ELSEVIER

European Journal of Pharmacology 354 (1998) 17–24

ejp

Effect of subchronic treatment with metrifonate and tacrine on brain cholinergic function in aged F344 rats

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Received 5 February 1998; revised 27 May 1998; accepted 3 June 1998

Abstract

The effects of 21-day treatment with the acetylcholinesterase inhibitors metrifonate (80 mg kg^{-1} per os (p.o.)) and tacrine (3 mg kg^{-1} p.o.), twice daily, on cortical and hippocampal cholinergic systems were investigated in aged rats (24–26 months). Extracellular acetylcholine levels were measured by transversal microdialysis in vivo; choline acetyltransferase and acetylcholinesterase activities were measured ex vivo by means of radiometric methods. Basal cortical and hippocampal extracellular acetylcholine levels, measured 18 h after the last metrifonate treatment, were about 15 and two folds higher, respectively, than in control and tacrine-treated rats. A challenge with metrifonate further increased cortical and hippocampal acetylcholine levels by about three and four times, respectively. Basal extracellular acetylcholine levels, measured 18 h after the last treatment with tacrine were not statistically different from those of the control rats. A challenge with tacrine increased cortical and hippocampal extracellular acetylcholine levels by about four and two times. A 75% inhibition of cholinesterase activity was found 18 h after the last metrifonate administration, while only a 15% inhibition was detectable 18 h after the last tacrine administration. The challenge with metrifonate or tacrine resulted in 90 and 80% cholinesterase inhibition, respectively. These results demonstrate that in aging rats a subchronic treatment with metrifonate results in a long-lasting, cholinesterase inhibition, and a persistent increase in acetylcholine extracellular levels which compensate for the age-associated cholinergic hypofunction. Metrifonate is therefore a potentially useful agent for the cholinergic deficit accompanying Alzheimer's disease.

Keywords: Aging; Acetylcholine release; Cholinesterase inhibitor; Alzheimer's disease; Microdialysis

1. Introduction

The forebrain cholinergic pathways projecting to the cortex and hippocampus play a key role in learning and memory mechanisms (Squire and Davis, 1981; Everitt and Robbins, 1997). The hypofunction of the forebrain cholinergic system is considered an important pathogenic element of age-associated cognitive impairments, including senile dementia of the Alzheimer's type (Bartus et al., 1982; Collerton, 1986). Since the incidence of Alzheimer's disease increases with the aging of the population, the need for drugs that might alleviate the disruptive effects of this degenerative disorder becomes more and more pressing. Many drugs have been proposed for the treatment of dementia (Sarter, 1991). However, the main group of compounds on which a large number of preclinical and clinical investigations are in progress is constituted by cholinesterase inhibitors (Giacobini, 1997), the rationale for the use of cholinesterase inhibitors in the treatment of Alzheimer's disease to prevent acetylcholine hydrolysis in the central nervous system. This leads to an increase in extracellular acetylcholine levels which may restore the cholinergic hypofunction. Among the many existing cholinesterase inhibitors, only tacrine and, more recently, donepezil (Bryson and Benfield, 1997) have been approved by the Food and Drug Administration for the symptomatic treatment of mild to moderate forms of Alzheimer's disease (Nightingale, 1997). However, the use of tacrine is hampered by its relevant toxicity (Davis and

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0014-2999/98/$19.00

Powchik, 1995), its short duration of action and narrow therapeutic window (Weinstock, 1995). For this reason, much effort is being dedicated to the development of novel cholinesterase inhibitors with longer duration of action that selectively inhibit brain cholinesterase and show fewer side effects. Among these second generation cholinesterase inhibitors (Giacobini, 1991), metrifonate, an organophosphorous compound inducing a long-lasting inhibition of cholinesterase, seems of particular interest since it has been used for a long time as an antihelminthic, and its safety in man is well known (Holmstedt et al., 1978; Webster, 1990). A preliminary report of its clinical efficacy in Alzheimer's disease (Becker et al., 1990) has been recently confirmed (Cummings et al., 1996, 1998). Metrifonate may be considered a pro-drug since in vivo, it is non-enzymatically transformed into the active cholinesterase inhibitor *O,O*-dimethyl 2,2-dichlorovinyl phosphate (DDVP) (Nordgren and Holmstedt, 1988) which is approximately 100 times more potent than metrifonate in vitro (Hinz et al., 1996b). The low levels of the active metabolite, slowly released from the parent compound, lead to delayed (Hinz et al., 1996a), long-lasting inhibition of cholinesterase in the brain (Soininen et al., 1990). On the contrary, tacrine leads to rapid inhibition of cholinesterase activity, followed by a rather quick recovery (for review, see Jaen and Davis, 1993). Studies in rodents have demonstrated that systemic administration of cholinesterase inhibitors such as physostigmine, heptylphysostigmine, tacrine (Messamore et al., 1993, Xiao et al., 1993; Cuadra et al., 1994) and metrifonate is followed by an increase in brain extracellular acetylcholine levels in young (Mori et al., 1995) and aged (Scali et al., 1997a) animals.

A large number of experimental data demonstrated a severe age-related brain cholinergic hypofunction in rats (for references, see Pepeu and Giovannelli, 1994). Old rats are therefore currently used in drug studies as a model for the impairment of the cholinergic system observed during normal aging and neuropathological conditions in man. Since there are no investigations of the changes induced in the forebrain cholinergic system by subchronic administration of metrifonate or tacrine in old rats, the aim of the present work was to measure the extracellular levels of acetylcholine in the cerebral cortex and hippocampus of rats receiving either drug orally for 3 weeks. The extent of cholinesterase inhibition was assessed, and in order to evaluate the functional consequences of a persistent raise in extracellular acetylcholine levels, choline acetyltransferase activity was measured and the effect of a challenge dose of metrifonate or tacrine was evaluated. The oral route was selected in order to mimic the administration route of these drugs in humans. An indication of the possible systemic toxic effects of the chronic administration of the two drugs was sought by measuring heart rate, blood pressure at the end of the treatment, and the changes in body weight.

2. Materials and methods

2.1. Animals

The experiments were performed on male Fisher 344 rats from Harlan-USA of 24–26 months of age. The rats were housed two per cage at 21–24°C, 40–60% humidity, on a 12-h light/dark cycle, light period starting at 0700, with food and water. All the experiments were carried out in compliance with the guidelines of the European Community's Council for Animal Experiments (DL 116/92).

2.2. Drug treatments

Rats were randomly allocated to one of the following experimental groups: metrifonate, tacrine, controls. Metrifonate (80 mg kg^{-1}), tacrine (3 mg kg^{-1}) or vehicle (5% Na-citrate buffer adjusted to pH 5.5–6) where administered per os (p.o.) for 21 days, twice daily (0800 and 1600). The two drugs were freshly dissolved in 5% Na-citrate buffer adjusted to pH 5.5–6 and a volume of 1 ml kg^{-1} was administered to all groups. In previous experiments (Scali et al., 1997a,b), single oral administrations of the doses of metrifonate and tacrine, used the present experiments, restored hippocampal and cortical extracellular levels of acetylcholine in old rats to young rat levels, and were well tolerated. Moreover, the dose of tacrine used was shown to be effective also by Aaltonen et al. (1991) and Riekkinen et al. (1992).

2.3. Cholinesterase activity

Whole brain cholinesterase activity was measured according to the radiometric method described by Johnson and Russell (1975), as modified by van der Staay et al. (1996).

Briefly, whole brains were homogenated in three weight in volume (w/v) of 0.9% NaCl solution. Enzyme activity was measured at 4°C in order to achieve a linear substrate conversion for up to 10 min of incubation. A total of 50 μl of the protein sample (50 μl of saline as a blank) was mixed with 10 μl of a 30-mM aqueous solution of acetylcholine iodide containing 100 000 dpm [^{3}H]acetylcholine iodide (New England Nuclear, Boston). After 6 min of incubation on ice, the reaction was stopped by adding 100 μl of an ice-cold cocktail containing 1 M chloroacetic acid, 0.5 M NaOH and 2 M NaCl. Finally, 4 ml of a polar scintillation cocktail (Quickscint 501, Zinsser, Frankfurt) was added to extract and the [^{3}H]acetate formed was quantified by β-scintillation counting.

2.4. Microdialysis and determination of extracellular acetylcholine levels

Extracellular acetylcholine levels were measured in the cerebral cortex and hippocampus by a microdialysis tech-

nique, as previously reported (Giovannini et al., 1991). Briefly, the rats were anaesthetised with ketamine (150 mg kg^{-1} i.p.) and placed in a stereotaxic frame. A microdialysis tube (AN 69 membrane, Hospal Dasco, Italy, molecular weight cutoff > 15 kDa), covered with super-epoxy glue except for a region corresponding to the length of parietal cortices and dorsal hippocampi (8 and 6 mm long, respectively), was inserted transversely through either of the two brain structures as described below. After sagittal cutting, the overlying skin and temporal muscles were retracted and folded away, and holes were drilled bilaterally at the level of the dorsal hippocampi (AP = −3.3, DV = −3.5) or parietal cortices (AP = −0.5, DV = −2.7); all coordinates (Paxinos and Watson, 1982) were taken over the bone and referred to bregma, with bregma and lambda on a horizontal plane. The microdialysis tube was then gently inserted through the holes using the micromanipulator of the stereotaxic instrument. The ends of the dialysis tube were bent upward, secured to the parietal skull with acrylic dental cement and the skin sutured. Rats were housed with free access to food and water to recover from surgery (1 rat $cage^{-1}$). The operation was carried out on day 20 from the beginning of the treatment, and on the day of the operation, the rats were treated twice with metrifonate, tacrine or vehicle, one in the morning before operation, and one in the afternoon, as usual.

On the following day, the membrane was perfused at a constant flow rate (3 μl min^{-1}) with Ringer solution (NaCl 147 mM, $CaCl_2$ 1.2 mM, KCl 4.0 mM) with no cholinesterase inhibitors added to the Ringer solution. The dialysate was collected at 20-min intervals for 5 h in minitubes containing 5 μl of 0.05 mM HCl to prevent hydrolysis of acetylcholine and directly assayed for acetylcholine. After a 1-h settling period and 18 h after the last treatment, three samples were collected in basal conditions. Rats were then challenged with metrifonate, tacrine or vehicle and samples were then collected for the following 4 h after drug treatment. At the end of the experiment, rats were treated with a further dose of metrifonate, tacrine or vehicle and killed 1 or 18 h after the last treatment. Brains were quickly removed and put on ice-cold saline. Brain structures (frontal neocortex and hippocampus) were quickly dissected and samples were stored in a deep freezer before analysis of ChAT activity and cholinesterase inhibition (see below).

Acetylcholine levels in the dialysates were assayed directly by a high-performance liquid chromatographic method with an enzyme reactor and an electrochemical detector as described by Damsma et al. (1987) and Giovannini et al. (1991). Briefly, acetylcholine was separated on a cation exchange column prepared by loading a reverse phase column (Chromspher 5 C18, Chrompack) with sodium lauryl sulfate (0.5 mg ml^{-1}). The mobile phase consisted of 0.2 M phosphate buffer (pH 8.0) containing 5 mM KCl, 1 mM tetra-methyl-ammonium (TMA) and 0.3 mM ethylenediaminetetraacetic acid (EDTA). The flow rate was 0.75 ml min^{-1}. Acetylcholine was hydrolyzed by acetylcholinesterase to acetate and choline in a postcolumn enzyme reactor; choline was oxidized by choline oxidase to produce betaine and hydrogen peroxide. Hydrogen peroxide was electrochemically detected by a platinum electrode at +500 mV. To evaluate the amount of acetylcholine in the samples, a linear regression curve was constructed with standards of acetylcholine and the peak heights of this compound in the samples were compared with those of the standards by means of an integrator (P.E. Nelson, model 1020). The detection limit was 50 fmol of acetylcholine.

2.5. Choline acetyltransferase activity

Frontal cortices and hippocampi were homogenized in 20 volumes of 10 mM EDTA buffer (pH 7.4) and 0.2% Triton X-100. Choline acetyltransferase activity was determined by measuring the conversion of 1-[^{14}C]acetyl-coenzyme A (Radiochemical Centre, Amersham, Arlington Heights, IL, specific activity 60 mCi $mmol^{-1}$) to [^{14}C]acetylcholine according to the method of Fonnum (1975). Incubation time was 15 min at 37°C. Choline acetyltransferase activity was expressed as μmol h^{-1} 100 mg $protein^{-1}$. Protein content in the homogenates was determined by the method of Lowry et al. (1951).

2.6. Heart frequency and blood pressure determination

Heart frequency and systolic blood pressure were measured in unanaesthetised animals using indirect tail-cuff plethysmography (BP Recorder, Basile, Comerio, IT). Rats were placed in a restraining sling and allowed 15 min to acclimate to the surroundings. Pressure determinations began when the animals were calm and heart frequency and blood pressure appeared to be steady. At least five repeated measurements were obtained from a single animal and they were averaged to obtain a single value (Cimini and Zambraski, 1985).

2.7. Drugs

Metrifonate ((*O,O*-dimethyl-2,2,2-trichloroethyl)-phosphonate) was supplied by Bayer/Troponwerke (Köln). Tetrahydroaminoacridine (Tacrine) was purchased from Sigma. All other chemicals were of analytical grade (Merck).

2.8. Statistical analysis

Extracellular acetylcholine levels were expressed as fmol μl^{-1}. The first four samples before treatment were taken as basal acetylcholine release (see Table 1 legend). Differences in drug effects on extracellular acetylcholine levels were evaluated using the mean maximal changes calculated for each animal between 120 and 200 min after drug or vehicle treatment.

Table 1
Effect of metrifonate and tacrine treatment on basal acetylcholine levels, expressed as fmol μl^{-1} in the cortex and hippocampus of aged rats

	Controls	Metrifonate	Tacrine
Cortex	1.97 ± 0.49 (5)	30.44 ± 8.8^{a} (6)	2.61 ± 0.66 (6)
Hippocampus	1.61 ± 0.37 (6)	4.09 ± 1.2^{a} (7)	1.84 ± 0.44 (5)

[a] $P < 0.05$ vs. control and tacrine treated rats (one-way ANOVA and Duncan's post-hoc test).
Number of rats in parentheses.

Statistical analysis was performed using the NCSS 5.0 program. Significance was calculated by means of two-way analysis of variance (ANOVA) or one-way ANOVA followed by Duncan's post-hoc test for multiple comparisons, where appropriate. The level of significance was set at $P < 0.05$.

3. Results

3.1. Systemic effects of drug treatments

Consistent with previous observations (Soininen et al., 1990; Blokland et al., 1995), the administration of metrifonate or tacrine to rats led to transient peripheral cholinergic activation as evidenced by tremor, defecation and fasciculations. However, upon repeated administrations, the signs of cholinergic overstimulation were greatly reduced compared to those observed after the first administration.

Rat body weight was measured daily. Body weights before treatment were: 441.2 ± 8.3 g ($n = 16$), 455.8 ± 8.3 g ($n = 17$), 433.6 ± 8.1 g ($n = 14$) for rats allocated to metrifonate, tacrine and control groups, respectively. One-way ANOVA showed no significant differences among the three experimental groups ($F_{2,44} = 1.859$; $P < 0.16$; n.s.). After 21 days, body weights decreased by 6 and 4% in metrifonate- and tacrine-treated rats, respectively, compared to vehicle treated rats ($F_{2,40} = 14.56$; $P < 0.01$).

Heart rate and systolic blood pressure were measured before treatment and at the 20th day of treatment, 2 h after the last drug administration, starting at 1000. Heart frequency and systolic blood pressure did not vary significantly after 20 days of metrifonate or tacrine treatment, compared to vehicle treated rats (data not shown).

3.2. Cortical and hippocampal extracellular acetylcholine levels and cholinesterase inhibition

Extracellular acetylcholine levels measured in the cortex and hippocampus of aged rats after 21 days of administration of metrifonate, tacrine or vehicle, 18 h after the last treatment and with no cholinesterase inhibitor added to the perfusing Ringer solution, are shown in Table 1 and Figs. 1 and 2. In the rats receiving metrifonate at the dose of 80 mg kg^{-1} p.o. twice per day, 18 h after the last administration, the basal cortical extracellular acetylcholine levels were about 15 times larger than those of the vehicle and tacrine-treated rats (3 mg kg^{-1} p.o. twice per day) ($F_{2,14} = 9.23$; $P < 0.005$, one-way ANOVA and Duncan's test). On the other hand, no difference was found between the basal cortical acetylcholine levels of tacrine and vehicle treated rats.

The effect of a challenge with metrifonate or tacrine on cortical acetylcholine levels of subchronically treated rats is shown in Fig. 1. Two-way ANOVA conducted with drug and time as factors indicate a statistical significant effect of both drug treatments ($F_{2,172} = 37.69$; $P < 0.00001$) and challenge ($F_{14,172} = 1.79$; $P < 0.05$). Metrifonate and tacrine increased cortical extracellular acetylcholine levels by about three and four times, respectively, with a peak 1 h after administration and a slow return to basal levels. Absolute cortical extracellular acetylcholine levels calculated at the plateau after metrifonate challenge (between dotted lines in Fig. 1) were on average around 90 fmol μl^{-1}, after tacrine they were around 9 fmol μl^{-1} and

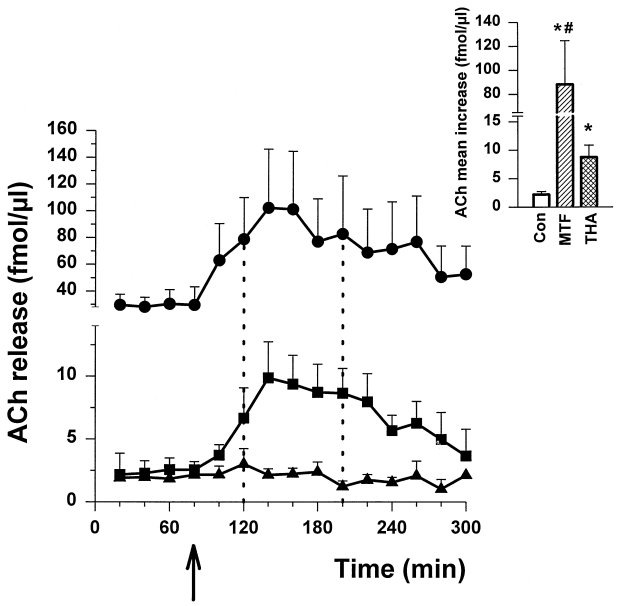

Fig. 1. Effect of a challenge with metrifonate (80 mg kg^{-1} p.o.), tacrine (3 mg kg^{-1} p.o.) or vehicle (1 ml kg^{-1} p.o.) on cortical acetylcholine release in aged rats treated for 21 days with metrifonate, tacrine or vehicle, respectively. Metrifonate, tacrine or vehicle were administered after collecting four basal samples, as shown by the arrow. Acetylcholine release is expressed as fmol μl^{-1}. Note the break of the *y*-axis. Metrifonate 80 mg kg^{-1} (●; $n = 6$); tacrine 3 mg kg^{-1} (■; $n = 6$); vehicle (▲; $n = 5$). Significant differences among the three experimental groups were calculated using two-way ANOVA with drug and time as factors (see details in Section 3). Inset: Absolute cortical extracellular acetylcholine levels at the plateau after metrifonate (MTF), tacrine (THA) or vehicle (Con), calculated between times 120 and 200 min, as shown by dotted lines. Significant differences in the acetylcholine maximum increases were calculated using one-way ANOVA ($F_{2,14} = 5.04$; $P < 0.02$) followed by Duncan's test for multiple comparisons ($* P < 0.05$ vs. controls; $\# P < 0.05$ vs. tacrine).

after vehicle they were around 2 fmol μl^{-1} (Fig. 1 inset: $F_{2,12} = 4.04$; $P < 0.05$, one-way ANOVA and Duncan's test).

In the hippocampus, the subchronic metrifonate treatment increased by twofold basal extracellular acetylcholine levels vs. tacrine (3 mg kg^{-1} p.o.) and vehicle treated rats ($F_{2,16} = 3.66$; $P < 0.05$), Table 1. The effect of a challenge with metrifonate or tacrine on hippocampal acetylcholine levels of subchronically treated rats is shown in Fig. 2. Two-way ANOVA conducted with drug and time as factors indicate a statistically significant effect of both drug treatments ($F_{2,159} = 14.44$; $P < 0.00001$) and challenge ($F_{14,159} = 1.84$; $P < 0.04$). Metrifonate and tacrine evoked a three- and a twofold increase in hippocampal extracellular acetylcholine levels, respectively, with a maximum 60 and 80 min after administration and a slow return to basal levels (Fig. 2). Absolute hippocampal extracellular acetylcholine levels calculated at the plateau after metrifonate challenge (between dotted lines in Fig. 2) were on average around 10 fmol μl^{-1}, after tacrine they were around 4 fmol μl^{-1}, and after vehicle they were around 1.5 fmol μl^{-1} (Fig. 2 inset: $F_{2,13} = 6.44$; $P < 0.01$, one-way ANOVA and Duncan's test).

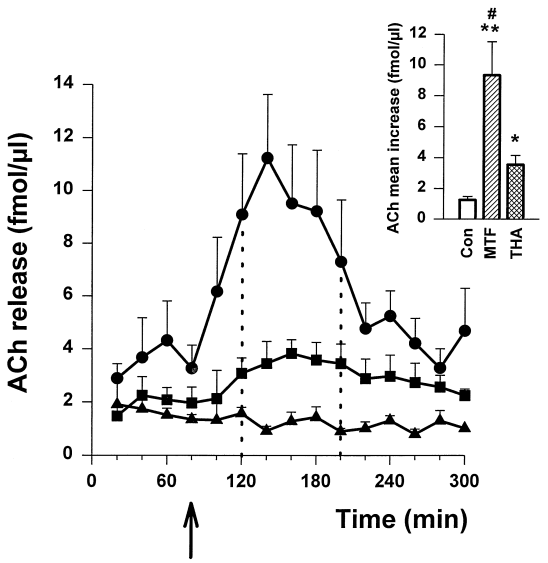

Fig. 2. Effect of a challenge with metrifonate (80 mg kg^{-1} p.o.), tacrine (3 mg kg^{-1} p.o.) or vehicle (1 ml kg^{-1} p.o.) on hippocampal acetylcholine release in aged rats treated for 21 days with metrifonate, tacrine or vehicle, respectively. Metrifonate, tacrine or vehicle were administered after collecting four basal samples, as shown by the arrow. Acetylcholine release is expressed as fmol μl^{-1}. Metrifonate 80 mg kg^{-1} (●; $n = 7$); tacrine 3 mg kg^{-1} (■; $n = 5$); vehicle (▲; $n = 6$). Significant differences among the three experimental groups were calculated using two-way ANOVA with drug and time as factors (see details in Section 3). Inset: Absolute hippocampal extracellular acetylcholine levels at the plateau after metrifonate (MTF), tacrine (THA) or vehicle (Con), calculated between times 120 and 200 min, as shown by dotted lines. Significant differences in the acetylcholine maximum increases were calculated using one-way ANOVA ($F_{2,16} = 9.10$; $P < 0.005$) followed by Duncan's test for multiple comparisons ($* P < 0.05$ vs. controls; $** P < 0.01$ vs. controls; $\# P < 0.05$ vs. tacrine).

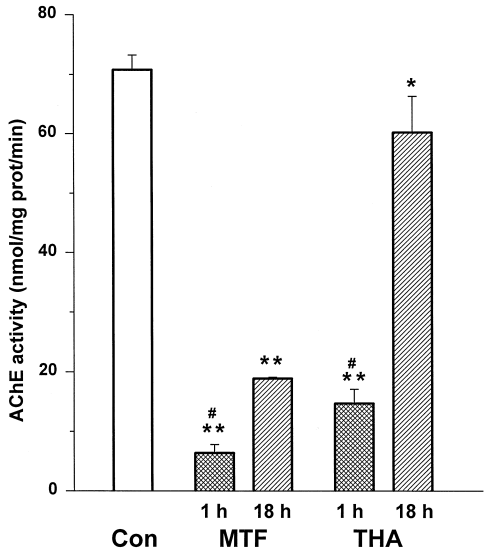

Fig. 3. Effect of metrifonate or tacrine treatment on cholinesterase activity in whole brain homogenates. Cholinesterase activity was detected 1 and 18 h after the last treatment. Con = vehicle; MTF = metrifonate; THA = tacrine. Number of rats ranging from four to 12. Significant differences among the five experimental groups were calculated using one-way ANOVA ($F_{4,34} = 85.25$; $P < 0.0001$) followed by Duncan's test for multiple comparisons ($* P < 0.05$ and $** P < 0.01$ vs. controls; $\# P < 0.05$ vs. respective 18-h treatment; no significant difference was observed between metrifonate and tacrine at 1 h after the last treatment).

Cholinesterase activity measured in whole brain homogenate 18 and 1 h after the last treatment with metrifonate and tacrine is shown in Fig. 3. 18 h after the last treatment, cholinesterase was still inhibited by about 75% in the metrifonate-treated, and by only 15% in the tacrine-treated rats. However, metrifonate and tacrine given 1 h after the last administration inhibited cholinesterase activity to comparable levels (90 and 80% inhibition vs. controls in metrifonate- or tacrine-treated rats, respectively). Nevertheless, the increase in acetylcholine extracellular level induced by the two drugs is markedly different as shown in Figs. 1 and 2. In a few animals, cholinesterase activity was measured in the cortex and hippocampus, separately, and the same degree of inhibition as in the whole brain was found (data not shown).

3.3. Cortical and hippocampal choline acetyltransferase activity

ChAT activity was measured in cortex and hippocampus and no difference was observed in either of the two brain regions at 1 or 18 h after metrifonate or tacrine treatment, compared to vehicle-treated rats ChAT activity values, expressed in $\mu mol\ h^{-1}\ mg^{-1}$ protein, in vehicle-treated rats were: cortex 1 h: 4.75 ± 0.85, cortex 18 h: $5.56. \pm 0.59$, hippocampus 1 h: 6.37 ± 1.05; hippocampus 18 h: 7.85 ± 1.97).

4. Discussion

Our experiments demonstrate that subchronic oral administration of metrifonate, at the dose of 80 mg kg^{-1} twice daily to aged rats, results in a profound, long-lasting cholinesterase inhibition paralleled by an increase in extracellular acetylcholine levels, both in the cortex and hippocampus.

The increase in acetylcholine level in the cortex was much larger than in the hippocampus. A challenge with metrifonate further inhibited cholinesterase activity and increased cortical and hippocampal extracellular acetylcholine levels. Conversely, 18 h after the last tacrine administration, there was only a 15% cholinesterase inhibition left which was not sufficient to induce an increase in extracellular acetylcholine levels above those of the control rats. Only after a challenge with tacrine, a significant increase in cortical and hippocampal extracellular acetylcholine levels was found.

Both cholinesterase inhibitors were well tolerated since they resulted only in a slight reduction in body weight while they did not affect heart rate and blood pressure.

The hypofunction of central cholinergic system is considered an important pathogenic element of the age-associated cognitive impairments and dementia (Bartus et al., 1982; Collerton, 1986). In the central nervous system of aged rodents, significant morphological changes of the forebrain cholinergic neurons, associated with their hypofunction, and behavioural impairments, have been repeatedly reported (Fischer et al., 1987; Wu et al., 1988; Scali et al., 1994).

The long-lasting increase of extracellular acetylcholine levels after metrifonate treatment reflects the persistent inhibition of cholinesterase activity caused by its active metabolite DDVP. DDVP behaves at first as a competitive inhibitor but then switches to noncompetitive inhibition of cholinesterase resulting in a stable drug–enzyme complex (Hinz et al., 1996b) that lasts for several hours. This mechanism of action of metrifonate can explain its long-acting cholinesterase inhibition and the ensuing persistent rise in extracellular acetylcholine levels. On the contrary, the 15% residual cholinesterase inhibition found after subchronic administration of tacrine was apparently not sufficient to affect extracellular acetylcholine levels which were similar to those of controls. Therefore, more frequent tacrine administrations are needed in order to achieve a constant cholinesterase inhibition accompanied by a persistent increase in acetylcholine levels.

One hour after the administration of the challenge, both drugs brought about a similar 80–90% cholinesterase inhibition. Nevertheless, the increase in absolute values in acetylcholine release was several folds larger after metrifonate than tacrine. As already reported by Becker and Giacobini (1988) and Messamore et al. (1993), there is not always a consistent relationship between the degree of cholinesterase inhibition and changes in brain acetylcholine concentration. Tacrine (for review, see Wagstaff and McTavish, 1994) can displace [^{3}H]quinuclidinyl benzilate from muscarinic receptors (Nillson et al., 1987; DeSarno et al., 1989) and inhibit high affinity choline uptake in vitro (Buyukuysal and Wurtman, 1989; Nielsen et al., 1989) and in the aged rat in vivo (Kristofikova et al., 1992). It is possible, therefore, that feedback inhibition via muscarinic presynaptic receptors (Nordberg et al., 1989; Svensson et al., 1996) and/or synthesis inhibition, via inhibition of high affinity choline uptake, could be the cause of the apparent discrepancy between the large inhibition of cholinesterase activity and the small increase in extracellular acetylcholine levels after tacrine treatment. It is pertinent to mention that Hodges et al. (1990) found in the rat that chronic treatment with tacrine did not significantly affect acetylcholine content in several brain areas, and that metrifonate is more effective than physostigmine and tacrine in increasing glucose utilisation, which is considered an indirect indication of the cholinergic function (Bassant et al., 1996).

Our findings do not demonstrate a central adaptation of the cortical cholinergic function induced by the persistent increase of acetylcholine levels, at least over 3-week treatment, since the challenge with metrifonate was followed by a further fourfold increase in acetylcholine levels, similar to that observed in aging rats after a single administration (Scali et al., 1997a). Furthermore, the large effect of metrifonate on the cortical cholinergic system did not lead to changes in choline acetyltransferase activity, or in the number or affinity of muscarinic or nicotinic receptors as demonstrated by Schmidt et al. (1997). However, an attenuation of the peripheral side effects induced by tacrine and metrifonate, namely tremor, defecation and fasciculations, was observed after repeated administrations, indicating an adaptation of the peripheral cholinergic system similar to that described in patients treated with cholinesterase inhibitors for myasthenia gravis (Taylor, 1996).

Our experiments show that the extracellular acetylcholine levels found in the cortex after 21 days of treatment with metrifonate were over 10 times larger than in the hippocampus. Scali et al. (1997a) demonstrated that an acute oral administration of the same dose of metrifonate increases cortical but not hippocampal extracellular acetylcholine levels in the aged rat while it inhibits at the same extent cortical and hippocampal cholinesterase activity. Therefore, repeated administrations of metrifonate are necessary in aged rats in order to induce a statistically significant increase in hippocampal extracellular acetylcholine levels. It is not easy to explain these regional differences between cortex and hippocampus, since the cholinesterase activity is inhibited by metrifonate at the same extent in both regions, as shown in a few animals in this work and after acute administration in previous experiments (Scali et al., 1997a). On the other hand, Bassant et al. (1996) reported that stimulation of local cerebral glucose utilisation by acute metrifonate administration occurs mainly in

the cortex. According to Cuadra et al. (1994) and Mori et al. (1995), regional differences in extracellular acetylcholine levels between the cortex and the hippocampus may be caused by the effects of cholinesterase inhibitors on other neurotransmitter systems.

Subchronic administration of metrifonate in the rat resulted in a persistent cholinesterase inhibition and a large increase in extracellular acetylcholine levels. It has been also demonstrated (Riekkinen et al., 1997) that cognitive improvement brought about by metrifonate becomes long-lasting in subchronically treated rats. Extrapolating these results to patients treated with metrifonate as a palliative treatment for dementia, it may be assumed that high levels of extracellular acetylcholine would be present in the cortex and hippocampus not only after administration but also in-between administrations of the drug. Maintaining high levels of extracellular acetylcholine in the cortex and hippocampus even in-between administrations might therefore functionally counterbalance the loss of cholinergic neurones occurring in Alzheimer's disease (Bowen et al., 1992, 1994). According to Everitt and Robbins (1997), attentional functions are involved in the modulation of short-term working memory and in conditional discrimination. High levels of extracellular acetylcholine after repeated treatment with metrifonate may restore these functions in demented patients and improve their cognitive performances (Cummings et al., 1998).

Acknowledgements

This investigation was supported by a grant from Bayer. We thank Mr. P. Ceccatelli and Mr. M. Beni for invaluable help in treating the animals and Mr. C.L. Susini for technical assistance.

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