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INHIBITION OF MAMMALIAN BRAIN ACETYLCHOLINESTERASE BY KETAMINE*

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Abstract—Ketamine caused a reversible inhibition of both membrane-bound and purified forms of acetylcholinesterase (AChE; EC 3.1.1.7.) prepared from beef brain caudate nucleus tissue. Apparent K_i values ($\times 10^{-4}$ M) ranged between 4·9 and 6·9 for the different enzyme forms. Inhibition was of the mixed kinetic type, which suggests interactions of the drug with both active site(s) and other anionic sites on the enzyme. Rat brain AChE was inhibited by ketamine *in vitro* at concentrations commensurate with brain levels of the drug determined after i.v. administration to rats. Gas chromatographic analyses demonstrated a 26 per cent increase in rat brain acetylcholine (ACh) 30 sec after the administration of ketamine (20 mg/kg, i.v.). ACh accumulation via inhibition of AChE may underlie certain pharmacological effects of ketamine which resemble cholinergic stimulation.

WHEN USED clinically as an anesthetic agent, ketamine causes certain pharmacological effects which resemble cholinergic stimulation. These include lacrimation and salivation,[1,2] increased skeletal muscle tone[2,3] and increased uterine tone.[4] The sialogogic actions, as well as certain central manifestations of disorientation on emergence from anesthesia, are antagonized by anticholinergic drugs.[5–7] Since the amine group of ketamine is partly charged at physiological pH, it is possible that the drug could cause acetycholine (ACh) accumulation via inhibition of acetylcholinesterase (AChE; EC 3.1.1.7.) In this study, the effects of ketamine on membrane-bound and purified forms of mammalian brain AChE were examined. Inhibition of the enzyme was related to the levels of ketamine achieved in brain tissue after administration of the drug *in vivo*, and to observed changes in the levels of ACh in the brain.

MATERIALS AND METHODS

Brain acetylcholinesterase preparations. Purified forms of AChE were prepared from bovine brain caudate nucleus tissue as previously described.[8,9] The procedure resulted in separation of three forms of the enzyme with estimated molecular weights of 390,000 (A), 270,000 (B) and 130,000 (C). These forms had average specific activities of 480 (A), 400 (B) and 575 (C) m-moles acetylthiocholine (ATC) hydrolyzed/mg of protein/hr.

Synaptosomal membrane fractions containing AChE activity were prepared from homogenates of bovine caudate nucleus tissue in 0·32 M sucrose using the method

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of DeRobertis *et al.*[10] Such preparations had an average AChE specific activity of 45 μmoles ATC hydrolyzed/mg of protein/hr.

In some experiments, AChE was prepared from homogenates of whole brain tissue from male Sprague–Dawley rats (100–120 g) following the procedure of DeRobertis *et al.*[10] to the separation of an M1 pellet. This was resuspended in 0·32 M sucrose (pH 7·0) for studies on a membrane-bound form of AChE. Soluble forms of rat brain AChE were obtained by mixing the M1 pellet in sucrose with added EDTA (0·5 mM) and imidazole buffer (5 mM) at 0–4° for 36 hr, followed by centrifugation at 100,000 *g* for 60 min to remove particulate material.

Estimation of brain ACh and choline. Male Sprague–Dawley rats (80–100 g) were injected via tail vein with either saline or ketamine (20 mg/kg). At selected time intervals, the rats were guillotined and the brains were removed rapidly (within 15–20 sec) and placed in liquid nitrogen. Brain levels of ACh and choline were determined simultaneously using the gas chromatographic procedure of Jenden *et al.*[11,12] with the following slight modifications. The dichloromethane extract (1 μl) was injected into a Varian 1700 gas chromatograph equipped with flame ionization detector and a 10 ft by 2 mm (i.d.) column containing Gas-Chrom Q coated with a 5% mixture of OV-101 and dodecyldimethylenetriamine succinamide. Operating conditions were: column temperature, 120°; injector and detector temperatures, 165° and 275° respectively; nitrogen, air and hydrogen flow rates, 17, 300 and 30 ml/min respectively. Under these conditions, the retention times of the demethylated derivatives of ACh, propionylcholine and pivaloylcholine were 145, 210 and 267 sec respectively. Concentrations of ACh and choline were calculated by peak height ratio analyses.

Other analytical methods. AChE activity was determined by the spectrophotometric method of Ellman *et al.*[13] at 25° using acetylthiocholine as substrate. Inhibition of AChE activity was plotted as Lineweaver–Burk plots with lines calculated by the least squares method of linear regression analysis. Apparent dissociation constants (K_i) were calculated from the slopes of the inhibited reactions at two substrate concentrations and confirmed by direct observation of Hunter and Downs plots.[14] Protein was estimated by the procedure of Lowry *et al.*[15] using bovine serum albumin standards. Brain levels of ketamine after intravenous administration to rats were determined by a modification of the gas chromatographic procedure of Chang and Glazko[16] as reported previously.[17]

Chemicals. All reagents used were of analytical grade. Acetylthiocholine (ATC) was obtained from Sigma Chemical Co. (St. Louis, Mo.). 5,5′-Dithio-*bis*-(2-nitrobenzoic acid), propionyl chloride and pivaloyl chloride were obtained from Aldrich Chemical Co. (Milwaukee, Wis.). Crystalline ketamine hydrochloride and its metabolites were donated by Parke Davis & Co. (Ann Arbor, Mich.) through the courtesy of Dr. A. J. Glazko. Acetonitrile (chromatoquality), dichloromethane (spectroquality), Pentane (spectroquality) and anhydrous ether (purified by passage over neutral Alumina, Biorad Lab., Richmond, Calif.) were obtained from Matheson, Coleman & Bell (New Jersey). *p*-Toluenesulfonic acid, silver salt, was obtained from Eastman Kodak Co. (Rochester, N.Y.). OV-101 was obtained from Applied Science Labs. (Pa.). Acetylcholine perchlorate ($AChClO_4$), pivaloylcholine perchlorate ($PivChClO_4$), choline sulfonate and succinamide polymer were synthesized according to Jenden *et al.*[11,12] A solution of sodium thiophenoxide was prepared in freshly distilled anhydrous butanone (J. T. Baker, AR) and stored frozen, sealed in ampules under nitrogen.

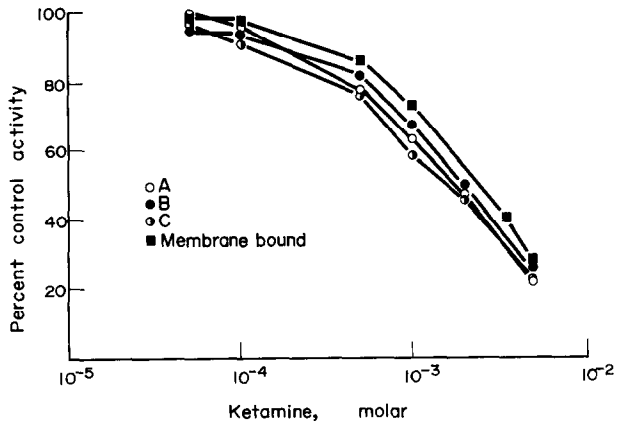

FIG. 1. Ketamine inhibition of bovine brain AChE. AChE activity was determined in the presence of ketamine by the spectrophotometric procedure of Ellman *et al.*[13] at pH 7·9 and substrate concentration of 1 mM ATC. Control activity, expressed as μmoles ATC hydrolyzed/mg of protein/hr, for the enzyme forms was: 480×10^3 (form A); 400×10^3 (form B); 575×10^3 (form C) and 45 (membrane-bound). Points represent mean values from three determinations (which differed by less than 4 per cent) at each inhibitor concentration.

RESULTS

Ketamine inhibition of bovine brain acetylcholinesterase. Membrane-bound (synaptosomal) and three purified forms of AChE from bovine brain tissue were inhibited by ketamine in a concentration-dependent manner (Fig. 1). The forms showed no marked differences in sensitivity to ketamine, apparent K_i values ($\times 10^{-4}$ M) being 4·9 for membrane-bound AChE and 6·9, 6·6 and 5·1 for purified forms C, B and A respectively. Inhibition was 90 per cent reversible after dialysis against 0·1 M phosphate buffer for 60 min to remove ketamine. Figure 2 is a Lineweaver–Burk plot for

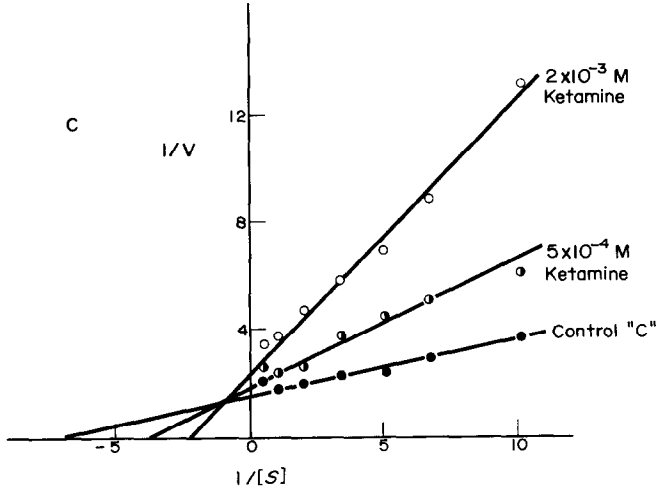

FIG. 2. Lineweaver–Burk plot of ketamine inhibition of purified AChE (form C). Enzyme activity was measured by the method of Ellman *et al.*[13] at substrate (ATC) concentrations from 0·1 to 5 mM at pH 7·9. Control activity at optimum substrate concentration (1 mM) was 575 m-moles ATC hydrolyzed/mg of protein/hr. Lines were plotted from calculations employing the least squares method of linear regression analysis.

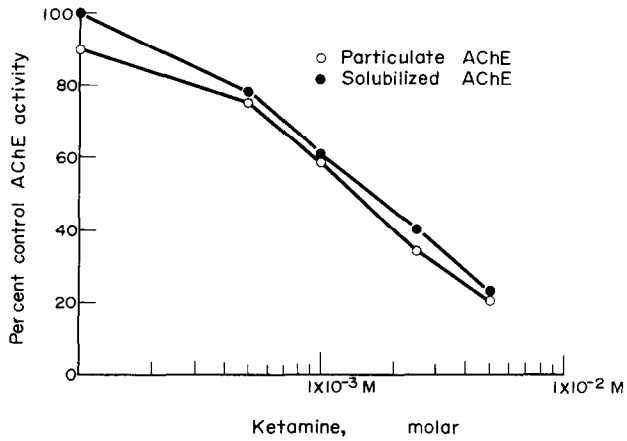

FIG. 3. Ketamine inhibition of rat brain AChE. Enzyme activity was estimated by the method of Ellman *et al.*[13] at pH 7·9 and optimal substrate concentration (1 mM ATC). Control activity was 9·4 and 14·3 μmoles ATC hydrolyzed/mg of protein/hr for the particulate and soluble form of the enzyme respectively. Points represent mean values from three determinations (which differed by less than 4 per cent) at each inhibitor concentration.

ketamine inhibition of AChE form C (mol. wt, 130,000), suggesting mixed kinetics of inhibition with changes in V_{max} and apparent K_m value. Similar data were obtained for the other forms of the enzyme. The demethylated metabolite of ketamine (metabolite I) also inhibited bovine brain AChE (data not shown), but at concentrations approximately 10 times that required for inhibition by ketamine.

Ketamine inhibition of rat brain acetylcholinesterase. Ketamine inhibited membrane-bound and solubilized forms of rat brain AChE in a concentration-dependent manner (Fig. 3). Approximately 20 and 40 per cent inhibition of both forms of the enzyme occurred at concentrations of 5×10^{-4} and 10^{-3} M ketamine respectively. The levels of ketamine achieved in rat brain were estimated after intravenous administration of the drug at a dose (20 mg/kg) that produced a loss of righting reflex of 6–8 min.[17] Table 1 shows brain levels of ketamine at three time intervals after administration of the drug with an estimation of "apparent molarity." The latter data are approximations which assume a specific gravity of 1·0 for brain tissue; they do not take into account possible regional differences in ketamine level or differences in the intra-extracellular distribution of the drug. Peak levels of ketamine achieved in rat brain tissue after administration *in vivo* were approximately 4×10^{-4} M.

TABLE 1. RAT BRAIN LEVELS OF KETAMINE AFTER INTRAVENOUS INJECTION*

Time after injection (min)	Ketamine (μg/g tissue)	"Apparent molarity" (M)
0·5	95·7 ± 12·6	$4{\cdot}1 \times 10^{-4}$
1·0	63·0 ± 2·8	$2{\cdot}7 \times 10^{-4}$
10·0	24·6 ± 3·9	$1{\cdot}0 \times 10^{-4}$

* Ketamine (20 mg/kg) was injected into the tail vein of rats. Brain tissue was assayed for ketamine at indicated times by the gas chromatographic procedure noted in Methods. Ketamine levels shown are mean values from four separate animals ± S.E.M.

TABLE 2. EFFECT OF KETAMINE ON RAT BRAIN ACh LEVELS*

Condition	Time after injection (min)	ACh (nmoles/g)	Choline (nmoles/g)	Increase in ACh (%)
Saline	0·5	17·75 ± 0·65	50·36 ± 0·58	
Ketamine	0·5	22·42 ± 0·41 ($P < 0·001$)	47·33 ± 3·57	26·3
Saline	1·0	18·69 ± 0·36	56·61 ± 6·27	
Ketamine	1·0	22·74 ± 1·20 ($P < 0·02$)	60·03 ± 1·12	21·7
Saline	10·0	20·39 ± 0·77	46·04 ± 7·65	
Ketamine	10·0	19·67 ± 0·99 ($P > 0·05$)	51·89 ± 2·1	

* Ketamine, 20 mg/kg, was injected into the tail vein of rats. Brain tissue was assayed for ACh and choline at indicated times by the gas chromatographic procedure noted in Methods. Levels shown are mean values from four to six separate animals ± S.E.M. P values were calculated by the paired Student's *t*-test.

Effect of ketamine on rat brain ACh levels. The whole brain levels of ACh and choline determined at three time periods after the intravenous administration of ketamine to rats are shown in Table 2. Control (saline-treated) levels of brain ACh are comparable to those reported previously.[18] Ketamine (20 mg/kg, i.v.) caused significant increases in brain ACh at 30 ($P < 0·001$) and 60 ($P < 0·02$) sec, but not at 10 min after administration. Brain choline levels exhibited some variability and were not changed significantly after ketamine treatment.

DISCUSSION

The anesthetic agent ketamine caused reversible inhibition of both membrane-bound and purified forms of mammalian brain AChE. The competitive component of inhibition may reflect an interaction of the positively charged nitrogen moiety of ketamine (pKa, 7·5) with the anionic region of the enzyme active site. The possibility of ketamine interactions with AChE at secondary anionic sites is suggested by the noncompetitive component of inhibition. Such interactions have been suggested for other inhibitors of AChE which possess charged nitrogen groups, including decamethonium and tubocurarine.[19,20]

Ketamine caused approximately 40 per cent inhibition of rat brain AChE *in vitro* at a concentration only 2·5 times that estimated to occur in brain tissue after intravenous administration of the drug (20 mg/kg) to the intact animal. The same dose of ketamine caused approximately 26 and 22 per cent increases in brain ACh levels at 30 and 60 sec after administration respectively. These data suggest that cholinergic stimulation via AChE inhibition could underlie certain of the pharmacological effects of ketamine. Other anesthetic agents, including barbiturates, chloral hydrate and ether, have been shown to elevate brain ACh levels when used under conditions of deep anesthesia.[21,22] In preliminary studies[23,24] ether and cyclopropane, both of which exert effects resembling cholinergic stimulation, were reported to cause inhibition of brain AChE *in vitro*. Since there have been no reports of AChE inhibition by barbiturates at concentrations similar to those occurring *in vivo* in the brain, it is possible that their effects on brain ACh levels result from mechanisms other than enzyme inhibition.

Similarities have been noted between AChE and cholinergic receptors in terms of ligand binding properties,[25] which suggest the possibility of a direct interaction of ketamine with certain cholinergic receptors. This may be the basis for the reported interactions between ketamine and certain neuromuscular blocking agents at the myoneural junction.[26]

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