ELSEVIER

European Journal of Pharmacology 281 (1995) 179–185

ejp

3,4-Diaminopyridine and choline increase in vivo acetylcholine release in rat striatum

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Received 8 March 1995; revised 10 April 1995; accepted 13 April 1995

Abstract

We investigated the effects of choline, 3,4-diaminopyridine and their combination on acetylcholine release from the corpus striatum of freely moving rats which were treated or not with atropine. Intraperitoneal administration of choline or intrastriatal administration of 3,4-diaminopyridine increased acetylcholine levels in striatal dialysates in a dose-dependent manner. When 3,4-diaminopyridine treatment was combined with choline, the observed effect was considerably greater than the sum of the increases produced by choline or 3,4-diaminopyridine alone. Administration of atropine (1 μM) in the dialysing medium was also found to be effective to stimulate striatal acetylcholine levels. 3,4-Diaminopyridine did not affect acetylcholine levels under these conditions. Whereas the choline-induced increase in acetylcholine release was significantly potentiated by atropine, co-administration of 3,4-diaminopyridine with choline failed to produce a further significant increase in the presence of atropine. These results suggest that a highly effective means for increasing acetylcholine release involves two concurrent treatments that increase neuronal choline levels and inhibition of the negative feedback modulation of acetylcholine release.

Keywords: Acetylcholine; Choline; 3,4-Diaminopyridine; Microdialysis

1. Introduction

In vivo (Casamenti et al., 1982; Damsma et al., 1988) and in vitro release of acetylcholine from peripheral (Vizi et al., 1977; Glavinovic, 1986; Roed, 1989) and central nerve endings (Dolezal and Tucek, 1983; Tapia et al., 1985; Foldes et al., 1988; Buyukuysal and Wurtman, 1990; Potter et al., 1989; Buyukuysal et al., 1991) can be enhanced by aminopyridines, which are thought to increase neurotransmitter release by blocking outward K^+ conductance (Yeh et al., 1976; Meves and Pichon, 1977; Molgo et al., 1977). This effect is dependent on the presence of calcium ions (Vizi et al., 1977; Tapia and Sitges, 1982; Dolezal and Tucek, 1983) as well as on the integrity of neuronal firing (Vizi et al., 1977; Dolezal and Tucek, 1983; Damsma et al., 1988; Buyukuysal and Wurtman, 1990). Hemicholinium-3, a specific inhibitor of high-affinity choline uptake, can decrease aminopyridine-induced acetylcholine release (Buyukuysal and Wurtman, 1990), indicating that its effect is also dependent on the availability of free choline levels in the medium.

Since both acetylcholine synthesis and release are dependent on available free choline levels, and since the effect of choline on acetylcholine release is enhanced when neuronal firing frequency is increased (Wurtman et al., 1981; Blusztajn and Wurtman, 1983; Tucek, 1984), it might be anticipated that the maximum amplification of acetylcholine release would be produced by administering a drug which increases neuronal activity along with choline or a choline source. In vitro studies clearly indicate that 3,4-diaminopyridine and choline can interact to augment acetylcholine release from rat striatal slices, both at rest and under depolarizing conditions (Buyukuysal et al., 1991). We now report that 3,4-diaminopyridine can also interact with choline to enhance acetylcholine release from conscious rats by a mechanism resembling an atropine-induced increase. This combination may have some usefulness in the long-term management of neurodegenerative cholinergic disorders.

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SSDI 0014-2999(95)00241-3

2. Material and methods

2.1. Microdialysis

The surgical procedures and experimental protocols were approved by the Uludag University Medical Center Institutional Review Board for Animal Research. Male Sprague-Dawley rats (weighing 300–350 g, from the Experimental Animals Breeding and Research Center, Uludag University, Bursa, Turkey) were anaesthetized with pentobarbital sodium (40 mg/kg) and placed in a stereotaxic frame. The skull was exposed and a hole was drilled over the right striatum: coordinates 1 mm anterior to bregma and 2.5 mm lateral (right) to the midline suture according to Paxinos and Watson (1986). A handmade probe (molecular weight cut-off of dialysis membrane was 10000 Da and length was 3 mm) was implanted to a depth of 8 mm (flat skull) and then fixed with two small screws to the skull. The location of the probe was confirmed by visual examination of the brain at the end of each experiment. Dialysis experiments were carried out 18–24 h after operation to avoid effects of anaesthesia and the dialysis probe was perfused with artificial cerebrospinal fluid (CSF) of the following composition: 120 mM NaCl, 3.5 mM KCl, 1.3 mM $CaCl_2$, 1.2 mM $MgSO_4$, 1.2 mM NaH_2PO_4, 25 mM $NaHCO_3$. This solution contained 10 μM of neostigmine bromide to improve analytical chemical detection of acetylcholine; the perfusion rate was adjusted to 2 μl/min.

Choline levels in striatal dialysates were compared with those of cerebellar or ventricular choline after the choline treatment. Rats were then anaesthesized with pentobarbital sodium (40 mg/kg) and placed in a stereotaxic frame. After exposure of the skull, two holes were drilled over both right striatum and lateral ventricle (or cerebellum). Coordinates of the probes were A −1 mm from bregma, L 1.5 mm (left) and V 4.2 mm for lateral ventricle, and A −9 mm from bregma, L 3 mm (left) and V 4.0 mm for cerebellum according to Paxinos and Watson (1986). The dialysis procedure was started just after implantation of the probes and dialysate samples were collected at 15-min intervals. When dialysate choline levels collected from each brain area reached steady state levels, 100 mg/kg choline was injected intraperitoneally. The depth of anaesthesia during the dialysis period was maintained with intraperitoneal injections of pentobarbital sodium.

2.2. Drugs and treatments

Choline chloride (from Sigma Chem. Co.) was dissolved in saline and injected intraperitoneally. The choline dose was calculated for the free base. Control animals received saline. 3,4-Diaminopyridine (from Aldrich Chem. Co.) was dissolved in distilled water and administered intrastriatally by adding it to the perfusion solution at the concentrations indicated in the text. When tested in combination, both drugs were administered simultaneously. Atropine (from Sigma Chem. Co.) was also added into the dialysing medium. Animals were dialysed with atropine-containing medium for 1 h and then 3,4-diaminopyridine or choline treatment was performed in the presence of atropine.

2.3. Analytical procedure

Dialysate samples were collected continuously at 15-min intervals and injected on a high pressure liquid chromatography (HPLC) system combined with an immobilized enzyme reactor and an electrochemical detector; acetylcholine and choline levels were measured according to the method of Damsma et al. (1985). Briefly, acetylcholine and choline were separated on a cation exchange column (from B.A.S.). An enzyme reactor containing acetylcholinesterase and choline oxidase (from B.A.S.) converted acetylcholine and choline to hydrogen peroxide. Hydrogen peroxide was then electrochemically detected with a platinum electrode at +0.500 V. The mobile phase consisting of 0.07 M Na_2HPO_4 (pH 8.3) and antibacterial Kathon (0.5%; from B.A.S.) was delivered by an HPLC pump (Shimadzu, Model LC-9A). The flow rate was 1.0 ml/min. Chromatograms were completed within 8 min, thereby allowing immediate analysis. The detection limit of the acetylcholine assay was about 100 fmol/injection.

2.4. Data analysis

Quantification of acetylcholine and choline levels in the dialysate samples was performed by comparison with peak heights of authentic standards. Since the baseline levels of acetylcholine varied between rats, the average amount of neurotransmitter output of the three pre-drug samples was taken as 100%, and release values obtained during drug treatment were expressed as percentages of the controls. The data are shown as means ± S.E.M.; statistical analysis was performed using the analysis of variance (ANOVA) followed by the Wilcoxon test for comparisons across the treatments. A P value of less than 0.05 was considered significant.

3. Results

3.1. Basal acetylcholine and choline levels

As indicated above, brain microdialysis was performed 18–24 h after implantation of the probe. In preliminary studies, we observed that acetylcholine levels in striatal dialysates reached steady state levels in

1–2 h. Thus, we started to collect the dialysate samples 2 h after the beginning of microdialysis. The basal acetylcholine and choline concentrations in striatal dialysates from untreated rats ($n = 8$) were 10.46 ± 2.4 pmol/15 min and 10.08 ± 1.2 pmol/15 min, respectively. These values were calculated by comparison of the peak heights of acetylcholine and choline standards without making any correction for the recovery from the probes. Although acetylcholine concentrations in dialysate samples were almost constant, choline levels showed a gradual decline during the overall dialysis period.

3.2. Effect of choline on acetylcholine and choline levels in striatal dialysates

Intraperitoneal injection of choline (50, 100 and 120 mg/kg as a free base) significantly increased acetylcholine levels in striatal dialysates during the first 15 min of the treatment. This effect was dose-dependent, reaching its maximum level in 30 min and persisting at significant levels during a 90-min dialysis period (Fig. 1). Choline levels, however, increased slightly with the 100 and 120 mg/kg choline treatment. In contrast to acetylcholine, this effect was seen only during the first 15 min of choline treatment after which choline levels

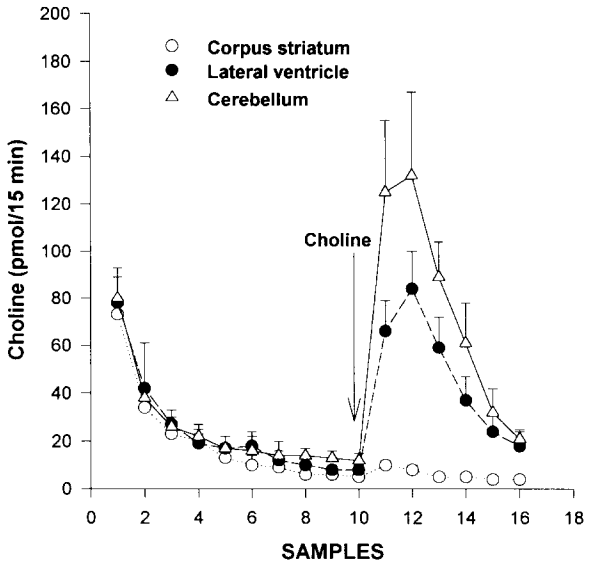

Fig. 2. Effect of intraperitoneal choline treatment on choline levels in dialysate samples collected from the striatum, lateral ventricle and cerebellum. Two probes were inserted in each rat brain as indicated in the text. The dialysing procedure was started just after implantation of the probes and samples were collected at 15-min intervals. After choline levels reached a stable baseline, 100 mg/kg of choline was injected intraperitoneally. Choline levels in each fraction were calculated by comparison of the peak heights of choline standards without making any correction for the recovery from the probes. The data are the means ± S.E.M. The number of experiments are: $n = 10$ (for striatum), $n = 8$ (for lateral ventricle) and $n = 4$ (for cerebellum).

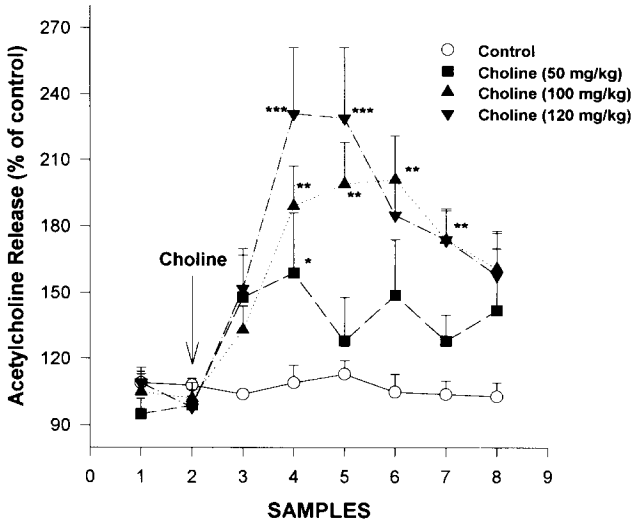

Fig. 1. Effect of different doses of choline given intraperitoneally on acetylcholine levels in striatal dialysates. Right striatum was dialysed 18–24 h after implantation of the probe. After basal acetylcholine levels reached a steady state level, choline or saline (control) was injected intraperitoneally and dialysis samples were collected at 15-min intervals. Acetylcholine levels in each fraction are expressed as percentages of basal values (an average of the last three samples). The data are the means ± S.E.M. The numbers of measurements are: $n = 8$ (control), $n = 7$ (choline 50 mg/kg), $n = 6$ (choline 100 mg/kg) and $n = 6$ (choline 120 mg/kg). $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$, significantly different from control values.

in striatal diaysates returned to their control levels (data not shown).

Choline levels in striatal, ventricular and cerebellar dialysates, collected just after implantation of the probes, were 83 ± 7 ($n = 10$), 88 ± 15 ($n = 8$) and 90 ± 9 ($n = 4$) pmol/15 min, respectively, and then showed a rapid decline during the dialysis period (Fig. 2). Intraperitoneal injection of choline (100 mg/kg) slightly and transiently increased the choline concentration in striatal dialysates. This treatment, however, caused a considerably greater increase in ventricular and cerebellar choline levels (Fig. 2).

3.3. Effect of 3,4-diaminopyridine

We tested the effect of three different doses of 3,4-diaminopyridine on acetylcholine levels in the striatal dialysates. While a 10 μM dose of 3,4-diaminopyridine caused no change, 50 and 100 μM doses of this drug significantly increased acetylcholine levels (Fig. 3).

3.4. Combined effect of choline and 3,4-diaminopyridine

In this series of experiments, choline (100 mg/kg) was injected in rats concurrently with 3,4-diaminopyri-

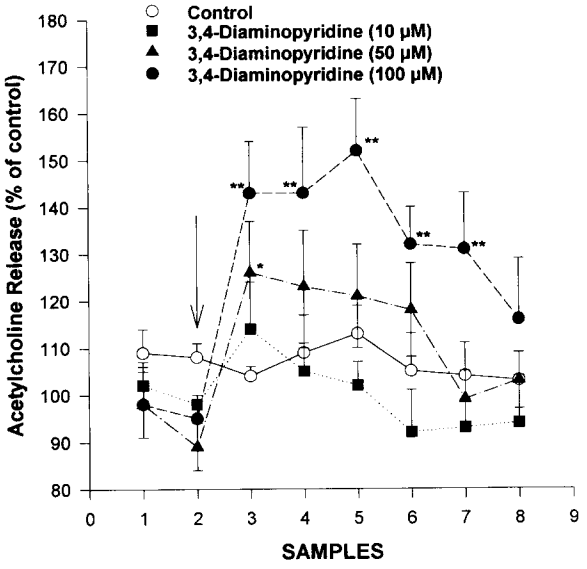

Fig. 3. Effect of 3,4-diaminopyridine on acetylcholine levels in striatal dialysates. After basal acetylcholine levels reached steady state levels, 3,4-diaminopyridine was added into the artificial CSF and the right striatum was dialysed with 3,4-diaminopyridine-containing medium. Samples were collected at 15-min intervals and acetylcholine levels were measured as indicated in the text. Acetylcholine levels in each fraction were expressed as percentages of the basal value of the last three fractions before 3,4-diaminopyridine administration. The data are the means ± S.E.M. The numbers of measurements are: $n = 8$ (control), $n = 4$ (10 μM), $n = 4$ (50 μM) and $n = 8$ (100 μM). $^{*}P < 0.05$, $^{**}P < 0.01$, significantly different from control values.

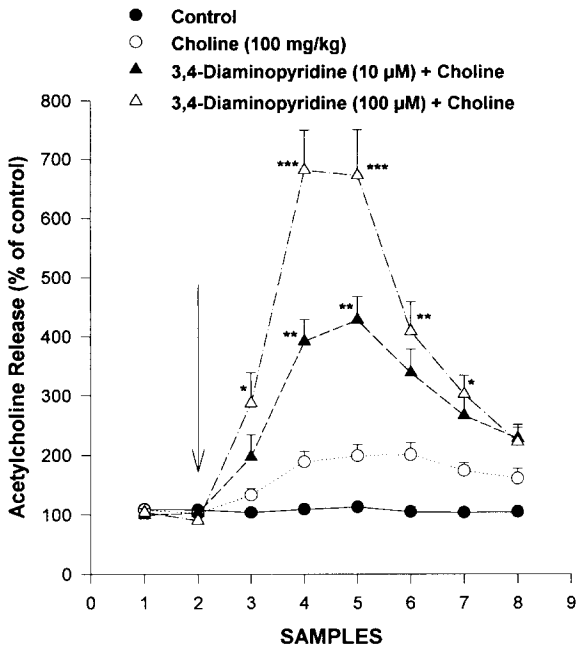

Fig. 4. Combined effect of 3,4-diaminopyridine and choline treatment on acetylcholine levels in striatal dialysates. After basal acetylcholine levels reached a steady state level, choline (100 mg/kg) was injected intraperitoneally and the dialysing medium was changed to the 3,4-diaminopyridine-containing artificial CSF. Samples were collected at 15-min intervals. Acetylcholine levels in each fraction were expressed as percentages of the basal value of the last three fractions before choline and 3,4-diaminopyridine treatment. The data are the means ± S.E.M. The numbers of measurements are 8 for each treatment. $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$, significantly different from corresponding values obtained after choline treatment alone.

dine administration. In choline-treated rats, a 10 μM dose of 3,4-diaminopyridine, which alone failed to affect striatal acetylcholine levels, now significantly increased the acetylcholine concentration in dialysate samples (Fig. 4). In the absence of 3,4-diaminopyridine, acetylcholine levels of striatal dialysates, collected over a 90-min period, rose to 172 ± 8% of the control value after choline treatment (Table 1, $P < 0.01$). On the

Table 1
Comparison of the effects of choline and 3,4-diaminopyridine alone and their combination on acetylcholine levels in striatal dialysates during a 90-min dialysing period

Treatment	Acetylcholine (% of control)	% Increase	(n)
Control	105 ± 7	–	8
Choline			
50 mg/kg	140 ± 17	33	4
100 mg/kg	172 ± 8 [a]	64	6
120 mg/kg	187 ± 15 [a]	78	6
3,4-diaminopyridine			
10 μM	100 ± 4	–	4
100 μM	136 ± 7 [b]	29	8
Choline (100 mg/kg) + 3,4-diaminopyridine (10 μM)	307 ± 31 [c]	192	8
Choline (100 mg/kg) + 3,4-diaminopyridine (100 μM)	438 ± 50 [c]	317	8

The averages acetylcholine levels of the three pre-treatment samples were taken as 100%, and acetylcholine values obtained after drug treatment were expressed as percentages of the controls. The values mentioned in this table were calculated by taking the mean of the six acetylcholine values obtained after drug treatment (which indicates a 90-min dialysing period). The data are given as means ± S.E.M. [a] $P < 0.01$, [b] $P < 0.05$ from control, [c] $P < 0.001$ from choline treatment. The numbers of treatments are shown in the column (n).

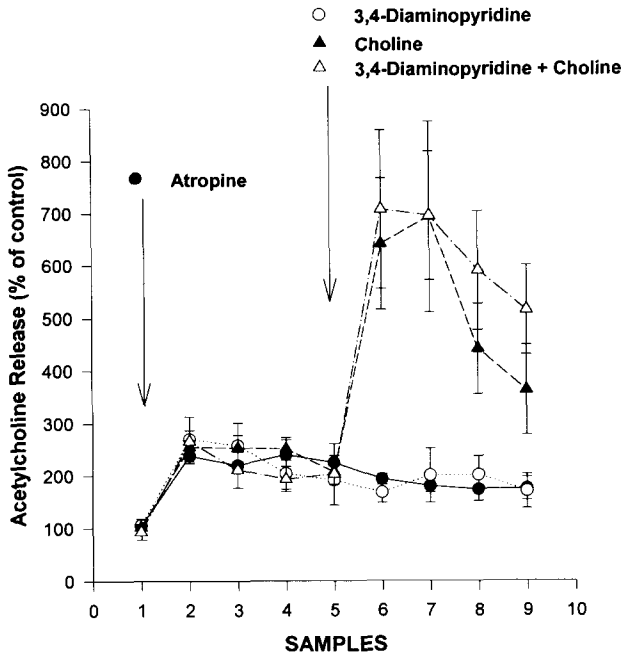

Fig. 5. Effect of 3,4-diaminopyridine, choline and their combination on acetylcholine levels of striatal dialysates collected from atropine-treated rats. After acetylcholine levels reached steady state level, atropine (1 μM) was added into the dialysing medium. Rats were dialysed with atropine-containing medium for 1 h and then received only choline (100 mg/kg i.p.), or 3,4-diaminopyridine (100 μM by adding to artificial CSF) or choline plus 3,4-diaminopyridine. Samples were collected at 15-min intervals and analysed as indicated in the text. The numbers of experiments are 6 for each group. The data are the means ± S.E.M. Values obtained after 3,4-diaminopyridine treatments were not significanly different from the corresponding control values ($P > 0.05$).

other hand, coadministration of 3,4-diaminopyridine (10 μM) with choline significantly potentiated choline's effect; under these conditions acetylcholine levels rose to 307 ± 31% of the control value ($P < 0.01$ from choline treatment alone). A similar interaction was also observed when the 3,4-diaminopyridine concentration in artificial CSF was increased to 100 μM (Fig. 4; Table 1).

3.5. Effect of choline and 3,4-diaminopyridine on acetylcholine release in atropine-treated animals

Administration of atropine (1 μM) in the dialysis medium significantly increased acetylcholine levels during the first 15 min of the dialysis period (266 ± 21% of the control value; $n = 16$). This increase declined slightly during a 60-min dialysis period. The addition of 3,4-diaminopyridine (100 μM) in the dialysing medium, a dose that stimulated acetylcholine release in control animals, failed to further affect the levels of acetylcholine (Fig. 5). Intraperitoneal choline treatment (100 mg/kg), however, significantly potentiated the atropine-induced increase. Choline's effect was not enhanced by coadministration of 3,4-diaminopyridine with choline to atropine-treated rats (Fig. 5).

4. Discussion

Our results clearly indicate that intraperitoneal injection of choline is able to enhance in vivo acetylcholine release from rat corpus striatum. This effect was dose-dependent and persisted at significant levels during a 90-min dialysis period. Although these data are in agreement with previous findings for in vitro (Maire and Wurtman, 1985; Ulus et al., 1989; Buyukuysal et al., 1991) and in vivo conditions (Koshimura et al., 1990; Johnson et al., 1992; Farber et al., 1993; Marshall and Wurtman, 1993), failure of choline given intraperitoneally to enhance the in vivo acetylcholine release has also been reported (Koshimura et al., 1990; Westerink and De Boer, 1990). In one of these studies, choline was given at a dose of 72 μmol/kg (7.5 mg/kg) – much less than the choline doses used in our study (50, 100 and 120 mg/kg). Thus, the absence of choline effect observed by Koshimura et al. (1990) can be attributed to its dose, which might have been insufficient to enhance striatal acetylcholine levels. Choline, however, at doses which reportedly failed to enhance basal acetylcholine levels (104 mg/kg, Westerink and De Boer, 1990), significantly increased the striatal acetylcholine levels in our study. The main differences between the two experiments are the types of probes inserted in the brain (transversal vs. U-shaped) and the neostigmine concentration added in the dialysis medium (0.1 μM vs. 10 μM). Differences in the neostigmine concentration rather than probe type might have contributed to the observed discrepancy in choline effect. The massive inhibition of acetycholinesterase by 10 μM of neostigmine might reduce the availability of free choline in the immediate vicinity of the cholinergic synapse. Thus, exogenous choline may be able to increase acetylcholine synthesis and release.

In contrast to those of acetylcholine, choline levels in striatal dialysates were slightly and transiently affected by choline treatment. A limited effect of choline treatment, intraperitoneally (Farber et al., 1993; Marshall and Wurtman, 1993) or even intracerebroventricularly (Koshimura et al., 1990), to affect the choline levels in striatal dialysates has also been reported previously. The enhancement of ventricular choline levels observed in this study indicates that intraperitoneal choline treatment sigificantly increases choline levels in cerebrospinal fluid (Fig. 2). Choline treatment, on the other hand, was also able to enhance choline levels in the dialysates collected from cerebellum, a brain area deprived of the high-affinity choline uptake mechanism. These results indicate that most of the choline

administered exogenously might be rapidly taken up into striatal tissue by the high-affinity choline uptake mechanism.

It is clear from our results that 3,4-diaminopyridine increased acetylcholine levels in striatal dialysates and its effect was significantly potentiated when combined with choline treatment. Because 3,4-diaminopyridine has no effect on [^{3}H]quiniclidinyl benzilate binding at the doses used in this study (Buyukuysal and Wurtman, unpublished data), its effect cannot be attributed to an antagonistic action on muscarinic receptors. The failure of both 3,4-diaminopyridine alone and its combination with choline to enhance acetylcholine release in the presence of atropine suggests that 3,4-diaminopyridine and atropine might enhance acetylcholine release, probably by a similar mechanism. This suggestion is supported by the previous findings from in vitro slice studies, indicating that presynaptic muscarinic receptors regulating acetylcholine release might be coupled to K^+ channels which are sensitive to aminopyridines (Fredholm, 1990; Allgaier et al., 1992; Zelles et al., 1992). In addition to opening of K channels, however, increased formation of cyclic GMP (Nordstrom and Bartfai, 1981), inhibition of adenylate cyclase (Alberts and Ögren, 1988) and closing of the calcium channels (Hamilton and Smith, 1991) are also reported as possible mechanisms to explain the inhibitory effect of muscarinic receptor stimulation on acetylcholine release.

In summary, one of the most effective treatments for increasing acetylcholine levels in the synaptic cleft is the inhibition of cholinesterase. Such a treatment, however, causes a massive inhibition of acetylcholine release by increasing negative feedback modulation and also reduces the availability of free choline in the immediate vicinity of the cholinergic synapse. Our results indicate that co-administration of 3,4-diaminopyridine with a choline source seems to be a useful adjuvant therapy for increasing the therapeutic efficacy of cholinesterase inhibitors. Although the therapeutic efficacy of aminopyridines has not been tested extensively, these compounds may have promise as therapeutic agents in neurodegenerative cholinergic disorders (Gibson and Peterson, 1986; Roberts, 1986). One potential disadvantage of the aminopyridines is the non-specificity of their sites of action (Tapia et al., 1985; Barnes et al., 1989; Damsma et al., 1988). Combining these compounds with a neurotransmitter precursor, such as choline, should increase the specificity of their synaptic action.

Acknowledgements

This study was supported by a grant from the Research Fund of Uludag University (1991/3). We wish to thank Dr. R.J. Wurtman for the valuable support given to this work and for his review of the manuscript.

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