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DIETHYL ETHER EFFECTS ON MUSCARINIC ACETYLCHOLINE RECEPTOR COMPLEXES IN RAT BRAINSTEM

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Abstract—The influence of diethyl ether on muscarinic acetylcholine receptor–G protein interactions was studied using membranes isolated from rat brainstem. Membranes were equilibrated with diethyl ether (0.5 to 10%) for 20 min before, and then during, the binding assay. The affinity, but not the number, of [^{3}H]*N*-methylscopolamine ([^{3}H]MS) binding sites was increased in the presence of diethyl ether (K_D in air = 0.41 nM, K_D in 2% diethyl ether = 0.21 nM). This increase in affinity reflected a decrease in the rapid dissociation rate constant (air $k_{-1} = 13 \times 10^{-3}$ min^{-1}, 2% diethyl ether $k_{-1} = 7 \times 10^{-4}$ min^{-1}) rather than a change in the association rate constant. Diethyl ether had no effect on the binding affinity of the muscarinic agonist carbamylcholine. However, the binding of a radiolabeled muscarinic agonist, [^{3}H]oxotremorine-M ([^{3}H]Oxo-M), to high affinity binding sites decreased about 25% in the presence of 2% diethyl ether. The ability of a guanine nucleotide to depress the high affinity binding of both carbamylcholine and [^{3}H]Oxo-M was decreased or eliminated by diethyl ether. Diethyl ether appears to interfere with muscarinic receptor–G protein interactions, perhaps by stabilizing receptor–G protein complexes or inhibiting the binding of guanine nucleotides.

Volatile general anesthetics alter muscarinic receptor-mediated control of cellular events in a variety of tissues. Muscarinic responses may be either augmented or depressed, depending upon the tissue involved. For example, halothane depresses muscarinic responses in the adrenal medulla [1], while the activity of cortical neurons can be either enhanced or inhibited by general anesthetics [2–4]. These effects may reflect interactions of the anesthetic with the receptor complex, or a nonspecific alteration of the membrane environment [5, 6].

We have demonstrated recently two effects of halothane on muscarinic acetylcholine receptors in rat brain and heart [7–9]: (1) halothane increases antagonist, but not agonist, binding affinity by decreasing the rate of dissociation, and (2) halothane decreases or eliminates the guanine nucleotide sensitivity of agonist binding. The latter effect indicates a disruption of muscarinic receptor–guanine nucleotide-dependent transducer protein (G protein) [10] interactions. This represents a novel mechanism for anesthetic disruption of intercellular chemical transmission.

In the present work, we investigated the effects of a second general anesthetic, diethyl ether, on muscarinic receptor complexes in rat brainstem. Compared to halothane, diethyl ether is less hydrophobic and is a 4-fold less potent general anesthetic which demonstrates significant sympathomimetic activity [11] perhaps related to a depression of parasympathetic tone [12]. Our results demonstrate that ether and halothane have similar effects on ligand binding to muscarinic receptors and receptor coupling to G proteins in rat brainstem.

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METHODS

Adult, male Wistar rats were decapitated, and their brainstems (midbrain-pons-medulla) were removed and homogenized in a Teflon–glass tissue grinder in 10 vol. of 50 mM Tris–HCl, pH 7.4, containing 2 mM $MgCl_2$ and 100 μM phenylmethylsulfonyl fluoride to prevent proteolysis. The homogenate was spun at 17,000 *g* for 20 min at 4°. The resulting pellet was resuspended in the Tris-magnesium buffer and used without further treatment.

Muscarinic receptor binding was measured at room temperature using [*N-methyl*-^{3}H]scopolamine methylchloride ([^{3}H]MS, 60–80 Ci/mmol, DuPont NEN, Boston, MA) by filtration procedures described in detail elsewhere [7, 13]. The number and affinity of [^{3}H]MS binding sites were determined by measuring equilibrium binding after a 90-min incubation with nine concentrations of [^{3}H]MS from 0.0032 to 32 nM. Binding was fitted by nonlinear regression analysis to a single receptor population model. The concentration of [^{3}H]MS sites was 0.62 ± 0.08 pmol/mg protein, and the [^{3}H]MS dissociation constant was 0.41 ± 0.05 nM.

Carbamylcholine binding was inferred from its capacity to inhibit the binding of [^{3}H]MS to brainstem membranes suspended at a concentration of 100 μg protein/ml in 2 ml of 50 mM Tris–HCl, pH

7.4, containing 2 mM $MgCl_2$. A concentration of [^{3}H]MS was used at which approximately 20% of the receptors were occupied by [^{3}H]MS at equilibrium (0.1 and 0.05 nM in the absence and presence of 2% diethyl ether respectively). Equilibrium data were fitted by iterative, nonlinear regression analysis 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, $[C]$ is the concentration of carbamylcholine, and B_H and $1 - B_H$ are the fractions of receptor possessing carbamylcholine dissociation constants of K_H and K_L respectively.

The association rate constant k_1 for [^{3}H]MS binding was calculated from the time course of binding using the integrated second-order rate equation [14]:

$$\ln\left(\frac{B_e \cdot [C_T - B_t \cdot B_e/R_T]}{C_T \cdot [B_e - B_t]}\right) = k_1 \cdot t \cdot \left(\frac{C_T \cdot R_T}{B_e}\right) - B_e,$$

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 concentration of [^{3}H]MS binding sites.

Dissociation rate constants (k_{-1} and k_{-2}) were determined by incubating neural membranes with 0.32 nM [^{3}H]MS for 90 min. Unlabeled methylscopolamine (100 μM) was then added, and receptor occupancy was measured by filtration at seven times over the next hour. [^{3}H]MS dissociation curves were biphasic and were resolved into two components using a model for dissociation from two independent receptor populations, as follows:

$$B = A \cdot e^{-k_{-1} \cdot t} + (1 - A) \cdot e^{-k_{-2} \cdot t},$$

where B is fractional binding at time at time t and A and $(1 - A)$ are the fractions of receptor displaying the fast and slow dissociation rate constants k_{-1} and k_{-2} respectively.

High affinity [^{3}H]oxotremorine-M ([^{3}H]Oxo-M; 85 Ci/mmol, DuPont NEN, Boston, MA) binding was measured at room temperature using a filtration assay. Suspended membranes (200 μg protein) were incubated with 3 nM [^{3}H]Oxo-M in 50 mM Tris–HCl, pH 7.4, containing 2 mM $MgCl_2$. After a 15-min incubation, samples were filtered under suction through Whatman GF/B glass filters treated with 1 mg/ml methylated bovine serum albumin and 5% Sigmacote (Sigma Chemical Co., St. Louis, MO) to reduce nonspecific binding. The filters were washed with 5 ml buffer, and their radioactivity content was determined by liquid scintillation counting.

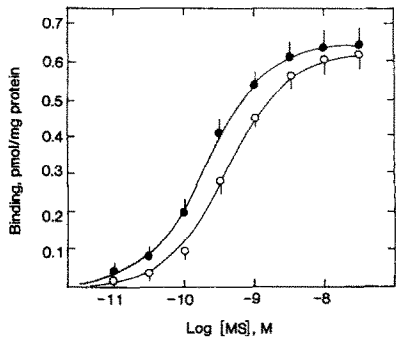

Fig. 1. Diethyl ether effects on [^{3}H]MS binding to muscarinic receptors from rat brainstem. The specific binding of [^{3}H]MS was measured in the absence (○) and presence (●) of 2% diethyl ether. Lines are drawn according to a nonlinear fit of the data to a single receptor population model which revealed the parameters (K_D and B_{max}) listed in Table 1. Each point and bar represent the mean and standard deviation from four experiments.

The influence of diethyl ether on muscarinic binding was determined using a calibrated Vernitrol vaporizer. Diethyl ether–air mixtures were blown over suspensions of neural membranes (1 mg protein/ml) for 20 min at room temperature. Binding assays were carried out at room temperature in a manifold in which the diethyl ether–air mixture was distributed at a rate of 69 ml/min per tube.

RESULTS

Influence of diethyl ether on [^{3}H]*MS binding.* Equilibration of brainstem membranes with 2% diethyl ether increased [^{3}H]MS binding affinity (K_D = 0.21 vs 0.41 nM; Fig. 1; Table 1). The number of [^{3}H]MS binding sites (0.62 to 0.65 pmol/mg protein) and the extent of nonspecific ligand binding were not affected by diethyl ether.

Table 1. Influence of diethyl ether on [^{3}H]MS binding to muscarinic receptors from rat brainstem

	Binding parameter			
Anesthetic	K_D	B_{max}	k_1	k_{-1}
None	0.41 ± 0.05	0.62 ± 0.08	3.9 ± 0.4	0.131 ± 0.018
Ether, 2%	0.21 ± 0.02*	0.65 ± 0.09	3.6 ± 0.2	0.076 ± 0.013*

All values are means ±SD (N = 4). K_D, apparent equilibrium dissociation constant in nM; B_{max}, density of [^{3}H]MS binding sites in pmol/mg protein; k_1, rate constant of association $\times 10^{-7}$ M^{-1} min^{-1}; k_{-1}, rate constant of dissociation for the rapid component of ligand dissociation, min^{-1}.

* Different from control measurements in the absence of ether ($P < 0.01$; Student's t-test).

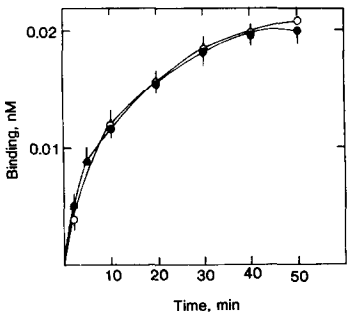

Fig. 2. Diethyl ether effects on the time course for [^{3}H]MS binding to brainstem muscarinic receptors. The specific binding of [^{3}H]MS was measured in the absence (○) and presence (●) of 2% diethyl ether at the times indicated on the abscissa. The initial concentration of [^{3}H]MS was 0.32 nM, the total concentration of [^{3}H]MS binding sites was 0.062 nM, and the concentration of [^{3}H]MS/receptor complexes at equilibrium was 0.026 nM. Kinetic constants were determined by regression analysis are listed in Table 1. Each point and bar represent the mean and standard deviation from three oxperiments.

Diethyl ether (2%) did not affect the rate constant of association of [^{3}H]MS (3.9 vs $3.6 \times 10^{-7}\,M^{-1}\,min^{-1}$; Fig. 2; Table 1). [^{3}H]MS dissociation from the receptor induced by atropine was biphasic, in agreement with previous reports [15, 16]. Dissociation kinetics were well-described by a model incorporating two receptor populations with different dissociation rate constants. The larger population (59 ± 3% of the total population) had a rapid rate of dissociation; the remaining receptors displayed a slow rate of dissociation (k_{-2} = $0.0058\,min^{-1}$). Diethyl ether did not affect the proportions of receptors displaying fast and slow rates of dissociation or the rate constant associated with the slow component of dissociation. However, diethyl ether (2%) decreased the rate constant associated with rapid ligand dissociation from 0.131 ± 0.018 to $0.076 \pm 0.013\,min^{-1}$ (Fig. 3; Table 1).

Influence of diethyl ether on agonist binding. Carbamylcholine binding to brainstem muscarinic receptors was determined in competition experiments with [^{3}H]MS (Fig. 4, Table 2). Carbamylcholine binding was well-described by a two receptor population model; 53% of the receptors displayed a high affinity for carbamylcholine ($K_H = 0.053 \pm 0.010\,\mu M$), while the remaining receptors displayed a dissociation constant (K_L) of $4.9 \pm 1.1\,\mu M$. Equilibration of the membranes with 2% diethyl ether did not change the distribution of receptors between high and low affinity conformations or K_H. K_L, however, was increased slightly from 4.9 to 7.5 μM (Table 2).

Carbamylcholine binding curves were shifted to the right when 10 μM Gpp(NH)p was included in the incubation medium (Fig. 4). Under these conditions, the binding was best described by a single receptor population model; nonlinear regression analysis indicated a dissociation constant of $3.1 \pm 0.6\,\mu M$ (Table 2). This conversion of high affinity sites to low affinity sites is evidence for transient receptor association with transducer G proteins (high affinity receptors are coupled to G proteins, low affinity receptors are not; see Discussion). The guanine nucleotide effect on carbamylcholine, however, was eliminated completely after equilibration of the membranes with 2% diethyl ether (Fig. 4; Table 2): Gpp(NH)p failed to change receptor affinity state or the dissociation constants associated with either the high or low affinity binding.

[^{3}H]Oxo-M was used as a direct probe for brainstem receptors with high affinity for agonists. At 3 nM, [^{3}H]Oxo-M labeled 12% of the [^{3}H]MS binding sites. Oxo-M/[^{3}H]Oxo-M competition studies indicated a single, high affinity receptor population (K_D = 3.7 nM). Equilibration of the membranes with 10% diethyl ether decreased the binding of 3 nM [^{3}H]Oxo-M by 30%; with 1% ether, [^{3}H]Oxo-M binding was depressed by 20% (Fig. 5). In the pres-

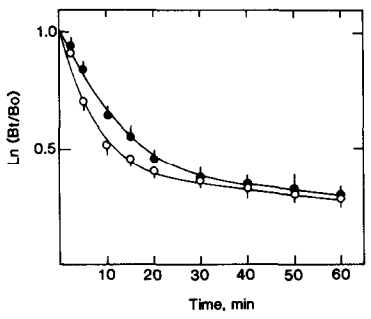

Fig. 3. Diethyl ether effects on the dissociation of [^{3}H]MS from brainstem muscarinic receptors. Brainstem membranes were incubated with 0.32 nM [^{3}H]MS for 90 min in the absence (○) and presence (●) of 2% diethyl ether. Unlabeled *N*-methylscopolamine (100 μM) was then added, and specific [^{3}H]MS binding was measured at the times indicated on the abscissa. Dissociation rate constants were determined by nonlinear regression analysis and are listed in Table 1. Each point and bar represent the mean and standard deviation from three experiments.

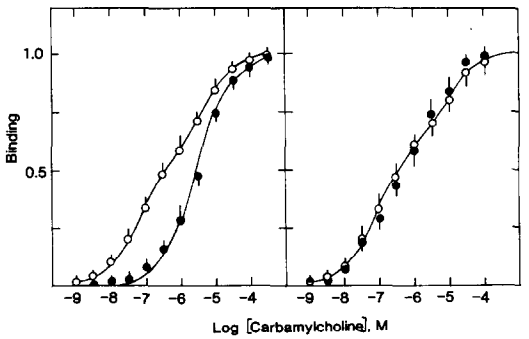

Fig. 4. Diethyl ether effects on agonist binding to brainstem muscarinic receptors. Carbamylcholine binding was measured in competition studies with [^{3}H]MS. Binding was measured in the absence (○) and presence (●) of 10 μM Gpp(NH)p in membranes that were equilibrated with air (left panel) or 2% diethyl ether (right panel). Lines were drawn according to nonlinear regression analysis using a two receptor population model, which revealed the parameters listed in Table 2. Each point and bar represent the mean and standard deviation from three experiments.

Table 2. Influence of diethyl ether on carbamylcholine binding to muscarinic receptors from rat brainstem

Anesthetic	Gpp(NH)p	Binding parameter		
		B_H	K_H (μM)	K_L (μM)
None	0	0.53 ± 0.04	0.053 ± 0.010	4.9 ± 1.1
	10	0		3.1 ± 0.6*
Ether, 2%	0	0.57 ± 0.05	0.07 ± 0.02	7.5 ± 2.6†
	10	0.58 ± 0.08	0.09 ± 0.04	7.8 ± 4.3

All values are means ±SD (N = 4). B_H, fraction of receptor displaying high affinity agonist binding; K_H and K_L, dissociation constants associated with high and low affinity carbamylcholine binding respectively.

* Different from control value in the absence of Gpp(NH)p ($P < 0.05$, Student's t-test).

† Different from control value in the absence of ether ($P < 0.01$).

ence of diethyl ether (1 or 10%), Oxo-M/[^{3}H]Oxo-M competition curves were still consistent with the presence of a single (albeit smaller) receptor population with a K_D of 3.9 nM.

Gpp(NH)p also decreased the level of 3 nM [^{3}H]Oxo-M binding (Fig. 5). This decrease is thought to reflect a conversion of receptors to a low affinity state (low affinity binding is not detected with the filtration assay used). Equilibration of the membranes with diethyl ether greatly decreased guanine nucleotide regulation of [^{3}H]Oxo-M binding (Fig. 5). Thus, in the absence of diethyl ether 10 μM Gpp(NH)p inhibited 3 nM [^{3}H]Oxo-M binding by 39%, while after equilibration with 5% diethyl ether only 20% of [^{3}H]Oxo-M binding was inhibited (Fig. 5).

This depression of the guanine nucleotide sensitivity of high affinity agonist binding by diethyl ether was also apparent in [^{3}H]Oxo-M/Gpp(NH)p titration curves (Fig. 6). In the absence of diethyl ether, Gpp(NH)p inhibited 3 nM [^{3}H]Oxo-M binding by up to 53% at 100 μM. In the presence of 2% diethyl ether, however, Gpp(NH)p inhibition curves were shifted to the right by approximately 30-fold. The shift with 2% diethyl ether was similar in magnitude to the shift caused by a second volatile anesthetic, halothane (2%) (Fig. 6; [7–9]).

DISCUSSION

Diethyl ether interfered with ligand binding to muscarinic acetylcholine receptors in rat brainstem as well as receptor interactions with transducer G proteins. These actions of diethyl ether resemble the actions of a second volatile anesthetic, halothane, in both brain and heart [7–9].

Diethyl ether increased antagonist ([^{3}H]MS) binding affinity without affecting agonist (carbamylcholine) affinity. While [^{3}H]MS binding kinetics are complex (e.g. multiple components of ligand dissociation are observed), the increase in affinity seemed to reflect a slowing in the rate of dissociation. The selective effect of diethyl ether on antagonist binding may reflect the relatively greater importance of hydrophobic interactions in antagonist as compared to agonist binding. Classical muscarinic antagonists are larger than agonists, possessing large lipophilic moieties that interact with hydrophobic

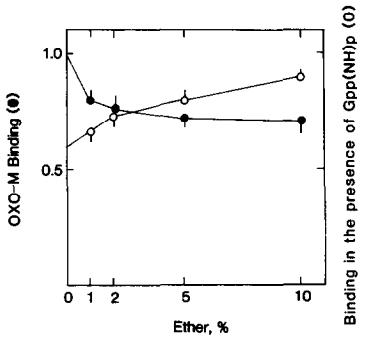

Fig. 5. Diethyl ether effects on [^{3}H]Oxo-binding to brainstem muscarinic receptors. The binding of 3 nM [^{3}H]Oxo-M was measured in the presence of the indicated concentrations of diethyl ether and is indicated as fractional binding on the left ordinate (●). The fraction of the specific binding of 3 nM [^{3}H]Oxo-M at each diethyl ether concentration which was observed in the presence of 10 μM Gpp(NH)p is indicated on the right ordinate (○). Each point and bar represent the mean and standard deviation from three experiments.

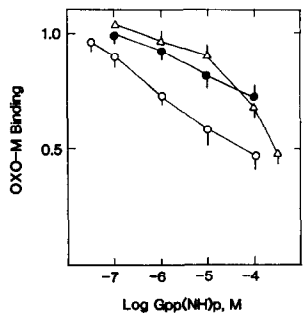

Fig. 6. Volatile anesthetic effects on guanine nucleotide regulation of [^{3}H]Oxo-M binding to brainstem muscarinic receptors. The binding of 3 nM [^{3}H]Oxo-M was measured in the presence of the concentrations of Gpp(NH)p indicated on the abscissa in membranes that were equilibrated with air (○), 2% diethyl ether (●) or 2% halothane (△). Each point and bar represent the mean and standard deviation from three experiments.

domains of the receptor protein or associated lipids. The close relationship of general anaesthetic potency and lipophilicity has implicated hydrophobic membrane domains as important sites for anesthetic action. It is likely that diethyl ether interacts with these domains in such a manner as to enhance the hydrophobic contribution to antagonist binding.

Diethyl ether did not affect agonist binding to brainstem muscarinic receptors as revealed by carbamylcholine/[^{3}H]MS competition experiments. The number of receptors displaying high affinity [^{3}H]Oxo-M binding, however, was decreased by diethyl ether. The reasons for this discrepancy are not clear. A decrease in [^{3}H]Oxo-M binding could reflect either an inhibition of ligand binding or a conversion of high affinity receptors to a low affinity conformation. However, the number of muscarinic binding sites that could be labeled with [^{3}H]MS was not affected by diethyl ether, and [^{3}H]MS binding affinity was increased. It is possible that differences in the nature of [^{3}H]Oxo-M and carbamylcholine binding account for this discrepancy. It is also possible that small qualitative differences in the nature of the sites labeled by [^{3}H]Oxo-M and [^{3}H]MS are not apparent using the present experimental approaches, e.g. [^{3}H]Oxo-M labels only a small fraction of the muscarinic receptor population that is sampled using [^{3}H]MS.

Diethyl ether interfered with guanine nucleotide regulation of agonist binding to the receptor. This was evident in the depression of the guanine nucleotide sensitivity of both carbamylcholine and [^{3}H]Oxo-M binding. The influence of guanine nucleotides on agonist binding is mediated by transducer G proteins which couple muscarinic (and other) receptors to effector mechanisms (e.g. ion channels, phospholipase C, and adenylate cyclase) in the postsynaptic cell [10, 17, 18]. Agonist binding to the receptor is believed to lead to the formation of a ternary complex with G protein. This induces an exchange of GTP for GDP on the alpha subunit of the G protein, which leads to an uncoupling of G protein subunits and a dissociation of receptor and G protein. G protein subunits then influence various cellular processes. Subsequent to GTP hydrolysis (by an inherent catalytic activity), the G proteins reassemble and are free to reassociate with the receptor [10, 19].

Muscarinic receptors are heterogeneous with respect to their affinity for agonists as determined in *in vitro* binding assays [20, 21], and guanine nucleotides convert receptors from a high affinity conformation to a low affinity state (e.g. [22]). This observation led to the proposal that receptors with high affinity for agonists are coupled to G proteins, whereas receptors with low agonist affinity are not. This was later confirmed in reconstitution experiments using isolated receptors and G proteins [12, 24]. In terms of the present results, it would appear that diethyl ether does not alter receptor coupling to G proteins insofar as the proportion of receptors with high and low affinity for agonists is not affected. The ability of guanine nucleotides to dissociate receptor–G protein complexes is, however, greatly depressed in the presence of diethyl ether. This "stabilization" of receptor–G protein complexes could be due to an interference with nucleotide binding or to an alteration of the lateral diffusion of macromolecules in the plane of the membrane. Other direct effects on either the receptor or G proteins are also possible. To define more precisely the site of anaesthetic action, we are presently examining the effects of anesthetics on several aspects of the G protein cycle, including GTPase activity (basal and receptor-stimulated), GDP release and GTP binding.

It should be noted that a number of other chemical and physical treatments also affect the stability of receptor–G protein complexes (See Ref. 25). For example, lowering the temperature to 4° converts receptors to a high affinity, guanine nucleotide-insensitive state, without affecting guanine nucleotide binding [26]. While diethyl ether also renders muscarinic receptors insensitive to guanine nucleotides, it does not affect the proportions of receptor displaying high and low affinity for agonists. Thus, the influence of diethyl ether on muscarinic complexes does not appear to derive from any simple alteration in the physical characteristics of the membrane.

As noted, in all of its actions on brainstem muscarinic receptors, diethyl ether resembles a second volatile anesthetic, halothane [7, 8]. Moreover diethyl ether and halothane were approximately equipotent at diminishing the sensitivity of [^{3}H]Oxo-M binding to a guanine nucleotide (Fig. 6), even though halothane is approximately 4-fold more potent and hydrophobic than diethyl ether.

The most prominent action of general anesthetics is a disruption of chemical communication between cells [5]. A specific interference with hormone/neurotransmitter receptor interactions with the ubiquitous transducer G proteins could contribute to the activity of volatile anesthetics in a wide variety of systems.

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