DIFFERENTIAL EFFECT OF *N*-ETHYL MALEIMIDE ON MUSCARINIC AGONIST BINDING IN RAT AND BOVINE BRAIN MEMBRANES

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1. Introduction

Muscarinic cholinergic receptors have now been characterized in a wide variety of tissues using various tritiated ligands [1–6]. Binding characteristics have proven to be similar in all of the different tissues studied to date for agonists as well as antagonists: antagonists are bound with high affinity, characterized by a Hill coefficient of 1, suggesting a single class of receptor sites, while agonists show a lower affinity with a Hill coefficient of <1, suggesting a possible heterogeneity of binding sites. Indeed, two or possibly more components have been recognized in the case of strong agonists [7]. Muscarinic receptors of rat brain have been converted from a state of low to a state of high affinity for agonists by the alkylating reagent *N*-ethyl maleimide without undergoing a change in antagonist affinity or a reduction of the total number of receptor sites [8,9]. We have found that the pharmacological characteristics of muscarinic receptors from bovine brain are similar to those of other mammalian species (unpublished results). Here I report the influence of several agents, including sulfhydryl reagents, on the binding properties of these receptors, using the potent muscarinic ligand [^{3}H]-quinuclidinyl benzilate. The effect of these compounds upon the binding was different from that described for the rat and in particular, no effect of NEM on agonist binding was observed.

Abbreviations: DTNB, 2,2′-dinitro-5,5′-dithiobenzoic acid; IAA, iodoacetamide; NEM, *N*-ethyl maleimide; pCMB, *para*-chloro-mercuribenzoic acid; DTE, 1,4-dithioerythritol; QNB, quinuclidinyl benzilate

2. Materials and methods

Ox brains were obtained fresh from the slaughterhouse within 4 h of death. Binding studies were performed using a synaptosomal-P_2 preparation prepared from the cerebral cortex at 0.02–0.06 mg protein/ml in 1 mM EGTA, 25 mM Na-phosphate buffer (pH 7.4). The binding assay was essentially as in [10].

The membranes (0.2 mg protein/ml) were incubated with the various reagents for 30 min at 22°C or 37°C as noted. The reagents were removed by centrifuging at 12 000 × *g* for 20 min. The pellets were rinsed with buffer resuspended and recentrifuged before use. When treatment with a second reagent followed, the procedure was repeated. Effects on specific binding were tested at 250 pM [^{3}H]QNB (16 Ci/mmol) in the presence and absence of 100 nM atropine. Inhibition curves were performed at 50 pM [^{3}H]QNB (44 Ci/mmol) at 22°C or 37°C as noted.

3. Results

3.1. *Specific [^{3}H]QNB binding*

Incubation of the membrane preparation with IAA, DTE or DTNB at ≤10 mM for 30 min at 22°C or 37°C had no effect on specific [^{3}H]QNB binding. The effects of NEM and pCMB varied with temperature: pCMB inhibited binding when incubated at both temperatures but to a much greater extent at 37°C (≤65% at 1 mM). This contrasts with the case of rat brain receptors for which NEM was found not to inhibit either antagonist or agonist binding.

Saturating concentrations of agonists (10^{-1} M) and antagonists (10^{-6} M) could protect quantitatively the receptor against the inhibitory effect of NEM (1 mM) and pCMB (0.1 mM). The effects of various

combinations of reagents on bovine brain are shown in table 1. NEM at 1 mM and pCMB at 0.1 mM reduced binding in bovine brain to 36% and 26% of control values, respectively, while DTE slightly increased binding. Reduction of disulfide bonds with 5 mM DTE before NEM or pCMB treatment slightly increased the inhibitory effect of these two reagents by ⩽10%, DTE was able to reverse the effects of pCMB by ~15–20%, while it further decreased binding in NEM-pretreated samples. The combination of NEM and pCMB treatments resulted in almost 100% inhibition of specific [^{3}H]QNB binding.

Table 1
Effects of various reagents on specific [^{3}H]QNB binding

Reagent	Ox brain (% control binding)
Control	100
1 mM NEM	36
0.1 mM pCMB	26
DTE (5 mM)	110
DTE–NEM	29
DTE–pCMB	20
NEM–DTE	14
pCMB–DTE	40
NEM–pCMB	3
pCMB–NEM	5

Membranes were incubated with the appropriate reagents for 30 min at 37°C. Reagents were removed by centrifugation, when a second treatment followed, the procedure was repeated as in section 2. Binding was assayed at 37°C with 250 pM [^{3}H]QNB ± 100 nM atropine. Data is represented as % specific [^{3}H]QNB binding. The values represented are from a typical experiment, absolute values varied ~10% from experiment to experiment, but the trends remained the same. Experiments were performed at least 3 times

3.2. *Agonist and antagonist binding*

In order to decide whether the inhibitory effect observed on [^{3}H]QNB binding resulted from a reduction in the number of active sites, or in their binding affinity, we examined the displacement of this ligand by a series of muscarinic agents. After pCMB treatment, the displacement curves obtained with atropine or carbamylcholine were identical with control curves. In contrast, DTE decreased the affinity of the receptor for carbamylcholine ~10-fold. Table 2 summarizes the inhibition constants of the receptor after these different treatments.

The effect of membrane preincubation with 1 mM NEM (30 min at 37°C) on carbamylcholine inhibition of [^{3}H]QNB binding is illustrated in fig.1 for rat and bovine brain preparations. Binding studies in this series of experiments were performed at 22°C and 37°C: there was little difference in the affinities of control preparations for agonists at these two temper-

Table 2
Effects of various reagents on agonist and antagonist inhibition of [^{3}H]QNB binding (50 pM) at 22°C and 37°C in rat and ox brain

Reagent	% Specific binding	Species	K_i (M) carbamylcholine		K_i (M) atropine	
			22°C	37°C	22°C	37°C
Control	100	Ox	1.1×10^{-5}	2.5×10^{-5}	1.4×10^{-10}	2×10^{-10}
		Rat	1.1×10^{-5}	1.4×10^{-5}	2×10^{-10}	2.5×10^{-10}
1 mM NEM	35 ± 10	Ox	1.1×10^{-5}	1.1×10^{-5}	1.1×10^{-10}	2.8×10^{-10}
	100	Rat	1.4×10^{-6}	1.1×10^{-5}	–	–
0.1 mM pCMB	25 ± 10	Ox	–	1.7×10^{-5}	–	5.7×10^{-10}
5 mM DTE	100 ± 10	Ox	–	1.1×10^{-4}	–	2.5×10^{-10}

Inhibition constants (K_i) for carbamylcholine were calculated from the curves shown in fig.1. The K_i was calculated from the equation:

$$K_i = \frac{ID_{50}}{1 + C/K_d}$$

where C is the concentration of radioactive ligand (50 pM) and K_d its dissociation constant (20 pM for [^{3}H]QNB as calculated from Scatchard analysis and kinetic data (not shown)). ID_{50} values were determined graphically. Atropine inhibition curves were performed the same day (curves not shown). Reagent incubation was at 37°C for 30 min. Binding assays were performed at 22°C and 37°C as noted. % specific binding indicates the % remaining sites after reagent treatment

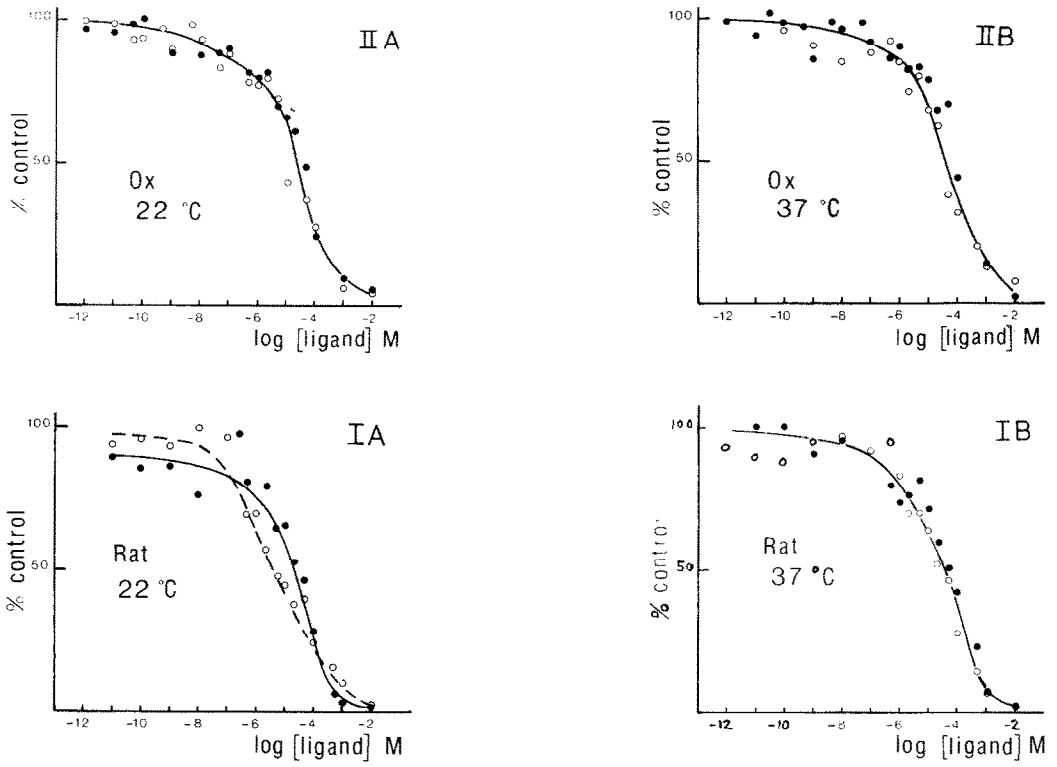


Fig.1. Effect of 1 nM NEM on carbamylcholine inhibition of [^{3}H]QNB binding at 22°C or 37°C in rat and bovine brain. The proportion of [^{3}H]QNB bound, relative to that observed in the presence of 50 pM [^{3}H]QNB alone, is plotted as a function of carbamylcholine concentration. The curves are thus normalised and do not reflect the inhibition observed after incubation with NEM (cf. table 1), which was performed at 37°C for 30 min. Inhibition curves (15 min with ligand, 45 min with 50 pM [^{3}H]QNB) were performed at 22°C or 37°C. (•———•) control; (○———○) 1 mM NEM-treated membranes.

atures. In all cases, the affinity of the remaining sites for antagonists (QNB and atropine) remained unchanged (data not shown). As seen in the fig.1, NEM treatment caused an increase in the affinity of the receptor from rat brain for carbamylcholine, shifting the K_i ~10-fold if binding was performed at 22°C. This effect was not observed however when the binding was performed at 37°C. For bovine brain membranes, no significant change could be detected at either 22°C or 37°C.

4. Discussion

We have studied the influence of various reagents on bovine brain muscarinic receptors. In agreement with the results in [9] we found that NEM pretreatment at 37°C of a rat brain membrane preparation increased receptor affinity for the agonist carbamylcholine, the K_i being shifted from 10^{-5}–10^{-6} M. However, this effect was only observed when binding was carried out at 22°C but not at 37°C. This indicates that not only the accessibility of affected groups is temperature dependent but also that their role in agonist binding is influenced by temperature. The increase in agonist affinity has been interpreted [8,9] as a reflection of the conversion of low affinity to high affinity conformation. Our results do not allow us to decide whether the characteristics of the site observed after NEM reaction are identical to those of the high affinity component of the control.

The effects observed with the various reagents examined on muscarinic binding in bovine brain differ in several respects from that obtained in rat brain. NEM causes an inhibition of specific QNB binding (without a corresponding change in affinity) in ox brain, but not in rat brain. In addition, we did not observe any significant change in agonist affinity after

NEM treatment, whether binding was performed at 22°C or 37°C. The protection observed in the presence of an excess of agonist (10^{-1} M carbamylcholine) or antagonist (10^{-6} M atropine) against inhibition by NEM and pCMB implies that the reactive groups, which are probably sulfhydryl groups, must be directly or allosterically involved in the binding. Treatment with DTE, which had little effect on binding in rat brain, caused a decrease in receptor affinity for agonists in ox brain. This suggests the presence of disulfide bonds in the receptor. DTE was also able to reverse ~20% of the inhibition caused by pCMB while it further inhibited binding in membranes pretreated with NEM. Finally, pretreatment with NEM did not protect against subsequent inhibition by pCMB and vice versa. Thus, the modifications observed with the sulfhydryl reagents reveal a clear difference between bovine and rat brain muscarinic receptors, in spite of the similarity of their pharmacological binding characteristics.

Our results indicate that it may be important to examine the biochemical properties of muscarinic receptors from different species before generalizing on the importance of sulfhydryl groups in receptor–ligand interaction. Such differences must be taken into account in biochemical studies of muscarinic receptors and may provide the basis for interesting comparisons regarding the functional role of essential groups in the receptor proteins.

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