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Investigating the interaction of McN-A-343 with the M_2 muscarinic receptor using its nitrogen mustard derivative

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ABSTRACT

We investigated whether the aziridinium ion formed from a nitrogen mustard derivative (4-[(2-bromoethyl)methyl-amino]-2-butynyl N-(3-chlorophenyl)carbamate; BR384) structurally related to McN-A-343 (4-(trimethyl-amino)-2-butynyl N-(3-chlorophenyl)carbamate) interacts allosterically or orthosterically with the M_2 muscarinic receptor. Chinese hamster ovary cells expressing the human M_2 muscarinic receptor were incubated with the aziridinium ion of BR384 in combination with McN-A-343 or other known orthosteric and allosteric ligands for various incubation times. After removing unreacted ligands, we measured the binding of [^{3}H]N-methylscopolamine to residual unalkylated receptors. Affinity constants, rate constants for alkylation, and cooperativity constants were estimated for the interacting ligands using a mathematical model. Receptor alkylation by BR384 was consistent with a two-step process. After rapidly equilibrating with the receptor (step one), the aziridinium ion–receptor complex became covalently linked with a first order rate constant of about 0.95 min^{-1} (step two). McN-A-343, acetylcholine and N-methylscopolamine competitively protected the M_2 receptor from irreversible alkylation by BR384. In contrast, the allosteric modulators, gallamine and WIN 51,708 (17-β-hydroxy-17-α-ethynyl-5-α-androstano[3,2-β]pyrimido[1,2-α]benzimidazole), allosterically inhibited or had no effect on, respectively, receptor alkylation by BR384. There was good agreement between affinity constants estimated from the kinetics of receptor alkylation and by displacement of [^{3}H]N-methylscopolamine binding. Our results suggest that BR384 covalently binds to the orthosteric site of the M_2 receptor and that McN-A-343 binds reversibly at the same locus. Our method of analyzing allosteric interactions does not suffer from the limitations of more conventional approaches and can be adapted to detect allosteric interactions at receptors other than the muscarinic subtypes.

1. Introduction

The compound 4-(trimethyl-amino)-2-butynyl N-(3-chlorophenyl)carbamate (McN-A-343) is a muscarinic ganglionic stimulant that causes a selective increase in blood pressure and heart rate *in vivo* [1]. It has little effect on the brain when administered peripherally because of its quaternary ammonium structure. In contrast, most quaternary muscarinic agonists elicit bradycardia and hypotension as well as salivation due to activation of the M_2 receptor in the sinoatrial node and M_3 receptors on the endothelium of blood vessels and in the salivary glands, respectively. The selectivity of McN-A-343 *in vivo* can be attributed to its greater agonist activity at M_1 and M_4 relative to the M_2 and M_3 receptor subtypes [2–4].

The mode of interaction of McN-A-343 with muscarinic receptor subtypes differs from that of related oxotremorine analogs because of the large 3-chlorophenylcarbamate moiety in McN-A-343 in place of the small pyrrolidino ring of oxotremorine (see Fig. 1). In binding experiments on cerebral cortex, high concentrations of McN-A-343 caused a complete displacement of the binding of the muscarinic antagonist [^{3}H]N-methylscopolamine ([^{3}H]NMS), whereas in heart McN-A-343 caused only partial inhibition [5]. These results are consistent with competitive and allosteric behavior, respectively, in the two tissues. This difference reflects a discrimination between muscarinic receptor subtypes, because the heart expresses M_2 receptors and the cerebral cortex mainly M_1 and M_4.

In a study on hemi-ligands based on the McN-A-343 structure, it was shown that the ethyl and methyl esters of 3-chlorophenylcarbamate behave as allosteric modulators, whereas trimethylammonium acts as a muscarinic agonist, presumably through interaction with aspartic acid 103 in the M_2 receptor [6] (D 3.32 using the numbering scheme of Ballesteros and Weinstein [7]). These two hemi-ligands are connected through a butyne chain in

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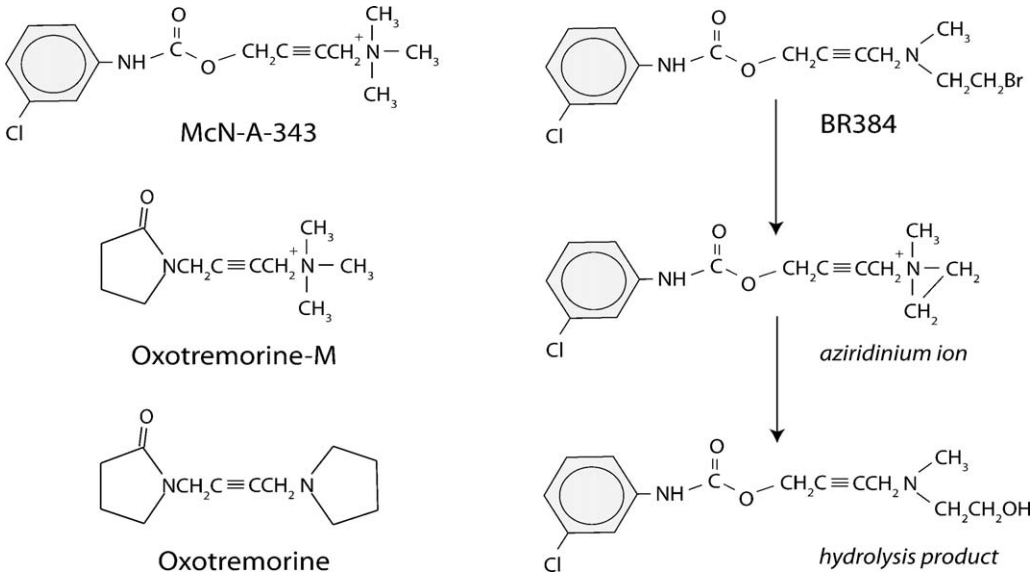

Fig. 1. Structures of McN-A-343, oxotremorine-M, oxotremorine, and BR384 and its transformation products in aqueous solution at neutral pH.

McN-A-343, suggesting that the intact molecule interacts simultaneously with allosteric and orthosteric sites.

Several investigators have shown that high concentrations of McN-A343 slow the dissociation of [^{3}H]NMS from the M_2 receptor [8,9]. This phenomenon is consistent with the trapping of [^{3}H]NMS by McN-A-343 when it occupies the allosteric site, because the allosteric site is located superficially to the orthosteric site in the normal cellular context. It has been suggested that McN-A-343 can bind independently to both sites with different affinities, but the symmetry of this model precludes the identification of which site it interacts with higher affinity in conventional kinetic experiments [10].

Mutagenesis of some residues in the M_2 receptor that are critical for orthosteric agonist activity have little effect on the activity of McN-A-343, whereas mutation of specific residues affecting the binding of allosteric antagonists enhance the activity of McN-A-343 [11]. These results also illustrate differences in how McN-A-343 and prototypic, acetylcholine-like orthosteric agonists interact with the M_2 receptor.

Irreversible ligands have advantages in identifying the mode of interaction of another ligand with a receptor. Their interaction with the receptor is consistent with a two-step process in which the reactive ligand first forms a reversible complex with the receptor (step one) followed by a subsequent alkylation step (step two) [12–14]. If a small agonist ligand with rapid binding kinetics is used as the alkylating agent, then it can achieve equilibrium quickly in the presence of an allosteric modulator or a competitive inhibitor during step one. The subsequent concentration-inhibition pattern of the modulator for inhibiting receptor alkylation (step two) reflects the nature of the reversible interactions during step one. Competitive inhibitors cause a concentration-dependent inhibition of alkylation and are capable of completely preventing alkylation at high concentrations. In contrast, any effect of an allosteric modulator reaches a limit at high concentrations [13]. We have found that McN-A-343 causes a competitive inhibition of receptor alkylation by acetylcholine mustard (AChM), indicating that the two ligands interact at the orthosteric site of the M_2 muscarinic receptor [13].

While these prior studies indicate that McN-A-343 binds to the orthosteric site of the muscarinic receptor, they do not rule out the possibility that it might also interact with other potential sites on the M_2 receptor linked allosterically to the orthosteric site. Having an irreversible analog of McN-A-343 would be useful for addressing this question in radioligand binding assays using both orthosteric and allosteric radioligands. Mutagenesis could also be used to identify the nucleophilic residue or residues that it alkylates.

To begin to address some of these questions, we have investigated the interaction of a nitrogen mustard derivative of McN-A-343 (4-[(2-bromoethyl)methyl-amino]-2-butynyl N-(3-chlorophenyl)carbamate; BR384) with the M_2 receptor. This compound has been shown to elicit immediate sympathetic effects on the cardiovascular system *in vivo* and bind irreversibly with muscarinic receptors, causing a long lasting inhibition of muscarinic responses [15]. In the present report, we describe a characterization of the interaction of BR384 with the human M_2 muscarinic receptor. We find that NMS, acetylcholine (ACh) and McN-A-343 competitively protect the M_2 receptor from irreversible alkylation by BR384, whereas gallamine and WIN 51,708 (17-β-hydroxy-17-α-ethynyl-5-α-androstano[3,2-β]pyrimido[1,2-α]benzimidazole) have partial or no protection consistent with an allosteric mechanism. Our results show that BR384 binds covalently to the orthosteric site of the M_2 muscarinic receptors, but do not rule out the possibility that it binds covalently or reversibly to additional sites.

2. Materials and methods

2.1. Cell culture

Chinese hamster ovary (CHO) cells stably expressing the human M_2 muscarinic receptor (CHO hM_2 cells) were obtained from Acadia Pharmaceuticals (San Diego, CA) and cultured in Dulbecco's Modified Eagle Medium with high glucose plus L-glutamine supplemented with 10% fetal calf serum, 3.7 g/l sodium bicarbonate, penicillin–streptomycin (100 units/ml and 100 μg/ml, respectively) and 0.4 mg/ml G418 disulfate salt at 37 °C in a humidified atmosphere with 5% CO_2/95% air.

2.2. Animals

Male Sprague–Dawley rats (200–250 g) were used as a source of tissue for the assay on homogenates of the rat heart.

2.3. Kinetics of M_2 muscarinic receptor alkylation by cyclized BR384

Our experiments for measuring the kinetics of receptor alkylation by BR384 involved three phases: (1) incubation of the