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Metrifonate Treatment Enhances Acquisition of Eyeblink Conditioning in Aging Rabbits

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KRONFORST-COLLINS, M. A., P. L. MORIEARTY, M. RALPH, R. E. BECKER, B. SCHMIDT, L. T. THOMPSON AND J. F. DISTERHOFT. *Metrifonate treatment enhances acquisition of eyeblink conditioning in aging rabbits.* PHARMACOL BIOCHEM BEHAV **56**(1) 103–110, 1997.—The cholinergic system is known to show deterioration during aging and Alzheimer's disease. In response, a therapeutic approach to Alzheimer's disease has been to attempt to compensate for the decrease in central cholinergic function by potentiating the activity of the remaining intact cholinergic cells with cholinesterase inhibitors. In this study treatment with the long-lasting cholinesterase inhibitor metrifonate enhanced acquisition of eyeblink conditioning in aging rabbits without producing interfering side effects. The effects of metrifonate on central and peripheral cholinesterase activity were evaluated, as was the involvement of plasma atropine esterase activity on the central and peripheral response to metrifonate. Results demonstrate that metrifonate can produce predictable, dose-dependent ChE inhibition. Associative learning in the aging rabbit was improved by metrifonate-induced steady state ChE inhibition within a range of 30–80%. Metrifonate was behaviorally effective in the absence of the severe side effects which typically plague cholinesterase inhibitors, suggesting that metrifonate is a possible treatment for the cognitive deficits resulting from normal aging and Alzheimer's disease.

Learning Eyeblink conditioning Aging Rabbits Cholinesterase Cholinesterase inhibitor Metrifonate Dichlorvos

THE hippocampus has been demonstrated in both clinical and animal studies to be a primary site of pathology in disorders of memory that accompany aging (20,44,55). The cholinergic system in particular is vulnerable to disorders associated with both aging and Alzheimer's disease (AD) (3,12,13,27,45). It is hypothesized that by improving cholinergic function, the characteristic loss of memory associated with aging and AD can be lessened (15,28,29). A recent therapeutic approach to AD has been to attempt to compensate for the loss of functional central cholinergic neurons by potentiating the activity of the remaining cholinergic neurons with cholinesterase inhibitors (14).

Several different cholinesterase (ChE) inhibitors have been evaluated in the treatment of AD. Two of the most widely used are physostigmine and tacrine (14,32,34). Treatment with these compounds is limited, however, by their short durations of action and tendencies to produce severe side effects (29, 42, 59). Pharmacological studies in the rat have shown that metrifonate, an organophosphorous ChE inhibitor, is longer lasting and less toxic than physostigmine and tacrine even at high doses (80 mg/kg) (28,29). ChE inhibition has been shown to last 4–5 times longer after administration of metrifonate than after physostigmine, and the degree of ChE inhibition has been shown to result in comparable increases in ACh levels (28). Metrifonate is transformed nonenzymatically to dichlorvos (phosphoric acid 2,2-dichloroethenyl dimethyl ester) which produces long lasting inhibition of both acetylcholinesterase (AChE) and butyrylcholinesterase (BuChE). Clinical studies in humans have demonstrated that it is possible to use metrifonate to obtain over 80% ChE inhibition in

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plasma and red blood cells (RBCs) without producing interfering side effects (4,5). Clinical testing of several ChE inhibitors in AD suggest that long lasting peripheral ChE inhibition in the range of 28 - 58% is associated with therapeutic benefits. Clinical benefits appear to decline, however, at higher and lower ends of the range with a maximum benefit occurring at approximately 43% ChE inhibition (6,24).

A widely used model for the study of associative learning in mammals is the classically conditioned eyeblink response task (16,25,53). Its advantages include: well-defined behavioral responses for both humans and rabbits (19), significant conditioning deficits in both humans and rabbits as a result of age (18,38,44,45,52,60), and an emerging understanding of the neural circuitry involved in this conditioning task (17,61). During eyeblink conditioning a tone conditioned stimulus (CS) is presented, followed by an airpuff unconditioned stimulus (US). Initially, the subject performs only unconditioned responses (URs) by blinking in response to the airpuff. After a sufficient number of pairings the subject learns to blink in response to the tone, generating a conditioned response (CR). Conditioning in this task has been shown to be dependent on an intact hippocampus in the rabbit when a 500 msec stimulus-free interstimulus interval is employed (36,48).

Several studies have demonstrated disruption of the eyeblink conditioning response in both humans and in rabbits after muscarinic cholinergic receptor blockade with scopolamine (30,35,37,46,47). This scopolamine-induced disruption was hypothesized to be mediated by the hippocampus, since the scopolamine disruption in rabbits only occurred in unoperated controls and in animals with neocortical lesions, while animals with hippocampal lesions were unaffected by scopolamine treatment (46). Scopolamine administration in rabbits was also shown to eliminate the characteristic firing patterns of pyramidal cells (40) which typically accompany eyeblink conditioning (7,8). Since central cholinergic blockade had been shown to disrupt eyeblink conditioning, we hypothesized that central cholinergic amplification with metrifonate treatment would enhance the performance of aging subjects in this task.

AChE distribution (21–23) and cholinergic function in signalling in the rabbit hippocampus (9,57,58) have been described, and the effects of aging on eyeblink conditioning of the rabbit have been established (26,52). Therefore, rabbit eyeblink conditioning was selected as a model to study the effects of metrifonate both on central cholinergic function and on learning. Central and peripheral ChE inhibition levels were examined to determine whether a correlation exists between them, and within what range of ChE inhibition would behavioral benefits be maximized. We describe here the use of chronic oral dosing of metrifonate to produce 30–80% steady state central and peripheral ChE inhibition, and the associated enhancement of eyeblink conditioning in aging rabbits.

METHODS

Aging (mean age = 33.71 ± 0.31 mo; $n = 24$) female albino rabbits (*Oryctolagus cuniculus*) were surgically fitted with restraining headbolts (16,52), and were divided into three metrifonate dosage groups (6 mg/kg, 12 mg/kg and 24 mg/kg) and a vehicle control group (100 mM sodium citrate [pH 6.0]). The experimenter was blind to the treatment condition of the rabbits. Baseline blood samples were taken, and oral administration of metrifonate was commenced one week prior to the start of behavioral conditioning. The subjects were weighed and administered the drug daily for five consecutive days followed by two days of no treatment, throughout the course of training for a maximum of six weeks. The drug or vehicle was administered orally through a syringe approximately 15 min prior to the start of training. On every fifth day of treatment blood samples were taken under Innovar-Vet anesthesia (0.125 ml/kg) 2 hrs after drug administration. Each blood sample was collected in two 1.5 ml aliquot tubes and mixed with 50 μl of heparin in each. Samples were centrifuged at 1000, 4°C for 15 min. The plasma and RBCs were then separated into 500 μl alliquots and stored at −80°C for later ChE inhibition assays.

During training in the eyeblink conditioning task subjects were secured in cloth bags and placed in Plexiglas restraining boxes. The training chamber was a single-walled, sound attenuated IAC acoustical chamber. Conditioning airpuffs were given to the right eye, and the right eyelid was held open with stainless steel dress hooks, which permitted detection of nictitating membrane (NM) extensions with non-invasive photoelectric detectors (51; see Fig. 1). Subjects were habituated to the behavioral apparatus for 1 h at least 24 h before the start of training.

Subjects were trained in the trace eyeblink conditioning paradigm which consisted of a 100 msec tone CS (85 dB, 6 kHz) presented via stereo headphones followed by a 150 msec corneal airpuff US (3 psi). The CS and US were separated by a 500 msec stimulus free trace period. Training sessions consisted of 80 CS-US paired trials presented at a random intertrial interval ranging from 30 to 60 sec (M = 45 sec). Experiments were controlled by a computer software system custom-designed to control associative learning tasks (2). A response was classified by the computer as a CR if a NM extension greater than 4 standard deviations above baseline and lasting for 10 successive 1 msec bins occurred during the interval between CS onset and US onset. Subjects were trained until reaching a learning criterion of 80% CRs occurring within a day of training or for a maximum of 25 days. Subjects that reached the learning criterion in less than 25 days of training were given several sessions of extinction testing, each of which consisted of 80 tone alone trials. Each daily training or extinction session lasted approximately one hour.

Mean learning curves were calculated for the four groups and were expressed as the percentage of CRs per training session. Since all of the subjects were not trained for an equal number of sessions, each subject's learning curve was normalized to the maximum number of possible training sessions, using the linear interpolation algorithms of Igor (WaveMetrics, Lake Oswego, Oregon), so that qualitative summaries of learning rates for animals requiring different numbers of trials to reach criterion could be made (50). Mean learning curves for each of the four groups were then calculated from the individual normalized learning curves. Behavioral data was analyzed with analyses of variance (ANOVA) (Statview, Abacus Concepts, Berkeley, CA) and Fishers' protected least significance post-tests, with a minimal criterion for statistical significance of $p < 0.05$. Behavioral data are cited as means ± standard error of the means.

At the end of training all subjects were deeply anesthetized and sacrificed by decapitation. The brains were quickly removed, frozen in liquid freon and then stored at −80°C. The frozen brains were later thawed at 4°C, dissected on the midline and the cerebellum removed. One hemisphere was weighed and homogenized in 8 ml/gm wet weight of Triton/EDTA (0.5% Triton × 100, 10 mM EDTA, pH 7.4). The homogenate was further diluted 1:40 with Triton/EDTA for assay.

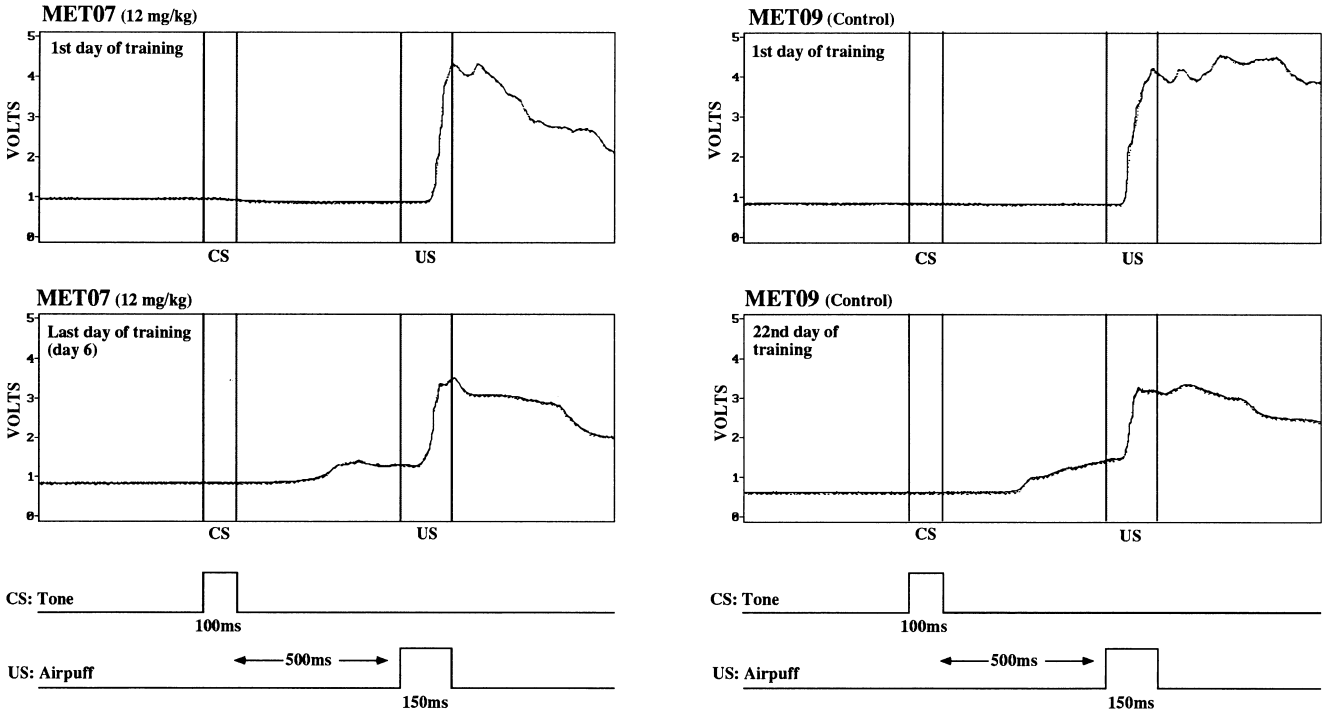

FIG. 1. Examples of typical responses on single trials shown by subjects during eyeblink conditioning, early (top) and late (bottom) in training. Examples were taken from both a 12 mg/kg subject (left) and a control subject (right). There were no significant differences between the average CR amplitudes, CR latencies or UR amplitudes of the different dosage groups.

ChE activity was determined by the radiometric method of Johnson and Russell (31) with slight modifications. Plasma was diluted 1:60 and RBC 1:200 with Triton/EDTA, for testing in a 5 min assay at 28°C. The substrate was a mixture of tritiated ACh (Dupont NEN) and cold ACh at 500 μM final concentration. Hemoglobin in diluted RBC samples was determined using a commercial kit (Sigma); RBC ChE activity was expressed as nmol/ml/min; brain activity as nmol/mg/min. Inhibition of plasma and RBC activity was determined as a percent of corresponding baseline activity of the same animal. For brain inhibition, mean ChE activity of brain tissue was determined for six age-matched, placebo-treated animals; inhibition in treated animals was expressed as a percent of this mean normal value.

Atropine esterase activity was estimated by a modification of the method of Tucker and Beattie (54). Plasma samples (50 μl) were each mixed with 50 μl of 0.4% cresol red, 1% atropine sulfate, pH 7.6 in a 650 μl plastic centrifuge tube, incubated at 37°C for 2 h, and then observed for color changes indicating presence of atropine esterase.

RESULTS

Daily Oral Administration of Metrifonate Enhanced Acquisition of Trace Eyeblink Conditioning in Aging Rabbits.

A 4 by 25 repeated measures ANOVA was conducted on the normalized average percent conditioned responses of the 4 dosage groups calculated for 25 blocks of 80 trials (80 trials a day for a maximum of 25 days). A significant result was noted in the drug treatment main effect [$F(3,20) = 3.09$, $p = 0.05$] as well as the effect of repeated testing sessions [$F(24, 480) = 35.06$, $p < 0.0001$]. Post-hoc Fishers' tests indicated that the 12 mg/kg group, in particular, showed a significant increase in percent conditioned responses over the course of training when compared with both the control ($p < 0.02$) and the 6 mg/kg groups ($p < 0.03$; Fig. 2A). When grouped together, all metrifonate treated subjects required significantly fewer trials (697.8 ± 134.8) than controls (1275.0 ± 245.2) to reach a learning criterion of 8 out of 10 conditioned responses [$F(1,22) = 4.48$, $p < 0.05$; Fig. 2B].The increased levels of learning were not due to generalized sensorimotor enhancement, because there were no significant differences between the average CR amplitudes [$F(3,20) = 2.02$, $p > 0.14$], CR latencies [$F(3,20) = 1.03$, $p > 0.40$], or UR amplitudes [$F(3, 20) = 2.3$, $p > 0.10$] observed for the four groups (Table 1). Administration of metrifonate in this study did not result in any of the side effects such as fasciculations, tremor, facial clonus, or increased defecation which were previously noted in rat studies (28,29).

Of the 24 subjects trained in the trace eyeblink conditioning task, 10 reached a criterion of 80% CRs per session and were then given at least 3 sessions of extinction testing. Planned comparisons were used to analyze the degree of extinction of each of the treatment groups by comparing the percentage of CRs exhibited by the subjects on the third day of extinction testing versus the percentage of CRs exhibited on the last day of training (Table 2). The metrifonate treated subjects in each of the 3 dosage groups (6 mg/kg, 12 mg/kg and 24 mg/kg) showed significantly fewer CRs on the third day of extinction testing than the last day of training [$F(1) = 4.77$, $p < 0.05$; $F(1) = 7.35$, $p < 0.02$; $F(1) = 5.91$, $p < 0.03$]. The control subjects, however, did not significantly extinguish the learned behavior by the third day of extinction testing ($p > 0.3$).

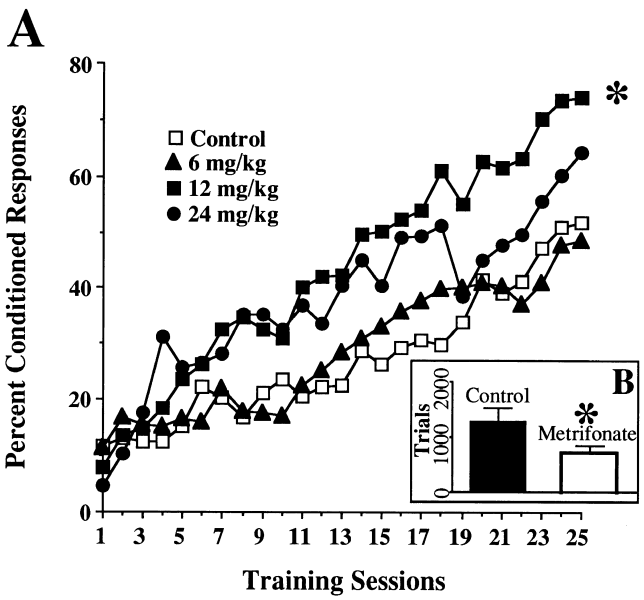

FIG. 2A. Normalized mean learning curves were calculated for the four groups and were expressed as the percentage of conditioned responses per training session. The 12 mg/kg group showed a significant increase in percent conditioned responses over the course of training when compared with the control ($p < 0.02$) and 6 mg/kg groups ($p < 0.03$), denoted by an asterisk. This learning facilitation was not due to a generalized sensorimotor enhancement because there were no significant differences between the mean CR amplitudes, CR latencies, or UR amplitudes of the different metrifonate dosage groups. The 24 mg/kg group showed an intermediate effect between the most effective 12 mg/kg dose and the other two groups. 2B. Chronic oral administration of metrifonate enhanced trace eyeblink conditioning in the aging rabbit. Metrifonate treated subjects (n = 18) required significantly ($p < 0.05$) fewer trials (697.8 ± 134.8) than control subjects (n = 6) (1275.0 ± 245.2) to reach a learning criterion of 8 conditioned responses occurring within 10 consecutive trials [$F(1, 22) = 4.48$, $p < 0.05$], denoted by an asterisk.

Preliminary experiments showed that pooled rabbit plasma and RBC ChE activities diverged markedly from Michaelis-Menton kinetics at substrate concentrations higher than 1 mM. Pooled brain tissue ChE showed substrate inhibition when ACh concentration exceeded 2 mM; activity was linear below this, with calculated $K_m = 35$ μM, $V_{max} = 10.53$ nmol/mg/min. Assay parameters were chosen within the linear range of enzyme activity for all tissues tested, and further reduction of activity (by dilution) did not result in deviation from expected values. Pre-treatment ChE velocities were RBC, 4.41 ± 0.24 nmol ACh/mg hemoglobin/min ($n = 24$); plasma, 343.8 ± 19.3 nmol/ml/min ($n = 24$); brain, 7.93 ± 0.40 nmol/mg/min ($n = 6$).

TABLE 1

MEAN RESPONSE AMPLITUDE AND ONSET LATENCY

Dosage Group	N	CR Amplitude (mV) M	CR Amplitude (mV) SD	UR Amplitude (mV) M	UR Amplitude (mV) SD	CR Onset Latency (ms) M	CR Onset Latency (ms) SD
Control	6	1944.8	247.9	1764.7	327.8	248.7	37.5
6 mg/kg	6	1871.4	216.8	1647.4	258.8	232.3	13.1
12 mg/kg	6	1928.4	154	1782.1	176.5	251.2	46.3
24 mg/kg	6	1672.4	232.5	2017.3	211.5	163.6	12.5

CR = conditioned response; UR = unconditioned response.

TABLE 2

MEAN PERCENTAGE OF CONDITIONED RESPONSES FOR SUBJECTS RECEIVING EXTINCTION TESTING

Dosage Group	N	% CRs on Last Day of EBC M	% CRs on Last Day of EBC SD	% CRs on 3rd Day of Extinction M	% CRs on 3rd Day of Extinction SD
Control	2	82.5	3.5	67.5	5.3
6 mg/kg	2	80	0	46.9*	39.8
12 mg/kg	4	84.1	4.9	55*	16.7
24 mg/kg	2	87.5	1.8	50.6*	15

CR = conditioned response; EBC = eyeblink conditioning. An asterisk denotes a significant decrease in the percent CRs elicited on the third day of extinction compared with the last day of training ($p < 0.05$).

Metrifonate treatment resulted in dose-related inhibition of ChE activity in RBCs and in plasma which reached near steady state values after two (plasma) or three (RBC) weeks of treatment (Fig. 3). Brain ChE inhibition was also clearly dose-related. A plot of log dose metrifonate versus percent brain ChE inhibition (Fig. 4) showed a highly significant correlation ($R = 0.92$, $p < 0.0001$) with the following equation: percent brain ChE inhibition = (77.6 × log metrifonate dose)-30.62.

Highly significant ($p < 0.001$) correlations were also observed between brain and peripheral ChE inhibition at the time of sacrifice (Fig. 5). Though plasma inhibition was lower than both brain and RBC inhibition, a slope close to 1.0 was calculated for the relation between each peripheral measure and the endpoint brain measures (percent brain ChE inhibition = (0.96 × plasma ChE inhibition) + 30.0; percent brain ChE inhibition = (0.96 × RBC ChE inhibition) + 10.6.

Eleven of the 24 rabbits had measurable atropine esterase activity (two to four atropine esterase positive animals in each treatment group). Presence of atropine esterase activity did not affect the response of the animals to metrifonate, either in the level of ChE inhibition observed or in the relationship between peripheral and central ChE inhibition.

DISCUSSION

Our results demonstrate that metrifonate can be administered to aging rabbits to obtain chronic dose-related brain ChE inhibition in a range of 30 - 80% and can enhance acquisition of trace eyeblink conditioning in aging animals without producing interfering side effects. Metrifonate treated groups taken as a whole showed behavioral improvement over the control group (Fig. 2B), with maximal behavioral benefits obtained with a metrifonate dose of 12 mg/kg (Fig. 2A). This correlates with a recent behavioral study which determined that daily p.o. treatment of 12.5 mg/kg of metrifonate in rats resulted in improved acquisition by young rats in the shuttle box two way active avoidance response and improved the platform escape response of young and aging rats in the Morris water maze task, while a daily dose of 24 mg/kg proved ineffective (56). In the present study the 12 mg/kg daily treatment resulted in steady state RBC ChE inhibition in a range of 35.3–41.3%.

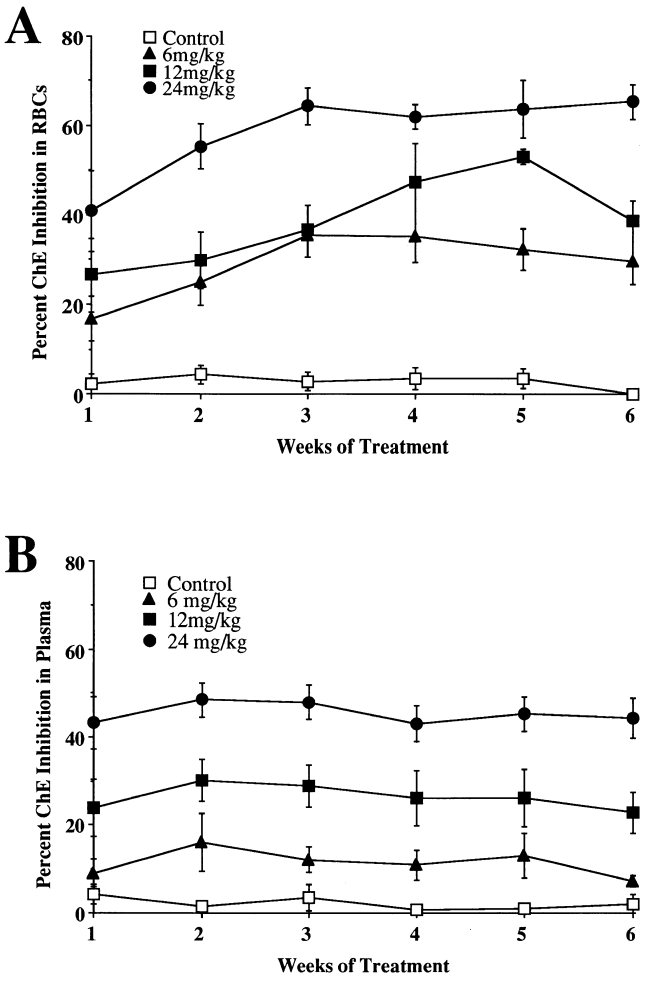

FIG. 3. Inhibition of red blood cell (A) and plasma (B) cholinesterase (ChE) measured weekly after daily oral treatment of metrifonate or vehicle. Subjects are grouped according to dosage. Data are mean ± SEM versus pretreatment enzyme activity of each animal (n = 6/ treatment group).

This coincides with the results of clinical studies (6, 24) which demonstrated that long-lasting ChE inhibition within a range of 28 - 58% with a peak at 43% was associated with maximum therapeutic benefit. The 6 mg/kg and 24 mg/kg groups, in which steady state ChE inhibition levels [(29.1% ± 2.3) and (58.4% ± 2.6), respectively] were at the far ends of the range, did not acquire the learning task as well as the 12 mg/kg group (Fig. 2A).

Of the 10 subjects that successfully acquired the trace eyeblink conditioning task to a behavioral criterion of 80% CRs within one training session, only those treated with metrifonate were able to extinguish the learned behavior (Table 2). This analysis was limited by the low number of subjects in each group, and should be considered preliminary. However, it was interesting that the extinction impairment exhibited by the two aging control subjects was similar to that observed in rabbits after hippocampal lesions (1, 36,41). Moyer and colleagues demonstrated that hippocampally-lesioned rabbits were able to acquire the 300 msec trace eyeblink conditioning task, but were not able to extinguish the learned behavior.

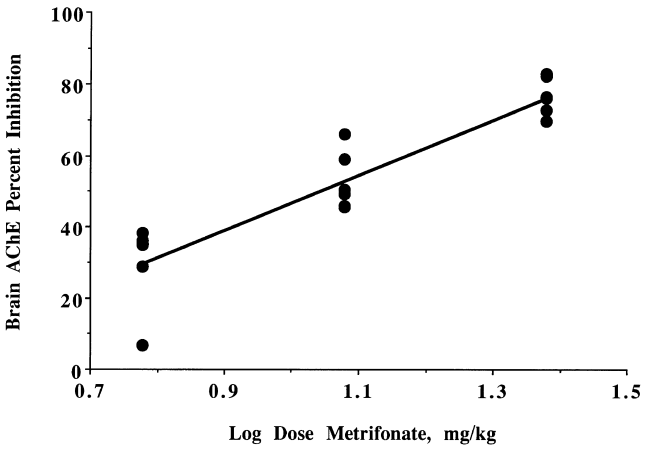

FIG. 4. Inhibition of brain acetylcholinesterase (AChE) at sacrifice as a function of log daily dose of metrifonate in rabbits (percent inhibition = (77.6 × log metrifonate dose) - 30.62; R = 0.92, $p < 0.0001$). AChE inhibition after treatment is expressed as a percent of mean brain AChE activity of six age-matched, placebo-treated animals.

This extinction deficit in trace eyeblink conditioning has also been observed in aging human subjects (10). These findings suggest that removal of the hippocampus or degeneration of this structure due to aging may affect a subject's ability to modify previously learned responses. Theoretical analyses have suggested that the ability to respond to stimuli with flexibility is a major function of the hippocampus (11). The fact that metrifonate treated subjects were able to extinguish the learned behavior faster than aging control animals and in a manner similar to young subjects provides further evidence that metrifonate treatment enhances learning in aging subjects and may alleviate some of the learning deficits that result from hippocampal degeneration.

Since the subjects were treated with metrifonate for only one week prior to the start of training, and steady state levels of inhibition were not reached until the second (plasma) or

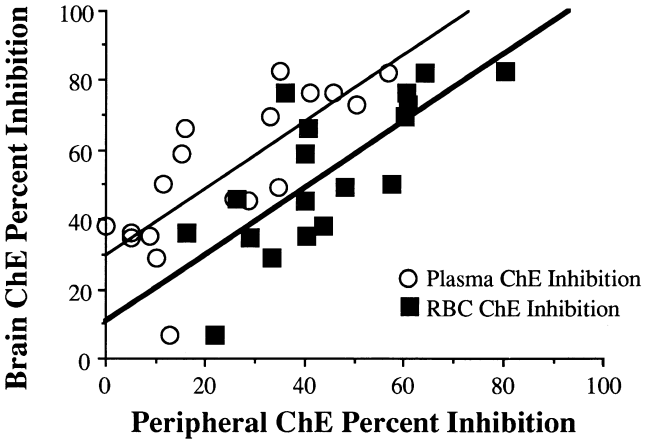

FIG. 5. Correlation between red blood cell (RBC) or plasma ChE inhibition and brain ChE inhibition after chronic oral metrifonate treatment of rabbits. Highly significant correlations (RBC: R = 0.76, $p < 0.001$; plasma: R = 0.77, $p < 0.001$) between peripheral and central ChE inhibition were observed (equations in text).

third (RBC) weeks of treatment (Fig. 3), maximal behavioral benefits and maximal behavioral separation of the dosage groups probably were not reached in the current study. We intend to pursue this in the future by extending the baseline treatment period for several weeks in order to establish steady state levels of ChE inhibition prior to the start of behavioral training.

Unlike the response of rat plasma enzymes which recover rapidly after dichlorvos or metrifonate inhibition (39,43,49), rabbit plasma ChE activity remained inhibited in a manner similar to brain enzyme. Either RBC or plasma enzyme activity can thus be used to monitor steady state drug effects in rabbits in vivo, as inhibition of both enzymes was significantly correlated with brain inhibition (Fig. 5). In addition, levels of ChE inhibition were not influenced by the presence or absence of atropine esterase in the animals. Since the muscarinic antagonist atropine has been used in investigations of the cholinergic system, either experimentally to manipulate cholinergic function or as a safety feature to avoid death by ChE inhibitor overdose, some investigators (e.g., 33) use only atropine esterase negative animals. Our results show that data obtained in such animals after ChE inhibition by metrifonate was similar to that from atropine esterase positive rabbits.

Muscarinic cholinergic receptor blockade has been demonstrated to disrupt eyeblink conditioning in rabbits (30,35,37,46,47). The improved eyeblink conditioning noted here in aging rabbits was correlated with ChE inhibition, which presumably enhances muscarinic cholinergic function. Furthermore, these results demonstrating improved learning in aging rabbits in response to amplification of the muscarinic cholinergic system are convergent with a recent study by Woodruff-Pak and colleagues which demonstrated facilitated eyeblink conditioning in aging rabbits after treatment with the nicotinic receptor agonist, GTS-21 (62). Together, these studies provide evidence supporting the cholinergic hypothesis of geriatric memory dysfunction (3). They also illustrate the importance of both the muscarinic and nicotinic cholinergic pathways in the modulation of hippocampal function and the resultant capacity for learning and memory in aging animals.

We observed several characteristics, including predictability of dose-response relationships, steady-state ChE inhibition and absence of side effects which suggest that metrifonate may help compensate for the central cholinergic system dysfunction which apparently contributes to impaired learning in aging rabbits (52). Furthermore, we observed a correlation between the most effective range of ChE inhibition for behavioral benefits in aging rabbits with those of human clinical studies. It is important to stress that the enhancement of acquisition rate was obtained in aging rabbits in the absence of obvious behavioral or physiological side effects. This is a relevant issue for ChE inhibitors, which tend to be plagued by negative side effects in behaviorally effective ranges (6, 32, 59). Thus, metrifonate may be effective as a possible treatment for aging or Alzheimer Disease-related cognitive deficits which could be used without producing uncomfortable or possibly life-threatening physiological side effects. This study also further substantiates the utility of the rabbit eyeblink conditioning paradigm as a model for the investigation of therapeutic agents to treat age-associated learning deficits.

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