Effect of TA-0910, a Novel Thyrotropin-Releasing Hormone Analog, on In Vivo Acetylcholine Release and Turnover in Rat Brain

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ABSTRACT—To examine the action of a novel thyrotropin-releasing hormone (TRH) analog, TA-0910 ((−)-N-[(S)-hexahydro-l-methyl-2,6-dioxo-4-pyrimidinylcarbonyl]-l-histidyl-l-prolinamide tetrahydrate), on the cerebral cholinergic systems, the release of acetylcholine (ACh) and choline in freely-moving rats and ACh accumulation in gamma-butyrolactone (GBL, a nerve impulse flow blocker) and physostigmine-treated rats were examined. TA-0910 (0.1 -1 mg/kg, i.p.) caused a marked dose-dependent increase in extracellular ACh levels and a decrease in choline levels in the hippocampus of freely moving rats. These effects were significantly stronger and longer-lasting than similar effects of TRH. TA-0910 (1, 3 mg/kg, i.p.) depressed the ACh accumulation in the cerebral cortex and hippocampus of GBL (1000 mg/kg, i.p.)-treated rats. Moreover, this analog (1, 3 mg/kg, i.p.) increased the accumulation rate of ACh in these regions in physostigmine (1 mg/kg, i.p.)-treated rats. TRH (30 mg/kg, i.p.) affected the ACh accumulation only in the hippocampus of the GBL-treated rats. These results suggest that TA-0910 not only enhances the release of ACh, but also accelerates the ACh turnover, i.e., ACh release and synthesis, at the cholinergic neuronal terminals in normal rats.

Keywords: TA-0910, Thyrotropin-releasing hormone, Microdialysis, Acetylcholine release, Acetylcholine turnover

Thyrotropin-releasing hormone (TRH: l-pyroglutamyl-l-histidyl-l-prolinamide) is widely distributed in the mammalian central nervous system (CNS) (1–3). TRH-binding sites are also observed in many parts of the CNS (4, 5). Apart from the thyroid stimulating hormone-releasing action, this peptide exerts several CNS actions, such as an increase in locomotor activity and antagonism to pentobarbital-induced sleep (6). These suggest that TRH may play a role as a neurotransmitter and/or neuromodulator (6).

TRH accelerates the acetylcholine (ACh) turnover rate in the parietal cortex of freely moving rats and in the hippocampus of pentobarbitalized rats (7, 8). The antagonistic action by TRH on the pentobarbital-induced sleep is blocked by atropine (9). Moreover, TRH and its analogs improve the memory impairment induced by lesions of the septo-hippocampal pathway (10). From these reports, TRH appears to be involved in the cerebral cholinergic system.

TA-0910 ((−)-N-[(S)-hexahydro-l-methyl-2,6-dioxo-4-pyrimidinylcarbonyl]-l-histidyl-l-prolinamide tetrahydrate), a new TRH analog, has various CNS activities, and these activities are greater than the corresponding activities of TRH, being 10 times higher by intravenous administration and 100 times higher by oral administration. Furthermore, the CNS actions of TA-0910 are about 8 times longer-lasting than those of TRH (11–13). TA-0910 also antagonizes pentobarbital-induced sleep, and this action of TA-0910 is counteracted by scopolamine (14). Moreover, this analog improves memory impairment induced by lesion of the basal forebrain, the nucleus of origin of the cortical cholinergic system (15). These observations suggest that TA-0910 may exert some effects on the cerebral cholinergic system.

The aim of the present study is to examine the effects of TA-0910 and TRH on the cerebral cholinergic system in vivo. Thus, we examined the effects by these drugs on ACh release in freely moving rats and ACh turnover in rats treated or untreated with the nerve impulse flow blocker gamma-butyrolactone (GBL) and the ACh esterase inhibitor physostigmine.
MATERIALS AND METHODS

Animals
Male Sprague-Dawley rats (Japan SLC, Hamamatsu), weighing 270–494 g, were housed under constant environmental conditions (23 ± 1°C, 55 ± 5%, lights on from 6:30 through 18:30) and given standard pellets (CRF-1; Oriental Yeast, Tokyo) and water ad libitum.

Drugs
TA-0910 and TRH (tartrate monohydrate) were synthesized at Tanabe Seiyaku Co., Ltd. Their chemical structures are shown in Fig. 1. The following compounds were commercially obtained: GBL (Research Biochemicals Inc., Natick, MA, USA); sodium pentobarbital, 1-octanesulfonate sodium salt (Nacalai Tesque, Kyoto); tetramethylammonium chloride (TMA), 1-decanesulfonate sodium (Tokyo Kasei, Tokyo) physostigmine hemisulfate, tetrodotoxin (TTX), atropin sulfate (Sigma Chemical, St. Louis, MO, USA); ethylenediamine tetraacetic acid disodium [EDTA-Na$_2$], Triton X-100 (Katayama Chemical, Tokyo); ethylhomocholine (Eicom, Kyoto). TA-0910 and TRH were dissolved in physiological saline (Otsuka Chemical, Tokyo) and injected intraperitoneally in a volume of 2 ml/kg body weight.

In vivo microdialysis study
The rats were anesthetized with sodium pentobarbital (40 mg/kg, i.p.) and placed in a stereotaxic frame (ST-7; Narishige, Tokyo). The skull was exposed and a small bur hole was drilled at the point (A: −5.8 mm, L: 4.8 mm from the bregma) to allow for the implantation of a guide cannula (0.55 mm in diameter) into the hippocampus. All measurements were done according to the brain atlas (16). The guide cannula was inserted to a depth of 4 mm from the brain surface and fixed with dental cement, with a screw acting as an anchor. More than 1 week after the implantation, the dialysis probe (0.5-mm diameter; 2-mm exposed membrane; Bioanalytica System (BAS), West Lafayette, IN, USA) was inserted into the hippocampus (to a depth of 6 mm from the brain surface) via the guide cannula under conscious conditions. One of the outlet tubes was connected to the perfusion pump (CMA-100, BAS) and the other to the auto-injector (AS-10, Eicom) of the HPLC apparatus (L-2000; Yanagimoto, Kyoto). The Ringer solution (147 mM NaCl, 2.3 mM CaCl$_2$, 4 mM KCl) containing 10$^{-5}$ M physostigmine was then perfused at a flow rate of 1.5 μl/min to the hippocampus via the dialysis probe. Perfused ACh esterase inhibitor inhibits the autoreceptor in the cholinergic neuronal terminals (17). Therefore, the probe was allowed to equilibrate for 2–3 hr before TA-0910 (0.1, 0.3 and 1.0 mg/kg, i.p.) and TRH (30 mg/kg, i.p.) administration took place. In some experiments, 15 min before the TA-0910 (1 mg/kg, i.v.) administration, TTX (5 × 10$^{-7}$ M) was perfused via the microdialysis probe. The microdialysates were collected automatically every 15 min into microtubes containing 10$^{-6}$ M ethylhomocholine as an internal standard. The results were calculated as pmol of ACh per 15-min fraction. The mean value at each time after drug treatment was compared with the baseline value (the average of four samples collected immediately before drug injection).

Ex vivo GBL treatment study
TA-0910 (1 and 3 mg/kg, i.p.) and TRH (30 mg/kg, i.p.) were administered 60 and 30 min, respectively, before the application of microwave irradiation (MWR: 5.2 KW, 0.8 sec; Toshiba, Tokyo). Physiological saline was administered to the control group. GBL (1000 mg/kg, i.p.) was injected 65 min before the MWR. In some experimental groups, GBL was not injected. The whole brain was then quickly removed from the skull, placed on ice, and divided into the cerebral cortex and hippocampus. Homogenates were obtained using a Polytron R (PT 10, setting 8, 20 sec × 2; Kinematica, Lucerne, Switzerland) from the brain tissues with ice-cold sodium phosphate buffer (SPB: 50 mM) containing 0.3% Triton-X 100 (pH 7.4 at 37°C) and then centrifuged (1600 × g, 5 min) after the addition of ethylhomocholine (20 mM).
supernatant, after filtration through a millipore filter (0.45 μm in diameter), served as the sample.

**Ex vivo physostigmine treatment study**

In place of GBL in the above experiment, physostigmine (1 mg/kg, i.p.) was injected 0, 3, 6, 9, 12, 15 or 18 min before the MWR application (time 0 means no treatment with physostigmine). From six to twelve animals were used to determine the ACh value in each experimental point. The ACh accumulation rate was assessed from the slope of the regression line obtained by plotting the ACh value as a function of time, and expressed as nmol/g tissue/min.

**Assay of ACh and choline by HPLC**

ACh and choline in the sample were assayed directly by HPLC with an electrochemical detector (ECD: ECD-100, Eicom) according to the method by Fujimori and Yamamoto (18). Acetylcholine esterase (E.C. 3.1.1.7) and choline oxidase (E.C. 1.1.3.17) were fixed on an immobilized enzyme reactor column (AC-Enzympak, Eicom). The column temperature was 33°C. The mobile phase consisted of 300 mg/l SDS, 40 mg/l EDTA-Na₂, and 0.6 mM TMA. The flow rate of the mobile phase was 1.0 ml/min. The applied potential at the working electrode was +450 mV versus Ag/AgCl₂.

**Statistical analyses**

All data are expressed as the mean±S.E.M. The data were analyzed by ANOVA followed by Dunnett’s or Scheffe’s multiple comparison test. The data on the slope of the regression line in the ex vivo physostigmine treatment study was not statistically analyzed.

**RESULTS**

**In vivo microdialysis study**

The basal levels of ACh and choline were constant after about 2 hr of perfusion. The average baseline values for ACh and choline release from the hippocampus before the drug administration were 6.64±0.40 and 73.22±3.22 pmol/15 min, respectively.

TA-0910 administration increased the extracellular ACh level in a long-lasting and dose-dependent manner. TA-0910, at the dose of 1 mg/kg, increased the release of ACh to higher than 400% between 45 and 120 min after administration, and the increase was still significant at 240 min after administration (Fig. 2). Concomitant with this effect, there was a significant decrease in the choline level by TA-0910 (Fig. 3), reflecting the change in the ACh level. TRH (30 mg/kg) significantly but transiently increased the release of ACh to about 280% at 15 min after administration (Fig. 2). TRH also caused a moderate

![Fig. 2. Effects of intraperitoneal administration of TA-0910 and TRH on in vivo acetylcholine (ACh) release from the hippocampus in conscious and freely moving rats. ACh release is expressed as percentages of the average of four consecutive values before drug administration. Each point represents the mean of 4 independent experiments; S.E.M. is shown by vertical bars. 〇 saline; ▲ TA-0910, 0.1 mg/kg; ■ TA-0910, 0.3 mg/kg; ○ TA-0910, 1.0 mg/kg; △ TRH, 30 mg/kg. *P<0.05, **P<0.01 compared with the control (one-way ANOVA followed by Dunnett’s test).](image)
decrease in the choline level (Fig. 3). The increase in ACh release caused by TA-0910 (1 mg/kg) was not observed under the perfusion of TTX via the microdialysis probe (Fig. 4).
Ex vivo GBL treatment study

The TA-0910 (1 and 3 mg/kg) and TRH (30 mg/kg) treatments did not affect the ACh level in the cerebral cortex and hippocampus of naive rats (Fig. 5). GBL (1000 mg/kg, i.p.) increased the ACh levels in the cerebral cortex and hippocampus to 67.3% and 53.2%, respectively (data not shown). TA-0910 (1 or 3 mg/kg) depressed the ACh levels elevated by GBL in the cerebral cortex and hippocampus (Fig. 6). TRH (30 mg/kg) depressed the ACh level only in the hippocampus (Fig. 6).

Ex vivo physostigmine treatment study

Physostigmine increased the ACh levels linearly between 3-9 min after treatment in the cerebral cortex and hippocampus of naive rats (data not shown). The accumulation rates in the cerebral cortex and hippocampus between 3-9 min after treatment were 1.491 and 1.214 nmol/g tissue/min, respectively (Table 1). TA-0910 at a dose of 1 and 3 mg/kg increased the ACh accumulation rate 1.35 and 1.38 times in the cerebral cortex and 1.43 and 1.62 times in the hippocampus, respectively (Table 1). In contrast to this, the accumulation rates of the TRH (30 mg/kg)-treated group in these brain regions were 1.472 and 1.283, respectively.

DISCUSSION

TA-0910 and TRH markedly facilitated the release of ACh from the hippocampus in freely moving rats in a dose-dependent manner. Such release is considered to be an index of the functional activity of cerebral cholinergic neurons (19). The ACh releasing action of TA-0910 was more potent and longer in duration than that of TRH in this experimental condition. Therefore, these results suggest that activation of the hippocampal cholinergic system by TA-0910 is stronger than that by TRH. We also observed that TA-0910 was 30-100 times more potent than TRH in antagonizing the pentobarbital-induced sleep in mice and improving the scopolamine-induced memory impairment in rats by intraperitoneal administration (11, 15). Although the differences in the affinity to the TRH-receptor is to be determined, this compound is more stable than TRH in the blood and brain (Y. Kodama et al., personal communication). Therefore, the potent and long-lasting effect may be due to a better penetration to the brain and lesser degradation after administration.

TTX infusion (10^-6-10^-7 M) via the microdialysis probe caused a drop in the output of basal ACh from the striatum of freely moving rats to undetectable values (20). The ACh-releasing action by TRH or a TRH analog is blocked by local application of TTX (21, 22). In the present study, the ACh release-enhancing effect of TA-0910 also disappeared by TTX infusion. A high concentration, 10 mM, of TA-0910 or TRH does not affect the ACh release from the hippocampal slice by high K^+ stimulation (I. Fukuchi et al., unpublished data). These observations suggest that the effect of TA-0910 depends on increased electrical activity on the cholinergic neurons themselves or of afferent fibers that innervated the cholinergic neurons. In fact, local injection of TRH or a TRH analog to the medial septum, where ACh-containing cell bodies innervating the hippocampus exist (23), enhance the ACh release in the hippocampus (24).
Enhancement of ACh release by a TRH analog in the frontal cortex is partially antagonized by haloperidol treatment (25). Moreover, while systemic administration of TRH induces an increase in the electrical activity of sensorimotor cortical neurons, microiontophoretic application of TRH causes a decrease in the firing rate in most such cells (26). The CNS actions by TA-0910 are thought to be via binding to the TRH receptors (27). From these observations, excitation of cortical neurons by systemic administration of TRH was assumed to be due to activation of extracortical neurons that exert a stimulatory effect on the cortex. Taken altogether, the ACh release-enhancing effect of TA-0910 in the hippocampus is thought to be due to an effect on the soma of cholinergic neurons and/or other neurons which innervated the cholinergic neurons.

Intraperitoneal administration of TA-0910, at the dose of 1 mg/kg, persistently enhanced ACh release from the hippocampus of freely moving rats for over 4 hr. On the other hand, TA-0910 at the relatively high dose of 3 mg/kg did not affect the ACh level in the hippocampus of normal rats in the ex vivo study. These observations suggest that this compound possesses not only an enhancing effect on ACh release, but also an accelerating effect on ACh synthesis in the cholinergic nerve terminals. In fact, TA-0910 (0.3 and 1 mg/kg) depressed the ACh accumulation in the hippocampus and cerebral cortex of GBL-treated rats and increased the ACh accumulation rate in physostigmine-treated rats. GBL, a CNS depressing drug, produced increases in ACh contents, decreases the ACh turnover rate and inhibits the high affinity uptake of choline by interrupting impulse flow in rat brain (28). The observation that TA-0910 depressed the ACh accumulation in GBL-treated rats indicates that this compound restores the depressed ACh turnover. On the other hand, physostigmine inhibits the conversion of the ACh released from the pre-synaptic nerve terminal to choline and increases the ACh level in the synaptic space. Acceleration by TA-0910 of the ACh accumulation in physostigmine-treated rats suggests that this compound also accelerates ACh turnover, i.e., ACh release and synthesis, in normal rats.

Both TA-0910 and TRH decreased the extracellular choline level in the hippocampus of freely moving rats. Another TRH analog, MK-771, enhances choline uptake in the hippocampus of septum-damaged rats (10) and also in the cerebral cortex and hippocampus of pentobarbital-treated rats (29, 30). TRH and its analogs are supposed to maintain the function of cholinergic neurons by enhancing ACh release and, secondarily, choline uptake (22). According to these reports, TA-0910 and TRH are thought to depress the choline level by enhancing choline uptake by pre-synaptic terminals.

TA-0910 not only strongly enhances ACh release, but also accelerates choline uptake and ACh synthesis, and thus stimulates the cholinergic turnover system. Moreover, this effect of TA-0910 was more potent and longer-lasting than that of TRH. Alzheimer’s disease (AD) is a neuro-degenerative disorder with progressive dementia accompanied by central cholinergic loss (31–33). Patients with AD showed a modest improvement in memory after an acute infusion of a high dose of TRH (34). TA-0910 improved memory impairment induced by central cholinergic lesion and scopolamine (15). Taken altogether, TA-0910 might improve the cognitive function in patients with AD.

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