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Effects of metrifonate on memory impairment and cholinergic dysfunction in rats

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Abstract

Metrifonate is an organophosphorous compound that has been used in the treatment of schistosomiasis. In this study, we investigated the effects of metrifonate on the impairment of learning and on central cholinergic dysfunction in scopolamine-treated and basal forebrain-lesioned rats. Oral administration of metrifonate (5.0–15.0 mg/kg) ameliorated the scopolamine- and basal forebrain lesion-induced learning impairment in the water maze and passive avoidance tasks. Metrifonate (50 and 100 mg/kg) also significantly increased extracellular acetylcholine levels but decreased choline levels in the cerebral cortex of the basal forebrain-lesioned rats. The basal forebrain lesion decreased the cholinesterase activity in the cerebral cortex, and metrifonate (100 mg/kg) further reduced the cholinesterase activity. However, cholinesterase inhibition was not observed at the dose that ameliorated learning impairments. These results indicated that metrifonate ameliorated the impairment of learning in both scopolamine-treated and basal forebrain-lesioned rats by not only increasing extracellular acetylcholine levels by inhibiting cholinesterase, but also by undefined other mechanism(s). This finding suggests the usefulness of metrifonate for the therapy of Alzheimer's disease.

Keywords: Alzheimer's disease; Acetylcholine; Basal forebrain lesion; Cholinesterase inhibitor; Scopolamine; (Rat)

1. Introduction

Alzheimer's disease, the most common dementia in the elderly, causes severe cognitive dysfunction. Hypofunction of various neuronal systems, particularly the cholinergic neuronal system, has been reported in these patients, as shown by a decrease in several cholinergic parameters, e.g., choline acetyltransferase and cholinesterase activity, choline acetyltransferase mRNA and acetylcholine receptors (Davies and Maloney, 1976; Rossor et al., 1982; Whitehouse et al., 1982; Coyle et al., 1983; Flynn and Mash, 1986; Strada et al., 1992). Since the cholinergic neuronal system plays an important role in learning and memory processes (Hepler et al., 1985; Nabeshima, 1993), this cholinergic hypofunction is thought to be related to the severity of cognitive dysfunction and memory loss in Alzheimer's disease patients (Sims et al., 1983; Bierer et al., 1995). Therefore, strategies for the therapy of Alzheimer's disease have largely focused on improving the cholinergic hypofunction.

There are various approaches for improving cholinergic function: increasing the synthesis of acetylcholine by administering precursors, stimulating acetylcholine receptors by administering acetylcholine agonists, and increasing extracellular acetylcholine and prolonging the effects of acetylcholine by administering cholinesterase inhibitors. The most promising results of the cholinergic approaches investigated thus far have been achieved by administering cholinesterase inhibitors such as tacrine. Several studies have reported the ameliorating effect of tacrine on impairment of learning and memory in experimental animals (Flood, 1985; Kwo-On-Yuen and Thal, 1989; Riekkinen et al., 1990; Nabeshima et al., 1991b). Moreover, some clinical studies have indicated that tacrine is effective in treating Alzheimer's disease (Summers et al., 1981, 1986). However, tacrine has been reported to be hepatotoxic (Kumar and Becker, 1989). The development of appropriate drugs for Alzheimer's disease that have no serious side-effects is necessary.

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Metrifonate (2,2,2-trichloro-1-hydroxyethyl dimethyl phosphonate) is an organophosphorous compound, but not a cholinesterase inhibitor per se. It is converted non-enzymatically in vivo to the active inhibitor dichlorvos (2,2-dichlorovinyl dimethyl phosphate; Nordgren et al., 1978). Metrifonate has been widely used for the treatment of schistosomiasis in humans since 1972. It causes no serious side-effects, although it markedly inhibits blood cholinesterase (Nordgren et al., 1981). Metrifonate shows long-lasting cholinesterase inhibition and few side-effects. Unni et al. (1994) have reported that, in humans without prior exposure to metrifonate, a single oral dose of metrifonate (5.0 mg/kg) decreased plasma and red blood cell cholinesterase activity to 88.2% and 25.2% of the control, respectively, and the $t_{1/2}$ of cholinesterase recovery was 9.0 days in plasma and 26.6 days in red blood cells. Although long-lasting cholinesterase inhibition was observed with a single dose of metrifonate, its $t_{1/2}$ in plasma was 2.3 h. This phenomenon reflects the irreversible cholinesterase inhibition exerted by metrifonate. The presence of minimal side-effects, based on the short drug $t_{1/2}$ and long cholinesterase recovery $t_{1/2}$, suggests the potential of metrifonate as a therapeutic drug for Alzheimer's disease. Additionally, Becker and Giacobini (1988) have suggested the usefulness of metrifonate for treatment of Alzheimer's disease in their clinical report.

It has been reported that metrifonate ameliorates the impairment of learning and memory in experimental animals (Blokland et al., 1996; Riekkinen et al., 1996; Van der Staay et al., 1996a,b). In these reports, the possibilities that mechanisms other than cholinesterase inhibition may be involved in ameliorating effects of metrifonate have been suggested. However, there are relatively few studies that simultaneously described the effects of metrifonate on both behavioral and neurochemical (i.e., cholinesterase inhibition and acetylcholine release) evaluations. In this study, to assess the possible use of metrifonate for the treatment of Alzheimer's disease and to identify the mechanisms of ameliorating effects of metrifonate, we investigated the effects of metrifonate on learning and memory, and on cholinergic dysfunction in rat amnesia induced by scopolamine or basal forebrain lesion.

2. Materials and methods

2.1. Animals

Male Wistar rats (Oriental Bioservice, Kyoto, Japan), weighing 280–320 g at the beginning of the experiments, were used. They were handled in accordance with the guidelines established by the Institute for Laboratory Animal Research of Nagoya University, and were housed in groups of three or four in a temperature- and light-controlled room (23°C, a 12-h cycle starting at 9:00 a.m.). They had free access to food and water. Each group consisted of 3–7 rats.

2.2. Basal forebrain lesion

The rats were anesthetized with sodium pentobarbital (35 mg/kg, i.p.) and fixed in a stereotaxic apparatus. Ibotenic acid (10 μg) dissolved in 0.5 μl of 50 mM sodium phosphate buffer (pH 7.4), was injected into the right basal forebrain (A −1.5, L 2.8, V 7.3 mm according to the atlas of Paxinos and Watson, 1986) to produce the lesion. One week later, the left side was lesioned. Sham-operated rats received 50 mM sodium phosphate buffer only (Nitta et al., 1993a,b). It has been reported that ibotenic acid-induced lesions are not selective for cholinergic neurons (Dunnett et al., 1987). However, we used this model in the present experiments, because no animal models of Alzheimer's disease have been developed yet and this model is easily prepared. Experiments were started 2 weeks after lesion of the left side. A decrease of choline acetyltransferase activity in the frontal and parietal cortex was observed at this time and lasted for over 3 months (Nitta et al., 1993a). Moreover, in our preliminary studies we could not observe significant changes in monoamine content in the brain.

2.3. Step-through passive avoidance task

A two-compartment (dark and illuminated) step-through passive avoidance apparatus (25 × 15 × 15 cm high) was used. The test consisted of an acquisition and a retention trial. In the acquisition trial, each rat was placed in the illuminated compartment; as soon as it entered the dark compartment through the guillotine door, the door was closed and an inescapable electric foot shock (3 mA, 5 s) was delivered through the grid floor. In the retention trial, given 24 h after the acquisition trial, the rat was placed in the illuminated compartment again and the time until it entered the dark compartment was measured as step-through latency. If the rat did not enter before 300 s had elapsed, a step-through latency of 300 s was assigned (Nitta et al., 1993a,b). We used a high intensity of electric shock because we let all control animals stay in the illuminated compartment until the cut-off time to facilitate detection of the impairment of learning in Alzheimer disease model animals.

2.4. Morris's water maze task

A circular water tank (140 cm diameter and 45 cm high) with a transparent platform set inside the tank (10 cm diameter, surface 2 cm below the surface of the water) was used (Morris, 1984). The pool was located in a large room, in which there were some cues external to the maze. The positions of these cues were not changed throughout the test. The test was conducted twice a day for 5 consecutive days, one session consisting of 2 trials (2 trials × 5 days). In each trial, the rat was placed in the water at one of five starting positions, with the sequence of the posi-

tions being selected randomly. The latency to escape onto the platform was measured. If the rat found the platform, it was allowed to remain for 15 s and was then returned to its home cage. If the rat could not find the platform within 90 s, the trial was terminated and a latency of 90 s was assigned (Nitta et al., 1993a,b).

2.5. Cholinesterase assay

Cholinesterase activity was measured by the method of Ellman et al. (1961) with a slight modification. The rats were killed by decapitation and the brain was removed and dissected. The brain tissues were homogenized with 25 mM phosphate buffer (pH 7.4) containing 0.5% Triton X-100. Centrifugation ($16\,000 \times g$) was performed for 20 min, and the supernatant was used for the enzyme solution. A mixture of 900 μl of substrate solution (5 mM acetylthiocholine and 0.5 mM dithiobisnitrobenzoic acid in 50 mM phosphate buffer, pH 7.4) was added to 100 μl of the enzyme solution, and this was incubated at 37°C for 30 min. The enzyme reaction was stopped by adding 2 ml of acetone. The mixture was cooled and centrifuged at 2000 $\times g$ for 15 min. The absorbance of the supernatant at 412 nm was measured. We had earlier confirmed that the reaction was linear under this condition and it is unlikely that substrate inhibition occurs under these conditions.

2.6. In vivo brain microdialysis

Brain microdialysis was performed by the method described previously (Hasegawa et al., 1993). Briefly, the rats were anesthetized with sodium pentobarbital (35 mg/kg, i.p.) and fixed in a stereotaxic apparatus. A guide cannula (AG-8; Eicom, Kyoto, Japan) was implanted diagonally (at a 30° angle against a cross-section) into the cerebral cortex (A −1.5, L 3.5, V 2.9 mm according to the atlas of Paxinos and Watson, 1986). Next day, Ringer's solution (147 mM NaCl, 4 mM KCl, 2.3 mM $CaCl_2$) containing 10^{-5} M physostigmine to inhibit the hydrolysis of acetylcholine was perfused through the dialysis probe (A-I-8-03, Eicom, Kyoto, Japan) at a constant flow rate of 2 μl/min. Perfusate was collected every 15 min and injected automatically into a high-performance liquid chromatography system with electrochemical detection. Acetylcholine and choline levels were monitored from 1 h before to 5 h after metrifonate administration. The mean concentration of each transmitter before drug treatment (4 measurements) was used as the basal value and the data were normalized with this basal value.

2.7. Experimental design

2.7.1. Effects of metrifonate on performance of the passive avoidance task in scopolamine-treated rats

We used two amnesic models in this study: one was scopolamine-treated rats and the other was basal forebrain-lesioned rats. In the first experiment, scopolamine-treated rats were tested in the passive avoidance task to assess the effective dose range of metrifonate, since this model and the task are more convenient and economical than the basal forebrain lesion model and the water maze task. Metrifonate and scopolamine (1.5 mg/kg) were co-administered 30 min before the acquisition trial.

2.7.2. Effects of metrifonate on performance of the water maze and passive avoidance tasks and on acetylcholine levels and cholinesterase activity in basal forebrain-lesioned rats

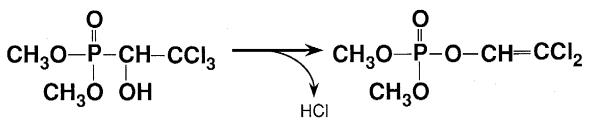
The second experiment was performed with basal forebrain-lesioned rats. Two weeks after the basal forebrain lesion (recovery period), behavioral tests (water maze and passive avoidance task), in vivo brain microdialysis and cholinesterase assay were carried out. As behavioral tests, the water maze task was performed first and then the passive avoidance task was carried out after the water maze task. Rats received metrifonate 30 min before each session of the water maze task and the acquisition trial of the passive avoidance task. For the cholinesterase assay, rats were decapitated 1 h after metrifonate administration.

2.8. Drugs

Scopolamine-HBr (Katayama Chemical Industries, Tokyo, Japan) or metrifonate (Bayer Yakuhin, Osaka, Japan) was dissolved in saline or 5% sodium citrate (pH 5.8, to ensure the change of metrifonate to dichlorvos, Fig. 1) and administered subcutaneously or orally, respectively. The reasons why we administered metrifonate and not dichlorvos are follows: (1) Metrifonate has already been used clinically; therefore, its safety for humans has been established. (2) Drug kinetics have shown that time to peak concentration and disappearance of dichlorvos in the brain is longer when it is administered as metrifonate than when it is administered as dichlorvos (Nordgren et al., 1978). Therefore, mild but long-lasting effects of cholinesterase inhibition are expected by metrifonate administration. The doses of metrifonate in behavioral tests were based on the work of Becker et al. (1990).

2.9. Statistical analysis

The data obtained from the water maze task and brain microdialysis were analyzed by two-way analysis of vari-



metrifonate
[2, 2, 2-trichloro-1-hydroxyethyl dimethyl phosphonate]

dichlorvos
[2,2-dichlorovinyl dimethyl phosphate]

Fig. 1. Chemical structures of metrifonate and dichlorvos.

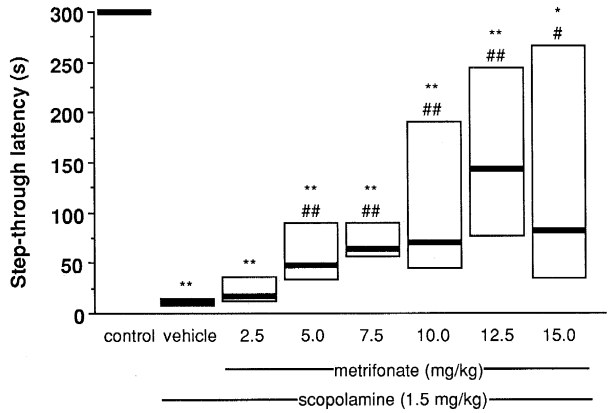


Fig. 2. Effects of metrifonate on performance of the passive avoidance task in scopolamine-treated rats. Values for step-through latency are shown as medians (horizontal bars) and interquartile ranges (vertical bars). Metrifonate was administered orally 30 min before the acquisition trial. $^{*}P < 0.05$, $^{**}P < 0.01$ vs. control; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ vs. vehicle.

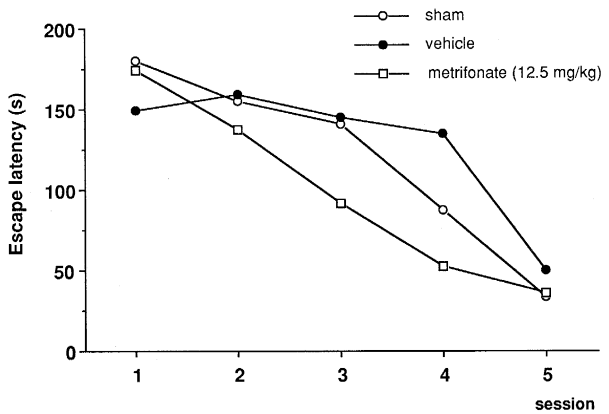


Fig. 3. Effects of metrifonate on performance in the Morris's water maze task in basal forebrain-lesioned rats. Metrifonate was administered orally 30 min before each trial. Each point represents the mean.

ance (ANOVA) and the data from the cholinesterase assay were analyzed by Student's *t*-test or one-way ANOVA. When significant differences were observed in ANOVA, Fisher's post-hoc test was used. In the passive avoidance task, the data were expressed in terms of medians and interquartile ranges and were analyzed by Mann-Whitney's *U*-test.

3. Results

3.1. Effects of metrifonate on impairment of performance in the passive avoidance task in scopolamine-treated rats

Step-through latency in the acquisition trial and general behavior were not changed by the treatment with scopolamine and metrifonate (data not shown). No differences were observed in the pain thresholds for electric foot shock (i.e., flinch, vocalization and jumping) among controls and drug-treated animals (data not shown). In the retention trial, control rats showed a long step-through latency, while the step-through latency of the scopolamine-treated rats was significantly shorter ($P < 0.01$). The administration of metrifonate (5.0, 7.5, 10.0, 12.5 and 15.0 mg/kg) significantly prolonged the step-through latency shortened by scopolamine (Fig. 2). The most effective dose of metrifonate was 12.5 mg/kg.

3.2. Effects of metrifonate on impairment of performance in Morris's water maze task in basal forebrain-lesioned rats

The mean values for the escape latencies of the 3 groups (to escape onto the hidden platform) in each session are shown in Fig. 3. The swimming distance and the latencies in the sham-operated group in the 1st session were not different from those in basal forebrain-lesioned group. The latencies of the 3 groups were decreased by repeated training. However, the latency of vehicle-treated rats with basal forebrain lesion was longer than that of the sham-operated rats ($F(1,4) = 12.62$, $P < 0.05$). Administration of metrifonate (12.5 mg/kg) ameliorated the basal forebrain lesion-induced impairment of learning ($F(1,4) = 5.040$, $P < 0.05$), and there was no significant difference between sham-operated and metrifonate-treated rats.

3.3. Effects of metrifonate on impairment of performance in the passive avoidance task in basal forebrain-lesioned rats

Sham-operated rats had a median step-through latency of 300 s. The basal forebrain lesion shortened the step-through latency to 52.4 s, indicating an impairment of learning. However, the administration of metrifonate (7.5

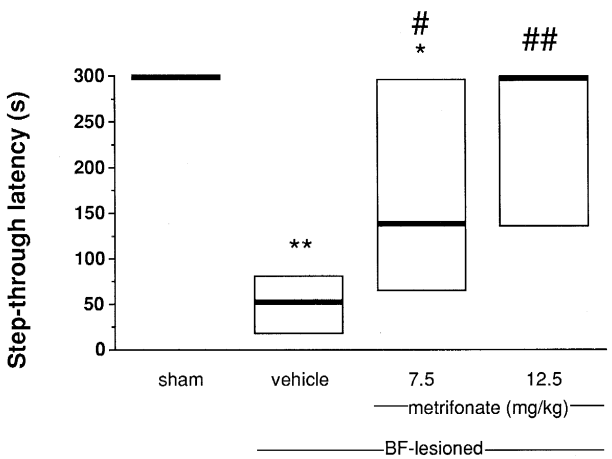


Fig. 4. Effects of metrifonate on performance in the passive avoidance task in basal forebrain-lesioned rats. Values for step-through latency are shown as medians (horizontal bars) and interquartile ranges (vertical bars). Metrifonate was administered orally 30 min before the acquisition trial. $^{*}P < 0.05$, $^{**}P < 0.01$ vs. sham, $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ vs. vehicle.

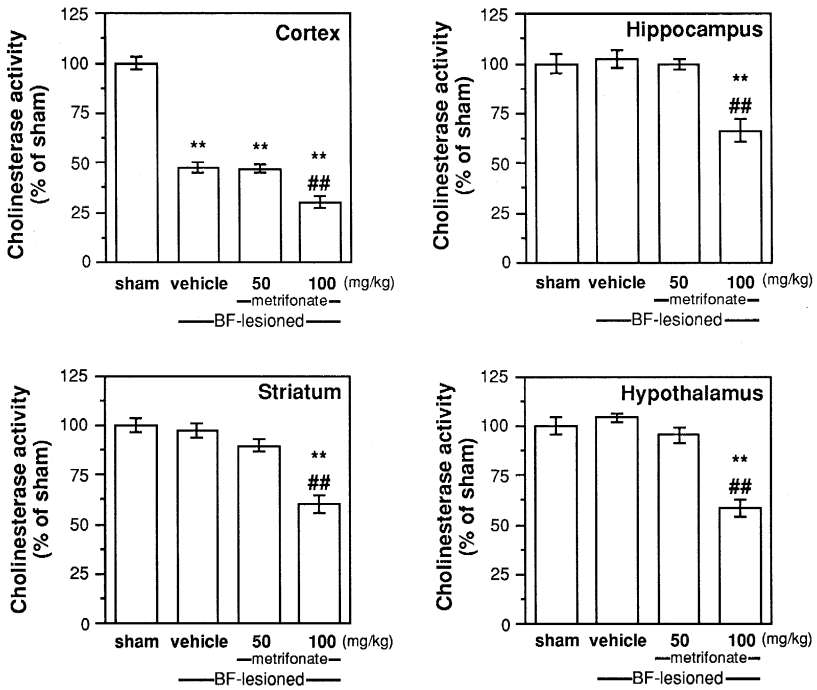


Fig. 5. Effects of oral administration of metrifonate on brain cholinesterase activity in basal forebrain-lesioned rats. Rats were decapitated 1 h after metrifonate administration. ** $P < 0.01$ vs. sham; $^{\#\#}$ $P < 0.01$ vs. vehicle.

and 12.5 mg/kg) 30 min before the acquisition trial prolonged the step-through latency to 138.27 and 298.97 s, respectively. There were significant differences in step-through latency between the vehicle-treated and the metrifonate (7.5 mg/kg)-treated rats ($P < 0.05$, Fig. 4). Metrifonate (12.5 mg/kg) reversed the shortened step-through latency induced by the basal forebrain lesion to the control level ($P < 0.01$, Fig. 4).

3.4. Effects of metrifonate on cholinesterase activity in basal forebrain-lesioned rats

Behavioral experiments showed beneficial effects of metrifonate, and therefore we investigated whether cholinesterase was decreased by metrifonate. In preliminary experiments, however, we could not find a significant change of cholinesterase activity 30 min after the oral administration of metrifonate (12.5 and 25.0 mg/kg, data not shown), and therefore we raised the dose of metrifonate (50 and 100 mg/kg). Fig. 5 shows the cholinesterase activity (percent of sham) in the cerebral cortex, hippocampus, striatum and hypothalamus of the sham-operated and the basal forebrain-lesioned rats. The cholinesterase activity in the sham-operated rats was: cortex, 907.03 ± 24.34; hippocampus, 1330.60 ± 69.12; striatum, 8322.37 ± 282.04 and hypothalamus, 1928.92 ± 80.24 μmol/h/mg protein. The basal forebrain lesion significantly reduced cholinesterase activity in the cerebral cortex ($P < 0.01$), but not in the hippocampus, striatum and hypothalamus. Metrifonate (100 mg/kg) further re-

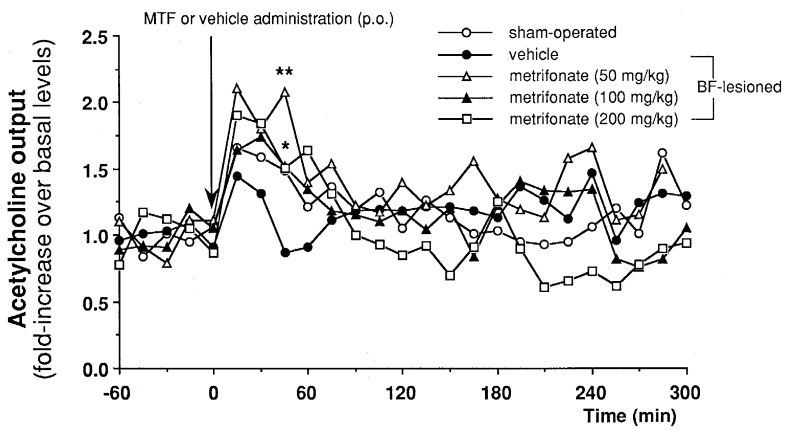


Fig. 6. Effects of metrifonate on extracellular acetylcholine levels in the cerebral cortex of basal forebrain-lesioned rats compared with sham-operated rats. Values were normalized as 'fold increase over basal levels'. * $P < 0.05$, ** $P < 0.01$ vs. vehicle.

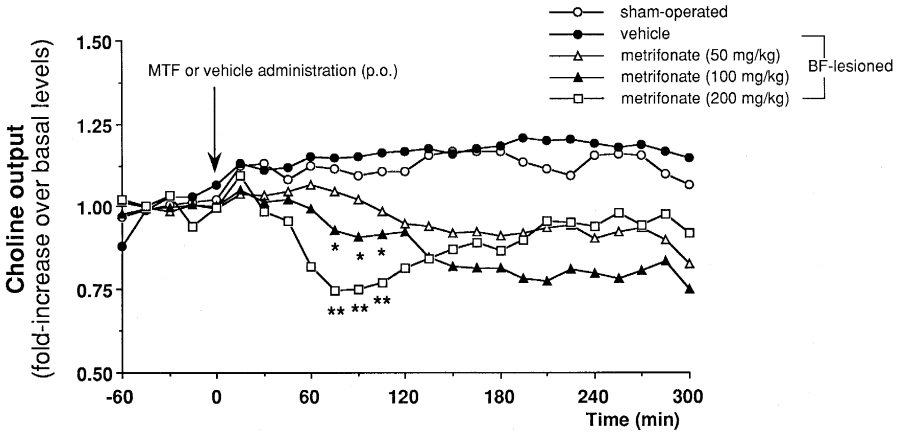


Fig. 7. Effects of metrifonate on extracellular choline levels in the cerebral cortex of basal forebrain-lesioned rats compared with sham-operated rats. Values were normalized as 'fold increase over basal levels'. * $P < 0.05$, ** $P < 0.01$ vs. vehicle.

duced cholinesterase activity in all regions tested compared with the vehicle-treated rats 1 h after administration ($P < 0.01$).

3.5. Effects of metrifonate on the release of acetylcholine and choline in basal forebrain-lesioned rats

The results of the cholinesterase assay showed that metrifonate (100 mg/kg) significantly decreased cholinesterase activity. Therefore, we investigated the effects of metrifonate on the release of acetylcholine and choline. The basal levels of acetylcholine and choline in the sham-operated and the basal forebrain-lesioned rats were: acetylcholine 1.65 ± 0.18 and 0.97 ± 0.07 pmol/15 min; choline 32.67 ± 1.44 and 31.62 ± 0.99 pmol/15 min, respectively. A significant difference was found in the basal release of acetylcholine between the sham-operated and the basal forebrain-lesioned rats ($P < 0.01$). Metrifonate (50, 100 and 200 mg/kg) significantly increased ($F(3,376) = 10.268$, $P < 0.01$) extracellular acetylcholine levels in the cortex compared to the vehicle-treated rats 45 min after administration (Fig. 6). In contrast to acetylcholine release, extracellular choline levels decreased significantly ($F(3,376) = 4.546$, $P < 0.01$) 45 min after metrifonate administration. The reduction lasted for more than 5 h (Fig. 7). The doses of metrifonate used in this experiment were high compared with those used in the behavioral experiments; however, severe behavioral abnormalities (e.g., salivation, muscular relaxation and respiratory insufficiency, which are side-effects of cholinesterase inhibitors) were not observed in our experimental conditions (data not shown).

4. Discussion

One of the most serious problems of Alzheimer's disease is cognitive dysfunction. It is generally agreed that the cholinergic neuronal system plays an important role in learning and memory. Moreover, postmortem pathological studies show that the cholinergic neuronal system is severely damaged in the Alzheimer's disease patient's brain (Whitehouse et al., 1982). These observations led to the hypothesis that the cognitive dysfunction in Alzheimer's disease may be due to cholinergic hypofunction. It has been reported that the severity of dementia is correlated with cholinergic hypofunction (Sims et al., 1983; Bierer et al., 1995). Therapeutic strategies for Alzheimer's disease have therefore focused on activating cholinergic neurotransmission. The administration of cholinesterase inhibitors to Alzheimer's disease patients is one of these strategies and is expected to be the most promising.

Although cholinesterase inhibitors (e.g., physostigmine, tacrine) ameliorate the impairment of learning in dementia animal models and in Alzheimer's disease patients (Summers et al., 1986; Flood, 1985; Kwo-On-Yuen and Thal, 1989; Riekkinen et al., 1990; Nabeshima et al., 1991a,b; Nitta et al., 1994), the use of these drugs entails some problems such as effective duration and side-effects (Kumar and Becker, 1989; Knapp et al., 1991; Somani et al., 1991). In contrast, metrifonate shows long-lasting cholinesterase inhibition and few side-effects (Unni et al., 1994). The presence of minimal side effects suggests the potential of metrifonate as a therapeutic drug for Alzheimer's disease. The most advantageous point is that metrifonate has been already clinically used in human and its safety has been confirmed.

In the first screening to determine the effective doses of metrifonate in the rats with scopolamine-induced amnesia, we found that metrifonate (5.0, 7.5, 10.0, 12.5 and 15.0 mg/kg) attenuated the impairment of performance in the passive avoidance task, the most effective dose being 12.5 mg/kg. Therefore, in the next series of experiments, we used this dose of metrifonate and investigated the effects of metrifonate in the basal forebrain-lesioned rats in Morris's water maze and passive avoidance tasks. The

cholinesterase assay showed that cholinergic neurons in the cortex, projecting from basal forebrain, were damaged in this model (Fig. 5), and impairments of learning were found (Figs. 3 and 4), in agreement with our previous experiments (Nabeshima et al., 1991a,b; Nitta et al., 1993b). Metrifonate improved the impairments of learning in both the water maze and the passive avoidance tasks in this model. These improvements of learning deficits can be partly explained by pharmacological properties of metrifonate, which include cholinesterase inhibition and an increase of acetylcholine levels, as shown in Figs. 5–7.

In this study, the doses that ameliorated the impairment of learning were lower than those that decreased the biochemical parameters such as cholinesterase activity. Doses that affect animal behavior often differ from those that cause biochemical changes (Yoshida and Suzuki, 1993; Ishii et al., 1994). Similar differences of effective doses of metrifonate between behavioral and biochemical parameters have been reported by Van der Staay et al. (1996b) and Hinz et al. (1995). Van der Staay et al. (1996a) found that 12.5 mg/kg but not higher doses of metrifonate improved the performance of the shuttle box avoidance task in rats. In opposition to their results, Hinz et al. (1995) reported that ex vivo cholinesterase inhibition was induced by much higher doses of metrifonate, with an ED_{50} of about 90 mg/kg. In the present behavioral study, the effective dose range was consistent with the results of Van der Staay et al. in the scopolamine-treated and basal forebrain-lesioned rats. Moreover, in our biochemical experiments, similar doses were required to change the acetylcholine release and cholinesterase activity. It is unlikely that the doses that decreased cholinesterase activity in our study substantially differ from those effective in the study of Hinz et al. (1995).

There are several explanations for this discrepancy in the effective doses between behavioral and biochemical studies.

(1) We found that 30 min after its administration (100 mg/kg), metrifonate did not decrease cholinesterase activity in the cerebral cortex, hippocampus, striatum and hypothalamus (data not shown) under our experimental conditions, although this dose reduced cholinesterase activity 1 h after administration. Thus, small undetectable changes in cholinesterase activity and/or in acetylcholine levels may be sufficient to ameliorate behavioral impairment.

(2) In the behavioral experiments, the basal forebrain-lesioned rats received metrifonate repeatedly. We did not investigate whether the repeated administration of metrifonate inhibited cholinesterase activity at the lower doses compared to those effective after single administration. As described in Section 1, the $t_{1/2}$ of cholinesterase recovery was 9.0 days. Therefore, there is a possibility that the long-lasting inhibition of cholinesterase induced by metrifonate may contribute to the ameliorating effects on the impairment of learning and memory.

(3) There is a possibility that metrifonate has other pharmacological actions not correlated to cholinesterase inhibition. It has been reported that cholinesterase inhibition decreased choline uptake by negative feedback in the presence of excess intraterminal acetylcholine (Yamada et al., 1983; Mattio et al., 1985). According to these reports, it is conceivable that the extracellular choline level is increased when metrifonate is administered; however, the present results show that metrifonate decreased extracellular choline levels and that the effect lasted for more than 5 h. In contrast, the increase in acetylcholine levels was transient. Moreover, we could not find detectable changes of cholinesterase activity in the brain of basal forebrain-lesioned rats 6 h after oral administration of metrifonate (100 mg/kg) (data not shown). Therefore, we can speculate that metrifonate decreases choline levels because of activation of choline uptake by unknown mechanisms. The existence of other unknown mechanisms of metrifonate besides cholinesterase inhibition, which contributes to the ameliorating effects on learning and memory, is also suggested by Van der Staay et al. (1996a,b). Further studies should be performed to determine the other pharmacological actions of metrifonate in the central nervous system in addition to cholinesterase inhibition.

(4) Amelioration of cognitive dysfunction by metrifonate might be due to activation of other neuronal systems as well as the cholinergic neuronal system. Mori et al. (1996) have reported that metrifonate increases brain monoamine levels. Thus activation of monoaminergic neuronal systems may additionally facilitate the beneficial effects of metrifonate on learning and memory in basal forebrain-lesioned rats, since the important role of monoaminergic neuronal systems on learning and memory has been demonstrated (Ichihara et al., 1992).

(5) A methodological problem could be responsible, in part, for this discrepancy. For the cholinesterase assay, samples had to be highly diluted and this procedure might cause spontaneous dissociation of inhibitor from the enzyme. Therefore, metrifonate could decrease cholinesterase activity at the higher doses, but not at the lower doses in ex vivo experiments. In the dialysis experiment, we cannot rule out the influence of 10^{-5} M physostigmine contained in Ringer's solution, since the dialysis membrane that we used could be penetrated by substances whose molecular weights were less than 50 000. It has been reported that local administration of cholinesterase inhibitors through the microdialysis probe affects the effect of systemically administered cholinesterase inhibitors (Szerb and Somogyi, 1973; De Boer et al., 1990; Kawashima et al., 1991, 1994; Messamore et al., 1993). Kawashima et al. (1994) have reported that tacrine (5 mg/kg, i.p.) could increase hippocampal acetylcholine 5.5-times higher than its basal levels and that the effect lasted for 4 h without cholinesterase inhibitor in the perfused solution. However, the same dose of tacrine could slightly increase acetylcholine and the effect was transient when the perfused solution contained a cholinesterase inhibitor. In the present

study, a transient effect of metrifonate on acetylcholine levels was also observed. These results suggest that physostigmine, which penetrated the membrane, might inhibit cholinesterase to some degree and thus increase basal acetylcholine during perfusion in the brain. The increased acetylcholine in the synaptic cleft would induce agonistic effects postsynaptically, but would also act presynaptically by slowing down the synthesis and release of acetylcholine (Szerb and Somogyi, 1973) due to activation of muscarinic autoreceptors (De Boer et al., 1990; Kawashima et al., 1991).

In the behavioral test with scopolamine-treated rats, 12.5 mg/kg of metrifonate was more effective than 15.0 mg/kg of metrifonate. Additionally, 50 mg/kg of metrifonate was more potent than 100 and 200 mg/kg of metrifonate on acetylcholine release. So it is conceivable that metrifonate has a bell-shaped dose-response curve, which is typical for cognitive enhancers. This hypothesis is consistent with the results of Van der Staay et al. (1996a,b). They also found that 12.5 but not 25.0 mg/kg of metrifonate improved the performance of rats in the shuttle box avoidance task.

Severe cholinergic side-effects were not observed after administration of metrifonate (100 and 200 mg/kg) in this study. Holmstedt et al. (1978) have reported that the oral LD_{50} doses of metrifonate ranged from about 400 to 600 mg/kg. In addition, Van der Staay et al. (1996a) have also noted that weak cholinergic side-effects were observed with 30 mg/kg of metrifonate. In their experiments, they used normal (intact) animals to evaluate the effects of metrifonate; however, in the present study we used basal forebrain-lesioned animals which had cholinergic dysfunction. This difference in subjects may be responsible for the lack of severe side-effects in spite of higher doses of metrifonate in the present study. As stated in Section 2, it has been reported that ibotenic acid-induced lesion was not selective for cholinergic neuron (Dunnett et al., 1987). We did not evaluate the areas lesioned by ibotenic acid histologically; however, in preliminary experiments at the dose we used, we had confirmed that the brain monoamine contents of basal forebrain-lesioned rats was not significantly different from that of sham-operated rats. In addition, at present, there is no adequate animal model of Alzheimer's disease. Further studies should be performed in various animal models to ascertain the beneficial effects of metrifonate on learning and memory.

As described above, metrifonate ameliorated the impairment of learning in both scopolamine-treated and basal forebrain-lesioned rats. These actions of metrifonate may be explained partly by the increase of extracellular acetylcholine levels and the inhibition of cholinesterase demonstrated in the brain microdialysis study and cholinesterase assay. The existence of unknown mechanisms other than cholinesterase inhibition by which metrifonate affects the impairment of learning and memory is also suggested. Since drugs for clinical use should be administered long-term, the long-term effects of metrifonate on behavior and on the central nervous system should be investigated. However, the present study, together with previous reports, suggests that metrifonate can be a useful therapeutic drug for Alzheimer's disease.

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