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Long-Term Central Cholinergic Hypofunction Induced in Mice by Ethylcholine Aziridinium Ion (AF64A) *in vivo*[1]

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ABSTRACT

Ethylcholine mustard aziridinium ion (AF64A), a neurotoxic choline analog, was evaluated for its interactions with the cholinergic system in mice. Parenterally administered AF64A was lethal (LD_{50} = 32.6 μmol/kg i.v.), and the lethality could be antagonized even at 8 LD_{50} doses by pretreatment with choline (714 μmol/kg i.p.) 2 min earlier. Mice that were protected by choline slowly developed neurological motor disturbances such as ataxia and hypokinesia, and lost weight. Intracerebroventricular administration of 65 nmol of AF64A was not acutely lethal, but produced similar delayed behavioral effects similar to those found after parenteral administration of AF64A. Seven days after a single injection of 65 nmol of AF64A i.c.v., there was a significant decrease in acetylcholine content in the cortex, striatum and hippocampus, but no change in choline levels. Acetylcholine content was still significantly reduced in the hippocampus at 3 weeks after this treatment. The reduction in activity of choline acetyltransferase and high-affinity choline transport paralleled the reduction in acetylcholine measured at 7 days post AF64A treatment, whereas muscarinic receptors in all three brain areas were unchanged. These combined data indicate that AF64A is a presynaptic chemical neurotoxin, capable of inducing a persistent deficiency in central cholinergic transmission.

Recent research has indicated that central cholinergic hypofunction may be involved primarily in the pathology of senile dementia of Alzheimer's type, and secondarily in other neurological and psychiatric disorders, *e.g.*, tardive dyskinesia, Huntington's disease, Friedreich's ataxia and Gilles de la Tourette's disease (Fisher and Hanin, 1980 and references cited; Corkin *et al.*, 1982). An adequate animal model for one or more of these diseases would be one in which the synthesis of ACh is permanently impaired *in vivo*. So far, there are no known pharmacological agents capable of inducing such a sustained decrease in central cholinergic function.

A long-term central cholinergic hypofunction could best be achieved through the development of a selective, irreversible neurotoxin (Fisher and Hanin, 1980). Such a potential cholinergic specific neurotoxin should be related structurally to Ch. Its site of action would then conceivably be directed to the Na^+-dependent HAChT, which is primarily localized at cholinergic nerve terminals and may be the regulatory step in ACh synthesis (Barker, 1979; Jope, 1979; Speth and Yamamura, 1979).

We report in this paper our studies using the compound ethylcholine mustard aziridinium ion, AF64A (fig. 1). This compound was selected because of its close chemical structural similarity to Ch and the presence in the molecule of a cytotoxic aziridinium moiety. We have synthesized this agent, and present in this paper pharmacological studies indicating that AF64A *in vivo* has the potential for inducing a long-term cholinergic hypofunction in mice.

Materials and Methods

Materials

AF64A and analogs. There are two mechanisms by which we obtained the aziridinium, AF64A. The first was by converting the corresponding ethylcholine mustard analog, AF64, to the aziridinium compound (fig. 2). AF64 had to be synthesized before AF64A could be made available for these studies. AF64 was isolated as the picrate salt after the acidic hydrolysis of acetylethylcholine mustard. Acetylethylcholine mustard, in turn, was prepared essentially according to the procedure described by Jackson and Hirst (1972) for ACh mustard. The hydrochloride salt of acetylethylcholine mustard was next precipitated as a white, hygroscopic solid in dry tetrahydrofuran and ether, then refluxed in aqueous hydrochloric acid for 10 to 20 hr, according to a modification of the method described by Hanby and Rydon (1947). In addition to obtaining AF64, a side product of this reaction was the piperazinium analog of AF64. Both products were isolated as the picrate salts.

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ABBREVIATIONS: ACh, acetylcholine; Ch, choline; HAChT, high-affinity Ch transport system; SIMS, secondary ion mass spectrometry; ChAT, choline acetyltransferase; QNB, quinuclidinyl benzilate.

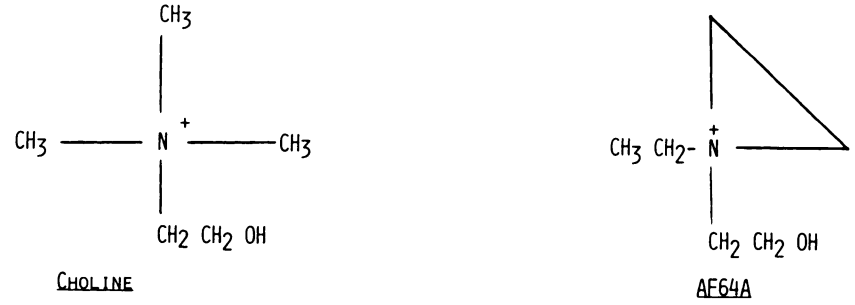

Fig. 1. Chemical structures for Ch and ethylcholine mustard aziridinium (AF64A).

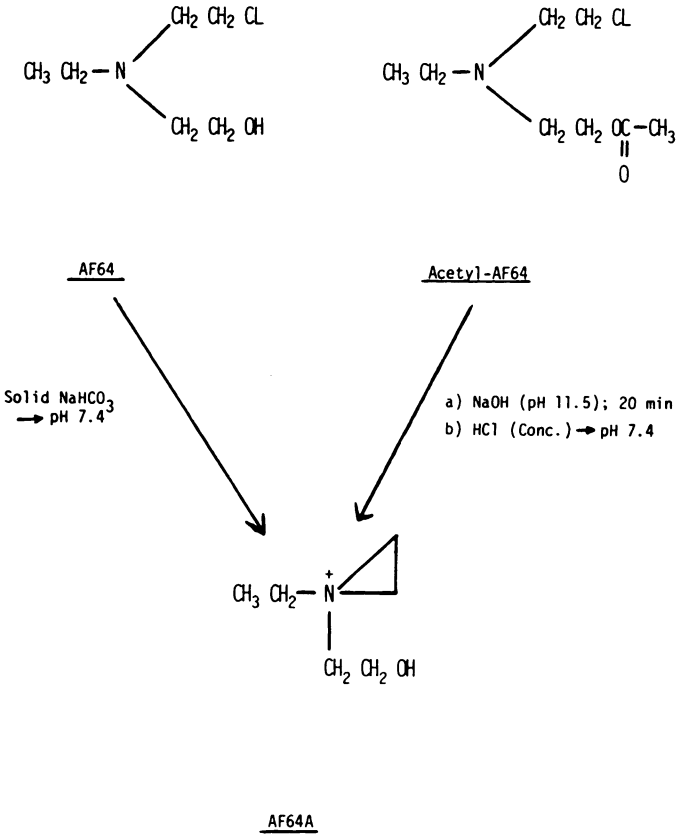

Fig. 2. Synthetic pathways used to generate AF64A. Acetyl-AF64, acetylethylcholine mustard HCl.

AF64A, the aziridinium specie of AF64, was then formed by dissolving AF64 (as the picrate salt) in 0.9% NaCl (saline) and adjusting the pH to 7.4 with solid $NaHCO_3$ (fig. 2). This solution was allowed to stand at room temperature for at least 60 min before injection into animals, in order to achieve maximal conversion of the mustard analog to the aziridinium form. AF64A obtained in this manner was used in all studies involving toxicity measurements.

The second mechanism by which AF64A was generated was directly from the ester. An aqueous solution of acetylethylcholine mustard HCl (Acetyl-AF64 in fig. 2) was treated with NaOH (10 N) at pH 11.5 for 20 min in order to convert the ester to AF64. The pH was next adjusted to 7.4 with concentrated HCl, essentially by the method described by Clement and Colhoun (1975a,b) for Ch mustard aziridinium ion. AF64A derived by this procedure (see general scheme depicted in fig. 2) was used in the remainder of the studies described.

Irrespective of the method by which AF64A was prepared, the aziridinium analog of the ethylcholine mustard showed the same structure. This was ascertained both by nuclear magnetic resonance and by SIMS.

Specifically, using nuclear magnetic resonance [at 100 MHz, δ, parts per million (D_2O)], the following values were obtained; 1.35 (t, CH_3); 3.14 (s, aziridinium, CH_2CH_2); 3.24 to 3.38 (m, CH_2-N-CH_2), and 3.82 (t, CH_2—O). These results provided a good indication of the identity of the aziridinium structure. Moreover, transformation of the mustard to the aziridinium structure was in excess of 95% complete under our experimental conditions (A. Fisher, S. Margalit and A. Vincze, manuscript in preparation).

SIMS analysis, conducted on lyophilized samples of the aziridinium compound, provided further confirmation of the identity and essential purity of the aziridinium formed according to the scheme described in figure 2. Specifically, the following SIMS spectra were obtained: m/z = 116 (cation); m/z = 87 (AF64A cation minus ethyl group); m/z = 72 ($\overset{+}{N}$-CH_2CH_3); and m/z = 56 ($\overset{+H}{N}$-CH_2) (Vincze *et al.*, 1981).

Moreover, we were able to show essentially complete conversion of the mustard to the aziridinium analog obtained by either of the approaches described in figure 2, using thin-layer chromatography. Samples were identified by thin-layer chromatography (developer $CHCl_3$/MeOH, 1:4; R_f 0.7) using the reagent 4-(paranitrobenzyl)pyridine (Sarquhar *et al.*, 1979).

Finally, both products of aziridinium formation exerted identical pharmacological properties. Therefore, this aziridinium analog will generally be referred to within the text as AF64A, regardless of its original precursor.

Other agents. Other drugs used in these studies were as follows: ACh perchlorate (K & K Laboratories, Inc., Plainview, NY); d_4-AChBr, d_9-AChBr, d_4-ChBr and D_9-ChBr (Merck Sharp & Dohme, Pointe Claire/Sorval, Quebec, Canada); hemicholinium-3 and oxotremorine sesquifumarate (Aldrich Chemical Company, Inc., Milwaukee, WI); Ch iodide (ICN Pharmaceuticals, Inc., Plainview, NY); [^{14}C]acetyl coenzyme A (58 mCi/mmol), [^{3}H]Ch (80 Ci/mmol) and [^{3}H]quinuclidinyl benzilate (33 Ci/mmol) (New England Nuclear, Boston, MA).

Animals. Female albino mice (20–30 g; Charles River Breeding Laboratories, Wilmington, MA), CD-1 strain, were used in all experiments. Animals were kept in their quarters for at least a week before use. Animal quarters were maintained at 20°C and subjected to a 12-hr light-dark cycle (light from 6:00 A.M. to 6:00 P.M.). Drug-treated and control animals were killed at approximately the same time of day to avoid possible circadian variations (Hanin *et al.*, 1970). At least five animals were used in each type of analysis, for each type of experimental manipulation (time, dose, route of administration), unless specified differently.

Drug administration. Intraperitoneal and i.v. injections of the drugs were administered in a volume of 0.1 ml/10 g of animal. Control animals were injected with saline solution (0.1 ml/10 g i.p. or i.v.). Doses of drugs injected are expressed in micromoles per kilogram for purposes of cross-comparison.

Unilateral i.c.v. injections in mice of either AF64A (65 nmol) or a vehicle solution were performed according to the method of Haley and McCormick (1957) as previously described (Mantione *et al.*, 1981a). The vehicle solution consisted of double distilled water to which an equivalent amount of base had been added, followed by adjustment of the pH by the same procedure as that used in preparing the AF64A solution.

Methods

Acute toxicity. AF64A was injected into mice by the i.p. and i.v.

routes, and its LD_{50} values were established. LD_{50} (i.p.) values at 95% confidence limits were derived according to Litchfield and Wilcoxon (1949). Mortality counts were taken after 24 hr. In surviving animals, incipient morbidity was recorded as changes in weight and gross behavioral effects for 1 to 2 weeks after administration of the compounds.

Antagonism of AF64A-induced lethality by Ch. These studies were conducted in order to determine whether AF64A competes with Ch for transport across specific barriers between blood and the eventual site of action of the compound. Ch (714 μmol/kg i.p.) was administered to mice at 1, 1.5, 2, 7, 10, 15, 20 and 40 min before administration of AF64A (261 μmol/kg i.v.). Mortality counts and time to death were recorded during 5 to 7 hr after injections.

ACh and Ch levels. Mice injected i.c.v. with AF64A (65 nmol/mouse) or vehicle were killed by microwave irradiation (2.5 sec.; 2.5 kw; 2.45 MHz) 3, 7 and 21 days postinjection. The whole brain was extirpated, and the cortex, both hippocampi and striata were dissected. Brain tissues were prepared for the analysis of ACh and Ch content using gas chromatography-mass spectrometry (Hanin and Skinner, 1975).

Na^+-dependent HAChT. Mice were killed by decapitation 7 days after i.c.v. injections of either AF64A (65 nmol/mouse) or vehicle. The cortex, hippocampi and striata from both sides of the brain were prepared as crude synaptosomal homogenates (P2 fraction) for HAChT measurements.

HAChT was determined by a method adapted from the procedures described by Yamamura and Snyder (1973) and by Kuhar *et al.* (1973). Synaptosomes were added to a 40-mM Tris physiological buffer (pH 7.5 at 30°C) containing: NaCl (125 mM); KCl (9.6 mM); $MgSO_4$ (4.2 mM); $CaCl_2$ (2.4 mM); dextrose (10 mM); 0.025 μM Ch and 0.2 to 0.3 μCi of [^{3}H]Ch. Duplicate samples were incubated at 30°C for 8 min. Ch transport was terminated by cooling the tubes on ice, followed by addition of 3 ml of cold incubation medium. The tissue was collected onto GF/F filters by vacuum filtration and rapidly washed with 9 ml of cold incubation buffer. Sample protein was measured according to Lowry *et al.* (1951). Na^+-dependent HAChT was defined as the amount of Ch accumulated into tissues incubated in buffered medium containing Na^+, minus the amount of Ch accumulated into identical samples incubated at 0°C. Under these conditions, HAChT was linear for up to 10 min using 0.5 to 5 mg of tissue at saturating Ch concentrations. Uptake is represented here as disintegrations per minute/8 min/milligram of protein, rather than as picomoles/8 min/milligram of protein because of the possible contribution of endogenous Ch to the incubation medium (Kuhar *et al.*, 1973).

ChAT activity. ChAT activity was measured by radiochemical assay, using the Spyker *et al.* (1972) modification of the method developed by McCaman and Hunt (1965). [1-^{14}C]acetyl-CoA was used as substrate, and sodium tetraphenylboron was used to isolate labeled ACh. Triton-X-100 was added to the synaptosomal homogenates (P2 pellet) to make a final concentration of 0.5%. Duplicate samples were incubated with labeled acetyl-CoA for 30 min at 38°C. Data are expressed as disintegrations per minute per milligram of protein/30 min.

[^{3}H]QNB binding. Mice were killed by decapitation 7 days after i.c.v. injections of either AF64A (65 nmol/mouse) or vehicle, and the cortex, hippocampi and striata were immediately wrapped in aluminum foil and cooled to −70°C. Crude tissue homogenates of specific brain areas were subsequently prepared according to the method described by Yamamura and Snyder (1974). Muscarinic receptor activity was measured by incubating triplicate samples containing 2.0 ml of 0.05 M Na/K-phosphate buffer at pH 7.4, 2.5 mg of tissue per ml and [^{3}H]QNB (1.7 nM) at 37°C for 60 min. Binding was terminated by filtering the samples over GF/B filters under vacuum, followed by washes with three 5-ml volumes of cold incubation buffer. Specific activity was calculated as the total amount of QNB bound minus nonspecific QNB bound (identical samples incubated in the presence of 100 μM oxotremorine).

Statistical analysis. Results are expressed as group mean ± S.E.M. The statistical comparisons between control and AF64A-treated animals were performed according to a two-tailed Student's *t* test.

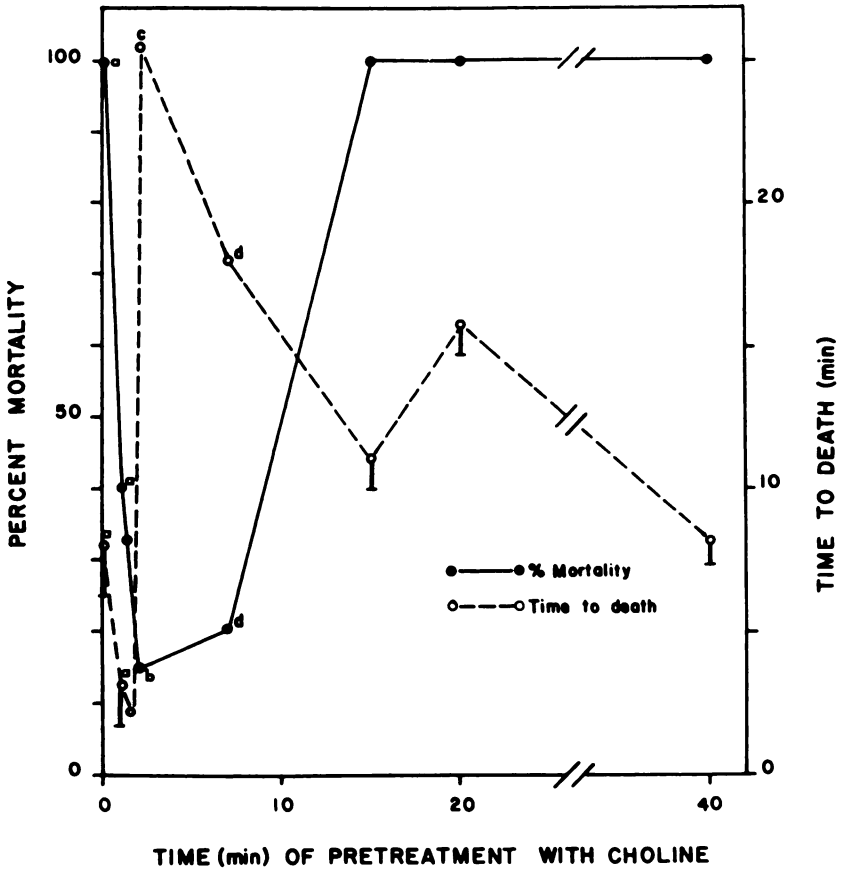

Fig. 3. Protection from AF64A-induced lethality (261 μmol/kg i.v.) in mice by pretreatment with Ch (714 μmol/kg i.p.). *N* = 6 mice per point except where mentioned otherwise: a, 10 mice; b, 66 mice; c, 16 mice; d, 5 mice; (in c and d, 1 mouse died in each group).

Results

Acute toxicity. AF64A-induced lethality in mice progressed behaviorally from an initial ataxia, leading to coarse tremor, exopthalamus, respiratory distress, violent convulsions and finally death. The symptoms were similar to those of hemicholinium-3 at lethal doses (0.35 μmol/kg i.p.). The mustard compound AF64 was less toxic (LD_{50} = 23 mg/kg i.p.) than its related aziridinium analog, AF64A (LD_{50} = 6 mg/kg i.p.; 5 mg/kg i.v.). Death, when it occurred, was induced within 5 to 15 min after i.v. injection, and within 20 to 30 min after i.p. injection of AF64A.

Mice that were injected i.c.v. with 65 nmol of AF64A developed overt neurological disturbances, such as hypokinesia and ataxia, and typically lost 10 to 20% of their original body weight over the course of the first 3 days. At this dose AF64A exhibited no acute lethality. After 7 days, however, there was a cumulative mortality of 30 to 50%, and those mice that remained alive lost about 25% of their weight compared with the vehicle-treated animals. By 21 days there were no overt behavioral differences and no differences in body weight in the surviving AF64A-treated mice when compared with the vehicle-treated animals. Vehicle-injected mice incurred no morbidity during all the time points studied and gained weight at a natural rate.

Antagonism of AF64A-induced lethality of Ch. The interaction between Ch and AF64A was time-dependent, as illustrated in figure 3. The maximal prophylactic effect of Ch against 8 LD_{50} doses of AF64A (i.v.) occurred when Ch was given 2 min before AF64A. Ch injected 2 min before AF64A not only antagonized the AF64A-induced lethality, but also delayed the time at which death occurred in those animals that were not completely protected by the Ch pretreatment. Interestingly enough, although at shorter times of Ch pretreatment (1 or 1.5 min) there was still a high level of protection against AF64A-induced lethality, mice dying during this manipulation died within a shorter time than the saline- or AF64A (8 LD_{50} doses i.v.)-treated mice.

Mice protected from AF64A by Ch, however, subsequently lost weight (approximately 10–20% of body weight per day), and 20 to 30% of the animals eventually died within 3 to 5 days. When mice were pretreated with the same dose of Ch, but with 4 LD_{50} doses of AF64A (130.5 μmol/kg i.v.), there was full protection against the lethal effect, and mice did not die on subsequent days.

ACh and Ch levels. Brain ACh levels were reduced after i.c.v. AF64A administration. This effect was most prominent in hippocampus and in cortex, whereas striatal ACh levels were more resistant to depletion after AF64A (fig. 4). ACh levels were significantly reduced in all three brain areas 7 days after administration of a single injection of 65 nmol of AF64A [hippocampus (−45%); cortex (−40%); striatum (−28%)]. By 21 days after this single i.c.v. dose of AF64A, ACh levels returned to normal in cortex and striatum, but remained significantly reduced (−50%) in the hippocampus. Ch levels, on the other hand, were not affected in the same brain areas from these animals. In general, there was no correlation between the steady-state levels of ACh or Ch and the general condition and overt behavior of the treated mice.

HAChT, ChAT and [^{3}H]QNB binding. Within 7 days, AF64A (65 nmol i.c.v.) significantly decreased HAChT in all three brain areas studied, without having any significant effect on the number of muscarinic receptors measured at a saturating ligand concentration (table 1). ChAT activity was also decreased significantly in the hippocampus and striatum and showed a tendency toward reduction in the cortex (table 1). The observed decreases in HAChT and ChAT were paralleled by a significant decrease in ACh levels in all three brain areas (table 1; fig. 4), indicating a generalized destruction of presynaptic cholinergic sites.

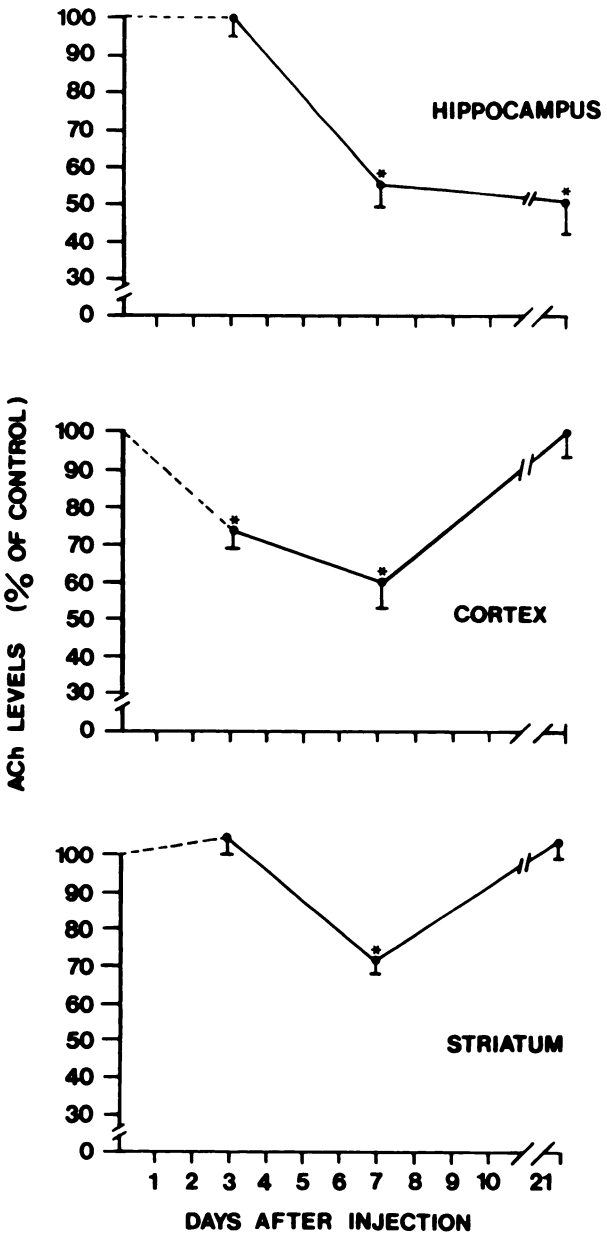

Fig. 4. AF64A-induced effects on steady-state levels of ACh in hippocampus, cortex and striatum at various times after i.c.v. injections of vehicle (control) or AF64A (65 nmol/mouse). Group means are expressed as percentage of control ± S.E.M., with five to eight animals used for each time point studied. Control levels of ACh (in nanomoles per gram) were: hippocampus, 33.67 ± 1.95 (N = 28); cortex, 18.27 ± 0.93 (N = 14); striatum, 86.00 ± 2.21 (N = 18). * $P < .001$.

Discussion

Our results indicate that AF64A could be considered a potent presynaptic and irreversible cholinergic neurotoxin capable of inducing in mice a long-term central cholinergic hypofunction.

TABLE 1

Effect of AF64A on ACh levels and neurochemical synaptic markers in selected brain areas from mice 7 days post i.c.v. AF64A or vehicle injection

See "Materials and Methods" for experimental details. The results are expressed as mean ± S.E.M.; number of animals used is in parentheses. AF64A did not affect Ch levels in all three brain areas studied. Levels of Ch (nanomoles per gram of wet tissue) in the vehicle-treated mice were: cortex, 17.96 ± 1.55 (14); hippocampus, 33.78 ± 2.42 (28); striatum, 55.19 ± 3.20 (18).

		Cortex	Hippocampus	Striatum
ACh (nmol/g wet tissue)	Vehicle	18.27 ± 0.93 (19)	33.57 ± 1.95 (28)	86.00 ± 2.21 (18)
	AF64A	11.31 ± 0.68 (17)***	19.73 ± 2.10 (26)***	61.58 ± 3.96 (15)***
	Δ%	−40	−45	−28
HAChT activity (dpm/8 min/mg protein)	Vehicle	4,220 ± 850 (5)	4,200 ± 700 (5)	23,380 ± 4,420 (5)
	AF64A	1,860 ± 490 (5)*	820 ± 130 (5)***	4,770 ± 400 (5)***
	Δ%	−56	−81	−80
ChAT activity (dpm/8 min/mg protein)	Vehicle	79.36 ± 12.70 (5)	24.48 ± 4.20 (9)	167.90 ± 19.96 (5)
	AF64A	51.46 ± 7.55 (5)****	11.00 ± 1.62 (7)**	102.90 ± 13.70 (5)*
	Δ%	−35	−55	−39
[^{3}H]ONB binding (B_{max}) (fmole/mg protein)	Vehicle	558.50 ± 98.64 (8)	543.50 ± 17.99 (8)	818.50 ± 48.80 (12)
	AF64A	543.50 ± 77.50 (8)	524.30 ± 22.70 (10)	848.30 ± 38.50 (12)
	Δ%	−2.7	−3.5	+3.6

* $P < .05$; ** $P < .02$; *** $P < .001$; **** $P < .1$.

In this study we have evaluated this compound with the emphasis on its toxicity and on its effects on several cholinergic markers in selected brain areas.

AF64A induces prolonged toxic effects. This was evident in mice that were pretreated with Ch (714 μmol/kg i.p.) before injection of 8 LD_{50} doses (i.v.) of AF64A and survived the lethal effect of this dose. AF64 also was toxic after i.p. injection into mice; its LD_{50}, however, was higher than that of AF64A. In this case AF64 probably acted as a prodrug to generate AF64A *in situ*, inasmuch as open chain mustard analogs rapidly cyclize in physiological buffers, forming the aziridinium ions, which appear to be the pharmacologically active forms (Rylett and Colhoun, 1980; our own unpublished data).

The structural similarity between AF64A and Ch is manifested *in vivo* in the antagonism of AF64A-induced lethality by Ch. These findings would imply that Ch and AF64A compete for transport mechanisms across some specific barriers between blood and the eventual site of action of these analogs, such as the nerve terminal HAChT system. Another site for such competition could be the blood-brain barrier. On the basis of a competitive interaction between plasma Ch and AF64A, less of the aziridinium compound would cross the transport barrier at 2 min after Ch administration, which is the time at which plasma Ch levels reach a peak. By the same token, as we have in fact demonstrated in these studies, the competitive effect would disappear at 15 to 30 min, when absorption of Ch from plasma to various storage compartments has significantly decreased plasma Ch (I. Hanin, unpublished observations; Freeman and Jenden, 1976; Ulus *et al.*, 1978; Barker, 1979).

In order to induce a persistent underactivity of central cholinergic neurotransmission of presynaptic origin, the activity of the cholinergic neurons should be irreversibly disrupted, leading eventually to a reduction in the steady-state levels of ACh (Fisher and Hanin, 1980). This type of cholinergic hypofunction was achieved in mice by direct i.c.v. injections of AF64A. In a recent report we demonstrated that a loss in the number of functional Na^+-dependent HAChT sites occurred in the cortex and hippocampus of mice given an i.c.v. injection of 65 nmol of AF64A 3 days earlier (Mantione *et al.*, 1981a). This prompted us to study steady-state levels of ACh and Ch during a 3-week time course after i.c.v. AF64A. Under these conditions AF64A caused a significant reduction in ACh levels, which in selected brain areas had different onsets and durations. This remarkable long-term decrease in ACh in selected brain areas is unique, in that usually drug manipulations are capable of decreasing ACh levels for a number of hours at most, but not for days, due to the strong regenerative powers of the intact central cholinergic systems (*e.g.*, Freeman *et al.*, 1979).

It is not clear yet why ACh levels returned to normal after 21 days in the cortex and striatum, but still showed a tendency to remain deficient in the hippocampus after AF64A treatment. One possible explanation could be the anatomical differences among cholinergic neurons in the striatum (mostly interneurons), cortex (a mixed population of long and short nerve fibers?) and hippocampus (long nerve fibers) (Kuhar, 1976; Sherman *et al.*, 1978).

Because at 7 days after AF64A i.c.v. injections all three brain areas studied showed a significant reduction in ACh levels, this time point was selected to analyze the effect of the compound on two presynaptic cholinergic markers, HAChT and ChAT, and on a postsynaptic cholinergic marker, the muscarinic receptor. It was found in cortex and hippocampus that a decrease in ACh levels was accompanied by a reduction in Ch transport as well as ChAT activity. The decrease in all three neurochemical parameters strongly suggests that AF64A is producing a long-term destruction of cholinergic nerve terminals or neurons in these brain areas. The fact that no significant changes were detected in the number of muscarinic receptors after AF64A i.c.v. further supports the hypothesis that AF64A interacts with presynaptic cholinergic neurons.

A similar correlation was not found in the striatum, where an almost 80% reduction in HAChT activity was followed by only a subtle decrease in ACh levels. This inconsistency in the coupling process between Ch uptake and/or ACh synthesis and levels in the striatum is not well understood. Possibly, different densities in cholinergic neurons, anatomical differences and probably even biochemical differences in the HAChT systems in the striatum may explain the anomalies observed between cholinergic function in striatal neurons, as opposed to those seen in the hippocampus and cortex (Kuhar, 1976; Sherman *et al.*, 1978; Mantione *et al.*, 1981b,c).

The decrease in ACh levels after AF64A treatment could occur either as the result of an irreversible alkylation of nucleophilic active sites on the Ch carrier and/or as a result of cholinergic nerve terminal degeneration. In the first case, AF64A might render the cholinergic nerve terminal deficient in

newly synthesized ACh, due to prevention of Ch from gaining access to sites of ACh synthesis. In the second case, the AF64A could be selectively accumulated into cholinergic neurons *via* the HAChT, conceivably resulting in high intracellular concentrations of this compound, which would disrupt fundamental metabolic processes required for viability.

In summary, our results reported in this paper indicate that AF64A is a unique presynaptic cholinergic chemical neurotoxin that induces in mice a state of long-term reduced cholinergic activity. AF64A thus could serve as a novel tool to develop animal models of human brain disorders in which a cholinergic hypofunction has been implicated.

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