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HALOTHANE EFFECTS ON MUSCARINIC ACETYLCHOLINE RECEPTOR COMPLEXES IN RAT BRAIN

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Abstract—Muscarinic acetylcholine receptors in membranes from rat cerebral cortex or brainstem were equilibrated with halothane (0.5 to 5%). Halothane did not affect the number of [^{3}H]methylscopolamine ([^{3}H]MS) binding sites. [^{3}H]MS binding affinity, however, was increased in the presence of halothane (K_D, air = 0.41 nM; K_D, 2% halothane = 0.26 nM). This increase reflected a decrease in the dissociation rate constant (from 13×10^{-3} min^{-1} to 6.5×10^{-3} min^{-1}) rather than a change in the bimolecular rate constant of association (1.8 and 1.9×10^7 M^{-1} min^{-1} in the absence and presence of 2% halothane respectively). Carbamylcholine affinity for brainstem or cortical muscarinic receptors was not affected by halothane. The ability of a guanine nucleotide to lower carbamylcholine affinity for brainstem receptors, however, was eliminated after equilibration with 2% halothane.

General anesthetics depress synaptic transmission in a variety of neural systems. This depression may reflect direct interactions with specific proteins or alterations of the physical state of the membrane, or both [1, 2].

Muscarinic acetylcholine receptor-mediated responses in the adrenal medulla are depressed by halothane [3], while muscarinic responses of cortical neurons have been reported to be either depressed [4, 5] or augmented [6] by general anesthetics.

To further define the neurochemical effects of halothane at muscarinic synapses, the influence of halothane on receptor binding interactions was examined in rat cerebral cortex and brainstem. Halothane was found to have allosteric effects on antagonist binding and to interfere with interactions between the receptor's binding and guanine nucleotide-dependent regulatory subunits.

METHODS

Adult male Wistar rats were killed by decapitation and the cerebral cortices and brainstems (midbrain-pons-medulla) homogenized separately in 10 vol. of 50 mM Tris–HCl, pH 7.4, containing 1 mM $MgCl_2$ and 100 μM phenylmethylsulfonyl fluoride (to prevent proteolysis). The homogenate was spun at 20,000 g for 20 min at 4°. The resulting pellet was resuspended in Tris–Mg^{2+} buffer and used without further treatment.

Muscarinic receptor binding was measured at room temperature using [N-*methyl*-^{3}H]scopolamine methyl chloride ([^{3}H]MS; 84 Ci/mmole; New England Nuclear) by filtration procedures described in detail elsewhere [7, 8]. The number and affinity of [^{3}H]MS binding sites were determined by measuring equilibrium after a 90-min incubation with seven concentrations of [^{3}H]MS from 0.01 to 2.2 nM. Binding data were fitted by nonlinear regression analyses to a one receptor population model. The concentration of [^{3}H]MS binding sites was 0.6 ± 0.1 (brainstem, N = 3) and 1.2 ± 0.1 (cortex, N = 3) pmoles/mg protein and the apparent [^{3}H]MS dissociation constant was 0.41 ± 0.04 nM.

Carbamylcholine binding was inferred from its ability to inhibit the binding of 100 pM [^{3}H]MS to neural membranes suspended at a concentration of 5–10 μg protein/ml in 5 ml of 50 mM Tris–HCl, pH 7.4, containing 1 mM $MgCl_2$. Under these assay conditions, the maximum fraction of receptors occupied by [^{3}H]MS was 19% and maximum tissue binding (specific and nonspecific) represented less than 5% of the added radioactivity. Equilibrium binding data were fitted by iterative, nonlinear regression analyses to a mass action expression for the case of two receptor populations which bind carbamylcholine with different affinities, as follows:

$$B = B_H \cdot [C]/([C] + K_H) + (1 - B_H) \cdot [C]/([C] + K_L),$$

where B is fractional receptor occupancy by carbamylcholine (i.e. fractional inhibition of specific [^{3}H]MS binding), $[C]$ is the concentration of carbamylcholine, and B_H and $1 - B_H$ are the fractions of receptors having dissociation constants of K_H and K_L respectively. Carbamylcholine dissociation constants were corrected for the shifts caused by ligand competition using the [^{3}H]MS dissociation constants listed in Table 1 [9].

The association rate constant (k_1) for [^{3}H]MS binding was calculated from the time course of association using the integrated second-order rate equation [9],

$$\ln\left[\frac{B_e \cdot [C_T - B_t \cdot B_e/R_T]}{C_T \cdot [B_e - B_t]}\right] = K_1 \cdot t\left[\frac{C_T R_T}{B_e}\right] - B_e$$

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where B_e and B_t are the concentrations of [^{3}H]MS–receptor complex at equilibrium and at time t, respectively, C_T is the total concentration of [^{3}H]-MS, and R_T is the total concentration of [^{3}H]MS binding sites. R_T was determined in independent equilibrium binding measurements (see above).

The dissociation rate constant (k_{-1}) was determined by incubating neural membranes with 320 pM [^{3}H]MS for 90 min. Unlabeled methylscopolamine (100 μM) was then added and receptor occupancy measured by filtration at seven times over the next hour. The dissociation rate constant was calculated by linear regression analyses according to the equation:

$$\ln(B_t/B_0) = -k_{-1} \cdot t,$$

where B_0 and B_t are the concentrations of [^{3}H]MS–receptor complexes before and at time t after the addition of unlabeled methylscopolamine respectively [9].

The influence of halothane on muscarinic binding was determined using a calibrated Vernitrol vaporizer. Halothane–air mixtures were blown over suspensions of neural membranes (1 mg protein/ml in 50 mM Tris–HCl, pH 7.4, containing 1 mM $MgCl_2$) for 20 min at room temperature. Binding assays were carried out in a manifold in which the halothane–air mixture was distributed at a rate of 69 ml/min/tube.

RESULTS

Influence of halothane on [^{3}H]MS binding. Equilibration of neural membranes from either the cortex or brainstem with halothane (0.5 to 10%) increased the binding of 0.32 nM [^{3}H]MS to muscarinic receptors (Fig. 1). Nonspecific binding measured in the presence of 10 μM atropine was not affected by halothane. The maximum increase in binding occurred with 2% halothane.

Saturation binding curves revealed a 60% higher affinity of [^{3}H]MS for cortical muscarinic receptors in the presence of 2% halothane (Fig. 2; Table 1). In either the presence or absence of halothane, [^{3}H]-MS binding was well-described by a model incorporating a single population of binding sites. Scatchard plots of the binding data were linear (Fig. 2b), also indicating a single receptor population. This increase in binding affinity was sufficient to account for the increase in the levels of 0.32 nM [^{3}H]MS binding illustrated in Fig. 1. Halothane increased the affinity of [^{3}H]MS for brainstem receptors to a similar extent (Table 1).

In a simple mass action binding relationship in which a ligand forms a reversible complex with a uniform set of noninteracting receptors, the equi-

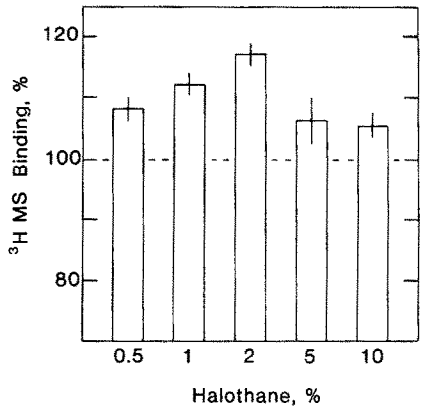

Fig. 1. Potentiation of [^{3}H]MS binding by halothane. Membranes prepared from rat cerebral cortex were equilibrated with the indicated concentrations of halothane. The specific binding of 0.32 nM [^{3}H]MS to muscarinic acetylcholine receptors was then measured. Binding is expressed as percent of control binding measured in the absence of halothane. The bars and lines represent the mean and standard deviations from four experiments, each performed in triplicate.

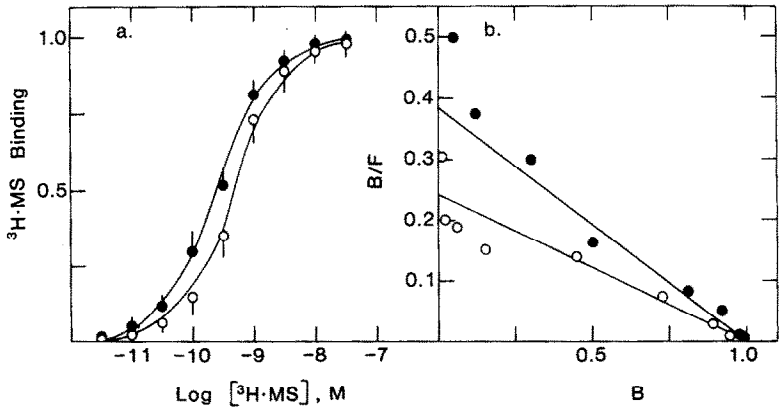

Fig. 2. Influence of halothane on [^{3}H]MS binding to muscarinic acetylcholine receptors from rat cortex. (Panel a) The specific (i.e. 10 μM atropine-sensitive) binding of [^{3}H]MS was determined in the absence (○) and presence (●) of 2% halothane. Binding is expressed as fractional receptor occupancy (B_{max} = 1.2 ± 0.1 pmoles/mg protein). Each point and bar represent the mean and standard deviation from three experiments. The line is drawn according to a nonlinear regression fit to a single receptor population which revealed the parameters listed in Table 1. (Panel b) Scatchard plot of the [^{3}H]MS binding data presented in "panel a". Key: (○), control; (●), 2% halothane; (B) fractional receptor occupancy; and (F) concentration of [^{3}H]MS in nM. All lines are drawn according to parameters derived from nonlinear regression analyses (Table 1).

Table 1. Muscarinic receptor binding parameters in the absence and presence of halothane

Tissue	Condition	Binding parameters					
		[^{3}H]MS			Carbamylcholine		
		K_D	k_1	k_{-1}	B_H	K_H	K_L
Cerebral cortex	Control	0.41 ± 0.03	1.8 ± 0.2	13.0 ± 1.1	0.20 ± 0.04	0.15 ± 0.05	23 ± 5
	Halothane (2%)	0.26 ± 0.04*	1.9 ± 0.2	6.5 ± 0.3*	0.21 ± 0.03	0.19 ± 0.03	27 ± 3
Brainstem	Control	0.42 ± 0.05			0.57 ± 0.05	0.05 ± 0.02	12 ± 4
	Control + Gpp(NH)p				0.40 ± 0.03	0.13 ± 0.04	40 ± 6
	Halothane (2%)	0.28 ± 0.03*			0.54 ± 0.04	0.06 ± 0.03	15 ± 3
	Halothane + Gpp(NH)p				0.51 ± 0.03	0.05 ± 0.02	18 ± 5

All values are means ± S.D., N = 3 or 4. K_D, equilibrium dissociation constant in nM; k_1, association rate constant × $10^7\,M^{-1}\,min^{-1}$; k_{-1}, dissociation rate constant × $10^{-3}\,min^{-1}$; B_H fraction of receptors displaying high-affinity agonist binding; K_H and K_L, equilibrium dissociation constants in μM associated with the high- and low-affinity receptor populations.

* Different from control value ($P < 0.01$, Student's t-test).

librium dissociation constant equals the ratio of the kinetic dissociation and association constants (i.e. $K_D = k_{-1}/k_1$). The binding of 0.3 nM [^{3}H]MS to cortical muscarinic receptors was measured in the absence and presence of 2% halothane at five times between 2 and 50 min (Fig. 3a). Linear regression analysis of binding plotted according to the integrated form of the second-order rate equation (Fig. 3b) revealed essentially similar association rate constants in the absence and presence of halothane (1.8 vs 1.9 × $10^7\,M^{-1}\,min^{-1}$ respectively; Table 1).

The dissociation of [^{3}H]MS from cortical muscarinic receptors was retarded in the presence of 2% halothane (Fig. 4). Linear regression analysis of the dissociation data plotted according to the integrated form of the rate equation (Fig. 4b) revealed kinetic dissociation constants of 13 × $10^{-3}\,min^{-1}$ and 6.5 × $10^{-3}\,min^{-1}$ in the absence and presence of halothane respectively. The equilibrium dissociation constants (K_D) calculated from the ratios of the kinetic constants (k_{-1} and k_1) were 0.73 and 0.35 nM in the absence and presence of halothane respectively. These dissociation constants are 35–78% higher than those revealed in equilibrium binding studies; the ability of halothane to enhance [^{3}H]-MS binding affinity, however, is evident in either analysis.

Influence of halothane on carbamylcholine binding. Carbamylcholine was used as a representative muscarinic agonist instead of acetylcholine since the rapid hydrolysis of acetylcholine would have dictated the use of cholinesterase inhibitors. Carbamycholine is a close structural analogue of acetylcholine and appears to differentiate the same populations of agonist binding sites, at least insofar as the proportions of high- and low-affinity agonist sites are similar when measured using either ligand [10].

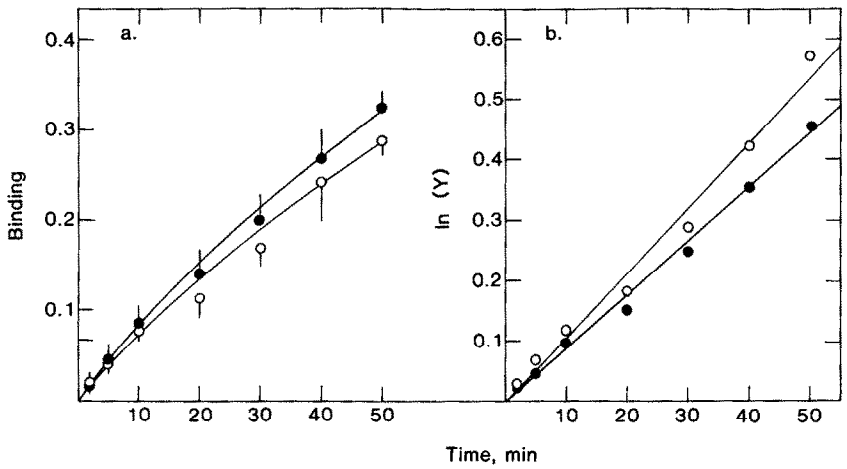

Fig. 3. Influence of halothane on the time course of [^{3}H]MS binding. (Panel a) The specific binding of 0.3 nM [^{3}H]MS (pmoles/mg protein) to muscarinic receptors in membranes derived from rat cerebral cortex was measured as a function of time. The membranes were equilibrated in air (○) or 2% halothane (●) before binding was assayed. Each point and bar represent the mean and standard deviation from three different determinations, each performed in duplicate. (Panel b) The time course data are replotted according to the second-order rate equation: $Y = \ln[B_e \cdot (C_T - B_t \cdot B_e/R_t)/C_t \cdot (B_e - B_t)]$. The lines are drawn according to linear regression analyses which revealed the kinetic association constants listed in Table 1.

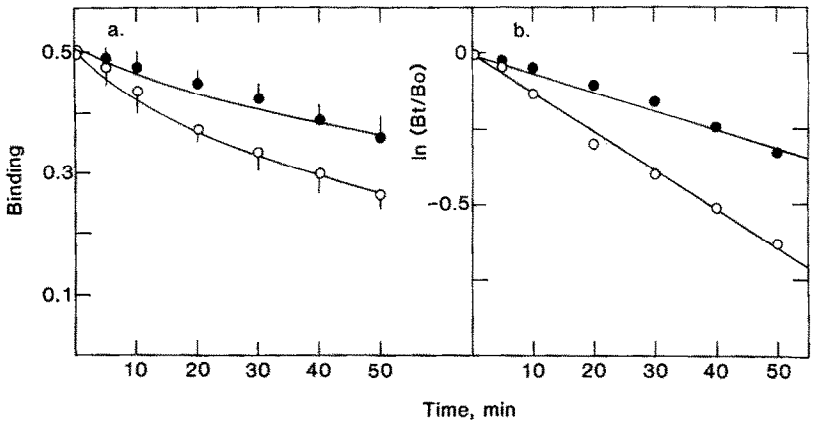

Fig. 4. Influence of halothane on the rate of [^{3}H]MS dissociation from muscarinic receptors. Membranes from rat cerebral cortex were equilibrated in air (○) or 2% halothane (●). (Panel a) [^{3}H]MS (0.32 nM) was added and allowed to come to equilibrium (90 min). [^{3}H]MS binding, expressed as pmoles/mg protein, was then measured at various times after the addition of 10 μM atropine. Each point and bar represent the mean and standard deviation from three separate experiments. (Panel b) The binding data are replotted according to the dissociation rate equation. The lines are drawn according to linear regression analyses which indicated the kinetic constants listed in Table 1.

Carbamylcholine binding to cortical muscarinic receptors was well-described by a two receptor population model (Fig. 5; Table 1). Twenty-one percent of the receptors displayed a carbamylcholine dissociation constant of 0.19 μM; the remaining receptors bound carbamylcholine with an apparent K_D of 27 μM. Carbamylcholine binding parameters were not altered after equilibration with 2% halothane (Fig. 5; Table 1).

Carbamylcholine binding to muscarinic receptors in brainstem membranes was measured in the presence and absence of 100 μM 5′-guanylylimidodiphosphate (Gpp(NH)p), a stable analogue of GTP (Fig. 6; Table 1). Guanine nucleotides influence muscarinic binding, particularly that of muscarinic agonists, as a consequence of interactions with a guanine nucleotide-dependent regulatory subunit which is closely associated with the binding subunit of the receptor [11]. Gpp(NH)p lowered carbamylcholine affinity for brainstem muscarinic receptors (Fig. 6a) primarily by decreasing the proportion of receptors displaying high-affinity binding (Table 1). Carbamylcholine binding was not affected by equilibrating the membranes with 2% halothane (Fig. 6b). The ability of Gpp(NH)p to lower carbamylcholine binding affinity, however, was eliminated by halothane (Fig. 6b; Table 1). The proportion of receptors displaying high-affinity binding and the affinity constants associated with the two receptor populations were unaffected by Gpp(NH)p after equilibration with halothane (Table 1).

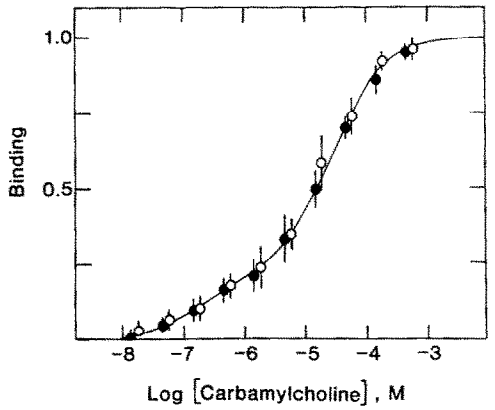

Fig. 5. Influence of halothane on carbamylcholine binding to muscarinic receptors from rat cerebral cortex. Carbamylcholine binding, expressed as fractional receptor occupancy, was inferred from its inhibition of [^{3}H]MS binding to receptors in membranes which were pre-equilibrated with air (○) or 2% halothane (●). Each point and bar represent the mean and standard deviation from four experiments. The line is drawn according to a nonlinear regression fit to a two receptor population model which revealed the parameters listed in Table 1.

DISCUSSION

Two effects of halothane on muscarinic receptors in rat brain were revealed in the present study. Halothane increases the affinity of the receptor for an antagonist ([^{3}H]MS) by slowing its rate of dissociation without affecting the receptor affinity for an agonist (carbamylcholine). In addition to this allosteric effect, halothane eliminates the ability of a guanine nucleotide (Gpp(NH)p) to lower agonist binding affinity.

Halothane increased antagonist, but not agonist, binding affinity. Hydrophobic binding interactions are generally more important in the binding of classical, high-affinity muscarinic antagonists (such as the belladonna alkaloids and glycolate esters) than muscarinic agonists, and the binding of many of these antagonists is entropically-driven [12]. It has been suggested that membrane lipids contribute to the maintenance of hydrophobic domains involved in the binding of muscarinic antagonists [12]. Enzymatic degradation or detergent mediated disaggregation of neural membranes, for example, alters antagonist

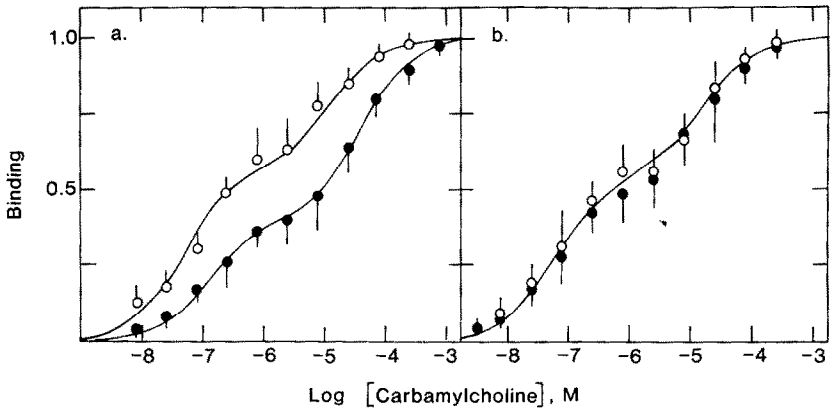

Fig. 6. Influence of halothane on carbamylcholine binding to muscarinic receptors from rat brainstem. Brainstem membranes were pre-equilibrated with air (a) or 2% halothane (b). Carbamylcholine binding was inferred from its inhibition of specific [^{3}H]MS binding, and is expressed as fractional receptor occupancy. Binding was measured in the absence (○) or presence (●) of 10 μM Gpp(NH)p. Each point and bar represent the mean and standard deviation from three experiments. Lines are drawn according to nonlinear regression fits to a two receptor population model which revealed the parameters listed in Table 1.

binding properties [12–14]. The correlation of general anesthetic potency and lipophilicity has implicated the lipid matrix of membranes and the hydrophobic regions of proteins as important sites of their action. Halothane interaction with hydrophobic membrane sites could account for its influence on antagonist binding by stabilizing hydrophobic molecular interactions.

The influences of guanine nucleotides on muscarinic binding are mediated by a group of regulatory proteins (G-proteins) which play an important role in coupling receptor binding subunits to certain metabolic processes (e.g. adenylate cyclase) in the cell [15].

The organization of muscarinic acetylcholine receptor complexes in neural membranes is incompletely understood. These complexes are composed of, at a minimum, a binding subunit which recognizes acetylcholine, effector structures, including the G-proteins, to which the binding subunit is transiently coupled, and closely associated membrane lipids. Subpopulations of muscarinic receptors have been defined on the basis of their ligand binding properties. For example, muscarinic receptors can be classified as guanine nucleotide-sensitive or -insensitive, as having high or low (or super-high) affinity for agonists [10], or as having high or low affinity for pirenzepine [16]. The binding subunit polypeptide in each class of muscarinic receptor appears to have the same molecular dimensions [17]. Thus, receptor subpopulations may largely reflect interactions of binding subunits with other structures within the membrane.

Guanine nucleotides convert muscarinic receptors from a state of high affinity for agonists to a low-affinity state, probably as a consequence of dissociating a part of the G-protein from the binding subunit [18]. The elimination by halothane of the guanine nucleotide effect may reflect its stabilization of the receptor–G-protein complex. Alternatively, the ability of G-proteins to bind Gpp(NH)p may have been eliminated. It is unclear whether this is due to a direct effect on the G-protein or to an alteration of the physical state of the membrane. A number of other treatments eliminate the guanine nucleotide sensitivity of muscarinic receptors, including heat (50°, 5 min; [19]), trystic digestion [20], exposure to urea [21], media of high pH [22] and low pH (B. L. Anthony and R. S. Aronstam, unpublished results), and endogenous proteolytic activity [23]. Most of these treatments, however, produce a lowering of agonist affinity, which was not seen with halothane. This suggests that halothane prevents the dissociation of the G-protein from the binding subunit, possibly by interfering with protein mobility in the plane of the membrane.

Not all muscarinic receptors are coupled to G-proteins (at least guanine nucleotides do not affect agonist binding to all muscarinic receptors). It is interesting to speculate that this may account for the variable effects of general anesthetics on the activity of single units in different areas of the central nervous system, where both stimulation and depression have been observed [4–6].

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