Effect of 1,3-Di-n-Butyl-7-(2-Oxopropyl)-Xanthine (Denbufylline) on Metabolism and Function of Cerebral Cholinergic Neurons

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ABSTRACT—Effect of denbufylline, a low K_m phosphodiesterase inhibitor, on cerebral cholinergic neurons was investigated using male Wistar rats. Single administrations of denbufylline (3, 10 and 30 mg/kg, p.o.) decreased cerebral cortical, hippocampal and striatal acetylcholine (ACh) contents in a dose-dependent manner. However, denbufylline (30 mg/kg, p.o.) had no effect on the activities of ACh synthesizing and degrading enzymes in these brain areas. In vitro addition of denbufylline (10^{-8} - 10^{-4} M) produced no significant change in [^{3}H]choline uptake in striatal slices, while denbufylline (10^{-4} M) increased high (20 mM) potassium-evoked endogenous ACh release from striatal slices. Denbufylline (30 mg/kg, p.o.) increased cyclic AMP (cAMP) contents in the cerebral cortex, hippocampus and striatum. In vitro addition of dibutyryl cAMP (5 \times 10^{-3} M) also accentuated the high (20 mM) potassium-evoked endogenous ACh release from striatal slices. These results suggest that denbufylline may induce the facilitation of ACh turnover by enhancing endogenous ACh release via the increase of cAMP content in the brain.

[1,3-Di-n-butyl-7-(2-oxopropyl)-xanthine] (denbufylline) is a new alkylxanthine analogue being developed for the treatment of occlusive arterial vascular diseases and as a drug possessing the capacity to enhance cerebral metabolism. This drug is known to be a potent inhibitor of low K_m phosphodiesterase (PDE) type III-1 (1, 2), a calcium/calmodulin- and cyclic GMP (cGMP)-independent and cAMP-selective type of PDE, and increases oxygen tension (Po_2), pH and contractility of ischemic and acidic fatigued skeletal muscle in animal models of peripheral vascular diseases (3). In addition, denbufylline and these alkylxanthine analogues are known to have various pharmacological actions such as the amelioration of learning and memory impairment (M. Katsura and K. Kuriyama, unpublished data), reduction of the viscosity of rat whole blood and increase of rat blood cell filterability (4–7). These results suggest that denbufylline may be effective for the treatment of cerebral disorders induced by hypoxic conditions such as cerebral ischemia and/or neuropsychiatric symptoms associated with senile dementia.

It is well-known that the function of cholinergic neurons in the brain is often deteriorated following cerebral ischemia and in patients with Alzheimer's disease (8–10). Although we have reported that denbufylline has the capacity to facilitate ACh release in the rat striatum (11), detailed mechanisms underlying this effect of denbufylline has not been clarified. In this study, therefore, we have examined the effects of denbufylline on the metabolism and release of ACh using the...
rat striatum as an experimental model. A possible correlation of denbufylline induced alterations in the function of cholinergic neurons with the increase of cAMP in the striatum were also examined.

MATERIALS AND METHODS

Animals
Male Wistar rats weighing 180–220 g were purchased from Shizuoka Laboratory Animal Center (Hamamatsu). They were used for the experiments after breeding for one week under conditions of solid feed (MF, Oriental Yeast Co., Ltd., Chiba) and 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 vehicle were orally administered to examine their effects on the cortical contents of ACh and cAMP, and the activities of ACh synthesizing and degrading enzymes. In the experiments of in vitro addition of denbufylline to examine its effects on 

Measurement of ACh and cAMP contents
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 the cerebral cortex, hippocampus and striatum were dissected out according to the method of Glowinski and Iversen (12).

Enzyme assay
The activity of choline acetyltransferase (CAT) was assayed by the formation of 

Measurements of \[^{3}H\]choline uptake and endogenous ACh release
Freshly prepared striatal tissues were sectioned at 200-μm thickness with a microslicer (DTK-1000, Dosaka EM Co., Ltd., Kyoto). Each striatal slice was placed in a small plastic vessel equipped with a nylon mesh at the bottom (17). The slices were preincubated for 20 min at 37°C in 5 ml/slice of oxygenated Kreb's-Ringer bicarbonate buffer (pH 7.4) containing 100 mM NaCl, 4.8 mM KCl, 1.2 mM KH₂PO₄, 1.2 mM MgSO₄·7H₂O, 2.7 mM CaCl₂·2H₂O, 25 mM NaHCO₃, and 10 mM d-glucose. After the preincubation, the slice was
incubated for 15 min in 1 ml of the incubation medium containing 1 μM \([\text{H}]\text{choline}\). The reaction was terminated by adding 5 ml of the ice-cold medium, and the slice was rinsed twice with 3 ml of the same medium. The radioactivity in the slice, which was solubilized with 0.2 ml of distilled water, was measured with a liquid scintillation spectrometer (model 3379, Packard) using Triton-toluene scintillant [0.5% 2,5-diphenyloxazone (PPO), 0.03% 1,4-di-(5-phenyl)oxazolyl)-benzene (POPOP) and 33% Triton X-100 in toluene].

To measure the release of endogenous ACh, the vessel containing striatal slices (4-5 slices/assay) was incubated for 15 min at 37°C in 5 ml of the incubation medium. After spontaneous ACh release from the striatum become constant (at 10 min after the incubation), the slices were transferred successively to vials containing 1.0 ml of 10^{-4} M physostigmine in the incubation medium at 1-min intervals. The slices were stimulated twice at 5 and 20 min by the addition of 20 mM KCl. Each incubation medium used for the measurement of ACh release was treated with 50 mM perchloric acid (PCA) to inactivate degrading enzymes and 50 mM KHCO_3 to neutralize the incubation medium. The data for the release of endogenous ACh was expressed as the ratio of \(S_2\) (second KCl-evoked ACh release) per \(S_1\) (first KCl-evoked ACh release), and the ratio was found to be approximately 1.0 under these experimental conditions.

The content of protein in each sample was determined by the method of Lowry et al. (18), with bovine serum albumin as a standard.

ACh determination

To examine cerebral ACh content, the extract of cerebral tissue homogenate or the incubated medium was injected into a high performance liquid chromatograph (HPLC) equipped with a precolumn (AC-ODS, Eicom), a separate column (Eicompak AC-Gel, Eicom), an immobilized enzyme column (AC-Enzympak, Eicom) and an 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-decansulfonate and 65 mg/l of tetramethylammonium chloride. The detector potential was maintained at 450 mV against an Ag/AgCl reference electrode (13, 19). The endogenous levels of ACh release in the brain was expressed as nmol/g wet weight or percentage of the mean of some control samples taken immediately before the addition of drug or potassium into the incubation medium.

Reagents

Reagents used were sodium 1-decansulfonate (Tokyo Kasei Kogyo Co., Ltd., Tokyo); tetramethylammonium chloride (Nacalai Tesque, Kyoto); physostigmine sulfate and tetraethylammonium chloride (Kanto Chemical Co., Ltd., Tokyo); dibutyryl cAMP (Sigma Chemical Co., St. Louis); [1-^{14}C]acetyl CoA (1.85 GBq/mmol) and [2,4-{3H}]choline (592.0 GBq/mmol, New England Nuclear, Boston). Other commercially available reagents used in this study were of the highest grade purity available.

Statistical analysis

Each value was expressed as the mean ± S.E.M. Statistical significance was determined with Student’s t-test.

RESULTS

Effect of denbufylline on cerebral ACh content

ACh contents in the cerebral cortex, hippocampus and striatum were reduced in a dose-dependent manner following the oral administration of denbufylline (3, 10 and 30 mg/kg), among which the dose of 30 mg/kg gave a statistically significant decrease as compared with each control (0 mg/kg) value (Table 1).

Effect of denbufylline on CAT and AChE activities

The administration of denbufylline (30 mg/kg, p.o.) had no significant effect on
Table 1. Effect of single administration of denbufylline on cerebral acetylcholine contents in rats

| Denbufylline (mg/kg) | Acetylcholine content (nmol/g wet weight) |
|---------------------|------------------------------------------|
|                     | Cerebral cortex | Hippocampus | Striatum |
| 0                   | 17.43 ± 0.87    | 26.41 ± 1.48 | 39.96 ± 2.18 |
| 3                   | 18.90 ± 0.24    | 25.23 ± 2.41 | 38.80 ± 4.36 |
| 10                  | 15.24 ± 1.76    | 22.38 ± 1.99 | 31.50 ± 5.89 |
| 30                  | 13.37 ± 0.57**  | 20.58 ± 0.57** | 29.78 ± 2.41** |

Rats were sacrificed by microwave irradiation on the head at 1 hour after the administration of denbufylline (0-30 mg/kg). **P < 0.01, compared with each control (0 mg/kg) value. Data are given as mean ± S.E.M. (N = 6-9).

CAT (nmol/mg protein/hr) and AChE (µmol/mg protein/hr) activities in the cerebral cortex [CAT (control: 59.90 ± 3.67, denbufylline: 68.16 ± 1.95), AChE (control: 0.80 ± 0.09, denbufylline: 0.83 ± 0.06)], hippocampus [CAT (control: 61.24 ± 2.07, denbufylline: 69.83 ± 3.76), AChE (control: 0.78 ± 0.04, denbufylline: 0.78 ± 0.02)] and striatum [CAT (control: 165.64 ± 7.87, denbufylline: 163.07 ± 4.58), AChE (control: 1.09 ± 0.01, denbufylline: 1.08 ± 0.04)].

Effect of denbufylline on [3H]choline uptake and endogenous ACh release

In vitro addition of denbufylline (10^{-8} - 10^{-4} M) had no significant effect on [3H]choline uptake into striatal slices (data not shown).

On the other hand, in vitro addition of denbufylline (10^{-4} M) induced a significant increase of the high (20 mM) KCl-evoked endogenous ACh release from striatal slices (Fig. 1) without altering the basal release (data not shown).

Effect of denbufylline on cerebral cAMP content

cAMP contents in the cerebral cortex, hippocampus and striatum were significantly increased following the oral administration of denbufylline (30 mg/kg, Fig. 2).

Effect of dibutyryl cAMP on endogenous ACh release

In vitro addition of dibutylr cAMP (5 × 10^{-3} M) did not induce any significant changes in the basal levels of ACh release, but induced a significant facilitation of the high (20 mM) KCl-evoked endogenous ACh release from striatal slices (Fig. 3).

DISCUSSION

Denbufylline is a newly-introduced alkylxanthine and is known as a potent inhibitor of low K_m phosphodiesterase type III-1 (1). This compound has been reported to have many pharmacological actions such as the amelioration of learning or memory impairment, increase of cerebral blood circulation, facilita-
effect of single administration of denbufylline on cerebral cyclic AMP content in rats. Rats were sacrificed by microwave irradiation on the head at 1 hour after the administration of denbufylline (30 mg/kg, p.o.). *P < 0.05, **P < 0.01, and ***P < 0.001, compared with each control value. Each column represents the mean ± S.E.M. (N = 5).

Fig. 3. Effect of in vitro addition of dibutyryl cyclic AMP (cAMP) on 20 mM KCl-evoked endogenous acetylcholine (ACh) release from rat striatal slices. Dibutyryl cAMP (5 X 10^-3 M) was added to the incubation medium 5 min before the second KCl stimulation. **P < 0.01, compared with the control value. Each column represents the mean ± S.E.M. (N = 3-4).

Denbufylline and Cerebral Cholinergic Neuron

Denbufylline has been reported to be an inhibitor of PDE type I (substrate: Ca^2+/calmodulin), II (substrate: Ca^2+/calmodulin and cGMP) and III (substrate: cAMP) subtypes have been proposed to exist in the brain. Denbufylline has been reported to be an inhibitor of PDE type I (substrate: Ca^2+/calmodulin), II (substrate: Ca^2+/calmodulin and cGMP) and III (substrate: cAMP) subtypes have been proposed to exist in the brain.

Single administration of denbufylline induced a significant reduction of ACh contents in the cerebral cortex, hippocampus and striatum in a dose-dependent manner (Table I). Under the same experimental conditions, denbufylline did not induce any significant changes in the activities of CAT and AChE in these brain areas. These results suggest that the denbufylline-induced decrease of ACh may not be due to the changes in enzyme activities involved in the synthesis and degradation of ACh. The high-affinity uptake process of choline, known as the rate-limiting step of ACh synthesis, was also not affected by in vitro addition of denbufylline. Therefore, the alteration in this uptake process is also unlikely to be involved in the cause of denbufylline-induced ACh decline in the brain. ACh release from brain slices has been determined mainly by the efflux of [3H]ACh formed from prelabeled [3H]choline (20–22), because endogenously released ACh is readily hydrolyzed by AChE. Recent observations, however, have indicated that the mechanism of endogenous ACh release from brain slices is different from that of preloaded [3H]ACh (23). Since recent advancements in HPLC procedures using electrochemical detection (13, 14) have made possible the simultaneous measurements of ACh and choline with reasonable sensitivity, we have used these procedures with a continuous tissue transfer system to minimize enzymatic degradation of released endogenous ACh by AChE. In vitro addition of denbufylline (10^-4 M) significantly enhanced 20 mM KCl (a submaximal dose for ACh release)-evoked endogenous ACh release from striatal slices under these experimental conditions (Fig. 1). These results suggest that denbufylline may accelerate the release of ACh in central cholinergic synapses.
type III-1 (1). Single administration of denbufylline (30 mg/kg, p.o.) significantly increased cerebral cAMP contents (Fig. 2). On the other hand, in vitro addition of dibutyryl cAMP (5 × 10^{-3} M) also exhibited a significant facilitating effect on 20 mM KCl-evoked release of endogenous ACh (Fig. 3). These results suggest that denbufylline may enhance the release of endogenous ACh via the increase of cAMP content in the striatum.

In the present study, we found that denbufylline facilitates ACh release at synapses via the increase of cAMP in the brain. Recently, the presence of muscarinic M1 and M2 receptor subtypes has been proposed in the CNS on the basis of their affinity for pirenzepine (24), and the modulation of striatal ACh release by muscarinic M2 type receptors has been demonstrated (23). Furthermore, this increase of ACh release in rat striatum was similarly found after the administration of a high dose of anticholinergic drugs such as atropine and scopolamine (25, 26). These drugs-induced increases in striatal ACh release are considered to be due to the attenuation of presynaptic control by muscarinic M2 autoreceptors and/or dopaminergic control by dopamine D2 receptors. Therefore, it may be possible that denbufylline also have a similar type of pharmacological actions at cholinergic synapses.

In general, it has been considered that the activation of D1 receptors leads to an increase of the production of cAMP, whereas D2 receptors inhibit the production of cAMP. In contrast, it has been shown that D2 receptors have no modulatory role on cAMP production in rat neostriatum and nucleus accumbens (27, 28). On the other hand, it has been well-documented that the release of ACh from striatal cholinergic interneurons is under tonic inhibitory influence of the nigrostriatal dopaminergic system (29), and this control is modulated by D2 receptors which have no coupling with adenylate cyclase (30, 31).

On the other hand, methylxanthine derivatives are considered to induce the elimination of adenosine A1 receptor-mediated inhibition of ACh release as well as the enhancement of adenosine A2 receptor-mediated cAMP accumulation (32). In addition, it has been reported that they enhance the electrically-evoked ACh release by antagonizing the action of endogenous adenosine at inhibitory adenosine A1 receptors (33). Considering these reports, it is necessary to employ further studies to determine whether or not denbufylline really acts on muscarinic M2 receptors, dopaminergic D2 receptors and/or adenosine A1 receptors. Such experiments are underway in our laboratory.

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