Effects of a Central Anticholinesterase, Methanesulfonyl Fluoride on The Cerebral Cholinergic System and Behavior in Mice: Comparison with an Organophosphate DDVP

Haruo Kobayashi,*[,a] Takuma Nakano,[a] Donald E. Moss,[b] and Tadahiko Suzuki[a]

[a]*Pharmacology, Departments of Veterinary Medicine, Faculty of Agriculture, Iwate University, Ueda, Morioka 020–8550, Japan and* [b]*Psychobiochemistry, Department of Psychology, University of Texas at El Paso, Texas 79968, U.S.A.*

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Mice were injected with an anticholinesterase, methanesulfonyl fluoride (MSF, 1.5 mg/kg) or *O,O*-dimethyl *O*-(2,2-dichlorovinyl) phosphate (DDVP, 10 mg/kg) singly or repeatedly and examined for synaptic activities on the cerebral cholinergic system and behavior. MSF inhibited the activity of cerebral acetylcholinesterase (AChE) more slowly but more irreversibly than DDVP. Although a single injection of DDVP increased the concentrations of total, extraterminal, intraterminal and cytoplasmic acetylcholine (ACh) remarkably shortly after injection, MSF was still as effective at 24 h as 3 h after administration in increasing the concentrations of fractional ACh. Repeated injection of MSF for 3 d showed a significant reduction in the activity of AChE one day after cessation with a slight recovery 5 d later. Repeated administration of DDVP for 10 days showed a less significant reduction in the activity of AChE one day after cessation with considerable recovery 14 d later. Although a single injection of DDVP showed suppressive effects on locomotor activity, rectal temperature and rotarod performance in mice, the administration of MSF did not produce any significant effects, while DDVP suppressed locomotor activity and rectal temperature during and after the term of repeated injection. MSF showed a significant suppressive effect only at the 3rd day without causing any other changes during or after the term of repeated injection. In conclusion, MSF causes similar, but longer lasting effects on cholinergic mechanisms than DDVP and has fewer suppressive effects on behavioral parameters than DDVP.

Key words —— methanesulfonyl fluoride, DDVP, Alzheimer's disease, anticholinesterase, behavior, cholinergic

INTRODUCTION

Alzheimer's disease typically involves significant loss of basal forebrain cholinergic neurons[1] which may be responsible, at least in part, for the cognitive impairment that is characteristic of this disease. Therefore, facilitation of cholinergic activity in the CNS through the use of short-acting cholinesterase (ChE) inhibitors, such as physostigmine, tacrine, donepezil, and rivastigmine, has become a strategy for the treatment of senile dementia of the Alzheimer's type (SDAT).[2–5] The efficacy of short-acting inhibitors, however, may be restricted by dose-limiting side effects that minimize the level of inhibition that can be obtained *in vivo*.[6,7] The undesirable characteristics of short-acting inhibitors seem to be related, in part, to the short duration of action and the lack of steady state inhibition.[8]

By the use of long-acting inhibitors, however, it has been found that a full range of acetylcholinesterase (AChE) inhibition in the brain (*e.g.*, up to 90%) may be produced without toxicity or interfering side effects.[6,7] Therefore, the use of long-acting inhibitors might provide a better test of the clinical improvement that might be expected from ChE inhibitors.[6,9]

A recent double-blind, placebo-controlled study of SDAT[6] tested the efficacy of 2,2,2-trichloro-1-hydroxyethyl dimethyl phosphonate (metrifonate), an organophosphate that is converted nonenzymatically into a long-acting irrevers-

*To whom correspondence should be addressed: Pharmacology, Departments of Veterinary Medicine, Faculty of Agriculture, Iwate University, Ueda, Morioka 020–8550, Japan. Tel.: +81-19-621-6213; Fax: +81-19-621-6215; E-mail: hk1664@iwate-u.ac.jp

ible ChE inhibitor, 2,2-dichlorovinyl dimethyl phosphate (DDVP, dichlorvos).[10] Metrifonate was first introduced as an insecticide[11] (as trichlorfon), then as a drug to treat schistosomiasis,[12] and lastly tested as a treatment for SDAT.[6] In spite of the expectation that a long-acting ChE inhibitor might produce better results, the outcome obtained with metrifonate as a treatment for SDAT was very disappointing. Becker *et al.*[6] concluded "from a clinical perspective ... acetylcholinesterase inhibition cannot significantly improve cognitive performance in SDAT."

In the first ever clinical use of a sulfonyl fluoride, a small double-blind, placebo-controlled trial with methanesulfonyl fluoride (MSF) showed significant clinical improvement in SDAT.[9] The strong positive outcome with MSF suggested that real improvement in cognitive functions in SDAT could, in fact, be expected from ChE inhibitors.

Although metrifonate (through conversion to DDVP[10]) and MSF are both long-acting inhibitors of acetylcholinesterase, they are different in many ways. For example, metrifonate is a stronger inhibitor of butyrylcholinesterase (BChE) than AChE whereas MSF is highly selective for AChE.[13,14] Although selectivity toward AChE compared to BChE might be the reason for different effects on cognition, there are other factors.

Another difference between metrifonate and sulfonyl fluorides is with regard to the duration of inhibition. In one study of the effect of metrifonate on rat brain ChE, brain enzyme inhibited to 74% immediately after intramuscular administration, decreased to 50% in three hours and 26% inhibition remained at six hours,[15] a half-time for recovery of only 4 h.

In patients who had prior exposure to metrifonate, inhibition of red blood cell (RBC) AChE from a test dose of metrifonate recovered with a half-time of only 7 h.[13] In patients with no prior exposure to metrifonate, recovery required a half-time of about 26 d.[13] By comparison, recovery of human RBC AChE inhibition produced by MSF required a half-time of 43 d, a half-time consistent with new synthesis of RBCs.[9]

The differing recovery rates of AChE activity after metrifonate has been explained as a rapid early partial recovery due to spontaneous reactivation followed by a slower phase consistent with new synthesis of the enzyme.[16] The pharmacodynamics of metrifonate and DDVP appear to be much more complex and variable than MSF.

Of course, ChE inhibitors are expected to affect acetylcholine levels. However, the relationship to ChE inhibition may not be direct. The availability of choline is regulated through high-affinity choline uptake (HACU), which appears to play an important role in the regulation of ACh synthesis in functioning cholinergic neurons. It has been reported, for example, that HACU, a rate-limiting step for the synthesis of ACh.[17,18] in the cerebral cortex and hippocampus was decreased when the activity of AChE was severely inhibited following the administration of soman.[19] Similar data, however, are not available regarding the effects of DDVP and MSF on HACU. Therefore, it was important to determine if DDVP or MSF affected total ACh or caused different distribution of ACh among fractional pools (*e.g.*, extraterminal ACh, cytoplasmic ACh, vesicular ACh).

In addition to direct effects on ChE activity, there may be other direct or indirect effects on receptors. Chronic administration of organophosphates (but not carbamates) or muscarinic agonists causes a decrease in the number of muscarinic receptor sites measured by high-affinity binding of antagonists such as quinuclidinyl benzilate (QNB) in rodent brain.[20–23] It is well known, for example that repeated doses of organophosphate ChE inhibitors induces tolerance by decreasing the density of muscarinic receptor sites in rodent brain tissue.[24–26] Such down-regulation of receptors might be caused by long-term accumulation of high levels of acetylcholine in the synaptic cleft. Similar experiments have not been conducted with a sulfonyl fluoride. Some ChE inhibitors may also bind directly to muscarinic AChR (mAChR).[20–27] Repeated doses of organophosphates and sulfonyl fluorides may have differential effects on mAChR binding. Therefore, an important part of the present investigation was to measure the effects of MSF and DDVP on mAChR binding.

It has also been reported that DDVP, but not the parent compound metrifonate, strongly inhibits rat brain mitochondrial cytochrome oxidase even at low doses (2% of LD_{50}) when given daily for 14 d.[28] Similar studies of MSF are not available.

In view of the apparent differences, it seemed

important to better characterize the actions of MSF and metrifonate (or DDVP) on the cholinergic system. It is hoped that such information will provide insights that will improve the practical uses of ChE inhibitors in treating central cholinergic dysfunction like that in SDAT.

The purpose of the present study was, therefore, to explore the differences between MSF and DDVP (the active metabolite of metrifonate) on the cerebral cholinergic system and behavior in mice. These effects were compared on acetylcholinesterase inhibition, concentrations of cerebral acetylcholine concentrations (total, extraterminal, intraterminal or cytoplasmic and vesicular pools), binding on muscarinic receptors, high affinity choline uptake, and behavior, including locomotor activity, rotarod performance, and rectal temperature.

MATERIALS AND METHODS

Animals and Administration —— Female ICR mice (8 to 12 weeks old) weighing 25 to 32 g were allowed free access to food and water in a room kept at 23± 1℃ with a 12-h light (8 : 00—20 : 00) : 12-h dark photoperiod.

Fifty mg of MSF (Aldrich, Milwaukee) was dissolved in xylene (0.5 ml), Sorpol-2934 (0.5 g) and diluted in sterile physiological saline to 0.15 mg/ml. Control mice received MSF-free solution. In one experiment, MSF was injected subcutaneously once (1.5 mg/kg). In a second experiment, it was injected for 3 consecutive days (1.5 mg/kg/d). Since this dose of MSF caused a long-lasting inhibition of brain AChE and was sometimes lethal following repeated injection for more than 5 d, it was only administered for 3 d in the second experiment.

DDVP (99.1%) was kindly supplied by Nippon Soda Co., Ltd., Tokyo. One g of DDVP was dissolved in xylene (0.59 ml) and Sorpol-2934 (0.21 ml ; Nippon Soda Co., Ltd.) and diluted with sterile distilled water to get 5.0 mg/ml solution. Control mice received DDVP-free solution. One ml of this dilution was added with 4 ml of sterile physiological saline to get 1.0 mg/ml solution. In one experiment, DDVP was injected subcutaneously once (10 mg/kg). The mice showed salivation, lacrimination, miosis, and trembling 10 to 60 minutes after 10 mg/kg DDVP and a higher dose could not be used. Since 10 mg/kg DDVP produced less ChE inhibition than the MSF dose (data presented in Results), in the second experiment DDVP was injected for ten consecutive days (10 mg/kg/d) in an attempt to accumulate about the same amount of inhibition as with MSF.

Acetylcholinesterase Activity —— As for all neurochemical assays, after decapitation the cerebral cortex was quickly dissected on a plate cooled with ice. For acetylcholinesterase assays, the cortex was homogenized in 0.1 M phosphate buffer (pH 8.0) using a polytron (Biotrona, Switzerland, setting 10). The activity of acetylcholinesterase in the homogenate was determined by the method of Ellman *et al.*[29] at 25℃ by incubation for 4 min, with 0.48 mM acetylthiocholine iodide as substrate.

Acetylcholine Content —— When rat brain tissue is homogenized in an isotonic sucrose solution containing antiChE, three fractions of ACh are detected.[30-32] Therefore, for the determination of ACh content, the cortex was plunged into 4 ml of an ice-cold 0.32 M sucrose solution containing 1 μmol physostigmine salicylate, and then homogenized in a glass/Teflon homogenizer. ACh was then quantified using the physostigmine-sensitized rectus abdominis muscle from the frog ; the sensitivity of this assay was 0.5 nmol ACh chloride/ml.[31] Two subcellular pools of ACh [extraterminal ACh and cytoplasmic ACh] and total ACh were determined experimentally.[31] Vesicular and intraterminal ACh were then calculated from the values obtained experimentally.

In Step I, to quantify extraterminal ACh, 1 ml of the cortex homogenate was mixed into 9 ml of physostigmine-free frog Ringer solution (25℃) immediately after preparation of the homogenate. The bioassay for extraterminal ACh was started within 1 min.

In Step II, to analyze cytoplasmic ACh, one part homogenate was suspended in two parts ice-cold water for hypotonic treatment for 5 min to rupture nerve terminals and release cytoplasmic ACh. This hypotonic suspension, which now made both extraterminal and cytoplasmic ACh available to act on the rectus abdominis, was then added to an equal volume of 29%-concentrated, physostigmine-free frog Ringer solution to restore isotonicity 1 min prior to the determination of ACh. The amount of cytoplasmic ACh was calculated by subtracting the amount of extraterminal ACh from the value obtained.

In Step III, for the determination of total ACh, the same Ringer solution as described in Step II was added to the remainder of the suspension. The resulting suspension was adjusted to pH 4 with 1 M HCl and immersed in boiling water for 2 min. After adjustment of the mixture to pH 6.8 with NaOH, the sample

was used for the determination of total ACh.

From values determined experimentally in Steps I, II and III, the amount of vesicular ACh was calculated by subtracting the amount of extraterminal and cytoplasmic ACh from the total amount of ACh. Intraterminal ACh was calculated as the sum of cytoplasmic ACh plus vesicular ACh.

Muscarinic Receptor Binding —— [^{3}H]QNB binding to mAChRs was assayed in cerebral cortex homogenized in 20 volumes of an ice-cold Tris-HCl buffer (20 mM Tris, 120 mM NaCl, 5 mM KCl, pH 7.4) with a polytron. Twenty μl of this homogenate and 960 μl of Tris-HCl buffer were incubated with 10 μl of [^{3}H]QNB (final concentrations: 0.067, 0.096, 0.29, 0.48 and 0.80 nM; 1613 GBq/mmol; NEN) in the presence or absence of 10^{-6} M atropine at 25℃ for 40 min. The binding was quantified by the method of Yamamura and Snyder[33]. Specific binding was determined by subtracting nonspecific binding in the presence of atropine from total binding in its absence. Scatchard analysis[34] of the specific-binding data was performed to determine the equilibrium dissociation constant (K_d) and the maximal binding capacity (B_{max}).

High Affinity Choline Uptake —— High affinity choline uptake (HACU) was assayed in a synaptosomal preparation from cerebral cortex by the method of Gray and Whittaker[35]. HACU was determined after incubation of the synaptosomal preparation at 37℃ for 5 min with 1.0 μM [^{3}H]choline chloride[36]. The material used was [1-methyl-^{3}H] choline chloride (3281.9 GBq/ mmol) purchased from New England Nuclear (NEN), Boston. The values of the apparent dissociation constant (K_m) and maximal velocity (V_{max}) were determined by Lineweaver-Burk analysis.

Quantification of protein —— Protein was quantified by the method of Lowry *et al.*[37] with bovine serum albumin as the standard.

Behavioral experiments —— Locomotor activity, rectal temperature and rotarod performance were determined in that order.[38] Briefly, one minute after placing a mouse on the field (30 × 45 cm) of Animex IIIA (Shimadzu Co., Kyoto), the locomotor activity of the mouse was measured for 5 min. Immediately after recording the activity, the rectal temperature of the animal was determined by a Telethermometer (Model MGA-III, Natsume, Tokyo). After recording the temperature, performance was assessed on the Rotarod (Model KN-75, Natsume, Tokyo). The animals were placed on a rod (30 mm in diameter) rotating at 5.5 rpm. The animals were previously trained on these apparatuses twice a day for 2 days in order to minimize exploratory behavior and so they could reliably stay on the rotating rod for longer than 180 sec.

Statistics —— Data are expressed as mean±S.E. The statistical significance of differences was determined by Student's t-test.

RESULTS

Behavioral Estimation

DDVP at 10 mg/kg produced salivation, lacrimation, miosis and trembling from 10 to 60 min after injection. MSF at 1.5 mg/kg did not produce any of these signs for the 12 h observation period. The equivalence of these doses will be compared in the results from the ChE assays.

As shown in Fig. 1A, B and C, a single dose of DDVP (10 mg/kg) produced marked suppression of locomotor activity, rectal temperature and rotarod performance for at least 2 h. A single dose of MSF (1.5 mg/kg) produced no significant changes as compared to the control mice.

As shown in Fig. 2A, a repeated administration of MSF showed a transient suppression of locomotor activity. As expected from the single injection experiment, DDVP suppressed the locomotor activity during and after repeated treatment (Fig. 3A). Repeated treatment with either MSF or DDVP did not produce significant changes in the rectal temperature (Figs. 2B, 3B) or rotarod performance (data not shown).

Acetylcholinesterase Activity

As shown in Fig. 4A, a single injection of MSF produced a peak of about 70% inhibition after about 6 h. A single injection of DDVP produced a peak of about 88% inhibition within less than 60 min. Cortical AChE recovered to about 80% activity by 24 h after DDVP, time of continued near maximum inhibition by MSF. In contrast to DDVP, cortical AChE activity recovered only to 70% activity 10 d after MSF.

Figure 4B shows the activity of cortical AChE 24 h and 5 d after the third daily injection of MSF and 24 h and 14 d after the tenth daily injection of DDVP. The activity of AChE 24 h after repeated injections of DDVP and MSF was 35% and 17%, respectively. The activity 5 d after repeated MSF treatment was about 32% and that 14 d after repeated injection of DDVP was approximately 70%.

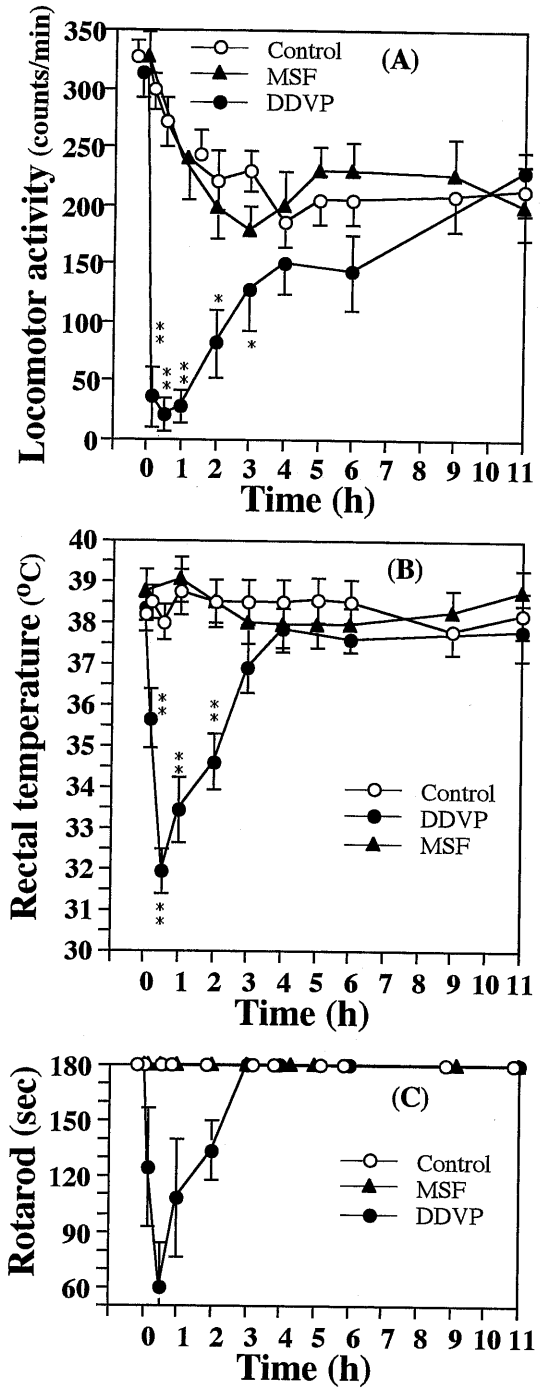

Fig. 1. Effects of Single Injection of MSF and DDVP on Locomotor Activity, Rectal Temperature and Rotarod Perfomance in Mice

(A) The locomotor activity of mice was determined for 5 min and indicated as counts per min. (B) The rectal temperature was expressed as ℃. (C) The rotarod performance of mice was scored. Data are expressed as mean±S.E.M. (n=8—9). Asterisks indicate experimental values that are significantly different from respective control values (*p<0.05; **p<0.01).

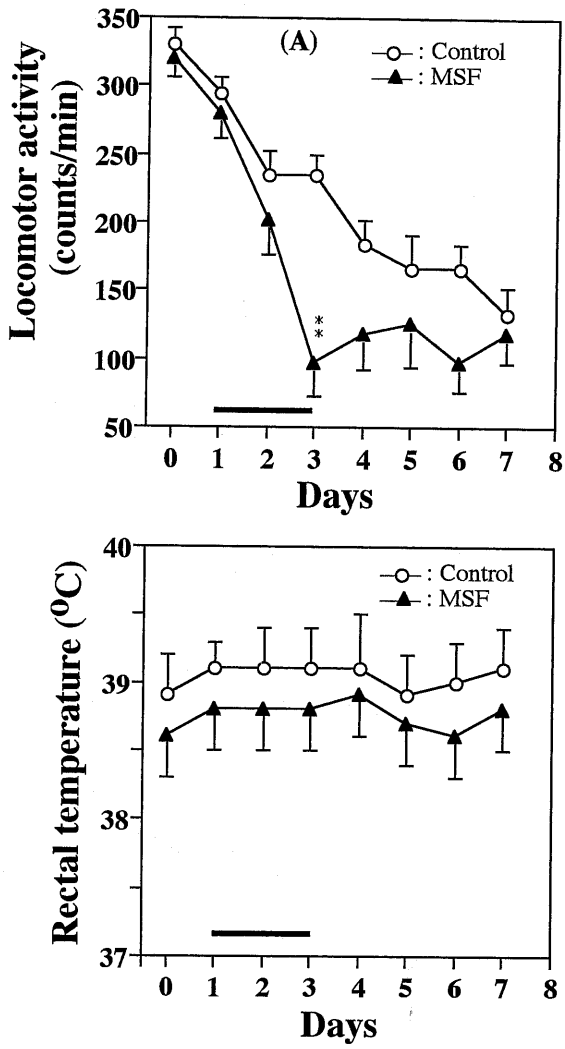

Fig. 2. Effects of Repeated Injection of MSF on Locomotor Activity and Rectal Temperature

Mice were injected with MSF for 3 d (▬).

(A) The locomotor activity of mice was determined for 5 min and indicated as counts per min. (B) The rectal temperature was expressed as ℃. Data are expressed as mean±S.E.M. (n=8—9). Asterisks indicate experimental values that are significantly different from respective control values (**p<0.01).

A semi-log plot (pseudo-first order) of AChE recovery (Fig. 5) at 30, 60, and 180 min and 24 h after a single injection of DDVP shows a half-time of 0.51 d. In contrast to recovery of AChE activity after a single injection of DDVP, AChE recovery at 360 min, 24, 48, 72, 192, and 240 h after a single injection of MSF shows a half-time of about 8 d for AChE recovery. Similar computations applied to the data shown in Fig. 4B suggests that recovery from inhibition produced by repeated doses of MSF and DDVP has a half-time of about 14 and 12 d, respectively.

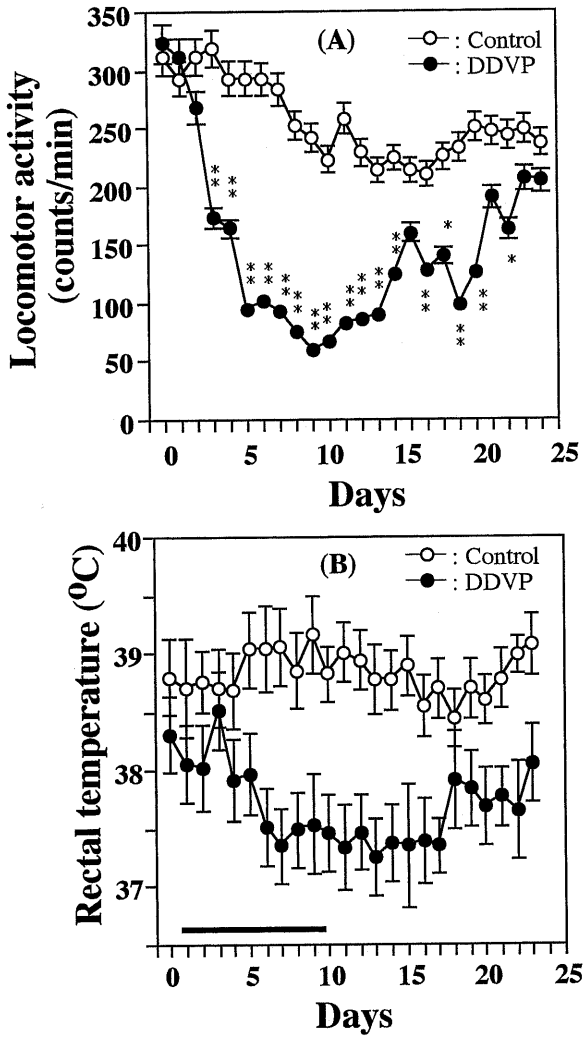

Fig. 3. Effects of Repeated Injection of DDVP on Locomotor Activity and Rectal Temperature

Mice were injected with DDVP for 10 d (▬). (A) The locomotor activity of mice was determined for 5 min and indicated as counts per min. (B) The rectal temperature was expressed as ℃. Data are expressed as mean±S.E.M. (n=8—9). Asterisks indicate experimental values that are significantly different from respective control values (*p< 0.05; **p<0.01).

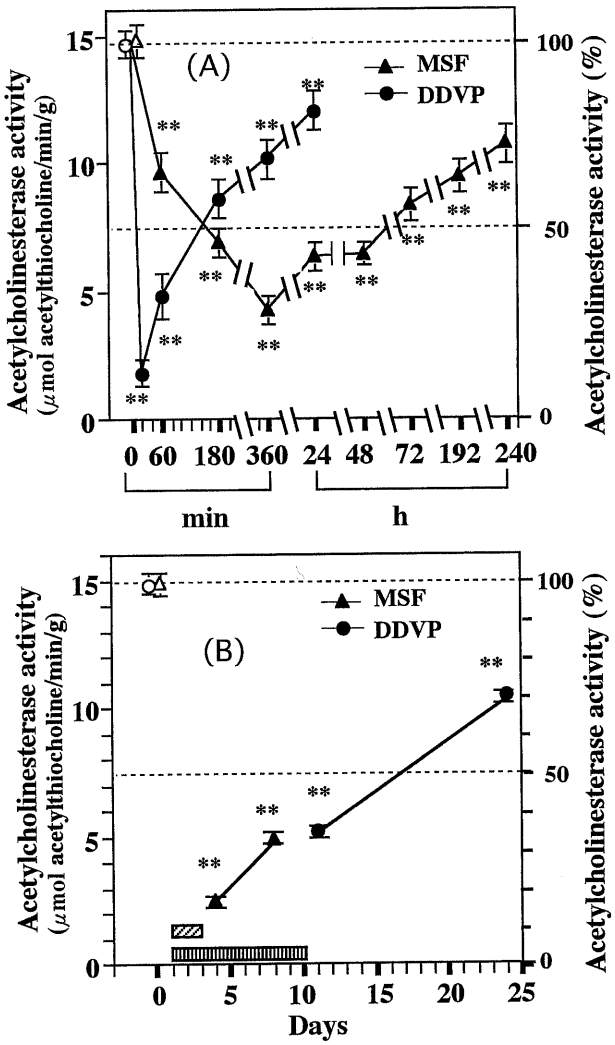

Fig. 4. Effects of Single or Repeated Injection of MSF and DDVP on the Activity of Acetylcholinesterase in the Cerebral Cortex of Mice

(A) Single injection. (B) Repeated injection.

The activity of AChE was determined after single injection of MSF (▲) or its vehicle (△) and DDVP (●) or its vehicle (○).

Data are expressed as mean±S.E.M. (single injection, n=8—10; repeated injection, n=5—10). Asterisks indicate experimental values that are significantly different from respective control values (**p<0.01).

Acetylcholine Content

Figure 6 shows cortical ACh content after a single injection of MSF or DDVP. MSF increased total ACh at both 3 and 24 h postinjection (Fig. 6A). DDVP also increased total ACh at 20 min but not at 24 h postinjection (Fig. 6B). The first time point (20 min or 3 h for DDVP and MSF, respectively) was selected to approximate the peak of AChE inhibition. The content of extraterminal ACh was increased by both MSF and DDVP at both times postinjection (Fig. 6A and 6B). MSF and DDVP both increased the concentration of intraterminal ACh at the first time point for each compound, primarily because of increases in cytoplasmic ACh (Fig. 6A and 6B).

Figure 7 shows cortical ACh content after repeated injections of MSF or DDVP. Total and extraterminal ACh were increased 1 d after repeated injections of MSF and DDVP. At this time point, cortical AChE was inhibited 83% and 65% by MSF and DDVP, respectively (Fig. 4B). Changes in total ACh 5 d or 14 d after the repeated treatment with MSF or DDVP were not significant. However, the content of extrater-

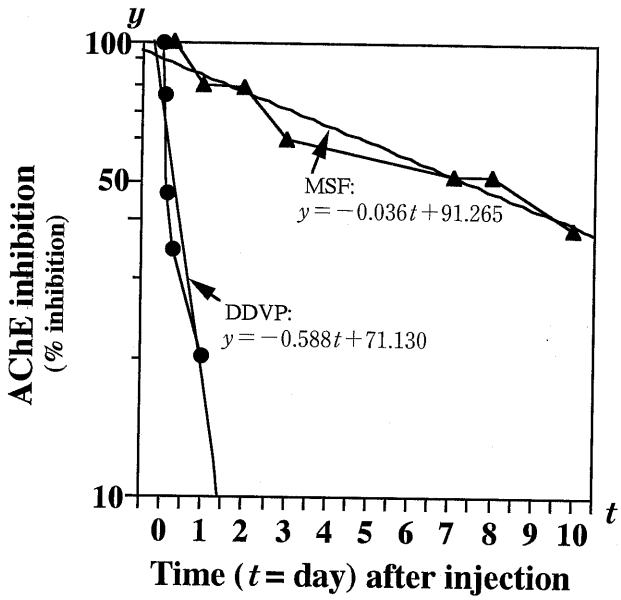

Fig. 5. Spontaneuos Reactivation of the Acetylcholinesterase Activity Inhibited by Single Injection of MSF and DDVP

Based on the values in Fig. 4A, the recovery of AChE activity inhibited by a single injection of DDVP or MSF was analyzed by a log-equation. The highest degree of enzyme inhibition was taken as 100% inhibition (log 100=2) and results were illustrated as other time intervals. The theoretical (straight line) and the experimental (plotted line) curves were compared. According to the theoretical curve, the half-life ($t_{1/2}$) for spontaneous reactivation and the time (t_0) required for complete recovery to the control level (0% inhibition) were calculated.

minal ACh remained significantly increased at 5 d and 14 d after the repeated injection of MSF or DDVP, respectively. No significant changes were seen in the concentration of intraterminal (cytoplasmic and vesicular) ACh after repeated administration of MSF or DDVP.

Table 1 shows that there was no change in K_d for QNB binding to cerebral membranes at any time point regardless of single or repeated injections of MSF or DDVP. There was, however, a significant reduction in B_{max} after even one injection of DDVP or after repeated injections of either MSF or DDVP. There was no change in B_{max} after a single injection of MSF.

Table 2 shows that there was a significant reduction in choline uptake after even one injection of DDVP or after repeated injections of either MSF or DDVP. There was no change in B_{max} after a single injection of MSF.

DISCUSSION

This study provides *ex vivo* and *in vivo* data concerning changes in subcellular cerebral ACh concentrations, AChE activity, HACU, mAChR binding, and behavior in mice after systemic administration of MSF and DDVP. There were many interesting differences between the effects of MSF and DDVP on both the neurochemistry of acetylcholine and behavior.

Although this was primarily a study of two irreversible ChE inhibitors, there were some interesting differences in the time course of AChE inhibition produced by DDVP and MSF. Since DDVP is an oxon-type organophosphate that can inhibit the activity of AChE without being oxidized, the inhibitory effect is expressed rapidly after exposure.[39] The maximal effect of DDVP to inhibit cerebral AChE was shown less than 1 h after a single injection, while that of MSF was between 3 and 24 h after a single administration.

The marked difference in the time-course of MSF and DDVP inhibition of AChE (Fig. 4A) was unexpected. If both of these compounds were truly stable irreversible inhibitors, then the time-course for recovery would depend upon new synthesis of AChE in the brain[7] which should be the same after both inhibitors. However, after single injections of DDVP or MSF, AChE activity recovered with half-times of 0.5 d and 8 d, respectively. These data suggest that DDVP-induced inhibition is at least partially reversible immediately after treatment.

The difference in recovery after each injection of DDVP or MSF made it very difficult to equate doses and effects over time when repeated injections were given. For example, repeated injections of MSF for 3 d inhibited cerebral AChE by about 83% and it was still inhibited about 67% 3 d after the final administration. On the other hand repeated injection of DDVP for 10 d, each inhibiting AChE more than each injection with MSF, inhibited cerebral AChE by only about 66% and it was still inhibited about 30% 14 d after the final administration. These results are consistent with significant reactivation of cerebral AChE after each injection of DDVP.

The difference between the half-times for AChE recovery after single injections of DDVP and MSF disappear after repeated daily injections (Fig. 4B) as shown by the half-times of 12 d

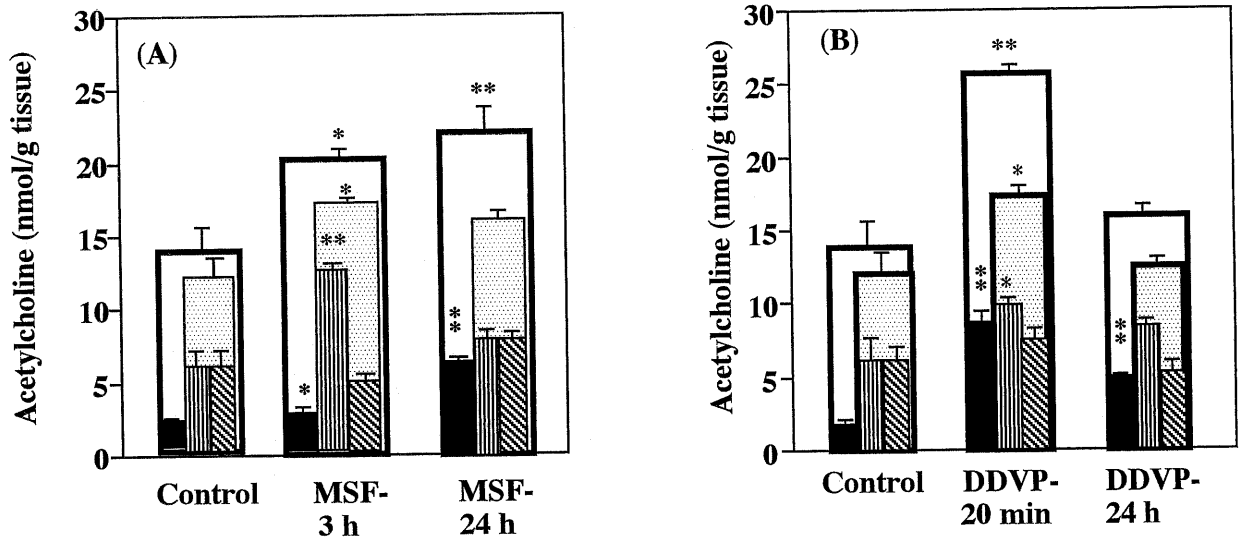

Fig. 6. Effects of Single Injection of MSF and DDVP on the Levels of Subcellular ACh in the Cerebral Cortex of Mice

A: The levels of subcellular ACh in the cerebral cortex of mice 3 h (MSF-3 h) and 24 h (MSF-24 h) after single injection of MSF. B: The levels of subcellular ACh in the cerebral cortex of mice 20 min (DDVP-20 min) and 24 h (DDVP-24 h) after single injection of DDVP. T-ACh (□) is the sum of extraterminal ACh (■) and intraterminal ACh (▨), which equals the sum of cytoplasmic ACh (▥) and vesicular ACh (▧).

Data are expressed as mean±S.E.M. ($n=5$). Asterisks indicate experimental values that are significantly different from respective control values (*$p<0.05$; **$p<0.01$).

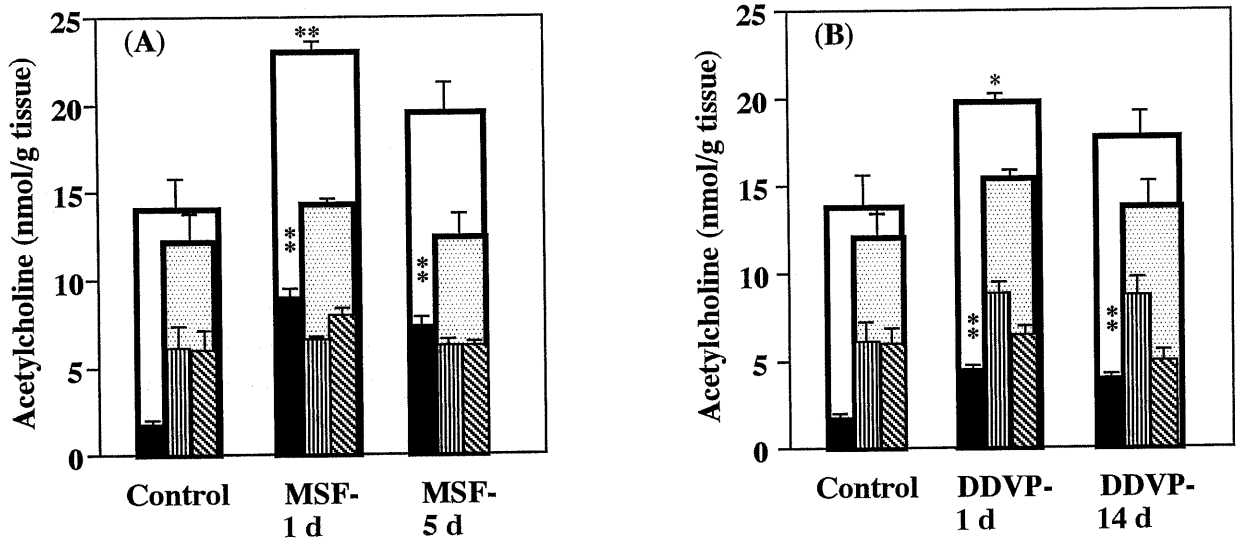

Fig. 7. Effects of Repeated Injection of MSF and DDVP on the Levels of Subcellular ACh in the Cerebral Cortex of Mice

A: The levels of subcellular ACh in the cerebral cortex of mice 1 d (MSF-1 d) and 5 d (MSF-5 d) after repeated injection of MSF. B: The levels of subcellular ACh in the cerebral cortex of mice 1 d (DDVP-1 d) and 14 d (DDVP-14 d) after repeated injection of DDVP. For further information see the legend to Fig. 6.

and 14 d for DDVP and MSF, respectively. These latter half-times for mouse cortex are virtually identical to the half-times of 11 and 12 d reported for resynthesis of AChE in whole rat brain[7] after MSF and rat cortex[40] after repeated injections of soman, another organophosphate ChE inhibitor. The half-times of 11—14 d probably reflects the rate at which AChE is synthesized in mouse and rat cortex.

Besides differing effects on AChE, another pronounced difference between MSF and DDVP was the appearance of marked salivation, lacrimation, miosis, and tremor after 10 to 60 min after DDVP but not after MSF. These signs involve both central and peripheral as well as muscarinic and nicotinic cholinergic mechanisms. For example, Sánchez and Meiser[41] reported that tremor is exclusively a centrally-mediated effect, whereas salivation involves both a central and a peripheral muscarinic component.

Table 1. The Binding of [^{3}H]Quinuclidinyl Benzilate (QNB) to the Cerebral Membrane Preparation from Mice Treated with MSF or DDVP

Treatment	[^{3}H]QNB binding	
	K_d (nM)	B_{max} (pmol/mg protein)
Control	0.10±0.02	0.96±0.06
MSF		
Single	0.08±0.02	0.94±0.04
Repeated	0.10±0.02	0.74±0.06*
DDVP		
Single	0.09±0.00	0.76±0.01*
Repeated	0.09±0.02	0.72±0.07*

Mice were killed 48 h and 24 h after single and repeated administration of MSF, respectively. Animals were killed 20 min and 24 h after single and repeated administration of DDVP, respectively. Data are expressed as mean±S.E.M. (n=3 to 8).
Significantly different from control value (p<0.05).

Table 2. The High-Affinity Uptake of Choline into the Cerebral Synaptosomal Preparation from Mice Treated with MSF or DDVP

Treatment	High-affinity choline uptake	
	Choline (pmol/mg protein)	Per cent
Control	25.3± 2.4	100
MSF		
Acute	22.8±10.2	90.1
Chronic	14.9± 1.6*	58.9
DDVP		
Acute	15.2± 1.5*	60.1
Chronic	12.8± 1.6*	50.6

Mice were killed 3 h and 24 h after single and repeated administration of MSF, respectively. Animals were killed 20 min and 24 h after single and repeated administration of DDVP, respectively. Data are expressed as mean±S.E.M. (n=3 to 8).
Significantly different from control value (p<0.05).

The appearance of salivation, lacrimation, miosis and tremor after a single injection of DDVP corresponded with the short-lived peak of ChE inhibition produced by this compound in the brain (Fig. 4A). These behaviors, however, suggest that DDVP has a greater effect on peripheral muscarinic and nicotinic cholinergic functions than MSF.

One reason why DDVP may affect peripheral cholinergic functions more than MSF may be related to the relative selectivity of these inhibitors for AChE and BChE. MSF is highly selective for inhibition of AChE, reacting with AChE about 20 times more rapidly than BChE.[14,42] By comparison, DDVP inhibits both AChE and BChE with about twice greater inhibition of BChE.[14] *In vivo*, DDVP produced 76.8 % inhibition of human plasma BChE with only 11% inhibition of erythrocyte AChE.[13] Because BChE is abundant in peripheral tissues,[43] an inhibitor of BChE will produce more severe peripheral cholinergic side effects. For example, moderate simultaneous inhibition of both AChE and BChE in smooth muscle, such as that produced by DDVP, produces more intense effects in peripheral smooth muscle than strong inhibition of either AChE or BChE alone.[44] In view of the differential effect of BChE inhibition, compared to AChE inhibition, it is not surprising that DDVP produced stronger peripheral effects.

Locomotor activity, rectal temperature and rotarod performance are known to involve central cholinergic mechanisms.[38] The experiments testing the effects of single injections of vehicle (control), MSF, and DDVP showed some striking differences (Fig. 1A, B, C). As expected from habituation and normal cholinergic function,[45] control mice showed reduced locomotor activity with repeated testing whereas measures of rectal temperature and rotarod performance were stable. MSF effects were not different from controls. In contrast, however, DDVP severely depressed locomotion and induced hypothermia and incoodination on the rotarod.

The temporal course of DDVP effects on locomotion, hypothermia and rotarod performance (Fig. 1) seems to correspond with the strong but short-lived peak of cortical AChE inhibition produced by DDVP after a single injection (Fig. 4A). In contrast, it is interesting that MSF, although producing slightly less peak inhibition, did not affect these behaviors after a single injection. It is important to note that repeated injections of MSF, producing about 83% inhibition (Fig. 4B), had only a small effect on locomotor behavior (Fig. 2). However, repeated injections of DDVP, which produced less inhibition (only about 66%, Fig. 4B), produced much more pronounced depression of locomotor behavior that persisted several days (Fig. 3). Therefore, there was poor correlation between degree of AChE inhibition and depression of locomotor behavior.

Differences between DDVP and MSF might, of course, also be explained on the basis of differences in effects on ACh. In control mice, the ratio of fractional pools of extraterminal ACh, cytoplasmic ACh, and vesicular ACh was about 12 : 44 : 44, respectively (Fig. 6). The total content of

the fractional ACh was increased by about 1.5 times 3 h after a single administration of MSF and about 1.8 times 20 min after a single administration of DDVP. The times were selected to correspond to the onset of cerebral AChE inhibition.

Treatment with a single injection of MSF or DDVP significantly increased the levels of extraterminal and cytoplasmic ACh in the cerebral cortex of treated mice (Fig. 6). The ratio of fractional pools of extraterminal ACh, cytoplasmic ACh, and vesicular ACh was about 13 : 61 : 25 in mice treated with MSF and about 33 : 39 : 28 in mice treated with DDVP. The greater increase in the level of extracellular ACh in mice injected with DDVP than with MSF seems to be due to a lower AChE activity.

The level of total ACh 24 h after administration of MSF was still significantly elevated in mice treated with MSF, but not DDVP. The content of extraterminal ACh after a single injection of MSF or DDVP was, however, still significantly higher than controls (Fig. 6). At 24 h after a single injection, the ratio of fractional pools of extraterminal ACh, cytoplasmic ACh, and vesicular ACh was about 29 : 36 : 35 in mice treated with MSF and about 24 : 51 : 25 in mice treated with DDVP. These data suggest that MSF is more effective in maintaining the level of cerebral ACh high than DDVP.

Repeated injection of MSF and DDVP increased the total content of the fractional ACh by about 1.6 times and 1.4 times as measured one day after the last injection, respectively (Fig. 7). Although the level of extracellular ACh was significantly higher in animals treated with either substance, neither cytoplasmic nor vesicular ACh was significantly different from controls. The level of extracellular ACh at 1 and 5 d after injection of MSF was greater than that at 1 and 14 d after injection of DDVP. The ratio of fractional pools of extraterminal ACh, cytoplasmic ACh, and vesicular ACh was about 38 : 28 : 34 in mice treated with MSF and about 22 : 44 : 34 in mice treated with DDVP (Fig. 7). Therefore, there were important differences in the effects of MSF and DDVP on fractional pools of ACh.

As shown in Table 1, MSF did not cause any changes in affinity (K_d) or density (B_{max}) of [^{3}H]-QNB binding to mAChR, 48 h after a single administration. A single administration of DDVP caused a significant decrease in the value of B_{max} without changing the K_d value. However, in the case of repeated administration of either MSF or DDVP, there was a significant decrease in the value of B_{max} without causing a change in the value of K_d.

When mAChR binding is considered in the context of ACh changes, it should be recalled that a single injection of DDVP produced a greater increase in the level of extracellular ACh at 20 min than at 24 h. On the other hand, an increase in the extracellular ACh by a single administrataion of MSF was greater at 24 h than at 3 h. It appears that a down-regulation of cerebral mAChR develops when a marked and rapid increase or a sustained increase in the level of ACh in synaptic clefts is brought about. The level of extraterminal ACh was significantly higher in mice treated repeatedly with MSF and DDVP. These results suggest that MSF induced a down-regulation of cerebral mAChR after repeated application but not after a single administration.

Comparison of these mAChR results with those of a reversible inhibitor such as tacrine shows similarities. Long-term administration of high dose (>5 mg/kg) of tacrine has been shown to cause down-regulation of mAChRs.[46,47] However, tacrine at lower doses (0.3 to 3 mg/kg), at which inhibition of AChE is not maximal, did not affect the density of mAChRs.[48] At therapeutic doses of MSF (0.18 mg/kg three times per week)[9] or metrifonate (2.1 mg/kg single dose weekly),[6] inhibition of cerebral AChE would not be maximal. The effect of therapeutic doses of MSF or DDVP on mAChR binding may not be similar to the effects observed in these experiments with high doses.

In view of the significant changes in ACh concentration, it was also important to assess the effects of MSF and DDVP on HACU. HACU appears to play an important role in the regulation of ACh synthesis, which is found in the nerve terminals or synaptosomes, and in functioning cholinergic neurons.[17,18] It is expected that HACU is suppressed when the synaptosomal ACh is saturated or activated when the ACh is deficient or the turnover rate of synaptosomal ACh is high.[49] It has been reported that HACU in the cerebral cortex and hippocampus was decreased when the activity of AChE was severely inhibited following the administration of organophosphates.[19,25,26]

In the present experiments, a single injection of DDVP decreased the HACU. However, MSF did not alter the HACU in spite of the significantly higher intrasynaptosomal level of ACh. It is suggested that a single injection of MSF can allow ACh to accumulate in the cerebral cortex without suppressing the synthesis or the turnover rate of ACh. On the other hand, repeated administration of either MSF or DDVP suppressed the cerebral HACU. A long-term accumulation of excess ACh following repeated administration of MSF and DDVP may have driven a negative feedback control of the synthesis of ACh. By comparison, it has been reported that repeated administration of tacrine decreased the HACU in a number of cortical regions of rats.[50]

In the present experiments, there were many interesting differences between MSF and DDVP. In addition to the different effects on the cholinergic system studied here, there could be other differences not related to cholinergic function. For example, Becker and Giacobini[8] have concluded that many effects of ChE inhibitors may not be related to ChE inhibition itself but to other drug-specific properties. DDVP may have drug-specific properties not shared by MSF. For example, organophosphates have long been known to produce a wide range of noncholinergic effects on the nervous system, perhaps by interacting with endogenous phosphorylation sites.[51,52] Protein phosphorylation regulates many processes in nerve cells, including modulating ion channels.[51,52] Sulfonyl fluorides cannot phosphorylate. Additional drug-specific properties of MSF or DDVP, not related to cholinergic function, cannot be ruled out as contributing to the differences observed here. However, the clear differences between MSF and DDVP in these experiments may explain widely dissimilar clinical results obtained with metrifonate and MSF. Metrifonate was reported to produce no clinical improvement in patients suffering with senile dementia,[6] whereas there was clear long-lasting improvement with MSF treatment.[9]

On the basis of the findings described above, it is concluded that single administration of MSF inhibited the activity of AChE more slowly and more irreversibly, and increased levels of intrasynaptosomal and extrasynaptosomal ACh in the cerebral cortex of mice more slowly than DDVP. A single injection of MSF, unlike DDVP, did not alter mAChR binding nor HACU and it did not produce signs of peripheral toxicity (salivation, lacrimation, miosis, and tremor) nor did it interfere with locomotor behavior, rectal temperature, or rotarod performance. Repeated daily injections of MSF at a high dose inhibited the activity of AChE severely and irreversibly, and accumulated the levels of intrasynaptosomal and extrasynaptosomal ACh in the cerebral cortex of mice which reduced binding ability of mAChRs and HACU. These results suggest that MSF might be useful in the treatment of SDAT at controlled doses given at appropriate dosing intervals.

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