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HALOTHANE ATTENUATION OF MUSCARINIC INHIBITION OF ADENYLATE CYCLASE IN RAT HEART

TANJORE K. NARAYANAN,* RAND A. CONFER,†‡ ROBERT L. DENNISON, JR.,*‡ BETTY L. ANTHONY* and ROBERT S. ARONSTAM*§

* Department of Pharmacology and Toxicology, and † Department of Anesthesiology, Medical College of Georgia, Augusta, GA 30912; and ‡ Anesthesia Service, Veterans Administration Medical Center, Augusta, GA 30910, U.S.A.

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Abstract—Halothane stimulated basal adenylate cyclase activity in rat cardiac membranes. Maximal stimulation (54%) was obtained after equilibrating the membranes with 2% halothane. Halothane did not affect the fractional stimulation of adenylate cyclase activity produced by either forskolin or isoproterenol. However, halothane decreased carbamylcholine inhibition of adenylate cyclase activity stimulated by both forskolin and isoproterenol. Maximal depression of carbamylcholine inhibition of stimulated cyclase activity was obtained after equilibration with 1% halothane. These results are consistent with evidence from ligand binding studies and indicate that halothane disrupts muscarinic receptor–G-protein interactions.

Volatile anesthetics depress cardiac function, and effects of halothane on autonomic regulation of cardiac function have been described [1, 2]. One of the most prominent actions of anesthetics is a disruption of chemical transmission between cells. While the molecular mechanisms underlying anesthetic actions are incompletely understood, it has been proposed that anesthetics act at hydrophobic sites in certain particularly sensitive proteins [3–5].

Cyclic nucleotides are important mediators of adrenergic and cholinergic regulation of cardiac function [6–9]. Halothane has been reported to affect the activity of adenylate cyclase, the enzyme which catalyzes the formation of 3′,5′-cyclic adenosine monophosphate, in a number of tissues. For example, halothane increases basal adenylate cyclase activity in rat cerebral cortical membranes [10] and increases basal and glucagon-stimulated cyclase activity in rat liver homogenates [11]. On the other hand, halothane inhibits myocardial adenylate cyclase activity in the dog [12].

We recently demonstrated in ligand binding studies that halothane alters the coupling of muscarinic receptors in rat brain and heart to the guanine nucleotide-dependent transducer proteins (G proteins) which mediate muscarinic control of several cellular processes [13–15]. G proteins are a family of closely related proteins involved in transmembrane signalling in many hormonal and neurotransmitter receptor systems [16, 17]. In the heart, inhibition of adenylate cyclase activity following muscarinic receptor activation is mediated by inhibitory G_i proteins. Accordingly, the present studies were designed to determine the consequences of halothane disruption of receptor–G-protein interactions on muscarinic control of adenylate cyclase activity. The effects of halothane on (1) basal adenylate cyclase activity, (2) forskolin and isoproterenol stimulation of adenylate cyclase, and (3) carbamylcholine inhibition of stimulated adenylate cyclase activity were determined in rat cardiac membranes.

METHODS

Adult male Wistar rats (120–150 g) were killed by decapitation. The hearts were cleaned of fat and blood vessels and homogenized in TED buffer, pH 7.5 (10 mM Tris–HCl; 1 mM EDTA; 1 mM dithiothreitol). The homogenate was centrifuged at 20,000 *g* for 10 min at 4°. The resulting pellet was washed twice with TED buffer and used without further treatment. Protein was estimated by a modification [13] of the Lowry method using bovine serum albumin as the standard.

Membrane suspensions in TED buffer were equilibrated with halothane vaporized from a vernitol vaporizer carried in air. The anesthetic concentration was monitored continuously, and the flow of gas was maintained at the upper phase of the membrane suspensions for 30 min at 4°. Control membranes were equilibrated with air at the same flow rate. After the 30-min equilibration, the membranes were assayed immediately for adenylate cyclase activity.

Adenylate cyclase activity was measured by the method of Salomon *et al.* [18] 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 dithiothreitol; 2 mM $MgSO_4$; 50 mM NaCl; 0.1 mM ATP; 1 mM cAMP; 1 μM GTP; 20 mM creatine phosphate; 10 units of creatinine phospholinase; 0.5 μCi [^{32}P]ATP; 100–200 μg protein. Assays were carried out in quad-

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

‖ Abbreviations: HEPES, 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid; and EGTA, ethyleneglycolbis-(amino-ethylether)tetra-acetate.

ruplicate; replicate values generally varied by less than 10%. The reaction was started by adding the membranes, and the incubation was continued at 30° for 10 min. The reaction was stopped by immersing the assay tube in a boiling water bath for 1 min followed by centrifuging at 4000 *g* for 15 min. Purification of cAMP was carried out by the method of Mao and Guidotti [19]. An aliquot of the supernatant fraction was loaded onto a neutral alumina column (0.7 × 4 cm) equilibrated with 0.06 M Tris–HCl, pH 7.5, and eluted with 5 ml of the equilibration buffer. The eluate was directly loaded onto a Bio-Rad AG 1 × 4 formate column (0.7 × 2 cm) equilibrated with water. The column was washed twice with 10 ml of water, and the bound cAMP was eluted with 5 ml of 1 N formic acid. An aliquot was counted for [^{32}P]cAMP content using a Beckman LS-1701 liquid scintillation counter. The recovery of cAMP during the purification procedure was routinely monitored using [^{3}H]cAMP, and was always between 70 and 80%.

RESULTS

Halothane increased basal adenylate cyclase activity by up to 54% (Fig. 1). The maximal effect was obtained after equilibrating the membranes with 2% halothane. This effect was diminished with higher halothane concentrations; at 8%, halothane had no effect on basal cyclase activity.

Forskolin stimulated adenylate cyclase in a concentration-dependent manner (Fig. 2). A 25-fold stimulation of basal activity was obtained with 100 μM forskolin. Halothane (2%) did not affect the degree of enzyme stimulation caused by forskolin (Fig. 2). However, since halothane increased the level of basal activity (Fig. 1), the absolute increase in enzyme activity caused by forskolin was proportionately greater in the presence of 0.5 to 4% halothane.

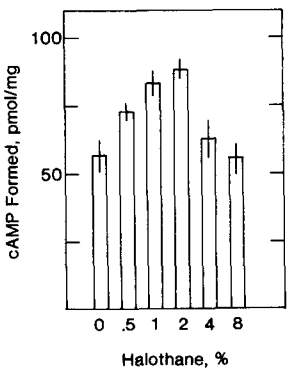

Fig. 1. Influence of halothane on basal adenylate cyclase activity in rat cardiac membranes. The formation of cAMP (pmol/mg protein during a 10-min incubation at 30°) was determined in quadruplicate after equilibrating the membrane for 30 min with the indicated mixtures of halothane and air. Replicate values varied by less than 10%. Each bar and line represent the mean and standard deviation from five experiments.

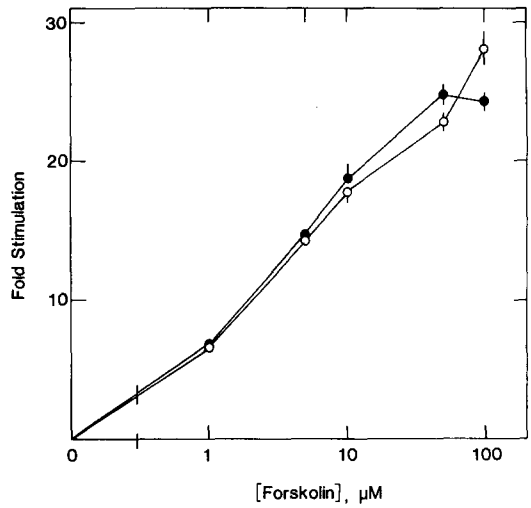

Fig. 2. Forskolin stimulation of adenylate cyclase activity in rat cardiac membranes. The formation of cAMP was measured in the presence of the indicated concentrations of forskolin, and is plotted as degree of stimulation of basal activity measured in the absence of forskolin. Enzyme activity was measured in membranes equilibrated for 30 min with air (○) or 2% halothane (●). Each point and bar represent the mean and standard deviation from three experiments.

Isoproterenol stimulated adenylate cyclase activity by up to 5-fold (Fig. 3). Stimulation was maximal with from 10 to 1000 μM isoproterenol. Halothane (0.5 to 4%) did not affect the fractional stimulation of basal adenylate cyclase activity caused by any concentration of isoproterenol (Fig. 3). Again, since halothane increased basal enzyme activity, the absolute increases in activity caused by isoproterenol were greater in the presence of halothane.

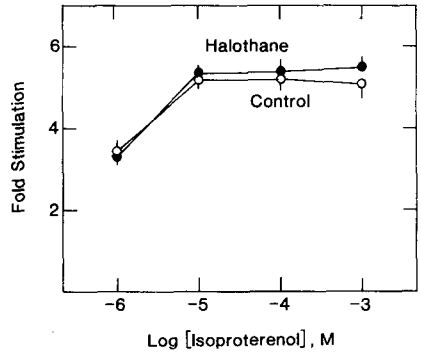

Fig. 3. Isoproterenol stimulation of adenylate cyclase activity in rat cardiac membranes. The formation of cAMP was measured in the presence of the indicated concentrations of isoproterenol, and is expressed as degree of stimulation of basal activity measured in the absence of isoproterenol. Enzyme activity was measured in membrane equilibrated for 30 min with air (○) or 4% halothane (●). Equilibration with 0.5 or 2% halothane was similarly without effect on the degree of stimulation by isoproterenol (not shown), although basal enzymatic activity was different (see Fig. 1). Each point and bar represent the mean and standard deviation from three experiments.

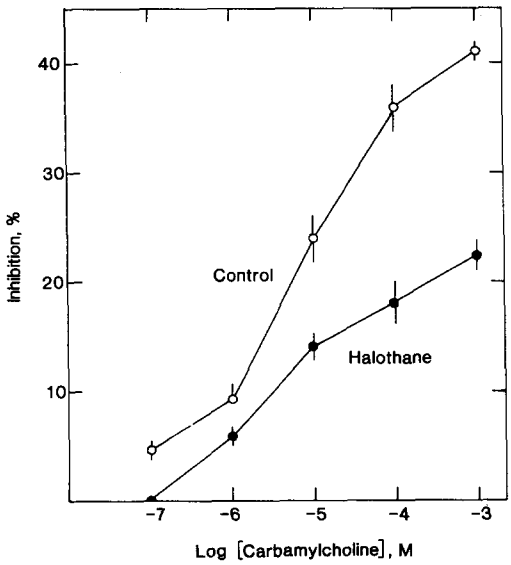

Fig. 4. Influence of halothane on carbamylcholine inhibition of forskolin-stimulated adenylate cyclase activity in rat cardiac membranes. The formation of cAMP was measured in the presence of 1 μM forskolin and the indicated concentrations of carbamylcholine. Carbamylcholine had no effect on basal cyclase activity. Enzyme activity was measured in membranes equilibrated for 30 min with air (○) or 4% halothane (●). Carbamylcholine inhibition was depressed to similar extents following equilibration with either 1 or 2% halothane (not shown). Each point and bar represent the mean and standard deviation from four experiments.

Carbamylcholine inhibited adenylate cyclase activity stimulated by 1 μM forskolin in a dose-dependent manner (Fig. 4). Maximal inhibition (41%) was obtained with the highest carbamylcholine concentration used (1 mM). Carbamylcholine inhibition was blocked by 10 μM atropine, indicating the muscarinic nature of the effect. Carbamylcholine had no effect on basal adenylate cyclase activity. Halothane (4%) attenuated the ability of carbamylcholine to inhibit forskolin activity by about 40% (Fig. 4). The effects of different concentrations of halothane on carbamylcholine inhibition of forskolin (1 μM)-stimulated adenylate cyclase activity are summarized in Fig. 5. Maximal attenuation of the carbamylcholine response was obtained after equilibration with 1% halothane.

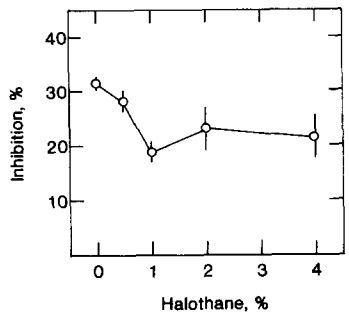

Fig. 5. Influence of halothane at different concentrations on carbamylcholine inhibition of forskolin-stimulated adenylate cyclase activity in rat cardiac membranes. Adenylate cyclase activity was measured in the presence of 1 μM forskolin and 30 μM carbamylcholine in membranes equilibrated with the indicated concentrations of halothane. The percent inhibition of forskolin-stimulated activity by carbamylcholine is indicated on the ordinate. The extent of carbamylcholine inhibition was lower with each concentration of halothane used. Each point and bar represent the mean and standard deviation from three experiments.

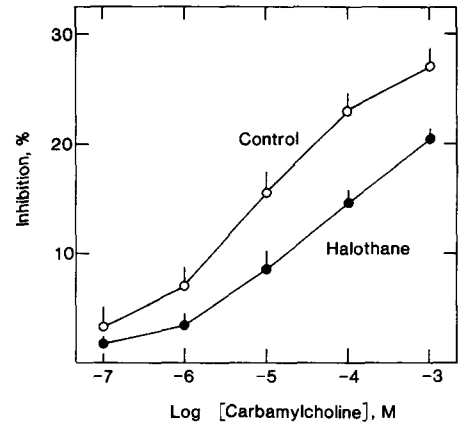

Fig. 6. Influence of halothane on carbamylcholine inhibition of isoproterenol-stimulated adenylate cyclase activity in rat cardiac membranes. The formation of cAMP was measured in the presence of 10 μM isoproterenol and the indicated concentrations of carbamylcholine. Fractional inhibition of isoproterenol-stimulated activity is indicated on the ordinate. Enzyme activity was measured in membranes equilibrated for 30 min with air (○) or 4% halothane (●). Carbamylcholine inhibition was depressed to similar extents by both 1 and 2% halothane (not shown). Each point and bar represent the mean and standard deviation from four experiments.

Carbamylcholine also inhibited adenylate cyclase activity stimulated by 10 μM isoproterenol in a dose-dependent manner (Fig. 6). At 1 mM, carbamylcholine inhibited 27% of isoproterenol-stimulated activity. Halothane attenuated carbamylcholine inhibition of isoproterenol-stimulated enzyme activity by about 35% at all carbamylcholine concentrations examined.

DISCUSSION

The effect of halothane on basal and stimulated adenylate cyclase activity has been studied in a number of tissues. Bernstein *et al.* [12] reported that halothane stimulates basal and glucagon-stimulated adenylate cyclase activity in rat liver homogenates, but has no effect on basal activity, and an inhibitory effect on glucagon-stimulated activity, in isolated liver membranes. Triner *et al.* [10] reported an increase in the V_{max} of rat brain adenylate cyclase in the presence of halothane. Gangt *et al.* [20, 21] reported a depressant effect of halothane on catecholamine-stimulated adenylate cyclase activity in cat and rat myocardial homogenates. In contrast, halothane has no effect on adenylate cyclase activity in myocardial sarcolemmal membranes in the dog [11]. These discrepancies have been attributed to either a loss of some component(s) during membrane isolation or to species differences.

The adenylate cyclase complex includes at least three structures: (1) a regulatory (receptor) unit with hormone/neurotransmitter binding sites facing the outside of the plasma membrane, (2) a guanine nucleotide-dependent transducer unit (G protein), and (3) a catalytic unit. Two types of G proteins mediate hormonal influences on cyclase activity: G_s which stimulates, and G_i which inhibits, enzyme activity. All three components of the adenylate cyclase system are present in the membrane preparations used in the present study. Stimulation of adenylate cyclase by forskolin (Fig. 2), whose action is mainly on the catalytic subunit, indicates the presence of the intact catalytic unit. Isoproterenol stimulation of adenylate cyclase (Fig. 3) indicates the presence of stimulatory β-receptors and functional G_s. Carbamylcholine inhibition of stimulated adenylate cyclase activity (Fig. 4) indicates the presence of inhibitory muscarinic receptors and functional G_i.

Two effects of halothane on adenylate cyclase activity on rat cardiac membranes were observed in the present experiments: (1) an increase in basal activity and (2) a depression of muscarinic inhibition of forskolin- and isoproterenol-stimulated activity. Halothane did not affect the degree of enzyme stimulation caused by either forskolin or isoproterenol. Halothane MAC (minimum aveolar concentration at 1 atmosphere pressure that produces immobility in 50% of subjects exposed to a noxious stimulus) is about 0.74% in humans and 1.1% in rats [22]. In the present experiments, the concentrations of halothane in the aqueous medium after equilibration for 20 min were approximately half that of the administered concentrations. Thus, the concentrations of halothane required to affect adenylate cyclase activity were well within the range encountered in the clinical situation.

The increase in basal activity engendered by halothane is in agreement with other reports [10, 12]. This stimulation may reflect anesthetic interactions with hydrophobic membrane components whereby conformational changes in membrane structure facilitate enzyme activity or expose additional catalytic sites. Alternately, halothane may affect the G protein cofactors. For example, if the GTPase activity of G_s were depressed by halothane, the G_s–enzyme complex would have a longer half-life. (GTP was included in all enzyme assays.) However, halothane did not enhance isoproterenol stimulation of cyclase activity, which is mediated by G_s. It is also possible that a selective effect of halothane on G_i could have upset the balance of G protein influences on the catalytic subunit.

Halothane did not affect stimulation of adenylate cyclase activity by either isoproterenol or forskolin. Isoproterenol stimulation is mediated by beta-adrenergic receptors via G_s; forskolin stimulation activates the catalytic subunit directly [23, 24], although its action may be affected by G proteins. The effects of halothane and isoproterenol and forskolin on enzyme activity are synergistic; the fractional stimulation by isoproterenol and forskolin is not changed by halothane, although the absolute increase in activity is greater. This suggests that mechanism of action of halothane is different from that of either stimulatory agent.

Halothane has a clear inhibitory effect on muscarinic regulation of stimulated adenylate cyclase activity. This could reflect an interference with either muscarinic binding or G_i protein action. We have reported that halothane increases the affinity of brain and cardiac muscarinic receptor for antagonists by slowing the rate of ligand dissociation [13–15]. The binding of muscarinic agonists, however, is not affected by halothane. A second prominent effect of halothane on muscarinic receptors is an elimination of guanine nucleotide regulation of agonist binding [13–15]. Guanine nucleotides convert muscarinic receptors from high-affinity to low-affinity agonist binding states. This effect is believed to reflect guanine nucleotide-engendered dissociation of receptor–G-protein complexes (receptor–G-protein complexes possess high affinity for agonists; uncoupled receptors have low affinity). These findings suggest that halothane disrupts receptor–G-protein interactions [13, 15]. Since agonist binding in the absence of exogenous guanine nucleotide is not affected, halothane does not simply inactivate G proteins or uncouple receptor–G-protein complexes. We have suggested that halothane stabilizes receptor–G-protein complexes, by either a physical action on the membrane or an inhibition of guanine nucleotide binding [13].

The present experiments are consistent with our earlier work, and indicate that halothane disrupts muscarinic control of adenylate cyclase activity in cardiac membranes. This effect appears to be specific for the muscarinic receptor and/or G_i components of the adenylate cyclase complex insofar as direct and adrenergic receptor/G_s-mediated stimulations of the catalytic subunit are not affected by halothane.

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