarpine (PILO)	3500 ± 450	3690 ± 230	619 ± 28	1106 ± 28	5.65	3.34
Non-selective ligands						
Choline (CHOLI)	507000 ± 85000	687000 ± 42000	147300 ± 9500	288000 ± 112000	3.44	2.38
Trimethylammonium (TRI)	1133000 ± 160000	1935000 ± 116000	706100 ± 33800	654200 ± 20800	1.60	2.96
Phenyltrimethylammonium (PHENYL)	23900 ± 3400	45200 ± 2800	13910 ± 660	13670 ± 590	1.72	3.30
Tetramethylammonium (TETRA)	145200 ± 22800	417900 ± 25200	105000 ± 7400	107900 ± 3400	1.38	3.87
Hexamethonium (HEXA)	57900 ± 8100	70600 ± 4700	11720 ± 580	10090 ± 280	4.94	7.00

K_i are the means of 3–10 independent determinations, carried out in duplicate and determined as indicated under Material and Methods.

* Binding assays were performed in the presence of physostigmine 10^{-6} M, an acetylcholinesterase inhibitor.

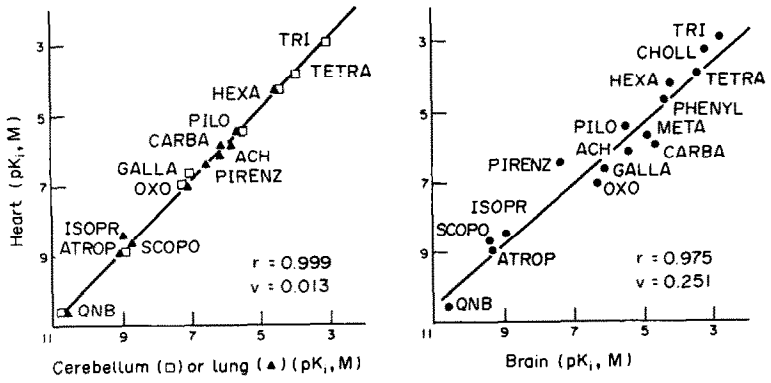

Fig. 3. Correlation between binding affinity constant (pK_i) at 37° of drugs for specific (^{3}H)-QNB binding sites from various tissues. Compounds and assay conditions were as reported in Table 2 and under Materials and Methods. Unweighted linear regression analysis of the data enabled the correlation coefficient (r) and the variance (v) to be calculated.

The binding isotherms of most muscarinic antagonists, such as atropine, and QNB, were consistent with a one-site model, whereas those of other ligands were complex, providing slope factors lower than 1 (Table 3). These binding data were tentatively analyzed in terms of a model with two independent sites (Table 4). The percentages of high and low affinity binding components were different from one ligand to the other. For instance, in brain, the high-affinity binding components of oxotremorine and carbachol were respectively 15 and 32% of the total binding.

The proportion of low- and high-affinity components of ligand binding was independent of the incubation temperature (Table 4). Table 5 shows that low- and high-affinity binding components were differently modified by temperature changes resulting in different K_i ratios at 37° and 2°. However, no consistency was observed according to the ligand or the tissue considered. The K_i-ratio of carbachol reached 50 for its high-affinity component in the heart, i.e. a very large increase in affinity when the incubation temperature was reduced from 37° to 2°. No such selective increase in the high affinity component of carbachol was observed with brain membrane, nor with other ligands in either tissue.

Effect of Gpp(NH)p on ligand binding in rat heart membrane

Adding the non-hydrolyzable GTP analogue did not affect the QNB binding, K_D and B_{max} were similar with and without Gpp(NH)p at 2° or 37°. Gpp(NH)p caused a concentration-dependent decrease (Fig. 4) in the capacity of carbachol to inhibit (^{3}H)QNB binding at 2° and 37° (Fig. 5). The greatest shift in binding isotherms was obtained with 10^{-4} M Gpp(NH)p. The slopes of the competitive binding curves increased with increasing concen-

Table 3. Slope factors of binding isotherms for muscarinic ligands. K_i are shown in Table 2

Compounds	Slope factors at 37° Heart	Brain	at 2° Heart	Brain
Antagonists				
QNB	0.93	0.96	0.98	0.99
Atropine (ATROP)	1.05	0.90	0.99	0.84
Scopolamine (SCOPO)	1.01	0.89	0.92	0.83
Isopropamide (ISOPR)	1.04	0.98	0.88	0.88
Pirenzepine (PIRENZ)	0.95	0.75	0.86	0.70
Gallamine (GALLA)	0.65	0.58	0.65	0.68
Agonists				
Oxotremorine (OXO)	0.60	0.73	0.43	0.79
Acetylcholine (ACH)	0.61	0.55	0.44	0.64
Carbachol (CARBA)	0.63	0.52	0.48	0.57
Acetyl-β-methylcholine (META)	0.54	0.59	0.45	0.62
Pilocarpine (PILO)	0.79	0.83	0.63	0.84
Non-selective ligands				
Choline (CHOLI)	0.65	0.88	0.60	1.06
Trimethylammonium (TRI)	0.72	0.92	0.75	0.93
Phenyltrimethylammonium (PHENYL)	0.75	0.89	0.74	0.92
Tetramethylammonium (TETRA)	0.63	0.87	0.54	0.86
Hexamethonium (HEXA)	0.74	0.93	0.70	0.84

Table 4. Apparent binding affinity constants and relative proportions of high- and low-affinity binding components of drugs in heart and brain membranes

Compounds	Binding site analysis		K_i (μM ± SD) Heart 2°	Heart 37°	Brain 2°	Brain 37°
Oxotremorine	1 site		0.014 ± 0.001	0.117 ± 0.017	0.081 ± 0.002	0.486 ± 0.030
	2 sites	High	0.001 ± 0.001 (44)	0.012 ± 0.006 (40)	0.002 ± 0.002 (14)	0.008 ± 0.003 (15)
		Low	0.092 ± 0.033 (56)	0.552 ± 0.180 (60)	0.157 ± 0.018 (86)	0.797 ± 0.066 (85)
Carbachol	1 site		0.161 ± 0.007	1.308 ± 0.174	2.823 ± 0.082	20.44 ± 1.23
	2 sites	High	0.005 ± 0.001 (48)	0.252 ± 0.081 (46)	0.088 ± 0.023 (31)	0.821 ± 0.175 (32)
		Low	1.157 ± 0.138 (52)	6.191 ± 1.559 (54)	9.934 ± 0.861 (69)	98.59 ± 11.52 (68)
Pilocarpine	1 site		0.619 ± 0.028	3.496 ± 0.457	1.106 ± 0.028	3.689 ± 0.230
	2 sites	High	0.052 ± 0.012 (35)	0.331 ± 0.119 (33)	n.s.	n.s.
		Low	1.916 ± 0.225 (65)	8.436 ± 2.476 (67)		
Pirenzepine	1 site		0.341 ± 0.018	0.397 ± 0.059	0.026 ± 0.001	0.039 ± 0.002
	2 sites	High	n.s.	n.s.	0.013 ± 0.002 (76)	0.022 ± 0.003 (75)
		Low			0.439 ± 0.159 (24)	0.491 ± 0.003 (25)
Gallamine	1 site		0.018 ± 0.001	0.214 ± 0.030	0.103 ± 0.003	0.933 ± 0.061
	2 sites	High	0.008 ± 0.001 (83)	0.106 ± 0.023 (82)	0.072 ± 0.006 (86)	0.504 ± 0.090 (83)
		Low	n.d. (17)	n.d. (18)	n.d. (14)	n.d. (17)

K_i of drugs for the whole population of (^{3}H)-QNB binding sites are taken from Table 2. Competitive binding curves were analyzed according to the two-site binding model (Materials and Methods). Values in parentheses are the relative proportions (expressed in %) of high and low affinity binding components. K_i for the high and low affinity binding components were determined with the method of Cheng and Prusoff (Materials and Methods). Table 5 shows the ratios between K_i at 37° and at 2°.

n.s. Competitive binding curves did not significantly fit to the two-site binding model.

n.d. The low affinity component of gallamine binding was irregular (see Fig. 2) and could not be analyzed accurately.

trations of guanine nucleotide, but never reached one. Gpp(NH)p reduced the apparent affinity of carbachol without significantly altering the affinity of atropine. In contrast, the slope of carbachol (Fig. 5) and of other agonists' competitive binding curves increased in the presence of Gpp(NH)p but never reached one. Indeed, Gpp(NH)p did not modify the proportions of high- and low-affinity components.

The effect of Gpp(NH)p is summarized in Table 6. The larger nucleotide-dependent shift was observed for the high-affinity component of carbachol at 2°. The affinities of oxotremorine and pilocarpine were less affected. The nucleotide prevented the temperature-dependent modification of the affinities of the binding components of agonists.

Table 5. Temperature-dependent modulation of the high and low affinity binding component for muscarinic ligands in rat heart and brain membranes

Compounds	K_i37°/K_i2° Heart 1 site	Heart High	Heart Low	Brain 1 site	Brain High	Brain Low
Oxotremorine	8.39	12.2	6.0	6.05	4.4	5.1
Carbachol	8.14	50.4	5.3	7.24	9.2	9.9
Pilocarpine	5.65	6.3	4.4	3.34	—	—
Pirenzepine	1.16	—	—	1.50	1.7	1.1
Gallamine	11.93	12.6	—	9.03	7.0	—

The ratios for K_i at 37° vs 2° are calculated from results shown in Table 4. They are typical temperature-dependent shifts of the binding isotherms as illustrated for gallamine and pirenzepine in Fig.2.

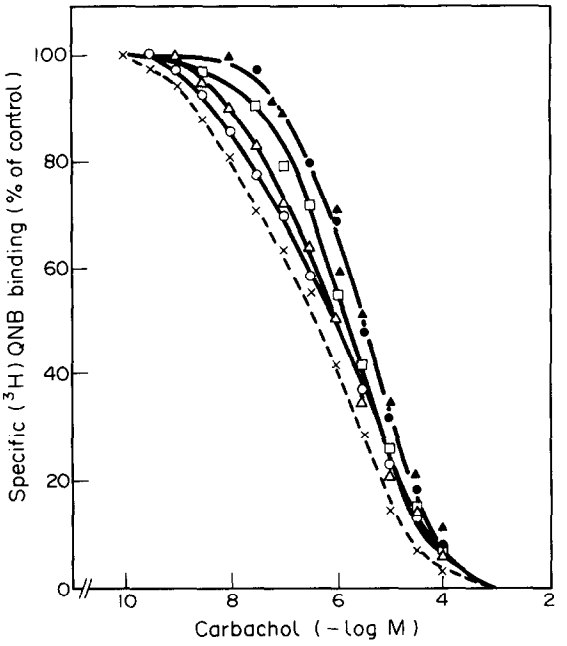

Fig. 4. Concentration/effect curve for inhibition of (^{3}H)-QNB binding to rat heart membranes at 2°. Cardiac membranes (50 μg protein/assay) were incubated with 100 pM (^{3}H)-QNB, various concentrations of Gpp(NH)p (×: control; ○: 3.10^{-7} M; △: 10^{-6} M; □: 10^{-5} M; ●: 10^{-4} M; ▲: 10^{-3} M) and with a range of carbachol concentrations. (^{3}H)-QNB bound in the presence of carbachol are represented as a function of Gpp(NH)p concentration. The data are for four experiments, in duplicate. The slope of the binding curve increases from 0.48 (control) to 0.62 (10^{-4} M Gpp(NH)p).

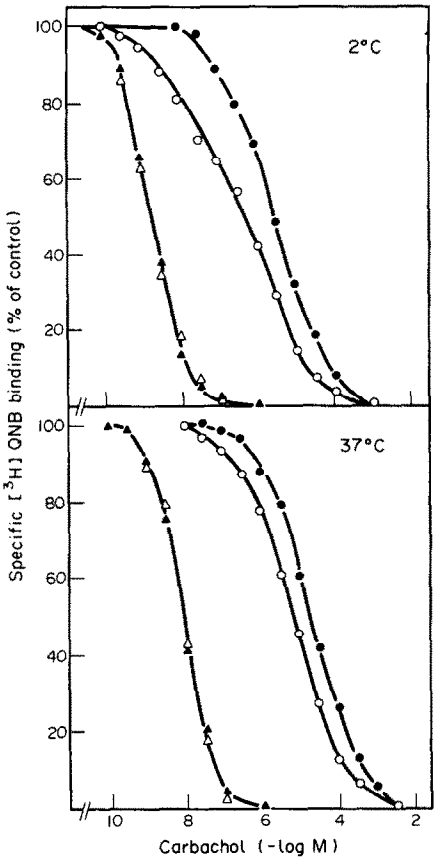

Fig. 5. Concentration dependence of carbachol- and atropine-induced inhibition of (^{3}H)-QNB binding to heart membrane in the presence and absence of Gpp(NH)p. Rat heart membranes (50 μg protein/assay) were incubated with 100 pM (^{3}H)-QNB and increasing concentration of carbachol and of atropine at 2° (top) and 37° (bottom) in the absence (○, △) or presence (●, ▲) of 100 μM Gpp(NH)p.

In the presence of Gpp(NH)p, the temperature-dependent modifications of the high-affinity binding components of carbachol and oxotremorine were similar.

DISCUSSION

The presence in a tissue of different receptors for one ligand can be detected by competitive binding experiments. However, whereas the shape of the binding curves may vary with the selectivity of the ligand used, the calculated percentage of each population of sites is a characteristic of the tissue and does remain similar whatever the selectivity of the ligand [29]. Thus, the observation in the present experiments of different percentages of sites from one ligand to the other, in both brain and heart membranes, does not tally with the concept of two different receptors. This major point in receptor subclassification has been often neglected. Moreover, if one considers muscarinic subtypes to be identical whatever their tissue origin, one expects to find general consistency in the binding properties of each population of sites. In fact, using similar experimental conditions we noted differences in affinity for the sites in heart and brain membranes. Thus, it has to be considered that the heterogeneity of the binding of muscarinic ligands is related to different conformational states of receptors. The possibility was studied of the conversion from one conformational state to the other being due to the presence of guanine nucleotides and/or to temperature changes.

Heterogeneity of agonist binding

Whereas temperature changes induced only moderate changes in the low agonist-affinity component of the binding isotherms, we observed much greater modifications in the high-affinity one. However, the proportions of each component did not differ at 2° or 37°, which tallies with previous observations at 25° and 37° [30]. Similarly, Phan *et al.* [21] reported that the Hill coefficient of carbachol binding to rat heart did not differ at 18° or 37°, despite their own results; they proposed that lowering temperature promoted the formation of a high-affinity conformer of the muscarinic receptor.

The temperature-induced changes we observed were reversible. The effect of the stable guanine nucleotide Gpp(NH)p was more obvious at the low temperature. Gpp(NH)p induced a decrease in the

Table 6. Gpp(NH)p and temperature modulation of the high and low affinity binding components for agonists in rat heart membranes

	$K_{i+Gpp(NH)p}/K_{i-Gpp(NH)p}$				$K_i37°/K_i2°$ with Gpp(NH)p	
	2°		37°			
Compounds	High	Low	High	Low	High	Low
Oxotremorine	19	1.7	8.8	2.5	5.5	9
Carbachol	50	6.0	4.4	3.9	4.4	3.5
Pilocarpine	20.2	3.8	6.9	5	2.2	5.9

These values were determined from binding isotherms at 2° or 37° and in the presence or absence of 100 μM Gpp(NH)p. K_i in the absence of Gpp(NH) are given in Table 4. The $K_{i+Gpp(NH)p}/K_{i-Gpp(NH)p}$ ratios are representative of the Gpp(NH)p-dependent shifts of the binding isotherms as illustrated for carbachol in Fig. 5. The temperature-dependent shifts of the binding isotherms in the presence of Gpp(NH)p have to be compared to control values (absence of Gpp(NH)p) indicated in Table 5.

high-affinity binding component of a ligand. The ligand displacement curves were right-shifted in the presence of Gpp(NH)p and the Hill coefficient increased slightly but never reached one. Indeed, the percentage of each binding component was not modified as, too, in other studies [18, 19], whereas some authors did report modifications of this percentage [14, 17, 31]. Thus, the present study does not support the proposal [14] that these discrepancies might be related to the different incubation temperatures used. The inability of guanine nucleotide to convert a heterogeneous binding process into a homogeneous one supports the hypothesis that the putative different conformational states of the receptor protein are not simply related to interactions with GTP-binding proteins. Conversion of high- to low-affinity sites cannot be assumed. Indeed, we showed that Gpp(NH)p inhibited both components of agonist binding.

Furthermore, we found that the high-affinity component of carbachol binding isotherm underwent greater modification than with oxotremorine and pilocarpine. These data tally with those of Ehlert [32] obtained on rabbit heart and concluding in good correlation between efficacy as measured by inhibition of adenylate cyclase and the influence of GTP on binding properties. Such a correlation is restricted to the high-affinity component of agonist binding isotherms, leading to the hypothesis that the corresponding conformational state of the receptor proteins might be related to interactions with adenylate cyclase and the inhibitory guanine nucleotide-binding protein.

Heterogeneity of antagonist binding

Pirenzepine binding depended little on temperature (Table 2), the percentages of high- and low-affinity binding components being unchanged. This does not tally with a recent preliminary report [22] concluding that the selectivity of pirenzepine binding is temperature-dependent. Indeed, this conclusion was based on the comparison of IC_{50} at 0° and 40°. The experimental conditions were not indicated. However, the determination of binding values from displacement curves at these two temperatures usually requires different concentrations of the labelled compound, and IC_{50} has to be converted as K_i to compare the affinities under the different conditions. Our results also disagree with another preliminary report [23]. We found that temperature changes influenced heart as well as brain muscarinic receptors (Table 2). Moreover, we cannot conclude any correlation between temperature changes and the agonist or antagonist behaviour of muscarinic ligands. The highest increase in affinity at 2° was observed with gallamine in both tissues. This nicotinic antagonist has been described as a non-competitive muscarinic antagonist [10] with a cardiac selectivity in agreement with present results (Table 1). Gallamine has been thought to bind to a site distinct from the conventional muscarinic ligand binding one, thereby modulating the binding of usual agonists and antagonists [11]. In contrast, competitive interaction with muscarinic ligand was observed by others [33]. Indeed, Dunlap and Brown [12] suggested that gallamine not only competes for QNB binding sites, but also binds at a secondary site of the receptor, involving an allosteric mechanism.

In conclusion, evaluating the binding characteristics for a large number of muscarinic compounds under identical conditions in different tissues and at different temperatures supports the hypothesis that binding heterogeneity does not depend only on the presence of two distinct receptors. The effects of Gpp(NH)p and of temperature changes do not enable any conversion to be envisaged between the putative different conformational states of the muscarinic receptors.

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