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# Halothane disruption of $\alpha_2$-adrenergic receptor-mediated inhibition of adenylate cyclase and receptor G-protein coupling in rat brain

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It is likely that a generalized depression of synaptic transmission underlies the elaboration and maintenance of the anesthetic state (e.g. [1]). It is also likely that the ultimate effect of general anesthetics is an alteration in the function of a specific protein(s) involved in synaptic transmission [2]. Guanine nucleotide-dependent transducer proteins (G proteins) are a closely related family of proteins involved in signal transduction between postsynaptic receptor and intracellular effector mechanism in a wide variety of neural systems [3, 4]. In view of their strategic location and critical function, G proteins are logical candidates for material sites in anesthetic action.

We have demonstrated recently that certain liquid volatile anesthetics alter the coupling of muscarinic acetylcholine receptors to G proteins [5–8]. This disruption is evidenced by a depression or elimination of the guanine nucleotide sensitivity of agonist binding to muscarinic receptors in rat brain. As a consequence of this action, muscarinic modulation of adenylate cyclase activity is attenuated [9].

To ascertain the generality of this effect with respect to receptor species, we examined the influence of halothane on $\alpha_2$-adrenergic receptor (1) regulation of adenylate cyclase activity and (2) sensitivity of agonist binding to guanine nucleotides. The results indicate that $\alpha_2$-adrenergic receptor–G protein interactions are disturbed by halothane in a functionally significant manner. These findings support our suggestion that interference with receptor–G protein coupling is a mechanism of some generality in the action of anesthetics and may contribute to the development of the anesthetic state.

## *Methods*

Male Wistar rats (125–150 g; Harlan Sprague–Dawley, Indianapolis, IN) were decapitated, and their cerebral cortices were removed and homogenized in 10 vol. of 50 mM Tris–HCl, pH 7.4, containing 2 mM $MgCl_2$ and 1 mM dithiothreitol (DDT)* using a Teflon–glass tissue grinder. The homogenate was spun at 17,000 *g* for 20 min at 4°. The pellet was resuspended and washed twice with the original buffer.

Adenylate cyclase activity was measured following the method of Salomon *et al.* [10] with minor modifications. Each assay tube contained the following reagents in a final volume of 250 μL: 25 mM HEPES, pH 7.5; 1.33 mM EGTA, 1 mM DTT; 2 mM MgSO4, 100 mM NaCl; 0.5 mM 3-isobutyl-1-methylxanthine; 0.1 mM ATP; 1 mM cAMP; 1 μM GTP; 20 mM creatine phosphate; 10 units of creatine phosphokinase; 0.5 μCi [$\alpha$-$^{32}$P]ATP; and 100–150 μg protein. Purification of cAMP was carried out by the method of Mao and Guidotti [11].

$\alpha_2$-Adrenergic receptor binding was measured in a medium containing 1 nM [*phenyl*-4-$^3$H]clonidine ([$^3$H])clonidine; 24 Ci/mmol, Amersham, Arlington Heights, IL), 50 mM Tris–HCl, pH 7.4, and 2 mM $MgCl_2$. After incubation for 45 min at room temperature, the reaction was terminated by filtration through glass fiber filters (No. 32, Schleicher & Schuell). The tubes and filters were washed twice with 5 mL Tris–magnesium buffer, and the radioactivity content of the filters was determined by liquid scintillation counting. Nonspecific binding (25–35% of total binding) was determined in the presence of 100 μM unlabeled clonidine. To evaluate the influence of guanine nucleotides on [$^3$H]clonidine binding, 5′-guanylylimidodiphosphate [Gpp(NH)p] was included in some assays.

The influence of halothane on [$^3$H]clonidine binding and adenylate cyclase activity was determined by equilibrating suspended membranes for 20 min with halothane–air mixtures provided by a calibrated Verritol vaporizer. Exposure to halothane was continued during the assay incubations using a manifold which delivered the air–gas mixture at a rate of 65 mL/min/tube.

## *Results and discussion*

Equilibration of cortical membranes with 1 and 2% (v/v) halothane increased basal adenylate cyclase activity by 38 and 42% respectively. Such an increase is in agreement with earlier work by ourselves [9] and others [12, 13]. Halothane did not affect the fractional stimulation of adenylate cyclase produced by forskolin (17 to 20-fold with 20 μM forskolin). The ability of clonidine to inhibit forskolin (5 μM)-stimulated enzyme activity, however, was greatly diminished in membranes equilibrated with 1% halothane (Fig. 1). Clonidine concentration–response curves were shifted to the right by almost two orders of magnitude.

The high-affinity binding of 1 nM [$^3$H]clonidine was inhibited by Gpp(NH)p with an $IC_{50}$ of 1.4 ± 0.3 μM (N = 5) (Fig. 2A). High-affinity, guanine nucleotide-sensitive agonist binding is thought to reflect binding to receptor–G protein complexes [4]; in the presence of guanine nucleotides, receptor–G protein complexes tend to dissociate,

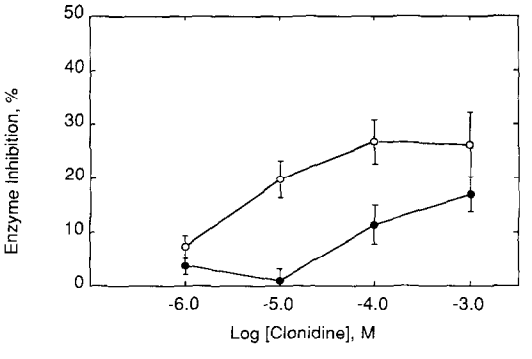


Fig. 1. Influence of halothane on clonidine inhibition of forskolin-stimulated adenylate cyclase activity in membranes prepared from rat cerebral cortex. Adenylate cyclase activity was measured in the presence of 5 μM forskolin and the indicated concentrations of clonidine in cortical membranes equilibrated with air (○) or 1% halothane (●). Fractional inhibition of forskolin-stimulated activity is indicated. Each point and bar represents the mean and standard deviation from five experiments.

* Abbreviations: DTT, dithiothreitol; Gpp-NH)p, 5′-guanylylimidodiphosphate; HEPES, *N*-[2-hydroxyethyl]piperazine-*N*′-[2-ethanesulfonic acid]; and EGTA, ethyleneglycolbis(aminoethylether)tetra-acetate.

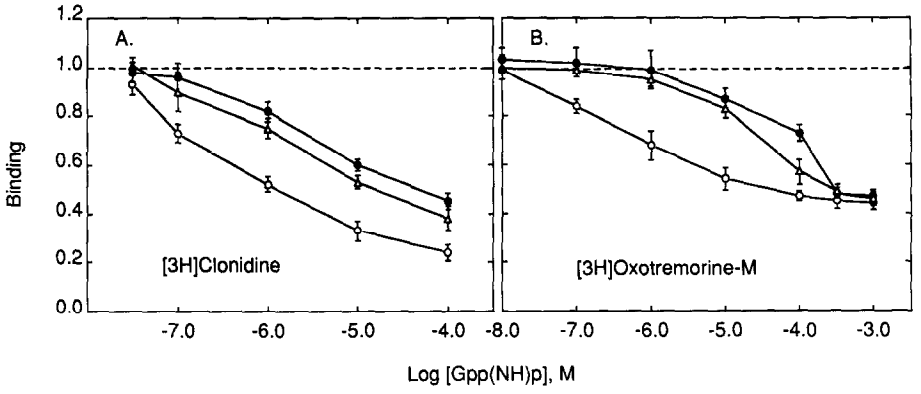


Fig. 2. Influence of halothane on high-affinity agonist binding to $\alpha_2$-adrenergic and muscarinic acetylcholine receptors. The high-affinity binding of 1 nM [$^3$H]clonidine to $\alpha_2$-adrenergic receptors in rat cerebral cortex (A) and 2 nM [$^3$H]oxotremorine-M to muscarinic acetylcholine receptors in rat brainstem (B) was measured in the presence of the indicated concentrations of Gpp(NH)p. Muscarinic receptor binding, included for comparison purposes, was performed as described by Dennison *et al.* [6]. Binding is plotted as fraction of specific binding measured in the absence of guanine nucleotide. Binding was measured in membranes equilibrated with air (○) or with 1 (△) or 2 (●)% halothane. Each point and bar represents the mean and standard deviation from four or five experiments.

leaving the receptor in a low-affinity, uncoupled state. This is reflected in the present assay as a decrease in [$^3$H]clonidine binding. Similar results were obtained with muscarinic acetylcholine receptors using [$^3$H]oxotremorine-M as the probe ([6]; Fig. 2B). Thus, the guanine nucleotide sensitivity of high-affinity agonist binding is one indication of functional receptor–G protein coupling.

Equilibration of the membranes with halothane (1 or 2%) greatly depressed the ability of Gpp(NH)p to inhibit [$^3$H]clonidine binding (Fig. 2A); inhibition curves were shifted to the right by 30 to 50-fold. This effect is similar in nature and extent to the effect of halothane on [$^3$H]oxotremorine-M binding to muscarinic receptors (Fig. 2B; [6]).

Insofar as the guanine nucleotide sensitivity of high-affinity agonist binding is a reflection of receptor–G protein coupling (or, more specifically, a guanine nucleotide-engendered dissociation of receptor binding and G protein subunits), the present results indicate that halothane interferes with $\alpha_2$-adrenergic receptor–G protein interactions. One possible explantion for these results is that halothane stabilizes receptor–G protein complexes, perhaps by inhibiting the release or subsequent binding of guanine nucleotides.

In these actions, $\alpha$-adrenergic receptors resemble muscarinic acetylcholine receptors. We have demonstrated previously that disruption of muscarinic receptor–G protein coupling is a common feature of volatile anesthetic agents [8]. The present results demonstrate that this interference is not restricted to muscarinic receptors, although the extent of the generality of the phenomenon remains to be established. In this regard, it is important to note that adrenergic ($\alpha$ and $\beta$) and muscarinic receptors are probably members of a multiple gene family of proteins with similar structures (e.g. [14]). Moreover, $\alpha_2$-adrenergic receptors resemble muscarinic receptors in that they efficiently couple to both the $G_i$ and $G_o$ species of G protein and mediate inhibition of adenylate cyclase [15, 16]. It is interesting to note that while halothane decreased carbamylcholine inhibition of adenylate cyclase activity, it did not affect the stimulation of adenylate cyclase produced by isoproterenol acting via $\beta$-adrenergic receptors coupled to stimulatory $G_s$ proteins [9]. This raises the possibility that anesthetic disruption of receptor–G protein interactions is restricted to receptors coupled to $G_i$ and $G_o$ proteins (or perhaps to the $G_i$ and $G_o$ proteins themselves).

In summary, in rat cerebral cortex, halothane depressed (1) clonidine inhibition of forskolin-stimulated adenylate cyclase activity and (2) the guanine nucleotide sensitivity of [$^3$H]clonidine binding to $\alpha_2$-adrenergic receptors. In these responses, $\alpha_2$-adrenergic receptors resemble muscarinic acetylcholine receptors in brain. It is suggested that halothane disrupts transmission at $\alpha_2$-adrenergic synapses by altering receptor–G protein interactions. Such an alteration may play an important role in the development of the anesthetic state.

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*Department of Pharmacology and Toxicology and Department of Anesthesiology Medical College of Georgia Augusta, GA 30912, U.S.A.*

MELISSA K. BAUMGARTNER
ROBERT L. DENNISON
TANJORE K. NARAYANAN
ROBERT S. ARONSTAM*

* Address all correspondence to: Robert S. Aronstam, Ph.D., Department of Pharmacology and Toxicology, Medical College of Georgia, Augusta, GA 30912.

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# Radioligand labeling of *N*-methyl-D-aspartic acid (NMDA) receptors by [$^3$H](±)-3-(2-carboxypiperazin-4-yl)propyl-1-phosphonic acid in brain synaptic membranes treated with Triton X-100

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Some free acidic amino acids endogenous to the brain are thought to play a role as excitatory synaptic neurotransmitters in the mammalian CNS [1, 2]. Synaptic receptors for these amino acids are classified into at least three different subgroups according to sensitivity to typical exogenous compounds, such as *N*-methyl-D-aspartic acid (NMDA), quisqualic acid (QA) and kainic acid (KA) [3, 4]. The NMDA-sensitive receptors are supposed to be involved in neuronal plasticity [5], convulsive seizures [6] or neuronal cell death in cerebral ischemia [7], hypoglycemia [8] and Alzheimer's disease [9].

However, biochemical labeling of this subclass is rather difficult. Some aminophosphonic acids with NMDA antagonistic activity, such as DL-2-amino-5-phosphonovaleric acid (AP5) and DL-2-amino-7-phosphonoheptanoic acid (AP7), as well as NMDA itself, have been shown to be unsuitable as a radioligand to label these NMDA sites [10–12]. A recent study introduced a novel antagonist highly selective to NMDA receptors, (±)-3-(2-carboxypiperazin-4-yl)propyl-1-phosphonic acid (CPP) [13]. Although this antagonist seems to label NMDA sites in brain synaptic membranes, the binding is detectable only when a centrifugation assay method is employed to separate the bound ligand from the free ligand [12]. A filtration assay method has been reported not to be useful for detecting [$^3$H]CPP binding in membranous preparations. Therefore, we have attempted in the present study to detect [$^3$H]CPP binding in brain synaptic membranes treated with Triton X-100 by employing a filtration assay method and we have demonstrated the usefulness of this method to determine NMDA-sensitive [$^3$H]L-glutamic acid (Glu) binding in these Triton membranes [14].

## *Materials and methods*

*Materials.* [$^3$H]CPP (*propyl*-1,2-[$^3$H]CPP, 30.7 Ci/mmol) was purchased from New England Nuclear (Boston, MA). DL-α-Amino-3-hydroxy-5-methylisoxazol-4-propionic acid (AMPA), willardiine (WIL), D- and L-AP5, D- and L-AP7 and CPP were supplied by Tocris Neuramin (Buckhurst Hill, U.K.). (+)-5-Methyl-10,11-dihydro-5*H*-dibenzo[*a*,*d*]-cyclohepten-5,10-imine maleate (MK-801) and phencyclidine (PCP) were donated by Dr L. L. Iversen (Merck Sharp & Dohme Research Laboratories, Harlow, U.K.) and Dr T Nabeshima (Meijo University, Nagoya, Japan) respectively. The other chemicals used were obtained from the Sigma Chemical Co. (St. Louis, MO).

*Membrane preparation.* Crude synaptic membrane fractions obtained from the brains of Wistar rats weighing 200–250 g [15] were washed four times by repeating the suspension in 50 mM Tris–acetate buffer (pH 7.4) [16] and subsequent centrifugation at 50,000 *g* for 20 min at 4° [17]. The final pellets were suspended, frozen, thawed, and treated with Triton X-100 [14].

*[$^3$H]CPP binding.* Membrane preparations (about 300 μg protein) were incubated with 10 nM [$^3$H]CPP in 0.5 mL of 50 mM Tris–acetate buffer (pH 7.4) at 2° for 10 min unless indicated otherwise. Incubation was terminated by the addition of 3 mL of ice-cold buffer and subsequent filtration