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Different effects of *N*-ethylmaleimide on M1 and M2 muscarine receptors in rat brain

(sulfhydryl groups/agonist–antagonist affinities/acetylcholine receptor subtypes)

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ABSTRACT ***N*-Ethylmaleimide (MalNEt) disclosed three differences between M2 muscarine receptors in membranes from the rat brainstem and M1 receptors in the hippocampus. At 0.1 mM, MalNEt completely interconverted the higher affinity state of M2 receptors for carbachol to a lower affinity state, while having no effect on the two affinity states of M1. This "uncoupling" effect is similar to that produced by guanine nucleotides and appears to be due to separation of an agonist–receptor complex from a guanine nucleotide-binding protein. Higher MalNEt concentrations (1–5 mM) increased the affinity of uncoupled M2 receptors, again without effect on M1 states. Finally, in MalNEt, the affinity of M2 receptors for carbachol was different from values for M1 receptors. Thus, MalNEt is an excellent agent for distinguishing M1 and M2 receptors and the two states of M2 receptors. MalNEt had no effect on the affinity or M1-selectivity of the antagonist pirenzepine.**

Although muscarine receptors in different tissues cannot so far be distinguished biochemically by size or charge or with antibodies (1), two classes of receptors are readily distinguished according to their pharmacological binding properties, locations, apparent mechanisms, and functions (2). One class ("M1"), characteristic of many neocortical, ganglion and glandular cells, remains after cholinergic denervation and in Alzheimer disease; the activation of these postsynaptic receptors modifies membrane phosphoinositides (3, 4) and ion fluxes (5, 6) and results in cellular excitation. These receptors show higher affinity for the antagonist pirenzepine than do M2 receptors (2, 7, 8). They have two affinity states for the agonist carbachol, which are interconverted [from "high" to "low" affinity, according to the terminology of Birdsall and co-workers (9, 10)] by removal of divalent cations (2). Another class ("M2") is characteristic of cholinergic nerves and nerve terminals and of peripheral muscles, including the heart (2); the activation of these receptors attenuates the activation of adenylate cyclase (11). These receptors also have two affinity states for carbachol, but these states (unlike those of M1) are interconverted by guanine nucleotides ["uncoupled" from "superhigh" to "high" affinity (9, 10)] (2, 12, 13).

Prior studies with muscarine receptors and *N*-ethylmaleimide (MalNEt) have shown that low concentrations of MalNEt diminish the affinity between cardiac and brainstem (M2) receptors and carbachol, mimicking the effects of 5′-guanylyl imidodiphosphate (p[NH]ppG) (14–16). Parallel effects of p[NH]ppG and MalNEt also have been noted for certain α-adrenoreceptors, dopamine receptors, opiate receptors, and other receptors (17–20), all of which are believed to act via the attenuation of adenylate cyclase (11). Concentrations of MalNEt of 1 mM or higher have an additional, unusual, effect of increasing the affinity of agonists for muscarine receptors in several tissues (21, 22). A general conclusion of those working with MalNEt or p[NH]ppG has been that interconversion of the states of muscarine receptors also produces the appearance of only one receptor type.

Here we show that MalNEt distinguishes clearly between M1 receptors in the rat hippocampus (80% of the total receptors; ref. 2) and M2 receptors in the brainstem (90% of the total receptors). At low concentrations, MalNEt uncoupled the higher affinity state of brainstem receptors for agonists, while having no effect on the two states of hippocampal receptors. It also accelerated the dissociation of the M2-selective agonist, [^{3}H]oxotremorine-M, from brainstem receptors. In addition, 1–5 mM MalNEt increased the affinity of carbachol for brainstem but not for hippocampal receptors. The agonist binding affinities of M1 and M2 receptors remained distinct in MalNEt. Thus, MalNEt is a highly effective agent for distinguishing M1 and M2 receptors.

MATERIALS AND METHODS

(−)-[^{3}H]Quinuclidinyl benzilate ([^{3}H]QNB, 33 Ci/mmol; 1 Ci = 37 GBq) was obtained from Amersham, and [^{3}H]oxotremorine-M acetate (83.3 Ci/mmol) was from New England Nuclear. MalNEt, carbamoylcholine (carbachol), and all other reagents were from Sigma. Pirenzepine was a gift from Boehringer Ingelheim.

Preparation of Membranes. Male Sprague–Dawley rats (200–250 g) were decapitated with a guillotine, and the hippocampus and "brainstem" (medulla, pons, cerebellum, and a piece of the midbrain including the superior colliculi) were removed. Tissues were homogenized with a Polytron blender in ice-cold 50 mM sodium phosphate/10 mM Na_3EDTA, pH 7.4 (19 ml/g of tissue) and were left on ice for 30 min. Unselected membranes containing 99% of the muscarine receptors in the tissues were recovered by centrifugation for 10 min at 48,000 × g_{max} and were resuspended with the blender in 20 mM Tris/1 mM $MnCl_2$ buffer, pH 7.4 (Tris/Mn buffer). In some experiments membranes were pretreated with 0.1–5 mM MalNEt for 30 min at 25°C and washed with Tris/Mn buffer before competition assays. Results in these experiments were identical to those where MalNEt was included in the incubation medium.

Competition Assays. Membranes from 5 mg of tissue were incubated in 10 ml of Tris/Mn buffer containing 1.0 nM (−)-[^{3}H]QNB and various concentrations of unlabeled competing ligand. This concentration of QNB saturates 99% of the receptors in the absence of counter ligands (2). Nonspecific binding was determined by the inclusion of 1 μM (±)-QNB and was usually 1–5% of total binding. After incubation at 37°C for 45–60 min, membranes were recovered on glass fiber filters (2.4 cm, 934 AH Reeve Angel) by vacuum filtration. Filters were washed three times with 4 ml of ice-cold

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Abbreviations: MalNEt, *N*-ethylmaleimide; p[NH]ppG, 5′-guanylyl imidodiphosphate.

buffer, dried, and placed in vials containing 4 ml of Liquiscint (National Diagnostics, Somerville, NJ). Radioactivity was determined by liquid scintillation spectrometry at a counting efficiency of 37.5%. Total and nonspecific binding were each assayed in triplicate, and the difference between the means was taken as binding to receptors. "Percent of sites occupied" in the figures equals the percentage inhibition of binding of [^{3}H]QNB.

Dissociation Rates. Membranes were labeled in 5 nM [^{3}H]oxotremorine-M at 25°C for 30 min in Tris/Mn buffer (1 ml for membranes for 50 mg of tissues). This concentration labels approximately half the total M2 receptors in membranes: those in the higher affinity state. Dissociation was initiated by 1:100 dilution of the labeled membranes in Tris/Mn buffer containing 1–5 μM (±)-QNB. Dissociation was stopped by filtration of membranes from 5 mg of tissue as described for competition assays.

RESULTS

Fig. 1 shows the pronounced effects of MalNEt on the affinity of carbachol for M2 muscarine receptors in membranes from the rat brainstem. MalNEt steepened competition curves between carbachol and [^{3}H]QNB and shifted them to the right. The observed IC_{50} values were $10^{-6.1}$, $10^{-5.2}$ and $10^{-4.7}$ M for 0, 0.01, and 0.1 mM MalNEt, respectively. A close approach to an isotherm was seen at 0.1 mM MalNEt, indicative of maximum uncoupling of higher affinity interactions between carbachol and M2 receptors. No further effect was seen with 0.1 mM p[NH]ppG (not shown). Affinity changes occurred without any alterations in the total number of QNB sites. In contrast, the maximum right-shift obtainable with p[NH]ppG alone occurred at 0.1 mM (not shown) and was no greater than that seen with 0.01 mM MalNEt (Fig. 1). Thus under these conditions MalNEt is significantly more effective than p[NH]ppG for uncoupling high-affinity complexes.

MalNEt accelerated the dissociation of another agonist, [^{3}H]oxotremorine-M, from M2 receptors in membranes from the rat brainstem (Fig. 2). Dissociation rates at 25°C in 0, 0.1 and 5 mM MalNEt were 0.0077, 0.0029, and 0.0012 sec^{-1}, respectively. The lower affinity of M2 receptors for carbachol at equilibrium in MalNEt (Fig. 1) correlates well with the faster dissociation of oxotremorine-M in MalNEt (Fig. 2).

Fig. 3 shows that MalNEt at 0.01–0.1 mM had little or no effect on the affinity of carbachol for M1 muscarine receptors in membranes from the rat hippocampus (Fig. 3). Occasionally a small right-shift was seen at the furthest left (bottom) part of these competition curves, where the few M2 receptors in the hippocampus show their binding (2). The competition curve for the hippocampus remained much flatter than that for the brainstem, indicating that higher and lower affinity states of M1 receptors are not interconverted by MalNEt (2).

Concentrations of MalNEt in excess of 0.1 mM had another selective effect on brainstem receptors. MalNEt at 5 mM increased the affinity of carbachol for brainstem receptors (Fig. 4), compared to results in 0.1 mM MalNEt. Since the brainstem curve did not flatten as it shifted leftwards, there is no reason to suspect recoupling of high-affinity carbachol–receptor complexes of the type seen in Fig. 1. In con-

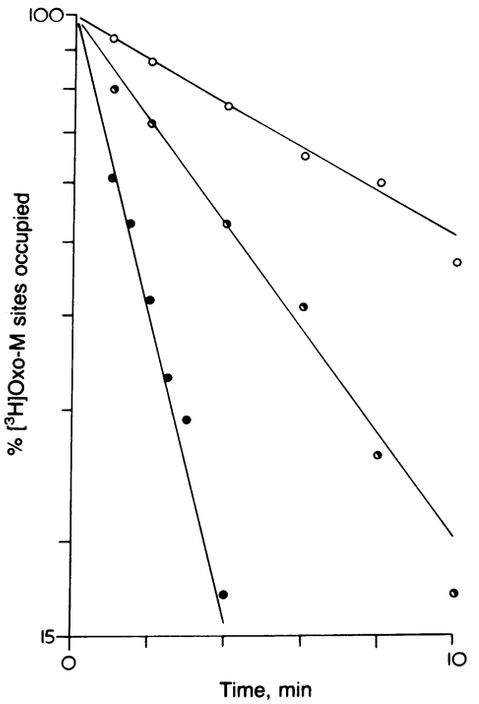

FIG. 2. Accelerating effects of MalNEt on the dissociation of [^{3}H]oxotremorine-M ([^{3}H]Oxo-M) from muscarine receptors in the rat brainstem. Labeled membranes were dispersed in Tris/Mn buffer at 25°C containing 5 μM (±)-QNB and MalNEt. MalNEt concentrations were 0 (○), 0.1 mM (◐), and 5 mM (●).

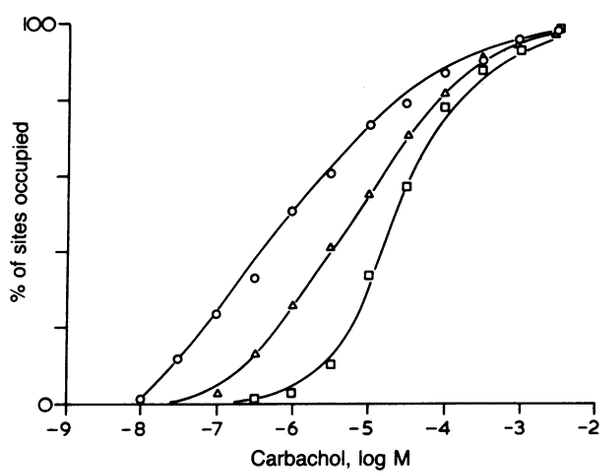

FIG. 1. Uncoupling effects of MalNEt on the affinity of carbachol for muscarine receptors in membranes from the rat brainstem. MalNEt concentrations were 0 (○), 0.01 mM (△), and 0.1 mM (□).

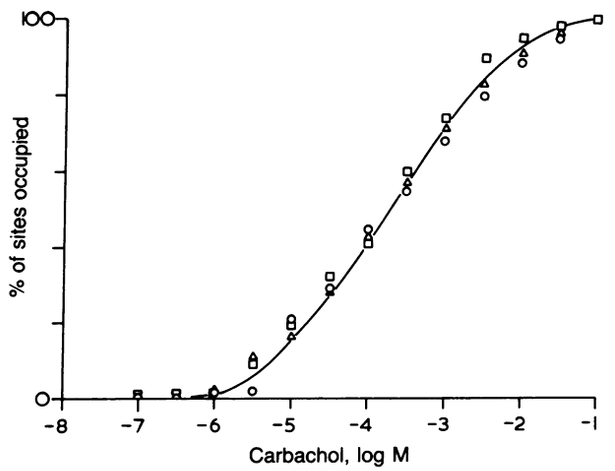

FIG. 3. Lack of effect of MalNEt on the affinity of carbachol for muscarine receptors in membranes from the rat hippocampus. Concentrations of MalNEt were as in Fig. 1.

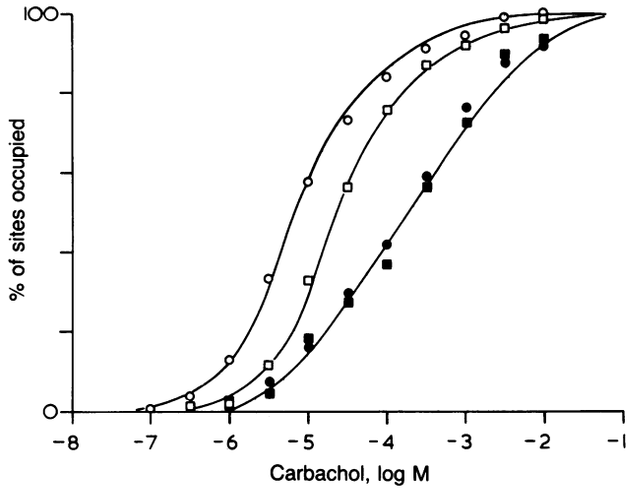

FIG. 4. Increased affinity of uncoupled brainstem receptors (○, □) for carbachol after pretreatment of membranes with 5.0 (○) MalNEt vs. 0.1 mM MalNEt (□). Affinities for hippocampal receptors (●, ■) were the same in 5.0 (●) or 0.1 mM MalNEt (■).

trast, 5 mM MalNEt had no effect on either the higher or lower affinity states of M1 receptors. Thus, MalNEt helps to distinguish M1 and M2 receptors by modifying affinities as well as by causing M2 uncoupling.

In contrast to the major effects of MalNEt on the binding of agonists, no significant changes were produced by MalNEt on the binding or selectivity of the M1-selective antagonist pirenzepine in the hippocampus or brainstem (Fig. 5). These results are in accord with our prior evidence that MalNEt does not modify the rates of association or dissociation of the antagonist [^{3}H]QNB to or from either M1 or M2 receptors, or the total amount of binding (2).

DISCUSSION

The present results concerning the selective uncoupling effects of low concentrations of MalNEt on brainstem muscarine receptors confirm and extend the work of Korn *et al.* (14), who demonstrated that 0.01–0.1 mM MalNEt diminished the affinity for oxotremorine of muscarine receptors in the rat cerebellum and brainstem more than the affinity in the hippocampus and cortex. However, these workers concluded that their very similar post-MalNEt affinities for oxotremorine in all four brain regions indicated that pre-MalNEt differences in affinities were the result of different degrees

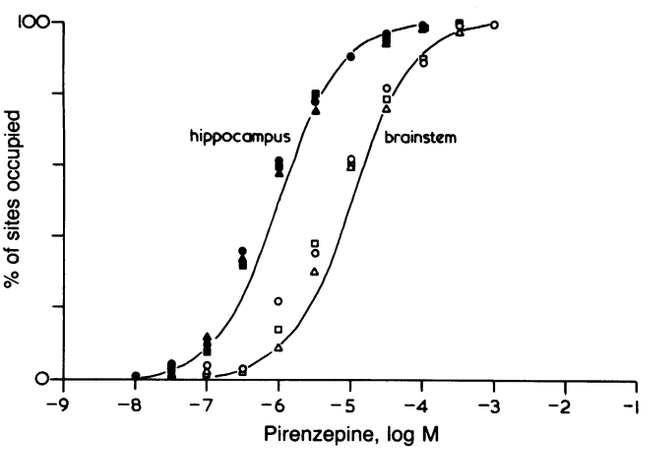

FIG. 5. Lack of effect of MalNEt on the affinity or M1-selectivity of pirenzepine for hippocampal and brainstem receptors. MalNEt concentrations were 0 (○, ●), 0.1 (△, ▲), and 5 mM (□, ■).

of coupling of muscarine receptors with a guanine nucleotide-binding protein. The much clearer distinction between brainstem and hippocampal receptors found in the present work is the result of using carbachol as the competitive ligand. In our hands carbachol is 6-fold more M2-selective than oxotremorine, and it produces much flatter binding curves for M1 receptors (2). Hence, it is apparent that MalNEt does not interconvert the higher and lower affinity states of M1 receptors in the hippocampus (Figs. 3 and 4), nor does MalNEt make the binding properties of uncoupled M2 receptors in the brainstem similar to the properties of either state of M1 receptors (Fig. 4). The latter distinction is more obvious in 1–5 mM MalNEt because of the left-shift seen in the brainstem (Fig. 4). The distinction between the receptors is still more obvious when EDTA is used additionally to uncouple the higher affinity state of hippocampal receptors (2).

In keeping with the fact that brainstem receptors show lower affinity for carbachol at equilibrium in 0.1 mM MalNEt than without it (Fig. 1), 0.1–5 mM MalNEt accelerated the dissociation of [^{3}H]oxotremorine-M from these receptors (Fig. 2). Because binding sites were occupied initially by the radioligand, the binding of MalNEt must have been elsewhere on the receptor and/or on its associated guanine nucleotide-binding protein (23, 24). After either MalNEt (at low concentrations) or p[NH]ppG has acted, it is clear that agonists do not bind with their previous high affinity to muscarine receptors in the porcine caudate nucleus (25), rat heart (15), or rat brainstem (2); and in our hands MalNEt is significantly more effective than p[NH]ppG. MalNEt and p[NH]ppG also appear to have similar uncoupling effects on other receptors that attenuate the activation of adenylate cyclase (17–20). In sharp contrast, MalNEt has been found to stabilize ternary complexes of agonist–β-adrenergic receptors–nucleotide-binding protein, and to slow the dissociation of β-adrenergic agonists (26, 27).

Whereas low concentrations of MalNEt selectively uncouple the higher agonist-affinity state of M2 receptors in the brainstem, 1–5 mM levels increase the affinity of the uncoupled receptors. This result has been observed by most investigators (13, 14, 21, 22) and may be a unique characteristic of muscarine receptors. All of the available evidence indicates that the left-shift for M2 receptors is not of the type produced by the formation of ternary complexes: agonist dissociation is increased (Fig. 2), the left-shift is parallel rather than flattened (Fig. 4), agonist-binding is no longer sensitive to guanine nucleotides (15, 25), and the ability of acetylcholine to cause muscle contraction is diminished (28). We conclude that the high-affinity state that is induced by MalNEt on M2 receptors is due to the binding of MalNEt to the receptor protein. It remains to be determined whether the effect is at the binding site.

MalNEt does not modify the selective binding of the antagonists pirenzepine (Fig. 5) or QNB (2). Nonetheless, MalNEt does have a significant effect on assays of M2 receptors in that it uncouples endogenous agonist–receptor complexes and makes all sites available for radioligands (2).

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