

Dopamine-sensitive Adenylate Cyclase Activity in the Rat Caudate Nucleus during Exposure to Halothane and Enflurane

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The effects of halothane and enflurane on the dopamine-sensitive adenylate cyclase and cyclic 3',5'-adenosine monophosphate (cAMP)-phosphodiesterase in the homogenate of rat caudate nuclei were determined. Halothane, in concentrations of 2 and 5 vol per cent, increased adenylate cyclase activity by 16 ($P < 0.005$) and 26 ($P < 0.001$) per cent, respectively, above a mean basal value of 316.5 ± 8.8 pmol cAMP/mg protein/3 min. Enflurane, in concentrations of 3 and 5 vol per cent, increased adenylate cyclase activity by 11 ($P < 0.05$) and 22 ($P < 0.02$) per cent, respectively, above a mean basal value of 343.9 ± 10.4 pmol cAMP/mg protein/3 min. The activity of adenylate cyclase already stimulated by dopamine (even with the maximally-stimulating concentration) was further increased by both anesthetics, suggesting that the anesthetics and dopamine activate the enzyme at different sites. Haloperidol and droperidol, known dopamine receptor blockers, reversed the effect of halothane or enflurane on the adenylate cyclase. The antagonistic effect of haloperidol on anesthetic-induced adenylate cyclase activity is probably not related to its property to inhibit dopamine effect. Both Km forms of cAMP-phosphodiesterase were significantly depressed by halothane (3 vol per cent) and enflurane (5 vol per cent). The results suggest that an action of halothane and enflurane on the enzymes of the cAMP system, altering the response of the postsynaptic membrane to the transmitter substance, may be related to some of their effects on the process of chemical transmission. (Key words: Anesthetics, volatile: halothane; enflurane. Ataractics, butyrophenones: haloperidol; droperidol. Enzymes, cAMP system. Metabolism, enzyme.)

HALOTHANE has been found to increase the activity of adenylate cyclase in the uterus,¹ bronchus,² liver,³ cerebral cortex, cerebellum,⁵ and platelets,** and to increase cyclic 3',5'-adenosine monophosphate (cAMP) formation in the aorta⁴ and cAMP content in cultured neuroblastoma cells⁵ and in the brain.⁶ Similarly, enflurane has been shown to increase

adenylate cyclase activity in bronchial tissue.² These findings and current concepts of the physiologic role of cAMP have led some investigators to suggest that the action of anesthetics on the cAMP system may be related to certain of their pharmacologic effects.^{1,4,††} It has even been postulated that "cAMP might be involved in the anesthetic process."⁵ There is growing evidence that cAMP is involved in synaptic transmission by mediating the action of certain neurotransmitters.⁷⁻¹¹ Since anesthetics have been shown to inhibit synaptic transmission¹²⁻¹⁴ and to affect the cAMP system in various tissues, it is possible that some of the effects of anesthetics on synaptic transmission are exerted through the cAMP system. The purpose of the present study was to determine whether halothane and enflurane have an effect on the enzymes of the cAMP system in the caudate nucleus, a suitable brain structure for the study of biochemical events modulating synaptic transmission since cAMP has been shown to mediate the action of dopamine in this tissue.

Materials and Methods

Caudate nuclei were obtained from Sherman female rats each weighing 180-200 g. The animals were sacrificed by decapitation and the brains rapidly removed and kept in iced saline solution while the nuclei were dissected out (putamen was not separated from caudate nucleus). The nuclei were then immediately homogenized at 4 C in an all-glass tissue grinder (seven strokes) in 100 volumes (v/w) of 50 mM Tris-HCl buffer (pH 7.5) containing EGTA, 2 mM, and theophylline, 20 mM. The homogenate was allowed to stand in an ice-water bath for 20 min before the adenylate cyclase assay was started.¹⁵

Adenylate cyclase activity was measured by the method of Krishna *et al.*¹⁶ modified by the addition of an ATP-regenerating system to maintain the substrate concentration constant. The assay mixture contained the following, in a total volume of 300 μ l: 50 mM Tris-HCl buffer (pH 7.5); ATP, 1 mM; 3-3.5 μ Ci of ³²P-ATP (25 Ci/mmol); MgSO₄, 3 mM; phosphoenolpyruvate, 3.3 mM; pyruvate kinase, 15 μ g/ml; 150 μ l of tissue homogenate (about 200 μ g protein) and

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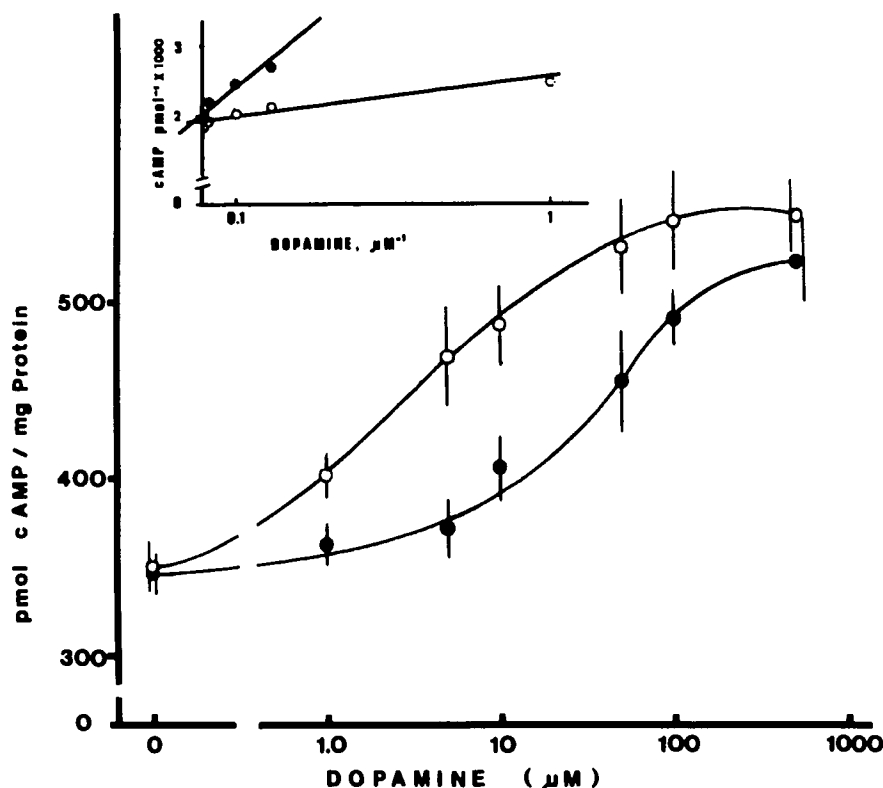


FIG. 1. Effect of dopamine in the absence (○ — ○) or presence (● — ●) of 0.5 μM haloperidol on adenylate cyclase activity in the rat caudate nucleus homogenate. Inset: double reciprocal plot of cAMP increase as a function of dopamine concentrations. ○ — ○; dopamine alone; ● — ●; dopamine plus 0.5 μM haloperidol. Points represent mean values \pm SEM from six experiments.

appropriate test substances when indicated. The test substances were added to the mixture immediately before the assay was started by the addition of the labeled ATP. The reaction was terminated after 3 min of incubation at 30 C by immersion of the test tubes in boiling water for 3 min. After cooling, a 100 μl volume of Tris-HCl buffer containing 250 μg of cAMP was added to each tube. The samples were centrifuged and cAMP in the supernatant was separated by ion-exchange (Dowex 50WX4, H^+ form) chromatography. Traces of contaminating nucleotides were precipitated twice with 0.2 ml each of ZnSO_4 (0.25M) and Ba(OH)_2 (0.25M). The radioactivity of the cAMP fraction was determined in an Intertechnique® liquid scintillation spectrometer using a Bray's solution. The recovery of ^{32}P -cAMP was determined by measuring the spectrophotometric absorption of cAMP at a wavelength of 259m μ . The results are expressed as pmol cAMP formed/mg protein/3 min, calculated from the specific activity of the substrate. All determinations were made in duplicate. When appropriate, before the reaction was started by the addition of ATP, aliquots of the homogenate, maintained at 4 C, were equilibrated for 20 min with the volatile anesthetics. Halothane or enflurane was vaporized from a Vernitrol® vaporizer, carried in 100 per cent oxygen, water-saturated and delivered to each test tube at a flow rate of 100 ml/min via polyethylene

tubing and a metal manifold. The flow of the anesthetic-oxygen mixture was maintained on the upper surface of the assay mixture and continued during the incubation. The concentration of anesthetic delivered was measured by means of a Narko-Test-M® analyzer. With each experiment a reagent blank was determined using denatured enzyme. Control samples (without anesthetics) were flushed with oxygen at the same flow rate.

Phosphodiesterase activity was measured by the method of Thompson and Appleman.¹⁷ In this procedure the substrate cAMP is hydrolyzed by phosphodiesterase in the presence of Mg^{++} to 5'-AMP, which is then quantitatively converted by a 5'-nucleotidase to adenosine. The tissue was homogenized in 50 mM Tris-HCl (pH 7.5) and the 500 $\times g$ supernatant was used as the enzyme source. The assay mixture (total volume, 400 μl) consisted of cAMP, in appropriate concentrations, 0.4 and 40 μM for low- and high-Km forms, respectively, labeled with about 200,000 cpm ^3H -cAMP (24 Ci/mM); MgCl_2 5 mM; mercaptoethanol, 3.75 mM; Tris-HCl, 50 mM, pH 7.5, and 50 or 100 μl of tissue homogenate (containing about 17–35 μg protein). The assay mixture, before addition of the homogenate, was equilibrated with the volatile anesthetics as described above, while control samples were flushed with oxygen for 20 min. The reaction was started by addition of the enzyme,

allowed to proceed for 5 min at 30 C, and stopped by immersion of the test tubes in a boiling-water bath. After cooling, the samples were incubated for 15 min at 30 C with an excess of 5'-nucleotidase from snake venom (*Crotalus atrox*, 80 μ g). 1-ml volume of a 25 per cent (v/v) slurry of an ion-exchange resin (Bio-Rad AG1-X2, chloride form) was then added and the samples thoroughly mixed with the resin, which separates adenosine from the nucleotides. After centrifugation, the radioactivity of the adenosine in the supernatant was determined in a liquid scintillation spectrometer. The 3 H-adenosine formed was corrected for recovery.¹⁸ For each experiment, reagent blanks were performed by replacing the enzyme in the assay mixture by the same volume of 50 mM Tris-HCl buffer or of denatured enzyme. The results are expressed as pmol cAMP hydrolyzed/mg protein/unit of time, calculated from the specific activity of the substrate. All determinations were made in quadruplicate.

Protein concentration was determined by the method of Lowry *et al.*¹⁹ All values are expressed as means \pm SEM. Significance of the results was evaluated by the student *t* test and a *P* value of <0.05 was regarded as significant.

Alpha 32 P-ATP was obtained from ICN and 3 H-cAMP, from New England Nuclear Corp; ATP, pyruvate kinase and phosphoenolpyruvate, from the Boehringer-Mannheim Corp; *Crotalus atrox* snake venom, from Sigma Chemical Co; cAMP and theophylline, from CalBioChem; halothane, from Ayerst Laboratories, Inc; enflurane, from Ohio Medical Products; haloperidol and droperidol, from McNeil Laboratories, Inc; dopamine hydrochloride, from Arnar Stone Laboratories.

Results

The response of the adenylate cyclase of the caudate nucleus to dopamine showed activities of a dopamine-sensitive cAMP-generating enzyme comparable to those reported by others.^{15,20} The adenylate cyclase was activated by dopamine in a dose-dependent manner, with a maximum increase to 61.4 ± 1.9 per cent above basal value (304.6 ± 8.2 pmol cAMP/mg protein/3 min, $n = 50$) obtained with dopamine, 100 μ M, and half-maximum activation with a concentration of 5 μ M. Haloperidol at a concentration that had no effect on the basal activity of the enzyme (0.5 μ M) significantly diminished the effect of dopamine (fig. 1). The inhibitory effect of haloperidol was overcome at higher concentrations of dopamine, which resulted in a shift of the dopamine dose-response curve to the right, indicating a competitive antagonism. The competitive nature of the halo-

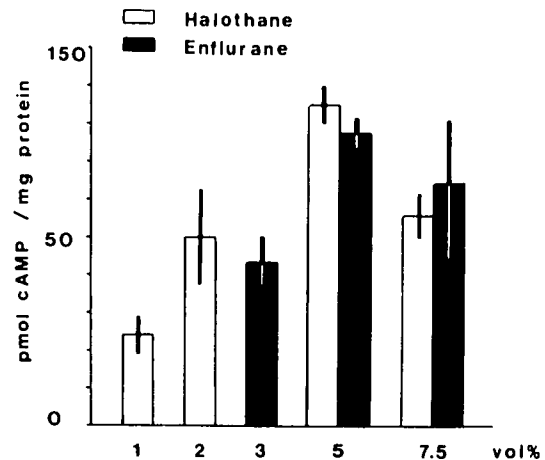


FIG. 2. Effects of halothane and enflurane on adenylate cyclase activity in rat caudate nucleus homogenates. Columns represent the mean net increases above basal activity (for halothane experiments, 316.5 ± 8.8 ; for enflurane experiments, 343.9 ± 10.4 pmol cAMP/mg protein/3 min) \pm SEM from four to eight experiments.

peridol and dopamine interaction is confirmed by the Lineweaver-Burk plot of the data (fig. 1, *inset*), where the two lines representing the enzymatic activity with dopamine alone and in the presence of haloperidol have a common intercept on the ordinate (V_{max}).

The activity of this dopamine-sensitive adenylate cyclase was increased by halothane and enflurane in proportion to their concentrations (fig. 2). Halothane, 2 and 5 vol per cent, significantly increased, 16 and 26 per cent, the adenylate cyclase activity above a mean basal value of 316.5 ± 8.8 pmol/mg protein/3 min. Enflurane, 3 and 5 vol per cent, produced significant increases of 11 and 22 per cent, respectively, above a mean basal value of 343.9 ± 10.4 pmol/mg protein/3 min. The activation of adenylate cyclase by both anesthetics appears to be maximal at about 5 vol per cent concentration, since at higher concentrations the effect on the enzyme was less.

Dopamine-induced adenylate cyclase activity, including that induced by the maximally-effective dopamine concentration, was further increased by both anesthetics. The effect of halothane, 2 and 5 vol per cent, on adenylate cyclase appeared to be additive or greater than additive with that of dopamine (fig. 3). Similarly, when enflurane, 3 and 5 vol per cent, and dopamine were combined, their effects on adenylate cyclase appeared to be additive (fig. 4).

Haloperidol, 0.05 μ M, without affecting basal adenylate cyclase activity, almost completely abolished the halothane-induced (5 vol per cent) increase in adenylate cyclase activity and diminished the effect of the anesthetic on the dopamine-stimulated

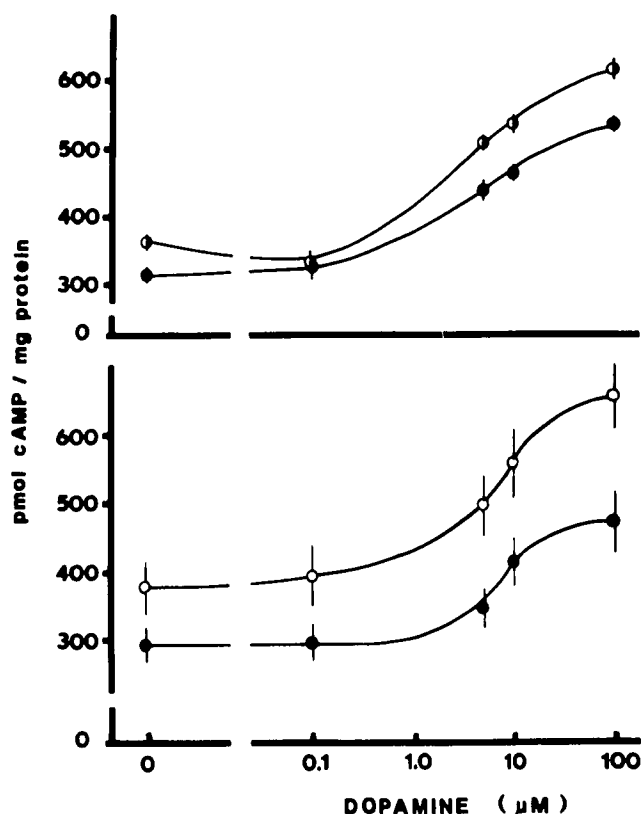


FIG. 3. Effect of halothane on the adenylate cyclase response to dopamine in the rat caudate nucleus homogenate. ●—●: dopamine alone; ◐—◐: dopamine plus halothane, 2 vol per cent; ○—○: dopamine plus halothane 5 vol per cent. Points represent mean values \pm SEM from four to six experiments.

adenylate cyclase (table 1). The effects of enflurane were similarly altered by haloperidol (data not shown). Droperidol, another butyrophenone derivative, also antagonized the effect of dopamine and the anesthetics on adenylate cyclase. A 10 μ M concentration of droperidol inhibited the dopamine (10 μ M)-induced adenylate cyclase activity significantly (table 2). Droperidol, at the same concentration, abolished the halothane-induced increase in adenylate cyclase activity and diminished the effect of the anesthetic on dopamine-stimulated adenylate cyclase activity (table 2).

Kinetic analysis of the rate of cAMP hydrolysis, measured at increasing substrate concentrations of 0.1 to 100 μ M, indicated that the nucleotide was hydrolyzed at two rates, each characterized by an apparent K_m . The two kinetic forms of the enzyme were distinguished by their affinities for cAMP, one with an apparent low K_m of 2–3 μ M, and the other with an apparent high K_m of 30–45 μ M. Halothane, 3 vol per cent, decreased significantly the activity of the low- K_m form, by 32 per cent, and

that of the high- K_m form by 14 per cent. Enflurane, 5 vol per cent, diminished significantly the activities of the low- and high- K_m forms, by 24 and 21 per cent, respectively (fig. 5).

Discussion

A dopamine-sensitive adenylate cyclase has been recently identified in several regions of the brain in a number of mammalian species, including man.^{15,21–24} It has been shown that dopamine receptors in the corpus striatum are coupled with adenylate cyclase, suggesting that the physiologic effects of dopamine are mediated through cAMP generated by adenylate cyclase in response to dopamine.^{15,25} Dopamine, released from the presynaptic terminals of the nigro-striatal pathway, binds to specific dopamine receptors, located on the membrane of the postsynaptic neuron, and activates adenylate cyclase. The stimulation of the dopamine-sensitive adenylate cyclase results in an increase of the cyclic nucleotide content in the postsynaptic neuron. The increase in cAMP is thought to result in the activation of another enzyme, protein kinase, responsible for phosphorylation of specific membrane proteins, which then presumably produce

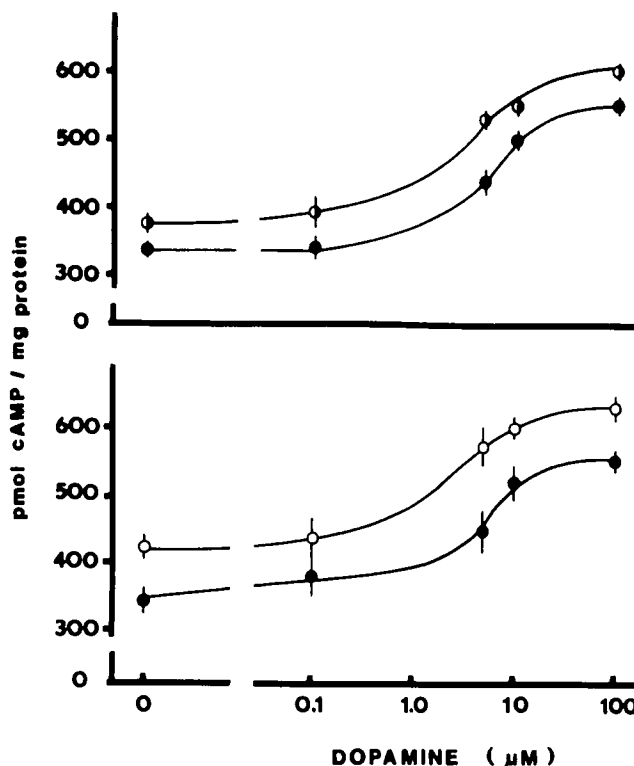


FIG. 4. Effect of enflurane on the adenylate cyclase response to dopamine in the rat caudate nucleus homogenate. ●—●: dopamine alone; ◐—◐: dopamine plus enflurane, 3 vol per cent; ○—○: dopamine plus enflurane, 5 vol per cent. Points represent mean values \pm SEM from six to eight experiments.

TABLE 1. Effects of Dopamine and Halothane on Adenylate Cyclase Activity in Caudate Nucleus Homogenates in the Absence (A) and Presence (B) of Haloperidol (0.05 μ M)

Drug	A		B		A minus B
	cAMP Formed (pmol/mg Protein/3 min)	Δ	cAMP Formed (pmol/mg Protein/3 min)	Δ	
None	372.1 \pm 11.5		362.8 \pm 12.2		9.4 \pm 6.8
Dopamine (10 μ M)	546.4 \pm 34.7	(174.3 \pm 18.2)*	489.3 \pm 30.7	(126.6 \pm 17.9)*	57.1 \pm 4.8*
Halothane (5 vol per cent)	463.7 \pm 22.0	(91.6 \pm 11.9)*	382.5 \pm 22.5	(19.7 \pm 13.5)	81.3 \pm 10.9*
Halothane + dopamine	759.0 \pm 27.3	(386.8 \pm 10.8)*	519.3 \pm 59.6	(156.5 \pm 52.6)*	239.7 \pm 37.8*

Values are means \pm SEM from three to six experiments.* $P < 0.05$.TABLE 2. Effects of Dopamine and Halothane on Adenylate Cyclase Activity in Caudate Nucleus Homogenates in the Absence (A) and Presence (B) of Droperidol (10 μ M)

Drug	A		B		A minus B
	cAMP Formed (pmol/mg Protein/3 min)	Δ	cAMP Formed (pmol/mg Protein/3 min)	Δ	
None	432.1 \pm 13.9		403.9 \pm 19.9		28.1 \pm 11.9
Dopamine (10 μ M)	627.5 \pm 55.9	(195.5 \pm 40.4)*	477.3 \pm 14.3	(73.3 \pm 20.6)*	150.2 \pm 41.8*
Halothane (5 vol per cent)	576.8 \pm 34.8	(144.8 \pm 26.2)*	416.4 \pm 25.0	(12.5 \pm 16.6)	160.4 \pm 14.6*
Halothane + dopamine	934.5 \pm 84.2	(502.4 \pm 76.2)*	512.8 \pm 20.5	(108.9 \pm 48.8)*	421.7 \pm 84.7*

Values are means \pm SEM from three to six experiments.* $P < 0.05$.

a decrease in membrane excitability.^{9,10} Thus, it has been postulated that the action of dopamine, mediated by the adenylate cyclase of the postsynaptic membrane, results in a decreased excitability of the postsynaptic membrane essential for the inhibitory function of the dopaminergic pathway in the caudate nucleus.²⁶ Since the purpose of the study was to determine the effects of halothane and enflurane on the cAMP-generating enzyme in a region of the brain where cAMP is known to have a role in synaptic transmission, a preparation containing the dopamine-sensitive adenylate cyclase of caudate nucleus was used in these experiments. The properties of the dopamine-sensitive adenylate cyclase (apparent affinity and maximum response, competitive inhibition of dopamine by haloperidol) found in our preparation were comparable to those reported by others.^{15,20,27}

Halothane and enflurane increased the activity of the dopamine-sensitive adenylate cyclase in a concentration-dependent fashion, reaching a maximum activity at a concentration of 5 vol per cent. The decrease in the activating effects of both anesthetics at higher concentrations is probably a manifestation of a toxic effect of the anesthetics on the enzyme. Activation of adenylate cyclase by halothane and enflurane has been shown previously in other tissues. The increases of cAMP content found in mouse neuroblastoma cells in culture⁵ and in the rat brain

exposed to halothane⁶ are also probably due to an activation of the cAMP-generating enzyme. On the other hand, Nahrwold *et al.*²⁸ have observed a decrease of cAMP content in the cerebral cortex and no alteration of the cyclic nucleotide content in the cerebellum and spinal cord in mice anesthetized with halothane, suggesting the possibility that halothane may have different effects on the cAMP system in different regions of the brain. Adenylate cyclase activity already stimulated by dopamine was also increased by both anesthetics. When dopamine was combined with halothane or with enflurane, their effects on adenylate cyclase appeared to be additive or greater than additive at all concentrations of dopamine, including the concentration producing the maximum effect on the enzyme. These results indicate that the anesthetics and dopamine activate the enzyme through actions exerted at different sites.

The mechanism by which halothane and enflurane activate adenylate cyclase is not clear at present. In a recent study attempts were made to locate the site of action of halothane on adenylate cyclase, and we have suggested that the catalytic unit of the enzyme is the most probable site.²⁹ However, since halothane has been shown to alter the response of adenylate cyclase to β -adrenergic agonists, prostaglandin, and glucagon, a halothane action exerted at the receptor sites is also possible. Halothane in-

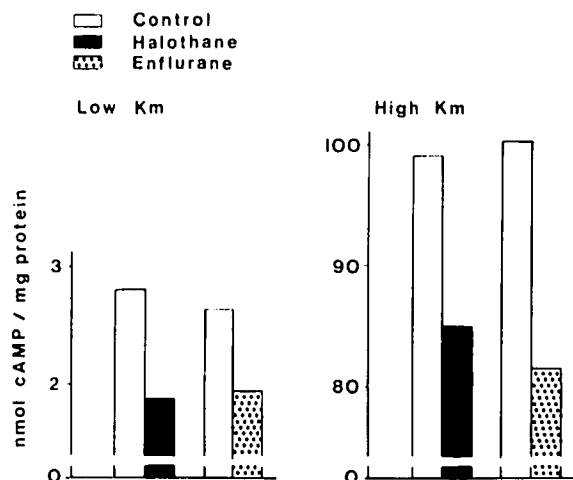


FIG. 5. Effects of halothane and enflurane on cAMP-phosphodiesterase activity in the rat caudate nucleus homogenate. The substrate concentration was $0.4 \mu\text{M}$ for the low Km activity and $40 \mu\text{M}$ for the high Km activity. Columns represent mean values from eight experiments.

creased the effects of isoproterenol and prostaglandin on uterine adenylate cyclase²⁷ and that of glucagon on liver adenylate cyclase,³ and, without affecting basal or sodium fluoride-induced activity, it diminished the β -adrenergic stimulatory effect on myocardial adenylate cyclase.^{‡‡}

The results of this study seem compatible with the suggestion that the effect of the anesthetics is exerted at the catalytic unit of the enzyme. We can speculate that halothane and enflurane produce an increased activity of the catalytic unit of adenylate cyclase, which could account for the higher rate of cAMP generation and for the greater response to dopamine when combined with halothane or enflurane. Such an effect on the catalytic unit of the enzyme could be due to conformational changes induced by the anesthetics, as suggested by Triner *et al.*²⁹

Haloperidol and droperidol were found to antagonize the effects of halothane and enflurane on adenylate cyclase and to diminish the effects of both anesthetics on the dopamine-activated adenylate cyclase. The nature of this interaction remains unclear. However, assuming that the anesthetics and dopamine act at different sites of the membrane-bound enzyme, the observation that haloperidol, at concentrations that did not completely block dopamine effect, abolished the maximum adenylate cyclase activity induced by halothane, suggests a non-competitive inhibition (in contrast to the competitive

interaction of haloperidol and dopamine). It would seem, then, that the antagonistic effect of the butyrophenone derivatives on halothane- and enflurane-induced activity of adenylate cyclase is not related to their property to inhibit the dopamine effect.

Halothane, 3 vol per cent, and enflurane, 5 vol per cent, decreased significantly both the high- and low-Km forms of cAMP-phosphodiesterase activity, as identified in our experiments. This inhibition of cAMP phosphodiesterase by halothane and enflurane would be expected to result in an accentuation of the effect of the anesthetics on the adenylate cyclase, leading to an increase in the intracellular cAMP content.

No attempt can be made to relate the described changes of enzymatic activity in caudate nucleus to the effects of halothane and enflurane in the central nervous system at present. However, our results show that halothane and enflurane activate dopamine-sensitive adenylate cyclase, modify the dopamine effect on the enzyme, and inhibit cAMP-phosphodiesterase. These effects may be considered post-synaptic events, because both enzymatic activities have been found mainly in the membranes of cells receiving the message carried by a transmitter. These membranes, in the case of caudate nucleus, also carry dopamine-specific receptors that have been shown to be intimately associated with dopamine-sensitive adenylate cyclase. Thus, the results indicate a possibility that the process of chemical transmission could be altered by the action of halothane or enflurane on the enzymes of the cAMP system in the membrane of post-synaptic neurons. Further investigation to determine whether these changes are reflected in a change in cellular cAMP content and whether they occur *in vivo* is warranted.

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