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The Effects of Halothane and Enflurane on Rat Brain Synaptosomal Sodium–Potassium-activated Adenosine Triphosphatase

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Synaptosomes, or nerve-ending particles, were isolated from the cerebral cortices of young rats by homogenization, differential centrifugation, and density-gradient centrifugation. The sodium–potassium-activated adenosine triphosphatase enzyme system [($Na^+ + K^+$)-ATPase] of these particles is believed to represent *in vitro* the sodium–potassium pump of the nerve terminal. Suspensions of synaptosomes were equilibrated with air containing various concentrations of halothane and enflurane, as determined by gas chromatography. Clinical concentrations of the anesthetics had no effect on ($Na^+ + K^+$)-ATPase activity. Fourteen per cent halothane and 14.8 per cent enflurane in the gas phase resulted in 12 and 10 per cent inhibition, respectively, of ($Na^+ + K^+$)-ATPase activity. These data confirm that interference with active cation transport by inhibition of neuronal ($Na^+ + K^+$)-ATPase is not related to the mechanism of halothane or enflurane anesthesia. (Key words: Anesthetics, volatile, halothane; Anesthetics, volatile, enflurane; Metabolism, enzymes, ATPase; Nerve, synaptosomal ATPase; Theories of anesthesia, ATPase.)

MANY STUDIES have explored the relationship between cellular cation transport and the anesthetic state. These have been reviewed recently.[1] Halsey *et al.* studied sodium transport in cold-stored erythrocytes.[2] They found that ether and chloroform had no effect on active sodium extrusion at concentrations that did not cause hemolysis. On the other hand, Andersen and Shim[3] found that several clinically useful anesthetics produced marked dose-related effects on the sodium current of isolated toad bladder. Because of these conflicting data, and in view of the fact that pharmacologic agents have been shown to affect sodium and potassium transport enzymes differently in various tissues of the same animal,[4] and in different structures of the same brain,[5] it is important to know the effects of anesthetics on active cation transport in neurons of the mammalian cerebral cortex.

Sodium–potassium-activated adenosine triphosphatase [($Na^+ + K^+$)-ATPase] is an enzyme system believed to represent *in vitro* the sodium–potassium pump of cell membranes.[6] This enzyme system actively transports potassium into the cell and actively extrudes sodium in order to maintain the intracellular sodium and potassium ion concentrations and the characteristic membrane potential. Ueda and Mietani[7] found that halothane and diethyl ether at clinical concentrations had no effect on brain microsomal ($Na^+ + K^+$)-ATPase. However, the microsomal fraction of brain homogenate contains membranes from glial cells in addition to nerve cell membranes. To assess the specific sensitivity to anesthetics of neuronal ($Na^+ + K^+$)-ATPase, we have isolated from rat brain cortex the nerve-ending (synaptosomal) fraction, which is relatively free of glial and blood vessel elements. We then studied the effects of enflurane and halothane on synaptosomal ($Na^+ + K^+$)-ATPase.

Methods and Materials

PREPARATION OF SYNAPTOSOMES

The preparation, similar to that reported by Autilio *et al.*,[8] is outlined in figure 1. Eight 18–21-day-old Osborn-Mendel rats (West Jersey Biological Supply Company) were

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used for each preparation. The rat was decapitated and its brain rapidly removed and placed on a chilled glass plate. The cerebellum and white matter were dissected off and the cortex was placed in ice-cold 0.32 M sucrose containing 1 mM Na_2EDTA. Subsequent procedures were performed at 0 C. The cerebral cortices of four rats at a time were homogenized in 10 volumes of the sucrose solution. Homogenization was performed in a glass–Teflon homogenizer (Arthur H. Thomas Co., catalogue no. 4288-C, size B), ground to 0.25 mm clearance, and motor driven at 900 rpm for ten up and down strokes. The homogenates from eight brains were placed in two centrifuge tubes and spun at 1,085 × *g* for ten minutes. The supernatants were drawn off with pipettes and centrifuged at 14,500 × *g* for 15 minutes to precipitate the crude mitochondrial fraction, which contains the synaptosomes also. The precipitate was resuspended with about 30 ml of the sucrose solution and centrifuged at 20,000 × *g* for 15 minutes. The precipitate was brought to a total volume of 12.5 ml with the sucrose solution and resuspended by hand in the glass–Teflon homogenizer. Six milliliters of the crude mitochondrial fraction were layered onto each of two previously prepared Ficoll density-gradient tubes (see below). These were centrifuged at 90,000 × g_{max} for 60 minutes in a Beckman L3-40 preparative ultracentrifuge equipped with an SW27 rotor. The material that layered in the two interfaces between 7.5 and 13 per cent Ficoll in each density-gradient tube was removed with a Pasteur pipette and placed in a centrifuge tube, to which was added about 25 ml of the sucrose solution. This was centrifuged at 27,000 × *g* for 20 minutes to precipitate the synaptosomes and separate them from the Ficoll. The synaptosomes were suspended in 2 ml of the sucrose solution and then lysed osmotically by slow addition of 18 ml 1 mM Na_2HPO_4 solution. The *p*H of the preparation was close to 7.40. Protein concentration was determined for each preparation by the method of Sutherland *et al.*,[9] using bovine serum albumin (Sigma Chemical Company) as standard. Typically, 6 g of cerebral cortex from the brains of eight rats yielded 23 mg of synaptosomal protein. The preparation was divided into 1.8-ml fractions and frozen at −20 C for future use.

Electron Microscopy

Unlysed synaptosomal fractions were processed for electron microscopy according to the method detailed by Autilio *et al.*[8] Briefly, pellets were fixed in 5 per cent glutaraldehyde, postfixed in 1 per cent osmium tetroxide, dehydrated in ethanol and embedded in Araldite. Contrast was enhanced by staining the sections with uranyl acetate and lead citrate.

Preparation of Density-gradient Tubes

Ficoll (Pharmacia Fine Chemicals, Inc.), a high-molecular-weight polysaccharide, was purified by dialysis against two changes of deionized water, one change of glass distilled water, and lyophilization. Decreasing concentrations of Ficoll in 0.32 M sucrose with 1 mM Na_2EDTA were layered in a 1 × 3-inch cellulose nitrate ultracentrifuge tube (Beckman catalogue no. 302236) as follows: 20 per cent, 4 ml; 13 per cent, 5 ml; 10 per cent, 5 ml; 7.5 per cent, 5 ml; 5 per cent, 5 ml. The tube was prepared at room temperature and cooled to 0 C for approximately one hour before use.

ATPase Reactions

The reactions took place in glass-stoppered tubes fitted with a gas inlet and shaken in a water bath at 37 C. The reaction medium of 1.5 ml contained approximately 0.09 mg of synaptosomal protein and the following: 50 mM Tris base, 2 mM $MgCl_2$, 150 mM NaCl, 10 mM KCl, 2 mM ATP, and 1 mM Na_2EDTA, adjusted with HCl to *p*H 7.40 at 37 C. Half of the reaction tubes contained also 1 mM ouabain (Sigma Chemical Co.), which inhibits completely the $(Na^+ + K^+)$-ATPase.[10] Both Tris–ATP and Na_2ATP (Sigma Chemical Co.) were used, with identical results. The reactions were run in triplicate. To test the effect of low potassium concentration, three experiments were done with 3 mM, instead of 10 mM, KCl. $(Na^+ + K^+)$-ATPase activity was computed by subtracting the ATP hydrolyzing activity in the presence of ouabain from that in the absence of ouabain. The ATP hydrolyzing activity that is not inhibited by ouabain is called Mg^{++}-ATPase. Therefore, $(Na^+ + K^+)$-ATPase = total ATPase − Mg^{++}-ATPase. The activity is expressed as micromoles of phos-

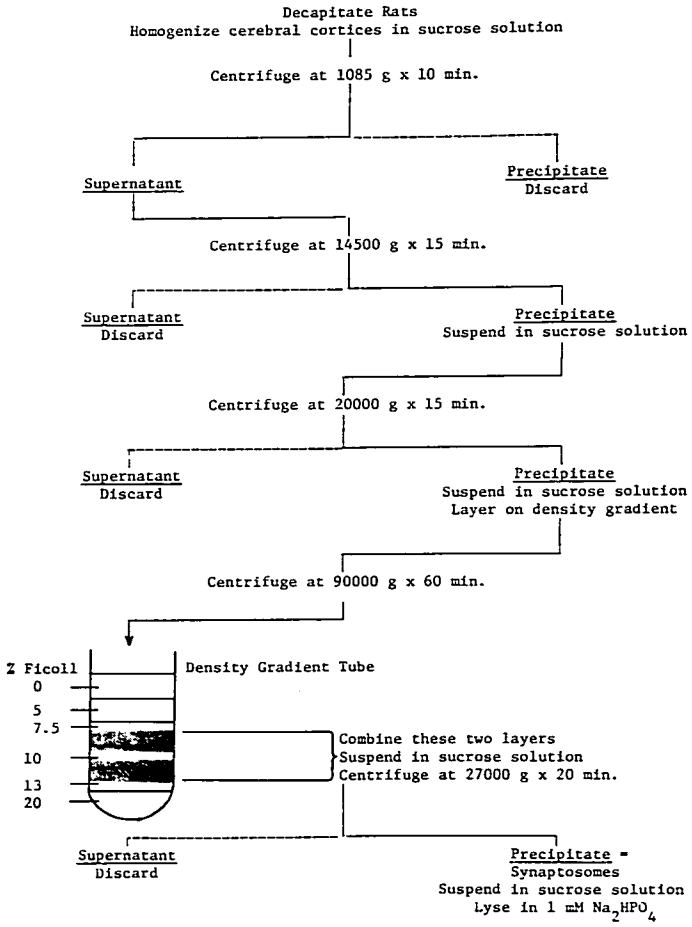

FIG. 1. Preparation of lysed synaptosomes. All procedures were performed at 0–4 C. The sucrose solution contained 0.32 M sucrose and 1 mM Na_2EDTA. The suspension of lysed synaptosomes was divided into 1.8-ml amounts and frozen for later use. See text for details.

phate released per milligram of synaptosomal protein per hour (μ moles phosphate·mg protein^{-1}·hr^{-1}).

ATP-free reaction mixture was equilibrated with humidified breathing-quality compressed air or with humidified anesthetic in air as delivered by a Dräger "Vapor" vaporizer. The tubes were equilibrated with the gas mixture at 37 C in a shaking water bath for 15 minutes. Preliminary tests showed that

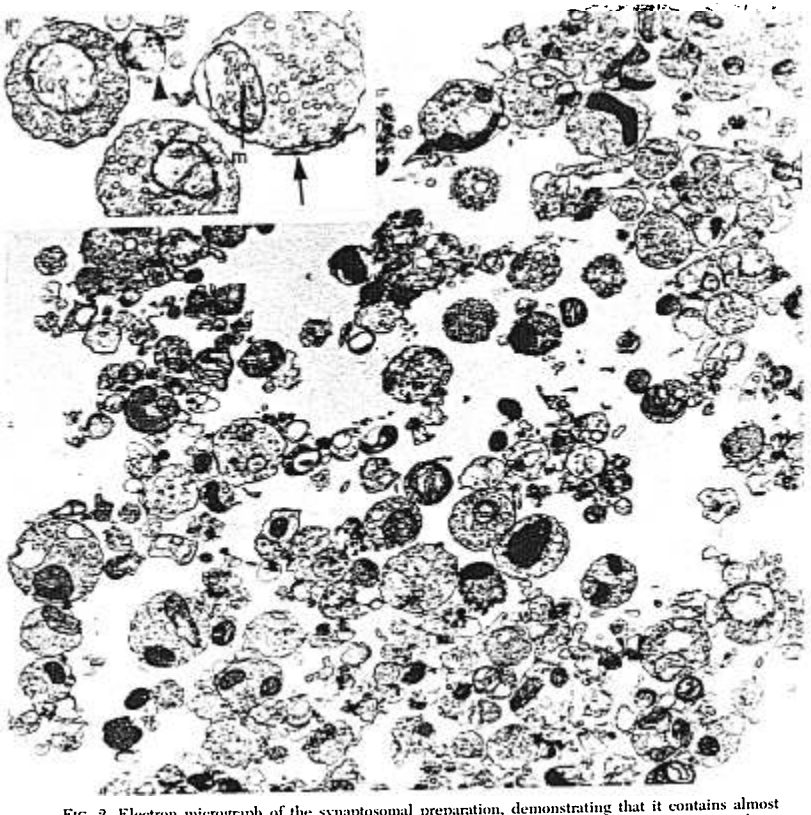

FIG. 2. Electron micrograph of the synaptosomal preparation, demonstrating that it contains almost exclusively membrane-bound rounded processes, most of which can be identified as presynaptic endings because of the presence of synaptic vesicles (×16,400, reduced from ×18,000.) *Inset:* An intrasynaptosomal mitochondrion (m) in a synaptosome that also contains many synaptic vesicles. Entire postsynaptic elements *(arrowhead)* or a portion of the postsynaptic membranes *(arrow)* were attached to some of the presynaptic endings (×42,000, reduced from ×46,000).

equilibration of the anesthetic with the medium occurred within 10 minutes in this system. The reaction was started by the addition of ATP and allowed to progress for 15 minutes, during which time the air or anesthetic in air was delivered continuously at a rate of 50 ml/min. The concentration of anesthetic coming out of the reaction tubes was determined for each experiment by gas chromatography. The reaction was stopped by addition of 0.5 ml 1.2 M perchloric acid containing 8 per cent silicotungstic acid. The tube was placed in an ice bath and analyzed immediately for inorganic phosphate by the method of Post and Sen.[11] This method utilizes rapid extraction of the phos-

TABLE 1. Synaptosomal ATPase Activities in Control Tubes and Tubes Equilibrated with Various Concentrations of Enflurane and Halothane

				Mg^{++} – ATPase‡				($Na^+ + K^+$) – ATPase‡			
		Concentration†		Control		Experimental		Control		Experimental	
	n*	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE
Enflurane§	11	0.3	0.01	6.08	0.33	5.65**	0.36	15.58	1.16	15.75	1.27
	7	0.7	0.03	6.09	0.41	5.79	0.45	10.10	0.61	9.82	0.61
	11	1.2	0.05	6.34	0.25	6.25	0.22	10.89	0.58	11.24	0.58
	6	2.4	0.15	6.72	0.45	6.52	0.43	13.93	2.22	14.16	2.02
	6	5.7	0.17	5.86	0.31	5.27**	0.29	13.17	1.17	13.76	1.02
	3	14.8	0.25	7.35	0.55	5.48**	0.14	17.78	0.48	15.99**	0.43
Halothane§	3	0.5	0.04	8.27	0.47	8.11	0.56	20.53	0.96	19.85	0.69
	3	1.2	0.07	8.31	0.32	7.99	0.43	21.05	0.73	20.71	0.93
	3	2.1	0.34	8.12	0.18	7.44**	0.21	20.76	0.48	20.79	0.18
	3	4.5	0.52	8.43	0.13	7.31**	0.33	21.97	0.65	21.68	0.24
	2	14.0	0.00	7.96	0.07	5.34**	0.02	19.16	0.46	16.86**	0.72
Halothane¶	3	4.3	0.52	8.21	0.46	6.60**	0.25	18.07	1.4	17.41	1.6

* Number of experiments; each experiment in triplicate.
† Concentration expressed as percentage in the vapor phase.
‡ Activity expressed as μ moles phosphate·mg protein^{-1}·hr^{-1}.
§ With 10 mM K^+ in the reaction medium.
¶ With 3 mM K^+ in the reaction medium.
** Significantly different from control, $P < 0.05$, t test for paired data.

phomolybdate complex into butyl acetate, thereby avoiding errors resulting from acid hydrolysis of ATP.

We analyzed the data by paired t tests between the mean ATPase activity of the control (air-treated) and experimental (anesthetic-treated) tubes of each experiment.

Results

Electron Microscopy

The morphology of the synaptosomal preparation was similar to that described by previous investigators.[8,12,13] The fraction consisted almost entirely of membrane-bound rounded structures averaging 0.8 μm in diameter. The great majority of these structures could be identified as presynaptic endings because of the presence of synaptic vesicles (fig. 2). Intrasynaptosomal mitochondria were evident in most of the particles. Complete postsynaptic elements or a portion of the postsynaptic membrane were seen attached to some of the presynaptic endings (fig. 2, *inset*).

ATPase Reactions

The ATPase activities are shown in table 1. The control ($Na^+ + K^+$)-ATPase activity is somewhat higher than that reported previously by us† and higher than that reported for a similar preparation by Abdel-Latif *et al.*[10] This may have resulted from the inclusion of disodium EDTA in our current preparation. EDTA might act by chelating calcium, which is a known inhibitor of this enzyme system.

The experimental ($Na^+ + K^+$)-ATPase activity was different from control activity at the .05 level only when the preparation was treated with 14 per cent halothane or 14.8 per cent enflurane. The Mg^{++}-ATPase activity (ouabain-insensitive activity) was inhibited by enflurane and halothane at lower concentrations than was the ($Na^+ + K^+$)-ATPase. In figure 3, the experimental ($Na^+ + K^+$)-ATPase activity is expressed as a percentage of the control activity and plotted as a function of anesthetic concentration.

† Levitt J: The effects of halothane on synaptosomal adenosine triphosphatase. Abstracts of Scientific Papers, 1972 Annual Meeting American Society of Anesthesiologists, pp 131–132.

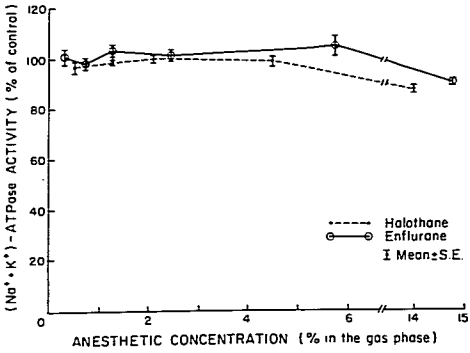

FIG. 3. Effects of enflurane and halothane on rat brain synaptosomal ($Na^+ + K^+$)-ATPase activity. Inhibition of enzymic activity occurs only at very high anesthetic concentrations.

In the three experiments performed at 3 mM potassium, a mean halothane concentration of 4.3 per cent did not cause significant inhibition of ($Na^+ + K^+$)-ATPase.

The effects of enflurane reported here are the results of 44 separate experiments performed on 11 synaptosome preparations. These were all experiments in which the control ($Na^+ + K^+$)-ATPase activity was greater than 8 μ moles $PO_4 \cdot mg\ protein^{-1} \cdot hr^{-1}$ and in which the difference between the highest and lowest triplicate reaction tubes did not exceed 25 per cent of the mean value.

The data on the effects of halothane are from 19 consecutive experiments performed on two synaptosome preparations. The halothane experiments were performed after completion of the enflurane experiments in order to confirm our previously reported results.‡ Because our recent preparations have had higher and more consistent activity than the previous preparations, we have not included the previous results in this report. Both the previous and the current work demonstrated the same lack of effect of halothane.

Discussion

The isolation of nerve ending particles (synaptosomes) was first described by Gray and Whittaker in 1962.[14] Since that time a great number of neurochemical and pharmacologic studies have been performed with this preparation. A synaptosome preparation is similar to a suspension of living nerve cells in a variety of its reactions.[15] Although there is always difficulty in relating what happens *in vivo* to the behavior of subcellular particles *in vitro*, the relative ease of administering drugs and measuring the products of reactions support the use of such particles. Synaptosomes have been shown to perform many of the reactions associated with neural tissue. These include protein synthesis,[8,12] acetylcholine synthesis and release,[16] and uptake of norepinephrine.[17] Depolarization of synaptosomes by electrical stimulation or by a rise in the potassium concentration of the bathing medium results in increased oxygen consumption[18] and release of acetylcholine[19] and neuroactive amino acids.[20] Moreover, the morphology of the particles did not change during the period of electrical stimulation.[21] In view of these properties, and the demonstration by Larabee and Posternak[22] of the greater sensitivity to anesthetics of synapses compared with nerve axons, synaptosomes should provide useful information in anesthetic research.

Halothane and enflurane were chosen for this study because of their diverse electroencephalographic effects in man. Although the effects are complex, halothane is not associated with seizure-like EEG patterns seen with enflurane anesthesia. Since intracerebral application of ouabain is known to cause central nervous system depression

followed by seizures in rodents,[23] we were interested in finding out whether enflurane had a ouabain-like effect on synaptosomal ($Na^+ + K^+$)-ATPase. We conclude that the electroencephalographic effects of enflurane are not caused by inhibition of this enzyme system.

Several attempts have been made to correlate the actions of general depressants with inhibition of sodium–potassium-activated ATPase. Using a beef-brain microsomal ATPase preparation, Israel and Salazar[24] studied the effects of several general depressants on ($Na^+ + K^+$)-ATPase. For ethyl ether, chloroform, and ethanol they found the ratios concentration for narcosis to concentration for 50 per cent enzymatic inhibition to be .20, .43, and .88, respectively. Because these ratios fell within a relatively narrow range, the authors suggested that inhibition of ($Na^+ + K^+$)-ATPase might be either a mechanism of anesthesia or a result of the interaction of anesthetic molecules with the nerve-cell membrane. Sun and Samorajski[25] studied the effects of ethanol on synaptosomal ATPase and acetylcholine esterase. Although much of their study involved drug concentrations considerably above the clinical range, they found the ($Na^+ + K^+$)-ATPase to be much more sensitive to ethanol than was the acetylcholine esterase. They also studied the effect of a series of aliphatic alcohols on synaptosomal ($Na^+ + K^+$)-ATPase activity. Of great interest was the fact that the inhibitory effect of these compounds was proportionate to their oil–water partition coefficients. They suggested "that the molecular properties which determine anesthetic potency might also determine the degree of inhibition of membrane-bound ATPase."

In the present study, we utilized the synaptosomal preparation to obtain ($Na^+ + K^+$)-ATPase from cortical nerve endings. Because of the limited permeability of the intact synaptosomal membrane to ATP, it was necessary to lyse the synaptosomes for maximal ($Na^+ + K^+$)-ATPase activity.[10] Our preparation, therefore, consisted of synaptosomal membranes and intra-synaptosomal particles, including synaptic vesicles and mitochondria of synaptosomal origin. Hosie,[26] using a similar preparation, studied the distribution of ($Na^+ + K^+$)-ATPase activity in subfractions derived from osmotically-disrupted synaptosomes. She found that 92 per cent of the recovered activity was associated with synaptosomal membranes, and only 4 per cent with mitochondria. Although our preparation was a mixture of nerve-terminal organelles, the enzyme system we studied was probably located in the synaptosomal membrane. Recently, preparations that consist of highly purified synaptosomal membranes with very high specific activity with respect to ($Na^+ + K^+$)-ATPase have been described.[27] It is not known whether these preparations would react differently to anesthetics than did the lysed synaptosomes used in this study. We doubt that the results would differ materially.

We have demonstrated that concentrations of as much as 5.7 per cent enflurane and 4.5 per cent halothane cause no significant change in synaptosomal ($Na^+ + K^+$)-ATPase activity. The anesthetics were delivered in the gas phase and equilibrated with the synaptosome preparation. These concentrations would be expected to yield tissue levels higher than necessary for clinical anesthesia. Fifteen per cent enflurane in the gas phase and 14 per cent halothane resulted in 10 and 12 per cent inhibition, respectively. Although these changes are significant statistically, their small magnitude in the face of high concentrations of anesthetic attest to the real insensitivity of this enzyme system to clinical anesthetics. Therefore, we agree with the recent conclusions of Seeman,[28] Ueda and Kamaya,[29] and Andersen and Amaranath[1] that inhibition of ($Na^+ + K^+$)-ATPase is probably not an important action of clinical anesthetics.

In these experiments the Mg^{++}-ATPase was more sensitive to enflurane and halothane than was the ($Na^+ + K^+$)-ATPase. The implications of this observation are not clear, because no transport function or other physiologic role has been attributed to Mg^{++}-ATPase.

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