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Effects of ketamine anesthesia on rat-brain membranes: fluidity changes and kinetics of acetylcholinesterase

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This investigation shows that the effects of general anesthetics previously observed in vitro on membrane fluidity and on enzymic activities and occurring at concentrations calculated to be clinically relevant can be reproduced in vivo in anesthetized animals. Anesthesia with 2-chlorophenyl-2-methylaminocyclohexanone (ketamine) induces a more fluid state of rat-brain synaptic and mitochondrial membranes, as shown by the rotational correlation times of the spin labels 16-doxylstearate and 5-doxylstearate. Changes in acetylcholinesterase activity, with a decrease in V_{max} and no change in the K_m for acetylcholine, closely follow the fluidity increase.

Introduction

General anesthetics include a variety of simple non-reactive substances which fit a strong correlation between anesthetic potency and lipid solubility; according to the majority of current theories, they act at a hydrophobic site in neural membranes by a physical mechanism [1,2]. It has generally been assumed that within the central nervous system, general anesthetics act by depressing synapses through interference with the process of chemical transmission [3]. Besides their site of action, also their mechanism of action is still uncertain at the molecular level and a variety of theories have been put forward. The majority of investigators believe that narcotics act by dissolving in a hydrophobic phase of a cellular membrane [4]; this phase may be represented either by lipids [5] or by the inner hydrophobic core of proteins [6]. It has also been suggested that anesthetics may act primarily by perturbing hydrogen bonds at the lipid-protein/water interface [7]. The original idea that general anesthetics act by increasing the fluidity of the lipid bilayer has been dismissed by several authors on the basis that not all agents enhancing fluidity have an anesthetic action, and above all that the fluidization observed by the increased mobility of spin labels in model lipid membranes occurs at anesthetic concentrations much above their estimated clinical concentrations [2,6]. Studies in our laboratory on membrane perturbation by general anesthetics, both in mitochondria and in synaptic membranes and on bilayers of lipid extracted therefrom, have shown that, although these molecules do not appreciably enhance the fluidity of the protein-free bilayers, they have a strong fluidizing effect on natural membranes containing

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their native protein complement [8–13]. Such an effect has been interpreted as a release, induced by anesthetics, of the immobilization exerted by integral proteins on the surrounding lipids. This effect is exerted at clinically relevant concentrations and appears related to a perturbation of lipid-protein interactions [14]. Other studies from our laboratory have demonstrated that anesthetics affect enzyme activities such as synaptosomal $(Na^+ + K^+)$-ATPase [15], erythrocyte acetylcholinesterase [16], or mitochondrial H^+-ATPase [17]; the inhibition kinetics may be in accordance with a conformational change of the enzymic proteins observed by us in synaptosomal membranes [18]. According to a working hypothesis elaborated by Lenaz et al. [14,19], the primary site of action of anesthetics is represented by lipid-protein interactions; the consequent conformational changes, induced by the alteration of the protein microenvironment, would give rise to functional changes in membrane-bound enzymes or carriers. The clinical significance of the in vitro studies may be limited by the difficulties in extrapolating the results to the anesthetized animal. For such reason we have undertaken a study in which we administered ketamine (a clinically used injection anesthetic) to rats, following changes occurring in brain synaptic and mitochondrial membranes at different times from the establishement of anesthesia. The parameters investigated were membrane fluidity (by use of lipid soluble spin labels) and synaptosomal acetylcholinesterase activity.

Materials and Methods

Ketamine (2-(*o*-chlorophenyl)-2-methylaminocyclohexanone) was purchased from Parke-Davis with the trade name of Ketalar. The trade material contains 1% benzethonium chloride as stabilizer. The spin labels 5-doxylstearate (5-NS) and 16-doxylstearate (16-NS) were obtained from Syva Co., Palo Alto, CA.

Preparative procedures

Male albino rats of the Wistar strain, weighing about 250–300 g were used in numbers of 16–18 for each experiment; half of each group were anesthetized with ketamine, and the others were used as controls.

The animals from the anesthetized group were administered intraperitoneally 50 mg of Ketalar per kg of body weight, and decapitated after either 30 min (under deep anesthesia), 1 h (recovering from anesthesia), or 24 and 48 h later. Correspondingly, the animals used as controls were administered benzethonium chloride at the same concentration contained in the ketalar experiments and decapitated as above. Synaptosomes and mitochondria were prepared from the brains (pools of two or three brains for each group) using the procedure of Whittaker et al. [20].

Protein was determined by the method of Lowry et al. [21] and lipid phosphorus by the method of Marinetti [22].

Spin labelling and ESR determinations

The lipid-soluble spin labels used were 5 or 16 (*N*-oxyl-4′,4′-dimethyloxazolidine) derivatives of stearic acid, denominated 5-NS and 16-NS, respectively. The labels were dissolved in absolute ethanol at a concentration of 10 mM and kept at $-20\,°C$. The labels were added to the membranes by vortexing, and incubated for 12 h, taking care that the label-to-phospholipid molar ratios were about 1 : 180 and that incorporation of the label was complete (no free label signal detectable). ESR spectra were recorded in Varian E-4 Spectrometer using a microwave frequency of 9.52 GHz; other parameters were: receiver gain, $4 \cdot 10^3$; modulation amplitude, 2 G; time constant, 0.5 s.

Changes in the mobility of spin labels are reflected by changes in the linewidths and peak distances in the ESR spectra [23]. In general, the ordering of the molecules is related to the position and the separation (hyperfine splitting) of the resonance lines, while the dynamic properties are reflected in their width. The ESR spectra of 5-NS exhibit clear probe ordering, proportional to the separation of two hyperfine extrema ($2T_{\parallel}$).

From the hyperfine values, order parameters S_n, corrected for polarity, were calculated. The ESR spectra of 16-NS consisted of three lines only, indicating that the segmental motion can be considered effectively isotropic; we have therefore used as an empirical measure of lipid fluidity a pseudoisotropic rotational correlation time, calculated according to the Kivelson formulation [24].

Although the parameters used to study 5-NS

and 16-NS mobility cannot be directly comparable, their changes depend on modification of the physical state of the environment in which they are localized.

Acetylcholinesterase activity

Acetylcholinesterase activity was assayed in isolated synaptosomal membranes by the method of Ellman et al. [25]; activity was expressed as μmol acetylcholine hydrolyzed per min per mg protein.

Results

Spin-label studies

The effects of ketamine addition in vitro on the physical state of synaptic membranes from pig brain [13] and on mitochondrial membranes from bovine heart [12] have been reported in previous investigations, and consist in both cases in a fluidization that is much more pronounced in the pseudoisotropic motion of 16-NS than in order parameter of 5-NS.

The results have been confirmed in the synaptosomal and mitochondrial membranes from the brains of the control rats in this experiment. As shown in Fig. 1, addition of progressive amounts of ketamine to synaptosomal membranes decreases the order parameter, S_n, of 5-NS, indicating disordering (Fig. 1A) and lowers the rotational correlation time τ_c of 16-NS, indicating an increase in the molecular motion of the probe (Fig. 1B). The fluidizing effects appear at ketamine concentration as low as 0.25 mM. Similar results have been observed in mitochondria (not shown). The plateau in the rotational correlation time was also found in previous studies (cf. Refs 10, 12, 13)

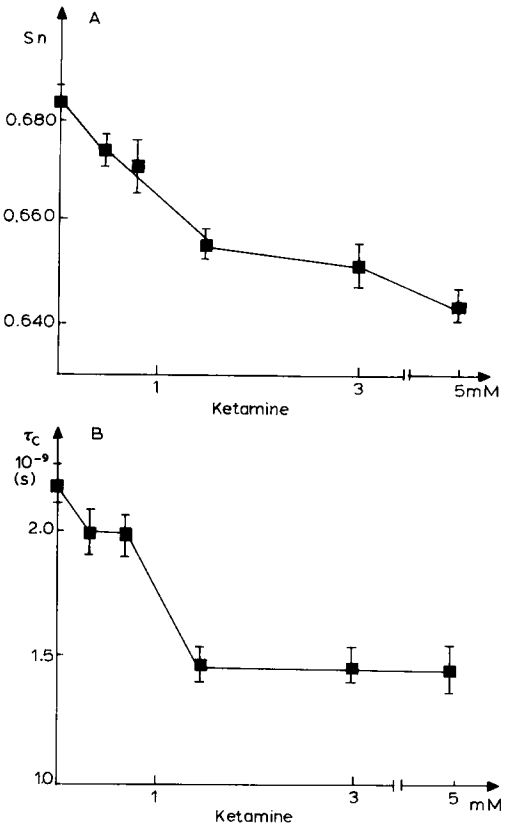

Fig. 1. Effect of ketamine on the fluidity of isolated rat brain synaptic membranes. (A) Order parameter of 5-doxylstearate. (B) Rotational correlation time of 16-doxylstearate.

and ascribed to the release of the immobilization induced by proteins on the surrounding lipids by low anesthetic concentration, followed by a much slighter effect on fluid bilayer lipids.

Table I reports the order parameters of 5-NS in synaptosomes and in mitochondria after in vivo

TABLE I

EFFECT OF KETAMINE ANESTHESIA ON THE ORDER PARAMETER OF 5-DOXYLSTEARATE IN RAT-BRAIN MEMBRANE FRACTIONS

Time after injections (h)	Synaptic membranes		Mitochondria	
	control	anesthetized	control	anesthetized
0.5	0.688 ± 0.0015	0.677 ± 0.002 *	0.695 ± 0.002	0.689 ± 0.002
1	0.688 ± 0.001	0.683 ± 0.003	–	–
24	0.687 ± 0.002	0.687 ± 0.0019	0.697 ± 0.003	0.689 ± 0.0025
48	0.689 ± 0.001	0.686 ± 0.0014	0.697 ± 0.003	0.689 ± 0.003

* Significant to $P < 0.005$ by Student's t-test.

TABLE II

EFFECT OF KETAMINE ANESTHESIA ON THE ROTATIONAL CORRELATION TIME ($\tau_c \cdot 10^{-9}$ S) OF 16-DOXYLSTEARATE IN RAT-BRAIN MEMBRANE FRACTION

Time after injections (h)	Synaptic membranes		Mitochondria	
	control	anesthetized	control	anesthetized
0.5	1.85 ± 0.033	1.51 ± 0.0033 *	1.98 ± 0.050	1.86 ± 0.060
1	1.85 ± 0.025	1.63 ± 0.030	–	–
24	1.82 ± 0.030	1.75 ± 0.080	1.99 ± 0.040	1.97 ± 0.050
48	1.82 ± 0.040	1.81 ± 0.020	1.99 ± 0.040	1.99 ± 0.050

* Significant to $P < 0.001$ by Student's t-test.

anesthesia. It appears that in synaptosomes from the treated animals (30 min after ketamine injection) is the order parameter significantly lower than in the control; in addition the order parameters approach normal values after recovery from the anesthesia (24 and 48 h after ketamine injection). At variance with synaptosomes, in mitochondria, the change is rather small and was shown to be not significant; no recovery is apparent after the rats have recovered from anesthesia.

The effects of ketamine anesthesia on the environment of 16-NS, which probes the depth of the membrane are illustrated in Table II. Both synaptosomes and mitochondria obtained from anesthetized animals appear to be more fluid with respect to their controls, as shown by the lower rotational correlation times.

After statistical examination, only in synaptosomes, however, the differences are significant to $P < 0.001$; even 1 h after the anesthesia, the patterns in the control and treated animals are no longer significantly different.

Acetylcholinesterase activity

Fig. 2 shows the effect of ketamine added in vitro to the synaptosomal membrane. The double-reciprocal plots indicate a non-competitive or mixed type of inhibition similar to that previously observed by us on erythrocyte ghost acetylcholinesterase [16]. In the in vivo experiment we have observed that during anesthesia the enzyme activity appears significantly lower than in the control (Table III); the inhibition appears reversed after recovery from anesthesia. The kinetics of the inhibition parallel those observed in vitro

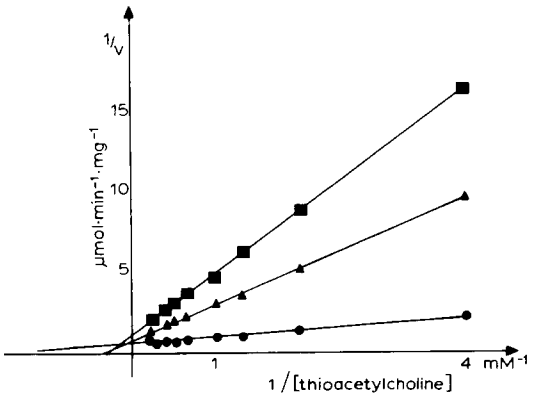

Fig. 2. Effect of ketamine on acetylcholinesterase activity of isolated rat brain synaptic membranes. ●, Control; ▲, 0.72 mM; ■, 1.45 mM.

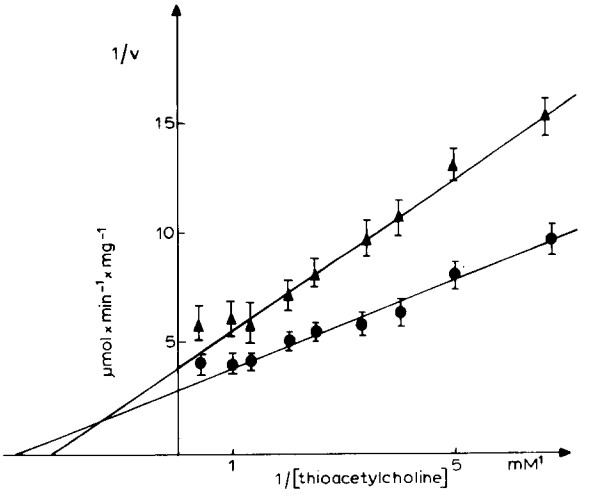

Fig. 3. Effect of ketamine anesthesia (30 min after injection) on acetylcholinesterase activity. ●, Control; 30 min anesthesia.

TABLE III

EFFECT OF ANESTHESIA ON ACETYLCHOLINESTERASE ACTIVITY

Acetylthiocholine was 0.5 mM.

	Acetylcholinesterase activity (μmol·min^{-1}·mg^{-1})			
	30 min	1 h	24 h	48 h
Control	0.171 ± 0.010	0.182 ± 0.011	0.176 ± 0.022	0.169 ± 0.023
Treated	0.125 ± 0.026 *	0.159 ± 0.012	0.167 ± 0.024	0.155 ± 0.015

* Significant to $P < 0.001$ by Student's t-test.

(Fig. 3); in other words, there is a decrease in the V_{max} in the anesthetized animals, whereas the K_m appears relatively unchanged.

Discussion

This investigation shows that the effects of several anesthetics previously observed in vitro on membrane fluidity and on enzymic activities and shown to occur at a concentration calculated to be clinically relevant [8–19] can be reproduced in vivo in anesthetized animals. We have employed for this study an injection anesthetic, ketamine, in order to avoid as far as possible the complications arising from the relatively low partition or the volatile character of many of the anesthetics used in previous investigations. Ketamine is an amphipathic molecule that, however, behaves as the other anesthetics studied as far as both membrane fluidization and enzymic activities are concerned [13]. This anesthetic appears to bind with quite high affinity to the membranes, since its effects are evident even after several manipulations and washings used for preparation of the memrbanes. The coincidence of the effects in vitro and in vivo suggest that they are exerted directly by the anesthetic by binding to the membrane, and not through secondary modifications (e.g., metabolic changes) and that they are occurring during clinical anesthesia. It is not possible to discriminate, however, whether the effects shown are related directly to the physiological state of anesthesia or are only concomitant features accompanying an as yet unknown primary change responsible for the anesthetic action.

The effects we have found, particularly in vivo, on membrane fluidity are more marked in the synaptosomal membranes than in mitochondria; this may be due either to the preferential localization of the drug in the synaptosomal (plasma) membranes than in the (intracellular) mitochondrial fraction; alternatively, this difference could reflect a preferential effect due, for example, to diversities in membrane composition.

Assuming a homogeneous distribution of the anesthetic in the total lipids of the animal and no loss, its concentration would correspond to about 2 mmol ketamine per mol lipid. This high apparent efficiency [4] may be due to a preferential uptake of the anesthetic by brain synaptosomal membranes.

The fluidization of synaptic membranes is well documented by the decrease of the pseudoisotropic rotational correlation time of the 'deep' probe 16-NS, as already observed in vitro. The membrane remains more fluid in the anesthetized rats even after the animals have awakened (although the changes may not be significant at 24 h or more), indicating that the anesthetic survives in the animals before it is removed or metabolized.

The fact that the membrane changes out-last the anesthetic state is of some interest; the most likely interpretation is the existence of a threshold of the fluidity change which is associated with anesthesia; alternatively, the changes are not of relevance to the molecular mechanism of anesthesia. In any case, the long duration of some effects of anesthetic addition is of considerable clinical interest.

The changes in acetylcholinesterase activity closely follow the fluidity increse, even if this does not establish a cause-to-effect relationship. Either

both modifications could derive independently from the presence of the anesthetic, or the enzymic changes could reflect the fluidity changes. The kinetics of the inhibition offer no clue, although they may reflect a structural change of the enzyme. Although the present experiments cannot discriminate among the two possibilities, we consider the latter hypothesis more likely by analogy with the relations existing between membrane fluidity and the kinetics of membrane-bound enzymes [26].

Although acetylcholinesterase is partly in the asymmetric high-salt-soluble form near the postsynaptic membrane [27], a portion of the enzyme may be present in detergent-soluble form [28] and sense the lipid environment of the membrane. The enzyme we have studied may be largely this membrane-bound form, since it has been studied in the synaptosomal fraction which should be devoid of the majority of the soluble form; therefore its sensitivity to anesthetics may reflect its lipid-dependence.

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