ABSTRACT—We examined the effects of tacrine (9-amino-1,2,3,4-tetrahydroacridine) on endogenous acetylcholine (ACh) release from rat hippocampal slices. Tacrine (more than 1 µM) increased the measurable amount of basal ACh release. On the other hand, in the presence of physostigmine (50 µM; under this condition, cholinesterase activity was inhibited), tacrine did not enhance the basal ACh release. Tacrine at more than 100 µM increased the submaximal electrical stimulation-evoked release of ACh in both the absence and presence of physostigmine (50 µM). This effect of tacrine was abolished by a combination of atropine (100 nM) and physostigmine. These results indicate that a high-dose of tacrine increases cholinergic neurotransmission not only by inhibition of cholinesterase but also by increasing ACh release through an atropine-like effect, perhaps by blockade of part of the process of muscarinic autoinhibition.

Keywords: Acetylcholine release, Atropine, Autoinhibition, Hippocampus, Tacrine

Tacrine (9-amino-1,2,3,4-tetrahydroacridine) has been investigated as a therapeutic agent for memory impairment in Alzheimer's disease (1). It is a centrally active cholinesterase inhibitor (2, 3), and other effects such as blockade of some types of cation channels (4–6) and inhibition of nicotinic (7, 8) and muscarinic ligand bindings (9, 10) have been reported. Due to its anticholinesterase activity, tacrine should increase the extracellular concentration of acetylcholine (ACh), like other cholinesterase inhibitors. It has been found that an increase in the extracellular concentration of ACh, or application of muscarinic agonists, inhibits ACh release from the nerve terminal (11, 12). Previous researchers have reported that tacrine inhibits depolarization-induced ACh release (13, 14), although these observations would be against the clinical usefulness of tacrine. In this study, we tested the effects of tacrine on ACh release from rat hippocampal slices to clarify its pharmacological profile.

MATERIALS AND METHODS

Experimental procedures and data analyses were carried out mainly in accordance with our previous methods (15).

Procedures for perfusion of rat hippocampal slices

Male Wistar rats (8- to 10-week-old) were decapitated, and the brain of each animal was quickly removed. The hippocampus was dissected out and sliced freehand with a stainless-steel razor blade on an ice-cold glass plate. Each slice was about 0.5-mm-thick, and the total wet tissue weight ranged from 10 to 35 mg. The hippocampal slices were placed in a perfusion chamber (0.3 ml volume) and perfused at a rate of 0.4 ml/min with artificial cerebrospinal fluid (ACSF) containing 2 µM choline chloride. The composition of the ACSF was as follows: 139 mM NaCl, 3.4 mM KCl (in high-K+ ACSF, 112 mM NaCl, 30 mM KCl), 1.26 mM CaCl2, 1.15 mM MgCl2, 21 mM NaHCO3, 0.6 mM NaH2PO4 and 10 mM glucose. All media used were saturated with 95 % O2 and perfused at a rate of 0.4 ml/min with artificial cerebrospinal fluid (ACSF) containing 2 µM choline chloride. The composition of the ACSF was as follows: 139 mM NaCl, 3.4 mM KCl (in high-K+ ACSF, 112 mM NaCl, 30 mM KCl), 1.26 mM CaCl2, 1.15 mM MgCl2, 21 mM NaHCO3, 0.6 mM NaH2PO4 and 10 mM glucose. All media used were saturated with 95% O2–5% CO2 and maintained at 37°C. After 90 min of conditioning perfusion, the perfusates were collected every 3 min throughout the subsequent experiments. After collection of 3 perfusates (basal release), the slices were subjected to 6-min periods of electrical field stimulation (duration of 2 msec, frequency of 3 Hz, rectangular pulse; stimulator: PAS-111, Star Medical, Tokyo, isolator: ME-6212, ME Commercial, Tokyo) using a Pt electrode. The current strength was raised from 1 to 25 mA with a 15-min interval between each stimulation period (see Fig. 1). At the end of this perfusion period, high-K+ ACSF was perfused for 6 min. In some experiments, sustained stimulation (2 msec duration, 3 Hz, rectangular pulse, 2 min) was applied, using a submaximal current (5 mA) in the presence of...
physostigmine (50 μM).

At the end of the experiment, the total wet tissue weight was determined.

All drugs used in this study were dissolved in ACSF and applied continuously throughout the experiment. Physostigmine and atropine in the doses used in this study (50 μM and 100 nM, respectively) induced maximum effects of cholinesterase inhibition and increase in ACh release, respectively (data not shown).

Procedures for ACh determination

The ACh content of the perfusates was determined by radioimmunoassay (15). The methods used for obtaining anti-ACh antibody and determination of its specificity have been described elsewhere (16).

A portion (100–400 μl) of each collected perfusate was incubated overnight with 50 μl of diluted anti-ACh antiserum in 0.15 M Tris-HCl buffer (pH 7.4) containing 0.4% bovine gamma-globulin and 0.05% isoflurophate, and 50 μl of tritiated ACh solution (about 4.2 pg, 5500 dpm) at 4°C. The antibody-bound tritiated ACh was separated from the free ACh by the ammonium sulfate method (17) and the radioactivity was counted with a liquid scintillation counter.

Data analysis

Released ACh was reported as pmol/g tissue per min (Figs. 1, 2 and 5). In Figs. 3 and 4, the mean ACh content of the first 3 fractions was regarded as the basal ACh release, and the amount of ACh released during the last 3 min of each 6-min stimulation period was regarded as the stimulation-evoked ACh release. The stimulation-evoked ACh release was expressed as a percentage of the basal ACh release for each set of perfusion conditions (ratio of stimulation/basal ACh release, S/B ratio) to allow comparison of the data obtained using different experimental conditions in Figs. 3 and 4.

Values are the means±S.E.M. Statistical differences were analyzed by ANOVA with Student’s t or Bonferroni’s test.

Materials

Acetyl[methyl-3H]choline chloride (3.02 TBq/mmol; Amersham, Buckinghamshire, UK), tacrine (Sigma, St. Louis, MO, USA), atropine sulfate and physostigmine sulfate (Wako, Osaka) were used in this study. Other chemicals were of reagent grade and obtained from commercial sources.

RESULTS

Figure 1 shows the typical pattern of ACh release (in the presence of 50 μM physostigmine).

Phyostigmine (50 μM) increased the measurable basal ACh release (Fig. 2). Tacrine (more than 1 μM) also increased the measurable basal ACh release, but in the presence of physostigmine (50 μM), it did not change the basal ACh release (Fig. 2).

Electrical stimulation induced a current strength-dependent ACh release under all the experimental condi-
Tacrine increases the S/B ratio (5 mA stimulation) with a maximal effect at 100 pM (Fig. 3). The dose-responsiveness of tacrine was similar to that reported by Dolezal and Tuček (18) and Tuček and Dolezal (14). In the present in vitro experiment, a higher concentration of tacrine and physostigmine than that estimated from clinical studies (1, 19) was required to produce the effects (e.g., cholinesterase inhibition occurred at 1 μM tacrine, as shown in Fig. 2). In the further experiments, 100 μM tacrine was used.

The effects of physostigmine (50 μM) and tacrine (100 μM) on the S/B ratio are shown in Fig. 4. Physostigmine did not affect the S/B ratio (see first and third column of each current strength). However, tacrine increased the S/B ratio significantly both in the absence and presence of physostigmine, as shown by the difference between the results with and without tacrine for each condition shown in Fig. 4. In high-K⁺-evoked ACh release, tacrine did not affect the S/B ratio in the absence of physostigmine, but significantly increased it in the presence of physostigmine (Fig. 4).

**Fig. 2.** Effects of tacrine (0.1–100 μM) on basal release of ACh from rat hippocampal slices in the absence (●) and presence (50 μM, ○) of physostigmine. Each value represents the mean ± S.E.M. of 6 separate experiments. *P < 0.05, **P < 0.01, compared with the control (tacrine 0).

**Fig. 3.** Effect of tacrine on the electrical stimulation (5 mA, duration 2 msec, frequency 3 Hz)-evoked ACh release represented as a percentage of basal release (S/B ratio) in the presence of physostigmine (50 μM). Each value represents the mean ± S.E.M. of 4 separate experiments. *P < 0.05, **P < 0.01, compared with the tacrine-free group.

**Fig. 4.** Effects of tacrine (100 μM) and physostigmine (50 μM) on stimulation-evoked ACh release (S/B ratio). Each value represents the mean ± S.E.M. of 6 separate experiments. Control (normal ACSF perfusion), open column; tacrine (100 μM), shaded column; physostigmine (50 μM), slashed column; combination of physostigmine and tacrine, filled column. *P < 0.05, **P < 0.01, compared between columns indicated.
Sustained electrical stimulations with submaximal current strength (5 mA, 3 Hz, 21 min) in the presence of physostigmine (50 μM) elicited a steady level of ACh release (Fig. 5A). Tacrine increased this stimulation-induced ACh release significantly (Fig. 5A). Atropine (100 nM) increased the ACh release induced by electrical stimulation (Fig. 5B). Tacrine did not affect ACh release in the presence of atropine and physostigmine (Fig. 5B).

**DISCUSSION**

Tacrine increased the measurable basal ACh release in the absence of physostigmine, but this effect disappeared in the presence of physostigmine. We have reported previously that atropine did not affect the basal release of ACh, and thus cholinergic autoinhibition of ACh release did not occur under resting conditions (15). Therefore, the effect of tacrine on basal ACh release is due to cholinesterase inhibition, and tacrine, in contrast to 4-aminopyridine (20), does not evoke ACh release directly.

The observation that physostigmine did not affect the S/B ratio indicates that, under the present experimental conditions, physostigmine did not affect the absolute amount of ACh released, but increased the detectable ACh content in the perfusate through cholinesterase inhibition. This agrees with previous observations (15). On the other hand, tacrine (100 μM) significantly increased the S/B ratio on electrical stimulation both in the absence and presence of physostigmine. Furthermore, as shown in Fig. 5A, tacrine did not affect basal ACh release, but significantly increased electrical stimulation-evoked ACh release in the presence of physostigmine. These results indicate that a high-dose of tacrine increases the ACh content of the perfusate not only by inhibition of cholinesterase activity but also by an increase in evoked ACh release.

We have also reported previously that atropine increased the S/B ratio during electrical stimulation by removal of cholinergic autoinhibition in both the absence and presence of physostigmine (15). In the presence of both physostigmine and atropine, tacrine did not induce surplus ACh release (Fig. 5B). In this experiment, we employed electrical stimulation at submaximal strength. Under these conditions, it is possible to induce surplus ACh release by, for example, direct membrane depolarization, as observed in high-K⁺ stimulation. Thus, it is suggested that tacrine increases stimulation-evoked ACh release partly through an atropine-like effect. It has been reported that tacrine inhibits muscarinic ligand binding and blocks the agonist-gated potassium channel (6, 9, 10). These observations predicted that tacrine would increase ACh release by blockade of the process of cholinergic autoinhibition, although previous researchers have shown that tacrine inhibits evoked ACh release (13, 14). The discrepancies between the previous and present results may be due to the experimental conditions employed. First, in the present study, we employed electrical stimulation. Its amplitude was at a submaximal level (15), and its frequency (3 Hz) was similar to the physiological burst frequency of rat septo-hippocampal neurons, which has been reported to be 3 to 10 Hz (21). Previous studies employed high-K⁺ stimulation. In the present study, tacrine had no effect on high-K⁺-evoked ACh release in the absence of physostigmine. We reported previously that in the absence of physostigmine, autoinhibition did not occur with high-
K⁺ stimulation, since this is an intense, non-physiological form of stimulation (15). The greater increase in the extracellular concentration of ACh, as induced by the presence of physostigmine, may be necessary to trigger cholinergic autoinhibition with high-K⁺ stimulation.

The second difference is the method used for ACh determination. In the present study, we determined released ACh directly by radioimmunoassay, as this method is able to determine the absolute amount of ACh released. Furthermore, because we used ACSF containing a physiological level of choline (2 μM) for perfusion, ACh was newly synthesized throughout the experiment. Thus, the present results reflect physiological ACh release. However, previous studies have employed a method where a radiolabeled precursor (choline) is preloaded. Although this method has many advantages, such as easy operation and high sensitivity, it is not able to determine the absolute amount of ACh released. The amount of labeled compound released may decline, while stored and newly synthesized ACh cannot be detected. The results obtained by this method may therefore reflect only a restricted proportion of ACh movement. Therefore, the present results would more likely reflect physiological phenomena.

The results of our investigation suggest that a high-dose of tacrine enhances central cholinergic activity by both inhibition of cholinesterase activity and increase in ACh release. The precise mechanism of the latter effect is not clear; one possible explanation is that tacrine shows an atropine-like effect, that is, blockade of part of the process of cholinergic autoinhibition. Although this effect requires a 100-fold higher dose of tacrine than the anti-cholinesterase effect and the dose (100 μM) seems to be higher than therapeutic doses, we can not rule out the effect in the case of chronic drug therapy. To clarify its mechanism of action, further studies will be required.

Acknowledgments

We thank Ms. Keiko Ouchi for her technical assistance. This work was supported in part by Grants-in-Aid for Encouragement of Young Scientists (No. 04258223) from the Ministry of Education, Science and Culture of Japan.

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