REGIONAL HETEROGENEITY OF MUSCARINIC RECEPTORS OF MOUSE BRAIN

Yoel KLOOG, Yaacov EGOZI and Mordechai SOKOLOVSKY
Department of Biochemistry, The George S. Wise Faculty of Life Sciences, Tel-Aviv University, Tel-Aviv, Israel

Received 15 September 1978

1. Introduction

In [1–3] binding to muscarinic receptors was performed on homogenate of whole mouse brain using the potent and highly specific antagonist *N*-[^{3}H]methyl-4-piperidylbenzilate ([^{3}H]-4NMPB). The non-linearity of Scatchard plots found in equilibrium studies, and the demonstration of biphasic kinetics, led to the formulation of the following scheme:

$$\begin{array}{ccccc} R & + & L & \rightleftarrows & RL \\ \uparrow & & & & \uparrow\downarrow \\ R^* & + & L & \rightleftarrows & RL^* \end{array}$$

This formulation was the simplest one that could accomodate the assumption of homogeneity of the receptors in the preparation. However, this cyclic scheme includes an irreversible step which contradicts the requirement of microscopic reversibility. This prompted us to question the assumption of receptor homogeneity in the mouse brain.

2. Materials and methods

Labelled and non-labelled compounds are the same as in [1–3]. Direct binding studies, competition experiments and the analysis of binding kinetics were carried out as detailed in [1–3]. Choline acetyltransferase and cholinesterase activities were determined according to [4,5].

Male ICR mice (20–25 g) were decapitated, brains were rapidly removed and the brain areas were dissected in a cold room after identification with the aid of [6]. After dissection, brain regions (from 15–25 mice for the thalamus and 10–15 mice for other regions) were homogenized in ice-cold 0.32 M sucrose using a motor-driven Teflon pestle (950 rev./min) in a glass homogenizer to yield a 10% homogenate (w/v).

Specific binding is defined as the total minus the non-specific, i.e., binding in the presence of 5×10^{-5} M of unlabelled ligand.

3. Results and discussion

The binding of [^{3}H]-4NMPB at 25°C was studied as in [1–3] in homogenates of the following brain regions: cortex, caudate putamen, hippocampus thalamus, medulla pons and cerebellum. The density of [^{3}H]-4NMPB binding sites together with the activities of choline acetyl-transferase and of cholinesterase found in the various regions are given in table 1. In agreement with neurophysiological studies [7,8], we found variations in the 4NMPB binding sites with at least a 13-fold variation between the putamen, containing the highest density of binding sites, and the cerebellum, the lowest. This distribution is in agreement with known distributions of muscarinic receptors in other mammalian brains. The other cholinergic markers studied here, closely parallel each other in the various regions (for comparison see also [8]) and correlate well with the 4NMPB binding in the same areas (table 1). However, as shown there are some discrepancies in these correlations. Thus, e.g., the CAT activity appears to be high within the medulla pons while its 4NMPB binding is relatively low as also was demonstrated in monkey brain [8]. Interpretation at this stage must be made cautiously since the transmitter synthesis takes place both in cell bodies and nerve terminals while receptor binding

Table 1
Regional distribution of specific [^{3}H]-4NMPB binding, choline acetyltransferase (CAT) and cholinesterase (ChE) activities in mouse brain regions

Region	[^{3}H]-4NMPB[a] binding (pmol/protein)	ChE[b] (nmol AcCh hydrolyzed/ mg protein/min)	CAT[b] (nmol AcCh synthesized/ mg protein/h)
Caudate putamen	1.54 ± 0.38	457 ± 60	23.0 ± 6
Hippocampus	1.05 ± 0.05	107 ± 10	10.1 ± 2
Cortex	0.91 ± 0.06	61 ± 6	8.0 ± 3
Thalamus	0.61 ± 0.10	98 ± 7	11.6 ± 3
Medulla pons	0.26 ± 0.05	65 ± 5	34.1 ± 5
Cerebellum	0.12 ± 0.01	12 ± 3	2.0 ± 1

[a] The values given are the mean of at least 4 separate binding curves ± SEM
[b] The values given are the mean ± SEM of 6 samples

presumably is largely associated with the post-synaptic membrane.

Equilibrium binding studies of [^{3}H]-4NMPB were performed with the homogenates of the cortex, thalamus and medulla pons, at 0.25–20 nM. Specific ligand binding exhibits the hyperbolic shape curve typical for saturation phenomena. Non-specific binding is much lower and varies linearly with ligand concentration. The binding data, when replotted according to Scatchard, gave straight lines for each of the regions examined indicating a single population of binding sites within each region at the concentration range investigated. The binding constants measured in these experiments are given in table 2.

The nature of the high affinity binding of [^{3}H]-4NMPB to the 3 regions was examined by competition experiments in which selected unlabelled muscarinic ligands inhibit the binding of [^{3}H]-4NMPB.

Table 2
Binding parameters for muscarinic antagonists and agonists determined by direct binding and competition with 2 nM [^{3}H]-4NMPB

Ligand	Binding parameter	Cortex	Thalamus	Medulla pons
^{3}H-4NMPB	K_d (nM)	0.4 ± 0.03	0.5 ± 0.06	0.82 ± 0.09
Scopolamine	K_d (nM)	0.62 ± 0.07	2.0 ± 0.15	2.6 ± 0.30
Carbamylcholine	I_{50} (μM)	170 ± 20	3.0 ± 0.23	4.7 ± 0.61
Arecoline	I_{50} (μM)	55 ± 6	9.1 ± 1.27	4.2 ± 0.54
Acetylcholine	I_{50} (μM)	30 ± 4	1.8 ± 0.25	3.0 ± 0.40

Values for K_d for agonists depend on their mode of binding and hence values of I_{50} are given; i.e., the concentration of drug causing 50% reduction in binding of [^{3}H]-4NMPB under the experimental conditions described. Values for K_d for antagonists were calculated as in [1,2], assuming a simple competitive interaction between 4NMPB and the competing ligand. The average values of I_{50} and binding constants determined, are the means ± SEM of 3–4 separate experiments, each performed in triplicate. For cholinesterase-sensitive agonists the assay contained 10^{-5} M physostigmine. This concentration of inhibitor did not affect the binding characteristics of [^{3}H]-4NMPB in the absence of agonists

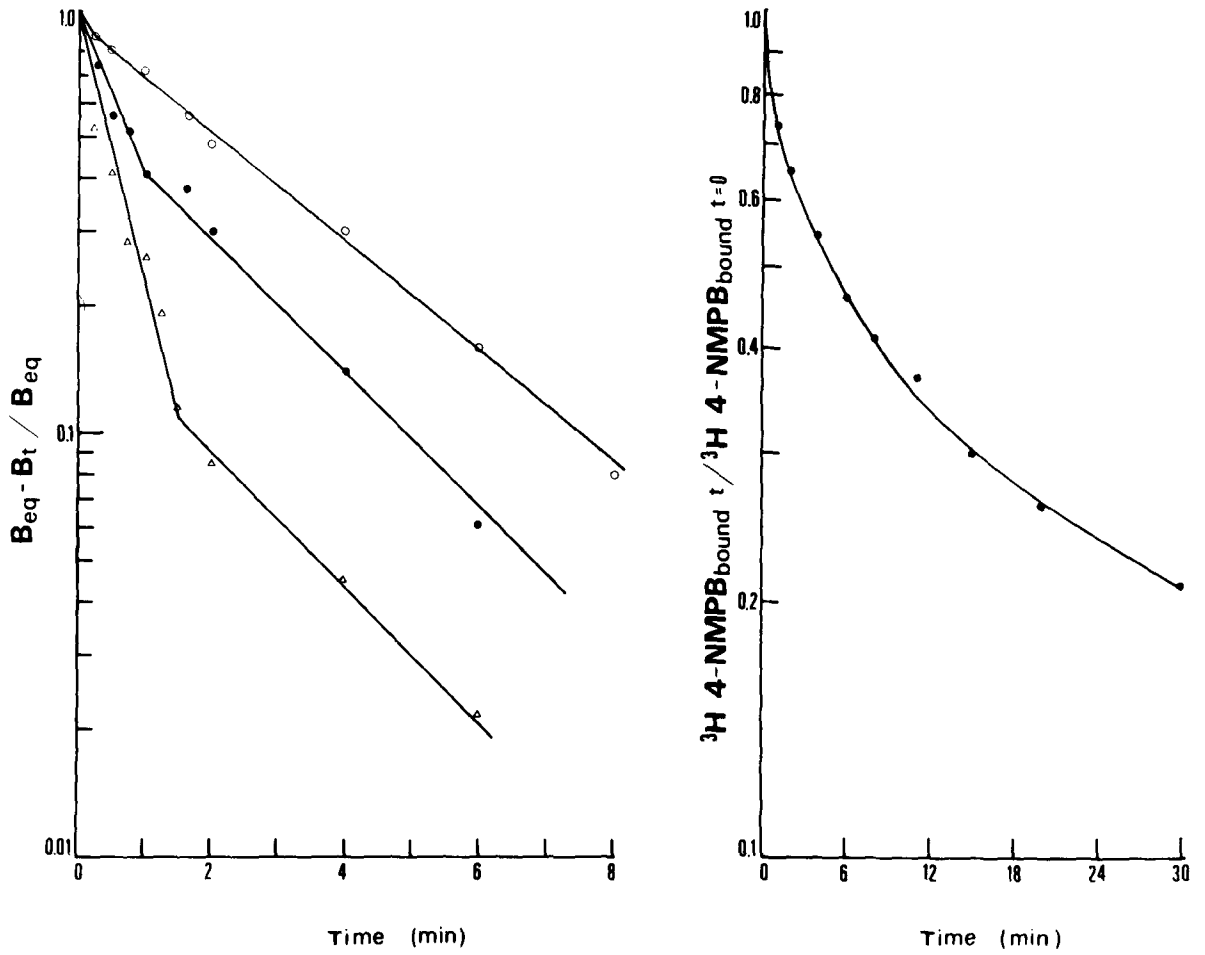

Fig.1. Left: Initial rate of [^{3}H]-4NMPB binding at different ligand concentrations to thalamus preparation. Homogenates (0.05 ml) were incubated with 0.5 nM (○), 2.5 nM (●) and 5.0 nM (△), [^{3}H]-4NMPB at 25°C in 2 ml modified Krebs solution for the various time intervals. Association was begun by addition of tissue (88 pM binding sites), terminated by rapid filtration, and specific binding was determined as in section 2. Each experimental point is the mean of triplicate samples whose standard error was < 5%. The data are plotted according to the equation:

$$\ln \frac{(B_{eq} - B_t)}{B_{eq}} = (k_1\,[4\text{NMPB}] + k_{-1}) \cdot t$$

where B_{eq} and B_t are the concentrations of the bound ligand at equilibrium and at time t.

Fig.1. Right: Dissociation of [^{3}H]-4NMPB from the muscarinic receptors in the thalamus preparation. Samples (0.05 ml) were incubated to equilibrium at 25°C in the presence of 5 nM [^{3}H]-4NMPB. Dissociation was begun by addition of 50 μM 4-NMPB to tubes and the samples were filtered immediately (zero time) and at the times indicated. Specific binding was determined as in the text. Each point is the mean of at least 3 experiments whose standard error was < 5%. ([^{3}H]-4NMPB$_{bound}$)$_{t=0}$ was 0.57 pmol/mg protein.

The binding constants and I_{50} values measured in these competition experiments are given in table 2. The data demonstrates some small but nonetheless reproducible differences between the K_d values of antagonists binding to the various regions, with lowest affinity at the medulla pons. On the other hand the I_{50} values for agonists reveal an opposite and more pronounced differences: compare the values of the medulla and thalamus with that of the cortex.

Binding was investigated further kinetically. Figure 1 (left) shows the initial rate of [^{3}H]-4NMPB binding to the thalamus homogenate at different ligand concentrations. Under such pseudo first order conditions the data should yield straight lines if the reaction is bimolecular. However, fig.1 demonstrates biphasic behavior. Similar biphasic behavior was observed for the binding of [^{3}H]-4NMPB to homogenates of the other two regions. The calculated $K_{on} \times 10^8$ (M^{-1} min^{-1}) are 4.03 for the medulla, 4.5 for the thalamus and 2.45 for the cortex.

An isotopic dilution technique is suitable to follow the course of dissociation of ligand–receptor complex. Figure 1 (right hand) demonstrates the first order plot for the dissociation of [^{3}H]-4NMPB from the thalamus preparation. The first order plot deviates from linearity. Similar biphasic behavior was observed for the dissociation from the other regions studied. The apparent half-life times of the dissociation of [^{3}H]-4NMPB from binding sites in the medulla pons, thalamus and cortex are 5 min, 5 min and 20 min, respectively. It is noteworthy that the trend in the kinetics experiments is consistent with the differences in K_d as discussed (vide supra). Thus, the weight of the evidence rests with a regional heterogeneity of muscarinic receptors in mouse brain. Therefore the irreversible cyclic scheme which assumes homogeneity is indeed inadequate. Two different schemes are more consistent with the kinetics experimental results. The first one is linear, i.e., $R + L \rightleftarrows RL \rightleftarrows R^*L$. The second one is again a cyclic scheme but it includes reversible reactions for all the steps:

$$\begin{array}{ccccc} R & + & L & \rightleftarrows & RL \\ \uparrow\downarrow & & & & \uparrow\downarrow \\ R^* & + & L & \rightleftarrows & R^*L \end{array}$$

Note that the transformation of $R \rightarrow R^*$ is much slower than the reverse reaction, therefore the reaction might be practically irreversible. Presently we are engaged in chase experiments with the purpose of establishing the forward and back rates of $R^* \rightarrow R$. This will allow us to distinguish between the two schemes presented here. Ligand-induced isomerization of the muscarinic receptors proposed [1–3] has now been reported independently for the chick heart [9], retina and neuroblastoma [10]. Recent characterizations of these receptors in the irides of rabbit [11] and cat [12] yielded similar results, thus increasing the variety of systems in which an isomerization step appears to be related to the interaction of antagonists with muscarinic receptors.

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