Biochemical Pharmacology, Vol. 36, No. 23, pp. 4119–4124, 1987.
Printed in Great Britain.

0006-2952/87 $3.00 + 0.00

DIFFERENT AGONIST BINDING PROPERTIES OF M1 AND M2 MUSCARINIC RECEPTORS IN CALF BRAIN CORTEX MEMBRANES

PATRICK VANDERHEYDEN,* GUY EBINGER† and GEORGES VAUQUELIN*

* Department Protein Chemistry, Instituut Moleculaire Biologie, Vrije Universiteit Brussel, B-1640 St. Genesius-Rode, and † Department of Neurology, University Hospital, Vrije Universiteit Brussel, B-1090 Brussels, Belgium

(*Received* December 1986; *accepted* 24 *June* 1987)

Abstract—The muscarinic antagonist 1-[benzilic 4,4′-^{3}H]-quinuclidinyl benzilate [^{3}H]-QNB) bound to a single class of non-cooperative sites in calf cerebral cortex membranes (K_D = 0.29 nM and B_{max} = 1.06 pM/mg protein). Computer-assisted analysis of the shallow pirenzepine/[^{3}H]-QNB competition binding curves indicated that 68% of these sites were of the M1-subtype and the remaining 32% of the M2 subtype. Respective K_i-values for pirenzepine were 27 nM and 1.14 μM. Binding characteristics of the antagonist atropine and of the agonist carbachol for M2 were evaluated by performing competition binding with 0.5 nM [^{3}H]-QNB in the presence of 2 μM pirenzepine. The binding characteristics for the M1 receptors were obtained indirectly by subtracting the curve for M2 from the total curve, or directly by competition binding with 0.3 nM [^{3}H]-pirenzepine. Atropine competition curves were steep for M1 and M2 and were not affected by 1 mM GTP nor by 1 mM N-ethylmaleimide. The carbachol competition curve was shallow for M2. The steep curves for M1 indicate that this receptor subclass was only composed of low agonist affinity sites. GTP, which caused a rightward shift and a steepening of the carbachol competition curve for M2, did not affect the curves for M1. *N*-ethylmaleimide provoked a leftward shift and a steepening of the carbachol competition curve for M2 and abolished GTP modulation. A leftward shift was also observed for M1, but of a smaller magnitude (i.e. 3–4-fold for M1 compared to 17-fold for M2). These data suggest that, in calf brain cortex, M1 and M2 receptors show different susceptibility towards GTP and *N*-ethylmaleimide modulation.

The great majority of acetylcholine effects in the central nervous system are mediated via interaction with muscarinic receptors [1]. These receptors can be easily identified and characterized by binding of radiolabelled muscarinic antagonists such as [^{3}H]-dexetimide, [^{3}H]-*N*-methylscopolamine and 1-(benzilic-4-4′-^{3}H)-quinuclidinyl benzilate ([^{3}H]-QNB) [2–4].

Muscarinic receptors were initially assumed to be composed of a single population of sites with regard to antagonist binding [3]. However, it is now generally accepted that they comprise two subtypes with respectively high and low affinity for the antagonist pirenzepine [5]. The high affinity sites, which can be selectively labelled with [^{3}H]-pirenzepine, are denoted as M1 receptors and are most abundant in cerebral cortex, striatum and hippocampus. Much lower affinity for pirenzepine is observed for M2 sites which are predominant in the heart, cerebellum and brain stem [6].

Heterogeneity for muscarinic receptors has also been observed for agonists. Shallow agonist competition curves are currently explained assuming the existence of different receptor subpopulations with superhigh, high and low agonist affinity [7]. Guanine nucleotides have been shown to provoke a marked rightward shift of agonist competition curves in the heart. Hence, it is suggested that the muscarinic receptors in the heart undergo functional coupling to the inhibitory regulatory component of the adenylate cyclase system (Gi) [9]. In contrast, only slight or even no effects of the nucleotides are observed on agonist binding in the cerebral cortex [8–12].

Muscarinic receptors appear also to display different susceptibility towards the alkylating reagent *N*-ethylmaleimide (NEM). This reagent causes a more pronounced leftward shift and steepening of the carbachol competition curve in the bovine heart as compared to the bovine forebrain [12]. These differences in NEM and guanine nucleotide action can be attributed to either different properties of M1- and M2-receptors or to the different cellular origin of the investigated membrane preparations.

To evaluate both possibilities, we investigated the effect of NEM and GTP on agonist and antagonist competition binding for the M2- and M1-sites in calf cerebral cortex membranes separately.

MATERIALS AND METHODS

Materials. Carbamylcholine chloride (carbachol) and atropine sulfate monohydrate were from Aldrich Europe (Beerse, Belgium). *N*-Ethylmaleimide (NEM) and neostygmine bromide were from Sigma Chemical Company (St. Louis, MO), guanosine-5′-triphosphate (GTP) from Boehringer Pharma (Mannheim, F.R.G.) and pirenzepine was purchased from Dr Karl Thomae GMBH (Biberach an der Riss, F.R.G.). *l*-[Benzilic-4-4′-^{3}H]-quinuclidinyl benzilate

([^{3}H]-QNB, 33 Ci/mmol) and [^{3}H]-pirenzepine (70 Ci/mmol) were supplied by NEN (Dreieich, F.R.G.).

Membrane preparations. Calf brains were obtained from a slaughterhouse and kept in ice. Further manipulations were performed within less than 1 hour. Samples of frontal cortex were dissected from coronal slices and homogenized in 10 mM sodium phosphate (pH 7.4)/145 mM NaCl (PBS) with a polytron mixer (15 sec) and with a Potter homogenizer (5 strokes at maximum speed). The homogenate was then centrifuged at 700 *g* for 15 min and subsequently at 10,000 *g* for 15 min. The resulting supernatant was further centrifuged at 29,000 *g* for 30 min and the pellet suspended in buffer (±10 mg protein/ml) was stored at −70° for up to 2 months without loss of muscarinic receptor binding characteristics. Protein determinations were performed by the method of Lowry *et al.* [13] using bovine serum albumin as the standard.

[^{3}H]-QNB and [^{3}H]-pirenzepine binding assay. Calf brain cortex membranes, at the final protein concentration of 0.2 mg/ml, were incubated with [^{3}H]-QNB or [^{3}H]-pirenzepine (respectively for 30 and 60 min) at 30° in a final volume of 1 ml sodium phosphate buffer (PBS). Under these conditions, binding reached equilibrium after 20 min for [^{3}H]-QNB and 40 min for [^{3}H]-pirenzepine. The acetylcholine esterase inhibitor neostygmine bromide (3 μM) was included in the assay medium to prevent a possible degradation of carbachol by this enzyme. For saturation binding experiments, the radioligand concentration ranged between 0.01 and 2 nM for [^{3}H]-QNB and between 0.5 and 10 nM for [^{3}H]-pirenzepine. The concentrations used in the competition binding experiments equalled 0.5 nM for [^{3}H]-QNB and 0.3 nM for [^{3}H]-pirenzepine. Non-specific binding was determined in the presence of 1 μM atropine. At the end of the incubation, 3 ml ice-cold buffer was added and the sample filtered through Whatmann GF/B glass of fiber filters. Filters were pretreated with 0.1% polyethyleneimine for the [^{3}H]-pirenzepine binding assays. The filters were washed twice with 3 ml of ice-cold PBS and counted after addition of 1 ml 0.1 M NaOH and 8 ml Picofluor 15 in a Packard liquid scintillation spectrometer. Each experiment involved triplicate determinations.

Calculation of antagonist- and agonist/[^{3}H]-QNB competition binding parameters for M1 and M2-muscarinic receptors. To label the total amount of muscarinic receptor, the cortical membranes were incubated with 0.5 nM [^{3}H]-QNB. Agonist and antagonist competition binding was performed by incubation with an increasing concentration of unlabelled competitor. The competition binding curve with unlabelled pirenzepine was analysed according to a two-site model by LIGAND as described by Munson and Rodbard [14]. 68 ± 5.1% of the sites showed high affinity for this drug (IC_{50} = 61 ± 13.1 nM) and were denoted as M1-sites. The remaining low-affinity sites (IC_{50} = 2.6 ± 0.44 μM) were denoted as M2-sites. [^{3}H]-QNB and pirenzepine interacted with each receptor class according to the law of mass action. Hence, for each receptor class, binding of the radioligand (B) in the presence of a given concentration pirenzepine (P) was related to binding of the radioligand in the absence of pirenzepine (B_0) by the following equation:

$$B = B_0/(1 + P/IC_{50}) \quad (1)$$

where IC_{50} is the pirenzepine concentration that caused half-maximal displacement of [^{3}H]-QNB (61 nM for M1-sites and 2.6 μM for M2-sites). By use of this equation, it could be calculated that 2 μM pirenzepine displaces 97% of [^{3}H]-QNB binding to the M1-sites. Hence, the M2-receptors represented 90% of the sites when the binding assay was carried out in the presence of 2 μM pirenzepine. Binding characteristics of unlabelled muscarinic ligands for the M2-sites can be evaluated with approximation under this condition. The competition binding curves for the M1-sites could be approximated as follows:

$$B_1 = (B_{tot} - 0.32 \times B_2)/0.68 \quad (2)$$

where B_{tot}, B_1 and B_2 represent [^{3}H]-QNB binding in the presence of a given concentration competitor for the total receptor population, for the M1-sites and for the M2-sites (determined in the presence of 2 μM pirenzepine), respectively.

RESULTS

Muscarinic acetylcholine receptors can be identified in calf brain cortex membranes by the specific and saturable binding of [^{3}H]-QNB. The Scatchard plot is linear and the Hill coefficient equals unity (nH = 1.1), which argues for the absence of cooperative interactions. The equilibrium dissociation constant (K_D) for [^{3}H]-QNB equals 0.29 ± 0.03 nM and the number of binding sites is 1.06 ± 0.132 pmol/mg protein (Table 1). The competition binding curve for the M1-selective antagonist pirenzepine is shallow (nH = 0.66) (Fig. 1). This can be attributed to the presence of two receptor subclasses with high- and low-affinity for pirenzepine (defined as M1- and M2-sites respectively). The curve can be analysed by

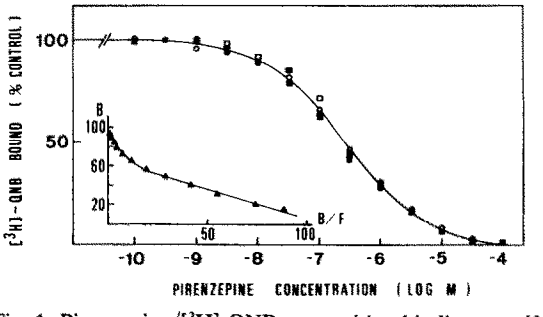

Fig. 1. Pirenzepine/[^{3}H]-QNB competition binding to calf brain cortex membranes: effects of NEM and GTP. Control (●—●), 1 mM GTP (○—○), 1 mM NEM (■—■) and NEM + GTP (□—□). The concentration of [^{3}H]-QNB was 0.5 nM. Values are means of three separate experiments. Insert: Hofstee plot of the competition binding data (control). B represents the percentage displacement of [^{3}H]-QNB and F the free pirenzepine concentration. The non-linear Hofstee plot was analysed according to a two-site model; 68 ± 5.1% of the total amount of binding sites exerted high affinity for pirenzepine (IC_{50} = 61 ± 5.1 nM) whereas 32 ± 5.1% had low affinity for pirenzepine (IC_{50} = 2.6 ± 0.45 μM).

Table 1. [^{3}H]-QNB and [^{3}H]-pirenzepine binding characteristics for calf brain cortex membranes: effects of NEM and GTP

Addition	[^{3}H]-QNB			[^{3}H]-pirenzepine		
	K_D (nM)	B_{max} (pM/mg)	r	K_D (nM)	B_{max} (pM/mg)	r
None	0.29 ± 0.03	1.061 ± 0.132	0.98	0.14 ± 0.01	0.92 ± 0.14	0.99
NEM (1 mM)	0.29 ± 0.03	1.011 ± 0.185	0.98	0.14 ± 0.06	0.80 ± 0.34	0.99
GTP (1 mM)	0.26 ± 0.02	1.019 ± 0.207	0.95	0.15 ± 0.03	0.92 ± 0.19	0.96
NEM + GTP	0.26 ± 0.05	1.015 ± 0.182	0.99	0.17 ± 0.04	0.96 ± 0.22	0.98

The K_D and B_{max} values are determined by Scatchard analysis of saturation binding data and r is the regression coefficient of the Scatchard plots. Values are means ± SD of three separate determinations.

the computerized method (LIGAND) of Munson and Rodbard [14] to yield the proportion of M1- (68 ± 5.1%) and M2-sites (32 ± 5.1%) as well as their IC_{50}-values for pirenzepine, i.e. 61 ± 5.1 nM and 2.6 ± 0.45 μM, respectively. The K_i values for pirenzepine, calculated from their IC_{50}-values according to the method of Cheng and Prusoff [15], are 27 ± 0.6 nM for M1- and 1.14 ± 0.198 μM for M2-sites. The M1 sites can also be directly labelled with [^{3}H]-pirenzepine (Table 1). The Scatchard plot is linear and the K_D for [^{3}H]-pirenzepine (14.9 ± 0.12 nM) is in good agreement with the value calculated from the unlabelled pirenzepine [^{3}H]-QNB competition curve, [^{3}H]-QNB, [^{3}H]-pirenzepine binding and pirenzepine/[^{3}H]-QNB competition binding is not affected by 1 mM GTP and 1 mM NEM, either alone nor in combination (Table 1 and Fig. 1).

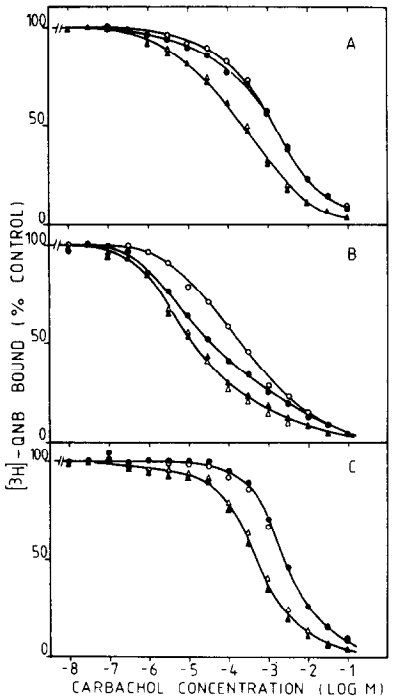

Fig. 2. Carbachol/[^{3}H]-QNB competition binding to the total receptor population (A), the M2-receptors (B) and the M1-receptor (C): effects of NEM and GTP. Control (●—●), 1 mM GTP (○—○), 1 mM NEM (▲—▲) and NEM + GTP (△—△). Competition binding for M2 was performed in the presence of 2 μM pirenzepine. The competition binding data for M1 were calculated by subtracting the curve for M2 from the total curve as described in the Methods.

The competition binding curve of the agonist carbachol with [^{3}H]-QNB is shallow (nH = 0.49) indicating the presence of multiple receptor subpopulations with different affinity for agonists as well (Fig. 2A). To assess whether these differences in agonist affinity can be attributed to the coexistence of M1- and M2-sites, the agonist binding characteristics are evaluated for M1 and M2 separately. As described in Materials and Methods, 90% of the [^{3}H]-QNB-labelled sites are of the M2-type when 2 μM pirenzepine is included in the incubation medium. Competition binding experiments, performed under this condition allow a good evaluation of the binding characteristics of M2-sites in the cortex. Binding characteristics of the M1-sites can then be calculated by subtracting the competition binding curves for M2 from the total curves (Materials and Methods). As shown in Figs 2B and C and Table 2, the carbachol/[^{3}H]-QNB competition binding curve is steep for the M1-sites (nH = 0.97) but is shallow for the M2-sites (nH = 0.38). The Hofstee plot of the competition curve for M1 is also linear (r = 0.97). Similar results for M1 are obtained when agonist competition binding was performed with 0.3 nM [^{3}H]-pirenzepine (Fig. 3 and Table 3).

Whereas M1 is composed of a uniform population of low-agonist affinity sites, M2 comprises high- as well as low-affinity sites. M1 and M2 are also different with respect to the ability of GTP to modulate the agonist binding: the nucleotide does not affect the carbachol competition binding curve for M1 (Tables 2 and 3), but causes a steepening and rightward shift of the curve for M2 (Table 2 and Fig. 2B). The alkylating reagent NEM, on the other hand, causes a leftward shift of the curves for M1 as well as M2. For M2, this shift is also accompanied by a steepening of the curve. Moreover, NEM impairs the ability of GTP to modulate the agonist binding for M2 (Table 2 and Fig. 2B). In the presence of GTP, NEM provoked a 3- to 5-fold shift for M1 and a 17-fold shift for M2.

Table 2. Apparent K_i values (in mM) of carbachol/[^{3}H]-QNB competition binding curves to the total receptor population (M1 + M2), the M1-receptors and the M2-receptors: effects of NEM and GTP

	M1 + M2		M1		M2	
Addition	K_i	nH	K_i	nH	K_i	nH
None	0.60 ± 0.19	0.49	0.67 ± 0.11	0.97	0.019 ± 0.005	0.38
NEM (1 mM)	0.11 ± 0.01	0.48	0.20 ± 0.03	0.84	0.007 ± 0.002	0.66
GTP (1 mM)	0.74 ± 0.24	0.54	0.83 ± 0.26	0.95	0.102 ± 0.018	0.59
NEM + GTP	0.13 ± 0.03	0.50	0.23 ± 0.04	0.92	0.006 ± 0.002	0.55

The corresponding curves are shown in Fig. 2. Values are means ± SD of three separate determinations.

Table 3. K_i values of carbachol and atropine/[^{3}H]-pirenzepine competition binding curves to M1-receptors: effects of NEM and GTP

	Carbachol			Atropine		
Addition	K_i (mM)	nH	r	K_i (nM)	nH	r
None	0.49 ± 0.20	0.94	0.97	0.31 ± 0.14	1.10	0.99
NEM	0.19 ± 0.02	0.95	0.99	0.35 ± 0.11	1.08	0.99
GTP	0.53 ± 0.25	0.95	0.99	0.45 ± 0.15	0.94	0.99
NEM + GTP	0.12 ± 0.02	1.09	0.94	0.30 ± 0.12	1.01	0.98

r represents the regression coefficient of the corresponding Hofstee plot. The displacement curves are shown in Fig. 3. Values are means ± SD of three separate determinations.

The competition curve for the antagonist atropine is steep for M1 as well as for M2 (Tables 3 and 4). As for pirenzepine, GTP and NEM does not affect the atropine binding characteristics for M1- and M2-sites.

DISCUSSION

The introduction of the antagonist pirenzepine has resulted in the classification of the muscarinic acetylcholine receptors into two subtypes, denoted as M1 and M2, respectively [5]. The M1-receptors have considerably higher affinity for pirenzepine than the M2-sites ($K_i = 2 \times 10^{-8}$ versus 1×10^{-6} M) [16]. This difference in affinity enables the calculation of the proportion of each receptor subclass in a given membrane preparation by two-site analysis of the pirenzepine competition binding curve with [^{3}H]-QNB. Alternatively, the availability of [^{3}H]-pirenzepine enabled the direct identification of M1-receptors in different tissues. It is now generally recognized that the M1-receptors are predominant in the cerebral cortex, hippocampus and striatum, whereas the heart, brain stem and cerebellum contain almost exclusively M2-receptors [6]. Membrane preparations from these tissues have been used for the characterization of each receptor subclass.

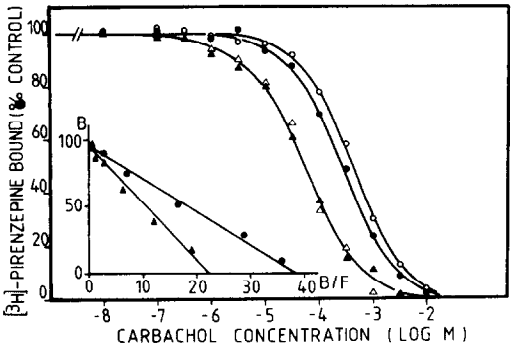

Fig. 3. Carbachol (A) and atropine (B)/[^{3}H]-pirenzepine competition binding to M1-receptors: effects of NEM and GTP. Control (●—●), 1 mM GTP (○—○), 1 mM NEM (▲—▲) and NEM + GTP (△—△). The concentration [^{3}H]-pirenzepine was 0.3 nM which selectively occupies M1-sites. Insert: Hofstee plot of the competition binding data (control). B represents the percentage displacement of [^{3}H]-pirenzepine and F the free concentration of the inhibitor. The regression coefficient of the Hofstee plots are given in Table 4.

In this study, we have investigated agonist- and antagonist-binding properties of M1- and M2-receptors in a membrane preparation from the same tissue, i.e. the bovine cerebral cortex. Computer analysis of pirenzepine/[^{3}H]-QNB competition binding curves reveal that these membranes contain 68% of M1-sites and 32% of M2-sites. The Scatchard plot of [^{3}H]-QNB saturation binding is linear, which indicates that both receptor subclasses have closely the same affinity for the radioligand (Table 1). Binding characteristics of the M2-sites were approximated by competition binding studies with [^{3}H]-QNB in the presence of 2 μM pirenzepine. Under these conditions, it can be calculated that 90% of the [^{3}H]-QNB labelled sites are of the M2-type. A further elevation of the pirenzepine concentration would

Table 4. K_i values (in nM) of atropine/[^{3}H]-QNB competition binding curves to the total receptor population (M1 + M2), the M1-receptors and the M2-receptors: effects of NEM and GTP

	M1 + M2		M1		M2	
Addition	K_i	nH	K_i	nH	K_i	nH
None	2.33 ± 1.27	1.10	1.43 ± 0.79	1.08	7.10 ± 1.23	0.90
NEM (1 mM)	2.46 ± 1.18	0.97	1.49 ± 0.78	1.20	7.01 ± 1.49	0.98
GTP (1 mM)	2.23 ± 0.49	1.02	1.36 ± 0.28	1.20	6.66 ± 0.48	0.93
NEM + GTP	2.16 ± 0.83	0.96	1.30 ± 0.10	1.10	5.74 ± 0.51	1.07

Values are means ± SD of three separate determinations.

have resulted in an even higher proportion of the [^{3}H]-QNB labelled M2-sites, but the concomitant decrease in total binding would have hampered an accurate evaluation of the competition binding data. The binding properties of the M1-sites were estimated by subtracting the competition binding curves in the presence of 2 μM pirenzepine from those obtained for the total muscarinic receptor population. Agonist- and antagonist-binding properties to M1-sites, evaluated by direct competition binding with 0.3 nM [^{3}H]-pirenzepine, were identical.

The carbachol/[^{3}H]-QNB competition binding curve for the M2-sites was shallow (Fig. 3B). A similar deviation from the mass action behaviour was also observed in M2-receptor prominent tissues such as the heart and cerebral basal arteries [12, 17]. This phenomenon had been attributed to the existence of multiple M2-receptor subpopulations with different agonist affinity but with the same affinity for the radioligand [7]. In contrast, the carbachol competition curve for M1 obeyed the law of mass action, the Hofstee plot was linear and the Hill coefficient of this curve was close to unity (Tables 2 and 3). Hence, the M1 receptors were only composed of sites with low affinity for the agonist (K_i = 0.67 ± 0.12 mM). Our agonist competition data were in agreement with recent autoradiographic studies [18] describing a correlation between high pirenzepine affinity and low agonist affinity.

Both muscarinic receptor subtypes also differed in their sensitivity towards GTP. Whereas GTP caused a rightward shift and a steepening of the agonist competition curve for M2, no effect could be detected for M1. These data indicated that M2-sites are entirely responsible for the reported GTP modulation of agonist competition binding for the total receptor population in brain cortex. The GTP sensitivity of M2 receptors occurred also in other tissues and it has been proposed that the nucleotide effect is associated with its ability to convert sites with high agonist affinity (Gi-compound receptors) into sites with low agonist affinity (uncoupled receptors) [8, 9]. In contrast, both competition studies with [^{3}H]-QNB and with [^{3}H]-pirenzepine revealed that GTP did not cause a significant decrease in the carbachol affinity for M1 in calf brain cortex membranes. However, Watson *et al.* found that Gpp(NH)p was able to provoke a small (2-fold) shift of the agonist/[^{3}H]-pirenzepine competition curves in rat cortex membranes [19]. This small shift can be explained by the involvement of a guanine regulatory protein (Gp) in M1-mediated stimulation of phosphatidyl inositol turnover. The discrepancy between the guanine nucleotide shifts on agonist binding to calf and rat brain cortex membranes might be imputed to different experimental conditions but equally well to species-dependent differences in the degree of M1-receptor–Gp coupling or to the sensitivity of guanine nucleotides.

The ability of the alkylating reagent NEM to shift agonist competition binding curves to the left in brain cortex membranes has already been documented. Different interpretations for this phenomenon have been presented, i.e. a conversion of low into high agonist affinity sites [20] and an increase in agonist affinity for the low affinity sites without alteration of the binding properties for high affinity sites [21]. These different interpretations may arise from the complexity of the system investigated, a problem which can be overcome by investigating NEM action on M1 and M2 separately. In this study, we show that NEM was indeed capable to increase the carbachol affinity for both M1 and M2. For the M2-sites, this shift was accompanied by a steepening of the curve and by the loss of GTP-modulation (Fig. 2B, Table 2). This phenomenon could be related to two different actions of NEM, i.e. uncoupling of the M2-receptor–Gi complex and an increase of the agonist affinity for the free receptors. This latter action has been illustrated by the ability of NEM to increase the agonist's affinity for M2-receptors in digitonin solubilized heart membranes [12]. When the agonist competition binding experiments for cortical M2-sites were carried out in the presence of GTP, the NEM-mediated increase in carbachol affinity was approximately 14-fold. An NEM shift of the same magnitude has also been observed in membrane preparations of M2-prominent tissues such as the heart, cerebral basal arteries and brain stem. In contrast, NEM, provoked only a 3–5-fold increase in the carbachol affinity for the M1-sites in the forebrain (Table 2). In addition, no modulation of agonist binding was reported for the M1-sites in the hippocampus [21]. These findings suggest that M1- and M2-sites may have a different sensitivity for the alkylating reagent NEM, independently of the tissue examined. The possibility that differences in NEM sensitivity reflect structural differences between M1- and M2-receptor merits further evaluation.

The [^{3}H]-QNB and [^{3}H]-pirenzepine saturation binding curves as well as the atropine- and

pirenzepine/[^{3}H]-QNB competition binding curves were superimposable for all the experimental conditions. These data are consistent with the earlier described inability of NEM and GTP to modulate antagonist binding to M1 and M2 [12].

Acknowledgements—This work was supported by Astra-Nobelpharma Belgium, Hässle Sweden, the Solvay-Tournay Foundation Belgium and by the Fonds voor Geneeskunig Wetenschappelijk Onderzoek Belgium, G.V. is Onderzoeksleider of the Nationaal Fonds voor Wetenschappelijk Onderzoek Belgium. The authors also wish to thank Mr J. P. De Backer for technical assistance and Mrs M. Doclo for secretarial assistance.

REFERENCES

1. G. J. Wastek and H. I. Yamamura in *Neurotransmitter Receptors* (Eds. H. I. Yamamura and S. J. Enna), Part 2, pp. 103–123, Chapman & Hall, London (1981).
2. N. J. M. Birdsall and E. C. Hulme, *J. Neurochem.* **27**, 7 (1976).
3. E. C. Hulme, N. J. M. Birdsall, A. S. V. Burgen and P. Metha, *Molec. Pharmac.* **14**, 737 (1978).
4. P. M. Laduron and P. F. M. Janssen, *J. Neurochem.* **33**, 1223 (1979).
5. R. Hammer, C. P. Berrie, N. J. M. Birdsall, A. S. V. Burgen and E. C. Hulme, *Nature, Lond.* **283**, 90 (1980).
6. M. Watson, H. I. Yamamura and W. R. Roeske, *Life Sci.* **32**, 3001 (1983).
7. N. J. M. Birdsall, A. S. V. Burgen and E. C. Hulme, *Molec. Pharmac.* **14**, 723 (1978).
8. C. P. Berrie, N. J. M. Birdsall, A. S. V. Burgen and E. C. Hulme, *Biochem. biophys. Res. Commun.* **87**, 1000 (1979).
9. M. Delhaye, J. M. Desmet, G. Taton, P. De Neef, J. C. Camus, J. Fontaine, M. Walebroeck, P. Robberecht and J. Christophe, *Archs Pharmac.* **325**, 170 (1984).
10. M. Sokolovsky, D. Gurwitz and R. Garlon, *Biochem. biophys. Res. Commun.* **94**, 487 (1980).
11. S. Uchida, K. Matsumoto, A. Mizushima, T. Osugi, H. Higuchi and H. Yoshida, *Eur. J. Pharmac.* **100**, 291 (1984).
12. P. Vanderheyden, L. Kanarek and G. Vauquelin, *Eur. J. Pharmac.* **125**, 127 (1986).
13. O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. biol. Chem.* **193**, 265 (1951).
14. P. J. Munson and D. Rodbard, *Analyt. Biochem.* **107**, 220 (1980).
15. Y-C. Cheng and W. H. Prusoff, *Biochem. Pharmac.* **22**, 3099 (1973).
16. M. Watson, W. R. Roeske and H. I. Yamamura, *J. Pharmac. exp. Ther.* **237**, 419 (1986).
17. P. Vanderheyden, G. Ebinger, J. P. De Backer and G. Vauquelin, *Life Sci.* **39**, 1517 (1986).
18. R. Cortes and J. M. Palacios, *Brain Res.* **262**, 227 (1986).
19. R. S. Aronstam, L. G. Abood and W. P. Hoss, *Molec. Pharmac.* **14**, 575 (1978).
20. P. Vanderheyden, C. Andre, J. P. De Backer and G. Vauquelin, *Biochem. Pharmac.* **33**, 2981 (1984).
21. D. D. Flynn and L. T. Potter, *Proc. natn. Acad. Sci. U.S.A.* **82**, 580 (1985).