Effect of [1,3-Di-n-Butyl-7-(2-Oxopropyl)-Xanthine] (Denbufylline), a Low Km Phosphodiesterase Inhibitor, on Striatal Acetylcholine Release in the Rat: Analysis Using Cerebral Microdialysis

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Accepted September 10, 1990

Abstract—Effect of oral and intraperitoneal administrations of [1,3-di-n-butyl-7-(2-oxopropyl)-xanthine] (denbufylline) on acetylcholine (ACh) content and release in the rat striatum was investigated. Denbufylline (3, 10 and 30 mg/kg, p.o.) decreased striatal ACh contents in a dose-dependent manner. Denbufylline administration (30 mg/kg, i.p.) produced no significant change in the spontaneous release of ACh, while it increased a high potassium-evoked ACh release in the striatum. These results suggest that denbufylline may be a drug inducing the increased release of ACh in the brain.

[1,3-Di-n-butyl-7-(2-oxopropyl)-xanthine] (denbufylline) is a novel alkylxanthine analogue being developed for the treatment of occlusive arterial vascular disease and for enhancing cerebral metabolism. This compound is a potent inhibitor of low Km cAMP phosphodiesterase and known to increase the oxygen tension (P_O2) and contractility of ischemic and/or fatigued skeletal muscle (1, 2). Denbufylline and other alkylxanthine analogues are also known to reduce the viscosity of rat whole blood and increase the filterability of rat blood cells (3–6). These results suggest that denbufylline may be effective for the treatment of cerebral disorders induced by hypoxic conditions such as cerebral ischemia as well as neuropsychiatric symptoms associated with these diseases.

Since it has been reported that the function of cholinergic neurons in the brain is often deteriorated following cerebral ischemia as well as in the brain of Alzheimer’s patients (7–9), we have examined the effect of denbufylline on cerebral cholinergic neurons using the rat striatum as an experimental model. The release of ACh in vivo was monitored by a microdialysis system in the presence and absence of high KCl stimulation.

Materials and Methods

Animals: Male Wistar rats weighing 200–250 g were purchased from Shizuoka Laboratory Animal Center (Hamamatsu). They were used for the experiments after breeding for a week, being given a diet of solid food (MF, Oriental Yeast Co., Ltd., Chiba) and allowed tap water ad libitum.

Drug treatment: Denbufylline (Smith Klein-Beecham Pharm. Co., Ltd., Tokyo) was suspended in distilled water with 1% (w/v) carboxymethylcellulose (CMC). Denbufylline and the same volume of its vehicle were orally administered to examine their effects on the striatal ACh content. On the other hand, the agents were administered intraperitoneally when examining their effects on ACh release in the striatum.

Measurement of ACh content: Rats were killed by focused microwave irradiation on the head (5 kW, 1.0 sec) at one hour after the oral administration of denbufylline, and only the striatum was dissected out according to the method of Giowinski and Iversen (10). The tissue samples were then subjected to the extraction of ACh according to the methods of Potter et al. (11) and Igarashi et al. (12). Namely, the weighed sample was homogenized in 2 ml of a 1 N formic acid : acetone...
mixture, and it was centrifuged at 1,700×g for 20 min at 4°C. The supernatant was washed with diethyl ether to obtain a freeze-dried sample. After resuspending the freeze-dried sample in distilled water, it was mixed with a solution containing KI solution and 1 mM tetraethylammonium chloride before centrifuging at 10,000×g for 3 min at room temperature. In order to remove excessive iodine, the pellets were dissolved in acetonitrile and anion-exchange gel (AG1-X8-Cl⁻) was added. The iodine-free supernatant was then dried under a N₂ stream and subjected to ACh determination.

**Procedures for cerebral microdialysis:** Rats were placed in a stereotaxic apparatus under halothane anesthesia. A dialysis probe was implanted into the striatum (3.5 mm right from the bregma; 0.2 mm posterior; 4.0 mm from the cerebral surface in depth) according to the rat brain atlas of Pllegrino and Cushman (13). The inserted probe (2 mm, BAS CMA/10) was perfused at a flow rate of 2 μl/min with Ringer solution containing 10⁻⁴ M physostigmine. The solution emerging from the other end of the tube was collected every 20 min into a minitube. Since ACh release from the rat striatum became constant at 90 min after the initiation of perfusion, a high concentration of KCl was added twice into the perfusion solution for 10 min at 20 min and 80 min later. The ratio of S₂ (second KCl-evoked release of ACh) to S₁ (first KCl-evoked release of ACh) was found to be approximately 1.0 under these experimental conditions. At the end of the experiment, the rat was perfused with 20% formalin-saline through the heart under diethyl ether anesthesia. Brain sections were then cut and examined micro- and/or macroscopically to determine the insertion site of the dialysis tube. Only samples having the tube in the appropriate location in the striatum were included in the biochemical analysis.

**ACh determination:** To measure ACh content, the collected perfusate fraction or the extract of tissue homogenate was injected into a high performance liquid chromatography (HPLC) apparatus equipped with a precolumn (AC-ODS, Eicom), separate column (Eicompak AC-Gel, Eicom), immobilized enzyme column (AC-Enzympak, Eicom) and electrochemical detector (VMD-101A, Yanaco) operating at a flow rate of 1.0 ml/min. The mobile phase consisted of 100 mM phosphate buffer solution (pH 8.0) containing 300 mg/l of sodium 1-decansulfonyl and 65 mg/l of tetramethylammonium chloride. The detector potential was maintained at 450 mV against an Ag/AgCl reference electrode (11, 14). The basal level of ACh release in the rat striatum was expressed as pmol/20 min or percentage of the mean of some control samples taken immediately before the injection of drugs or addition of KCl into the perfusion solution.

**Reagents:** Reagents used were sodium 1-decansulfonylate (Tokyo Kasei Kogyo Co., Ltd., Tokyo), tetramethylammonium chloride (Nacalai Tesque, Kyoto), and physostigmine sulfate and tetraethylammonium chloride (Kanto Chemical Co., Ltd., Tokyo). Other reagents used in this study were the highest grade available commercially.

**Statistical analysis:** Each value was expressed as the mean±S.E.M., and statistical significance was determined using Student’s t-test.

**Results**

**Effect of denbufylline on striatal ACh content:** ACh content in the striatum showed a dose-dependent decrease following the oral administrations of denbufylline (3, 10 and 30 mg/kg), among which 30 mg/kg gave a statistically significant decrease as compared with the control (0 mg/kg) group (Table 1).

**Effect of denbufylline on basal ACh release in the striatum:** The basal ACh release in the rat striatum was found to be stable at 90 min after the initiation of perfusion of Ringer solution containing 10⁻⁴ M physostigmine. The steady-state level of ACh release was found to be 3.05±0.28 (n=12). The single administration of denbufylline (30 mg/kg) did not induce any significant changes in the basal level of ACh release in the striatum (Table 2).

**Effect of denbufylline on high potassium-evoked ACh release in the striatum:** In order to evaluate the potassium-evoked enhancement of ACh release in the rat striatum, various concentrations of KCl were added into the perfusion system. As shown in Fig. 1, 50 mM
Table 1. Effect of single administration of denbufylline on striatal acetylcholine content in rats

| Denbufylline (mg/kg) | Acetylcholine content (nmol/g wet weight) |
|---------------------|------------------------------------------|
| 0                   | 39.96±2.18                              |
| 3                   | 38.79±4.36                              |
| 10                  | 31.50±4.89                              |
| 30                  | 29.76±2.41**                            |

Denbufylline (3–30 mg/kg) were given orally. **P<0.01, compared with the control (0 mg/kg) value. Data are given as means±S.E.M. (n=6–9).

Table 2. Effect of single administration of denbufylline on spontaneous release of acetylcholine (ACh) in rat striatum

| Time after administration (min) | Acetylcholine (pmol/20 µl/20 min) | Denbufylline |
|---------------------------------|-----------------------------------|--------------|
| 0                               | 3.47±0.22                        | 3.28±0.10    |
| 30                              | 3.14±0.18                        | 3.04±0.17    |
| 60                              | 3.07±0.08                        | 3.16±0.20    |
| 90                              | 3.17±0.08                        | 3.10±0.09    |

Denbufylline (30 mg/kg) was given intraperitoneally after the spontaneous release of ACh measured by microdialysis was found to be stabilized. Data are given as means±S.E.M. (n=3).

Fig. 1. Effects of perfusion with various concentrations of potassium on the acetylcholine release in rat striatum. Fifty mM (○——○) and 100 mM (●——●) KCl were added into the perfusion solution for 10 min. Shaded bars (□) indicate the duration of KCl stimulation. The S2/S1 ratio of 100 mM KCl-stimulation was 0.960±0.015 (n=3). Each point represents the means±S.E.M. (n=3).

Fig. 2. Effect of denbufylline on high potassium-evoked acetylcholine release in rat striatum. Denbufylline (30 mg/kg) or its vehicle (control) were given intraperitoneally 30 min before the application of the second stimulation of potassium. **P<0.01, compared with the control value. Each column represents the means±S.E.M. (n=4).

Effect of denbufylline on potassium-evoked acetylcholine release. Furthermore, 50 and 100 mM potassium-evoked ACh release gave an S2/S1 ratio of almost one. Therefore, the 100 mM potassium-evoked ACh release was used to evaluate the effect of denbufylline.

or 100 mM KCl was added into the perfusion solution for 10 min at 20 min and 80 min after the stabilization of basal ACh release. The addition of 50 mM KCl induced a relatively small percent of increase (approximately 160%), while that of 100 mM KCl gave a 340% increase as compared with the basal release.
evoked ACh release in the striatum: Denbufylline (30 mg/kg, i.p.) administration significantly increased the S2/S1 ratio (control: 0.959±0.012, denbufylline: 1.225±0.056) in the potassium (100 mM)-evoked ACh release as shown in Fig. 2.

Discussion
Denbufylline is a newly-introduced alkylxanthine derivative and has been shown to have various pharmacological actions such as the amelioration of learning or memory impairment, increase of cerebral blood circulation, facilitation of cerebral glucose metabolism and improvement of intracerebral oxygen tension (Po2) (1-6). These results suggest that this drug may also affect the cerebral metabolism of various neurotransmitters. Therefore, the effect of denbufylline on the function and metabolism of the cerebral cholinergic system was investigated using the rat striatum as an experimental model.

Following the oral administration of denbufylline, a dose-dependent decrease in the striatal ACh content was detected (Table 1).

Since in vivo microdialysis to determine neurotransmitter release under a free-moving condition has been widely acknowledged as useful experimental system to measure the functional state of neurotransmitters (ACh, amino acids, amines and neuronal peptides) in the brain (15-19), the effect of denbufylline on ACh release was analyzed using this system. We have examined the release within 3 hours, because the basal level of ACh release was reported to be stable for at least 5 hours under our experimental conditions (16, 19, 20). The administration of denbufylline, however, produced no change in the basal release of ACh from the rat striatum (Table 2).

To elucidate the most suitable concentration of KCL for evoking ACh release from the rat striatum (Fig. 2), various concentrations of KCL were added to the perfusion system. Among the various concentrations of KCL tested, 100 mM KCL was found to be the best for evaluating the effect of denbufylline on the potassium-evoked ACh release, since the percent increase evoked by the repetitive potassium stimulation gave a similar value. A similar result was also reported in the case of high potassium-evoked release of ACh from striatal slices in vitro (21). Denbufylline pretreatment produced a significant increase in the S2/S1 ratio. These results suggest that denbufylline is likely to induce the decrease of cerebral ACh contents by increasing ACh release at synapses. This effect seems to be also similar to the increase of ACh release in the striatum following the administration of a high dose of anticholinergic drugs such as atropine and scopolamine (15, 20, 22). These drug-induced increases in striatal ACh release are considered to be due to the attenuation of the presynaptic control by muscarinic autoreceptors (23) and/or by the attenuation of the dopaminergic control via dopamine D-2 receptors (24).

On the other hand, it has been reported that propentofylline, a methylxanthine derivative, possesses protective action against ischemic cell damages. Furthermore, this protective action of the methylxanthine derivative is considered to be due to the elimination of adenosine A1 receptor-mediated inhibition of ACh release as well as the enhancement of adenosine A2 receptor-mediated cyclic AMP accumulation (25). The stimulation of cyclic AMP-accumulation by forskolin has been also reported to enhance ACh release (26). In addition, it has been reported that caffeine and aminophylline also enhance the electrically-evoked ACh release by antagonizing the action of endogenous adenosine at inhibitory adenosine A1 receptors (27). Denbufylline may have a similar type of pharmacological action at the cerebral cholinergic nerve terminals. Detailed analyses on these points are underway in our laboratory.

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