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Effects of Halothane on the Cyclic 3',5'-Adenosine Monophosphate (Cyclic AMP) System in Rat Uterine Muscle

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The effects of halothane on the cyclic 3',5'-adenosine monophosphate (cyclic AMP) system and on smooth muscle contractility were studied *in vitro* in rat uterus. Halothane increased the activities of both adenyl cyclase and phosphodiesterase in a dose-dependent fashion. Halothane stimulated the adenyl cyclase activity to a greater extent than that of phosphodiesterase. Uterine tissue levels of endogenous cyclic AMP were also increased by halothane. Acetylcholine-induced contraction of the rat uterus was inhibited by halothane in concentrations similar to that affecting the cyclic AMP system. These results suggest that the action of halothane in relaxing the uterine muscle is related to an increased intracellular cyclic AMP owing to increased activity of adenyl cyclase. In contrast to epinephrine, these effects of halothane were not antagonized by beta-adrenergic blocking compounds, propranolol and MJ 1999, indicating that the action of halothane on uterine smooth muscle is not mediated through the beta-adrenergic receptors as previously suggested. (Key words: Halothane; Cyclic AMP; Adenyl cyclase; Phosphodiesterase; Uterine muscle relaxation.)

THE RELAXING EFFECT of halothane in the uterus, blood vessels, and bronchus is well documented clinically and experimentally. However, the mechanism of this action is not known. Studies with isolated uterine segments *in vitro* demonstrated that intact innervation is not essential for this action of halothane.[1] Ngai *et al.*[2, 3] showed that halothane does not affect the release, uptake and turnover of norepinephrine in the heart and brain, thus making unlikely the possibility that catecholamines would mediate the uterine or other smooth muscle relaxing action of halothane. A direct action of halothane on the target organ, therefore, has been considered as a possible mechanism of action. Klide *et al.*[4] suggested that halothane causes relaxation of isolated rabbit uteri by stimulating beta-adrenergic receptors.

It has been shown recently that the epinephrine-induced relaxation of the rat uterus is accompanied by increased adenyl cyclase activity and cyclic AMP level, suggesting that the action of epinephrine is mediated by cyclic AMP.[5] The tissue levels of cyclic AMP are regulated by adenyl cyclase and phosphodiesterase. The former catalyzes the formation of cyclic AMP from ATP, and the latter catalyzes the inactivation of cyclic AMP to 5'-adenosine monophosphate. Further studies of the cyclic AMP system showed that the increase in intracellular cyclic AMP brought about by adenyl cyclase stimulation, phosphodiesterase inhibition, or addition of exogenous cyclic AMP results in decreased tone and contractility of the smooth muscle. These findings led to the suggestion that the cyclic AMP system is part of the regulatory mechanism of smooth muscle function.[6]

With this information, it seemed relevant to explore the action of halothane on the cyclic AMP system. We report the results of experiments performed in the rat uterus *in vitro*,

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TABLE 1. Effects of Halothane on Phosphodiesterase Activity of Rat Uterine Homogenates*

	cAMP (μmoles/mg N_2/min)	Per Cent Increase of Activity	P
Control	1,862 ± 25		
Halothane (v/v)			
2 per cent	1,968 ± 30	5.7 ± 1.1	<0.05
5 per cent	2,070 ± 39	12.0 ± 1.5	<0.001
10 per cent	2,194 ± 89	19.0 ± 3.0	<0.001

* Expressed as amount of cyclic AMP consumed, means ± SE from seven experiments.

which was used as our first model for smooth muscle.

Methods

Young Sherman female virgin rats, each weighing 125–150 g, were pretreated for 1–2 days with diethylstilbestrol (60 μg/100 g) given intraperitoneally. Vaginal smears were examined microscopically. Only animals found in mid-estrus, as evidenced by clustering of epithelial cells, were used. They were decapitated and the uterus rapidly removed.

For the measurement of adenyl cyclase and phosphodiesterase activity, the uterus was homogenized in an all-glass homogenizer with 20 vol of 0.05 M THAM (*p*H 7.5) per gram of tissue at 4 C. Phosphodiesterase activity was determined from the decrease of labelled substrate, ^{3}H-cyclic AMP. The reaction was started by adding 0.15 ml homogenate to a test tube containing 10 mM $MgSO_4$, 10 mμ moles of cyclic AMP (final concentration 33 μM), and 2 μCi ^{3}H-cyclic AMP (New England Nuclear Corp., Boston, Mass.). After 8 minutes of incubation in a water bath at 37 C, the reaction was stopped by boiling for 5 minutes. Cyclic AMP was isolated on an ion-exchange resin (Dowex 50W-X4, H^+ form, 35 mm × 20 mm^2) column and precipitated twice with zinc sulfate and barium hydroxide according to the method of Krishna.[7]

Adenyl cyclase activity was measured by Krishna's method[7] with the following modifications: phosphoenolpyruvate coupled with pyruvate kinase was used as a regenerating system to maintain the ATP concentration constant, and instead of inhibiting phosphodiesterase with theophylline, an excess of cyclic AMP was added.[8] The substrate for the reaction was 1 mM ATP–Na_3H_2O in the presence of 10 mM $MgSO_4$ and 3.5 to 5 μCi ^{32}P-ATP (International Chemical and Nuclear Corp., Irvine, Calif.). The reaction was started by adding 0.15 ml of the homogenate; the mixture was incubated at 37 C for 10 minutes and the reaction stopped by boiling for 5 minutes. Again, the same ion-exchange resin

TABLE 2. Effects of Halothane on Adenyl Cyclase Activity of Rat Uterine Homogenates*

	cAMP (μmoles/mg N_2/10 min)	Per Cent Increase of Activity	P
Control	398 ± 15	17.4 ± 5.8	<0.02
Halothane, 2 per cent	465 ± 21		
Control	372 ± 21	35.5 ± 5.3	<0.002
Halothane, 5 per cent	500 ± 26		
Control	386 ± 47		
Halothane, 10 per cent	590 ± 59	55.5 ± 15.0	<0.002

* Expressed as cyclic AMP formed. Values are means ± SE from seven experiments in each pair of control and experimental preparations.

TABLE 3. Effects of Beta-adrenergic Blockade on Halothane-induced Increases in Cyclic AMP Formation (μmoles Cyclic AMP/mg N_2/10 min)*

Control	Halothane (v/v)	Halothane + Beta-adrenergic Blockers
388 ± 20	(2 per cent) 462 ± 26	477 ± 34†
354 ± 31	(5 per cent) 506 ± 41	463 ± 29†
420 ± 44	(2 per cent) 483 ± 30	489 ± 26‡
412 ± 18	(5 per cent) 558 ± 68	513 ± 20‡

* Values are means ± SE from four experiments. Differences between control and halothane groups are significant ($P < 0.05$); differences between halothane and halothane with beta-adrenergic blockers are not significant.
† Propranolol, 10μM.
‡ MJ 1999, 100 μM.

from Nagarse and Co., Ltd. (Osaka, Japan). column was used to separate cyclic AMP. Radioactivity in the cyclic AMP-containing fraction was counted in Bray's solution [9] using an Intertechnique liquid scintillation spectrometer. The activity of phosphodiesterase or adenyl cyclase was expressed in $\mu\mu$moles of cyclic AMP consumed or formed per mg of tissue nitrogen, determined by Ferrari's method.[10]

For the determination of cyclic AMP formation in intact tissue, uterine segments were incubated for 10 minutes in Krebs-Ringer bicarbonate (KRB) solution with or without 10 mM theophylline. Immediately after incubation, the tissue was frozen in liquid nitrogen and homogenized in 5 per cent trichloroacetic acid. The homogenate was centrifuged at 6,000 *g* for 10 minutes at 0 C. The supernatant was extracted with ether, evaporated to dryness, and taken up in an appropriate volume of water. Cyclic AMP level was then determined with the protein kinase-binding method of Gilman.[11]

The effect of halothane on uterine contractility was studied in uterine horns suspended under 1 g tension in a tissue bath of KRB medium (*p*H 7.4) containing 10 mM glucose. Contractions were produced by increasing concentrations of acetylcholine (0.25–64 μM), measured isometrically by a force-displacement transducer, and recorded on a Grass polygraph.[12]

For the measurement of adenyl cyclase and phosphodiesterase activity, halothane vapor was carried in air to the reaction tubes for 15 minutes before and during the incubation. For the endogenous cyclic AMP and functional studies, a mixture of 95 per cent O_2 and 5 per cent CO_2 was used as the carrier. In the functional studies, the halothane mixture was bubbled through the fluid for 30 minutes before and during the experiment. The concentration of halothane was monitored by an ultraviolet analyzer and the vapor pressure in the reaction tube or tissue bath was determined by gas chromatography. Equilibration of halothane vapor took approximately 4 minutes in the systems used for enzyme activity and endogenous cyclic AMP assay, and 20 minutes for the functional studies. Air or a corresponding gas mixture was used for concurrent control studies.

Student's *t* test and *t* test for paired data were used to assess the differences between control and experimental values.

Results

Effects of Halothane on Phosphodiesterase, Adenyl Cyclase and Endogenous Cyclic AMP

Basal phosphodiesterase activity in the broken-cell preparation was stable for more than 90 minutes after homogenization, the time needed for each experiment. Table 1 shows the effects of halothane on phosphodiesterase activity. The increases in enzymic activity in the presence of halothane were relatively small, but were significant, consistent, and dose-dependent.

Adenyl cyclase activity in the homogenate decreased gradually. Therefore, it was necessary to measure control activity simultaneously with each assay. The effects of increasing concentrations of halothane on adenyl cyclase are shown in table 2; 2 per cent halothane increased the activity of adenyl cyclase by 67 $\mu\mu$moles/mg N_2/10 min; 5 per cent halothane by 128 $\mu\mu$moles/mg N_2/10 min; 10 per cent halothane by 204 $\mu\mu$moles/mg N_2/10 min.

When the effects of halothane are expressed as per cent change from control phosphodiesterase and adenyl cyclase activities, halothane increased the activity of adenyl cyclase more than that of phosphodiesterase (fig. 1). In intact uterine tissue halothane increased the tissue level of cyclic AMP. In the presence of 10 mM theophylline, 5 per cent halothane in eight experiments increased the intracellular cyclic AMP by 32 per cent (from a control of 2.07 ± 0.17 to 2.74 ± 0.25 $\mu\mu$moles/mg tissue/10 min, $P < 0.02$). In four experiments without theophylline in the medium, 5 per cent halothane also changed the level of cyclic AMP from a control of 1.08 ± 0.16 to 1.70 ± 0.76 $\mu\mu$moles/mg tissue/10 min, but this was not statistically significant.

Effects of Halothane and Dibutyryl Cyclic AMP on the Contractile Response of Rat Uterus

The uterine horn, suspended in KRB under 1 g tension, was challenged with increasing concentrations of acetylcholine until the maxi-

mum contractile response was reached. After the preparation in the tissue bath was equilibrated with 2 per cent halothane vapor, the contractile response to acetylcholine was diminished. The dose of acetylcholine needed to produce the same degree of contraction as in the control situation increased approximately fourfold. The maximum contraction induced by acetylcholine was decreased by 25 per cent. A higher concentration of halothane (5 per cent) further decreased the contractile response of the uterus to acetylcholine. The maximum contraction produced by 64 μM acetylcholine decreased by more than 50 per cent (fig. 2). After halothane was discontinued, the contractile response to acetylcholine returned to control values within 60 minutes.

An increase in intracellular cyclic AMP brought about by the addition of exogenous cyclic AMP also decreased uterine contractility (fig. 3). Dibutyryl cyclic AMP, an analog, was shown to mimic the effect of cyclic AMP.[12] The acetylcholine-induced contractile response of the uterus was diminished in the presence of 1 mM dibutyryl cyclic AMP.

Beta-adrenergic Blocking Compounds and the Effects of Halothane on Adenyl Cyclase and Contractility of the Rat Uterus

The stimulatory effect of halothane on adenyl cyclase was not inhibited by the beta-adrenergic blocking compounds propranolol (10 μM) and MJ 1999 (100 μM) (table 3). Correspondingly, propranolol and MJ 1999 failed to counteract the halothane-induced relaxation of the rat uterus (figs. 4 and 5). Both these compounds were used in concentrations which inhibited the stimulatory effect of epinephrine on adenyl cyclase. Propranolol has been reported previously to block epinephrine-induced stimulation of adenyl cyclase, as well as its relaxing effect in rat uterus.[14]

Discussion

The purpose of the present study was to determine whether the smooth muscle-relaxing action of halothane is related to changes of cyclic AMP in the cell.

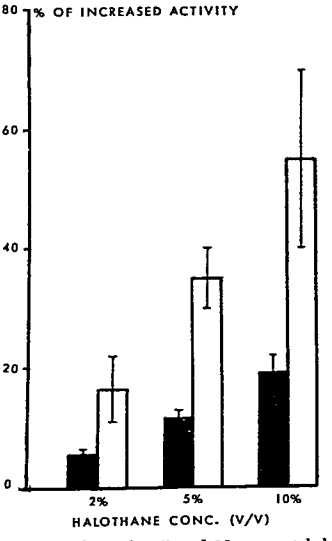

FIG. 1. Effects of 2, 5, and 10 per cent halothane in air on phosphodiesterase (solid bars) and adenyl cyclase (open bars) activities of uterine muscle homogenates as compared with controls. Each value represents the mean ± SE of seven experiments.

Our results confirmed that halothane relaxed the rat uterine horn directly and corroborated the findings of Munson *et al.*[1] in human uterine muscle. We also found that halothane increased the activities of both adenyl cyclase and phosphodiesterase in a dose-dependent manner. In the broken-cell preparation, halothane increased adenyl cyclase activity more than phosphodiesterase activity. These results suggested that, with halothane, the rate of cyclic AMP formation would be higher than the rate of its inactivation; therefore, the net result would be an increase in tissue cyclic AMP level. In order to substantiate such an assumption, the effect of halothane on cyclic AMP level in intact tissue was measured. We found that halothane indeed increased the cyclic AMP level in intact rat uterus. The increase in cyclic AMP caused by halothane in the presence of theophylline

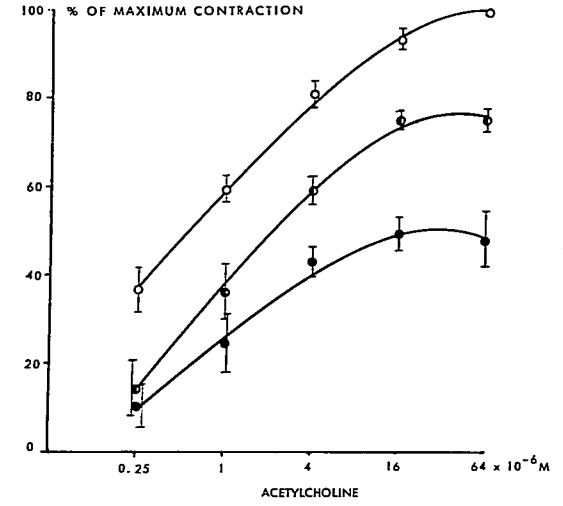

FIG. 2. Effects of halothane (2 and 5 per cent) on acetylcholine-induced contractions of rat uterine horn *in vitro*. Mean ± SE of seven experiments. Open circles, control; half-solid circles, 2 per cent halothane; solid circles, 5 per cent halothane.

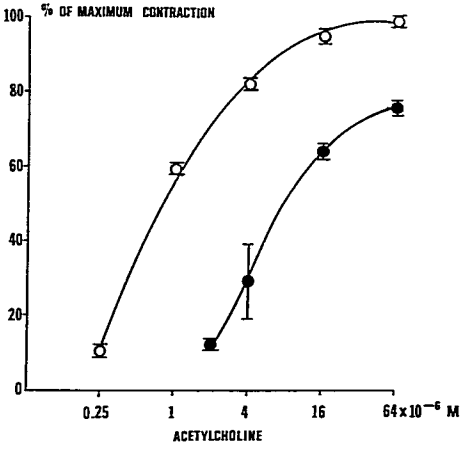

FIG. 3. Effects of 1 mM dibutyryl cyclic AMP on acetylcholine-induced contractions of rat uterine horn. Mean ± SE of four experiments. Open circles, control; solid circles, 1 mM dibutyryl cyclic AMP.

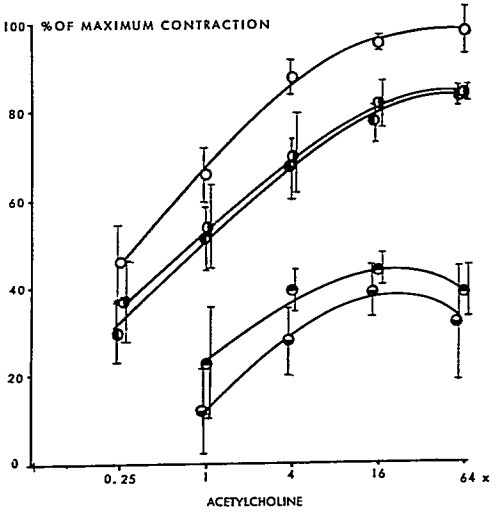

FIG. 4. Effects of propranolol and halothane on acetylcholine-induced uterine contractions. Mean ± SE of four experiments. Open circles, control; solid right side, 2 per cent halothane; solid left side, 2 per cent halothane + 10 μM propranolol; solid bottom half, 5 per cent halothane; solid top half, 5 per cent + 10 μM propranolol.

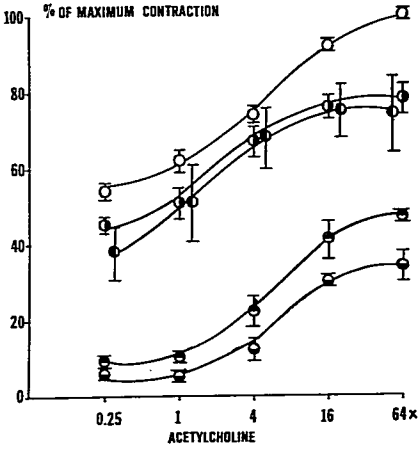

FIG. 5. Effects of MJ 1999 and halothane on acetylcholine-induced uterine contractions. Mean ± SE of four experiments. Open circles, control; solid right side, 2 per cent halothane; solid left side, 2 per cent halothane + 100 μM MJ 1999; solid bottom half, 5 per cent halothane; solid top half, 5 per cent halothane + 100 μM MJ 1999.

reflects mainly the stimulatory effect on adenyl cyclase. In the absence of theophylline, the change was not significant. Since the activities of both enzymes regulating the cyclic AMP level were increased by halothane, it is possible that the turnover of cyclic AMP was enhanced without significant alteration of its level. Further investigations are needed to clarify this point.

The contractility of the rat uterine horn was decreased by halothane in a dose-dependent fashion in concentrations similar to those found to act on the cyclic AMP system. The relaxing effect of halothane was mimicked by exogenous dibutyryl cyclic AMP. These data support the hypothesis that cyclic AMP is involved in the relaxing effect of halothane, but offer no conclusive direct evidence.

Since Klide *et al.*[4] reported that MJ 1999 decreased the relaxing effect of halothane on rabbit uterine muscle, two beta-adrenergic blocking compounds, propranolol and MJ 1999, were tested. In contrast to their antagonistic effects on epinephrine, these compounds altered neither the stimulatory effect of halothane on adenyl cyclase nor its relaxing action. The discrepancy of our results with those reported by Klide may be explained by the findings of Zauder *et al.*,[15] who presented evidence that MJ 1999 alone increased the tone and motility of rabbit uterus. Therefore, the relaxing effect of halothane might have been counteracted by the oxytocic activity of MJ 1999 and not necessarily by its beta-adrenergic blocking effect. Our biochemical and functional data with both beta-adrenergic blocking agents showed that these agents do not influence the actions of halothane. At least by this criterion, halothane cannot be characterized as a beta-adrenergic agonist.

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