SUT-8701, a Cholecystokinin Analog, Prevents the Cholinergic Degeneration in the Rat Cerebral Cortex Following Basal Forebrain Lesioning

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ABSTRACT—SUT-8701 is a cholecystokinin octapeptide (CCK8) analog and a more lipophilic peptide than CCK8. We previously demonstrated that intra-ventricularly administered CCK8 protected against the degeneration of the cholinergic neurons in the cortex of the nucleus basalis magnocellularis (nbm)-lesioned rat. We determined whether SUT-8701 and CCK8 have the ability to protect against cholinergic degeneration in the cerebral cortex of nbm-lesioned rats. Systemically administered SUT-8701 (0.1–1 µg/day/animal, s.c.) preserved choline acetyltransferase activity and K+-evoked acetylcholine release in nbm-lesioned rats. SUT-8701 was more potent than CCK8. However, SUT-8701 was much less potent than CCK8 in satiety action. The affinity of SUT-8701 to the cholecystokinin (CCK) receptors assessed by using [125I]-CCK8 was almost the same as that of CCK8 in the mouse cerebral cortex, but was 107 times less than that of CCK8 in guinea pig pancreas. These results suggest that SUT-8701 may be effective in slowing down the degenerative processes in Alzheimer’s disease by preserving the integrity of cholinergic neurons in the nucleus basalis.

Keywords: Alzheimer’s disease, Acetylcholine release and choline acetyltransferase activity, Cholecystokinin octapeptide (CCK8), SUT-8701, Receptor binding

Cholecystokinin octapeptide (CCK8) was found to be a gastric peptide in peripheral tissue (1). More recently, much attention has been paid to the possible role of CCK8 as a neurotransmitter or neuromodulator in the central nervous system. CCK8 binding sites are present in high density in the cerebral cortex (2). CCK8 increased 2-deoxy-glucose uptake (3) and modified dopamine (4) and acetylcholine (ACh) release (5). It also has a profound effect on memory function (6). We reported that the central administration of CCK8 protects against the degeneration of cholinergic neurons in the rat cerebral cortex following lesion of the nucleus basalis magnocellularis (nbm) (7). This observation is important because lesion of the rat nbm provides an animal model of cholinergic dysfunction in the cerebral cortex of patients with Alzheimer’s disease (AD), in that such a lesion mimics some of the neurochemical pathologies associated with AD. Thus, lesioning of the nbm causes a dramatic decrease in the activity of choline acetyltransferase (ChAT) and acetylcholinesterase (AChE) (8, 9). High affinity choline uptake (10) and ACh release (11) were also significantly decreased in the nbm-lesioned rat. Similar deficits in the ChAT (12) and AChE activity (13) and diminished ACh release (14) were observed in postmortem and biopsied samples from AD brain. Learning and memory impairment, characteristic of AD, were also produced in nbm-lesioned animals (15). Deficits in ChAT activity significantly correlate with impairment in T-maze reinforced alternation performance following the nbm-lesion (16). Wallace et al. reported the synthesis of amyloid precursor protein in the cerebral cortex of rats with lesions of the nbm (17).

Our previous results as well as findings reported by other laboratories indicate that CCK8 may be a candidate drug for the therapy of AD. However, CCK8 has great difficulty crossing the blood brain barrier, and in addition, it is rapidly degraded in the bloodstream. CCK8 also has a potent satiety action as a side effect through a peripheral
cholecystokinin (CCK) receptor. For these reasons, we synthesized SUT-8701, a CCK8 analogue, as a more lipophilic and stable peptide than CCK8. The structure of SUT-8701 is shown in Fig. 1.

In the present study, we compared the abilities of systematically administered CCK8 and SUT-8701 to protect against the cholinergic degeneration in the nbm-lesioned rat cerebral cortex by measuring K+-evoked ACh release and ChAT activity. We have also examined the binding of SUT-8701 to CCK8 receptors in the guinea pig pancreas and mouse cerebral cortex by displacement of $^{125}\text{I}$-CCK8 in a binding assay. We also examined the satiety action of SUT-8701 and CCK8 using the method of suppression of food intake in mice.

MATERIALS AND METHODS

Synthesis

SUT 8701 was prepared as shown in Fig. 2 (18). The N-terminal tripeptide ester and C-terminal tetrapeptide amide were elongated stepwise by the mixed anhydride method using dimethylphosphinothioic chloride (19). The tert-butoxycarbonyl (Boc) group was used as the amino protecting group, except for the case of tryptophan, for which the dimethylphosphinothioyl (Mpt) group (20) was used. The dimethylphosphinyl (Dmp) group was introduced by using the corresponding chloride (21) at the tripeptide stage, which was then catalytically hydrogenated to produce the N-terminal tripeptide acid. The C-terminal tetrapeptide amide hydrochloride was then prepared by removal of the Mpt group with HCl in ethyl acetate. Both segments were coupled by the mixed anhydride method to produce the protected heptapeptide amide. The protecting group was removed by catalytic hydrogenation.

Animals

Male Sprague-Dawley rats (Sankyo Lab. Co., Tokyo), weighing 250–280 g at the start of the experiments, were used in nbm-lesion studies. Male ddY mice (Sankyo Lab. Co.), weighing 16–18 g, were used for the central type CCK (CCKB) receptor binding assay and measurement of the food intake study. Male guinea pigs (Sankyo Lab. Co.), weighing 180–220 g were used for the peripheral type CCK (CCKA) receptor binding assay. All animals were housed in the cages maintained at 25 ± 2°C with a 12
hr/12 hr light-dark cycle (lights on at 08.00-20.00 hr). The animals had free access to laboratory chow (CE-2 for rats and mice; CR-3 for guinea pig, Nihon Clea Co., Tokyo) and water throughout the experiments except when otherwise mentioned.

**Nucleus basalis magnocellularis lesion**

Each rat was anesthetized with sodium pentobarbital (50 mg/kg, i.p.) and mounted in a stereotaxic apparatus (David Kopf Instrument, Tujunga, CA, USA). Unilateral lesions of the nbm were performed by injection of ibotenic acid (Sigma, St. Louis, MO, USA), 5 µg/1 µl, in a vehicle of sterile, filtered 200 mM sodium phosphate buffer, pH 7.4. Ibotenic acid was delivered with a graduated microliter glass pipette with an external tip diameter of approximately 75 µm mounted in a pipette holder. The nbm was stereotaxically localized using the following coordinates: AP = -2.3 mm from bregma, ML = ±3.7 mm, 7.5 mm below the dura (Fig. 3) (22). The toxin was delivered over a period of 10 min, and the micropipette was left in place for a further 5 min following the injection. The vehicle was injected contralaterally. After that, an osmotic mini-pump (Alzet® MODEL 2002, 200 µl reservoir volume, Alza Corporation, Palo Alto, CA, USA) filled with CCK8 (1 µg/12 µl, Peptide Institute, Osaka), SUT-8701 (0.1-1 µg/12 µl) or vehicle was placed subcutaneously (s.c.) on the back of the rat immediately after nbm lesion. SUT-8701 was dissolved in 5% DMSO saline, and CCK8 was dissolved in saline. The mini-pump assured constant delivery of the test substance for two weeks at a pumping rate of 0.5 µl/hr.

**Dissection**

Rats were sacrificed by decapitation at two weeks after nbm-lesion. The brains were rapidly removed and placed in ice cold Krebs Ringer bicarbonate buffer of the following composition: 118 mM NaCl, 4.70 mM KCl, 1.50 mM MgSO4, 1.15 mM KH2PO4, 1.25 mM CaCl2, 25 mM NaHCO3 and 11.1 mM glucose, bubbled with 95% O2 and 5% CO2. The brain was immediately sliced into 400 μm sections with a McIlwain tissue chopper (Brinkman, The Mickle Laboratory Engineering Co., Ltd., Gomshall, Surrey, England). Each of ten sections ranging from +2.0 mm bregma to −2.0 mm bregma was dissected free and placed on a parafilm covered microscope slide. Three micro-punches (2.0 mm diameter) were taken from each hemisphere of the cortex (CC-1 to CC-5, Fig. 3). A total of sixty punches were taken; 30 were frozen on dry ice for later determination of ChAT activity, and the other 30 punches were immediately used for measurement of ACh release.

**Acetylcholine release**

The release of [3H]-ACh was measured as previously described (23). The micro-punches were incubated with Krebs Ringer bicarbonate buffer containing 0.1 µM [3H]-
Choline chloride (80 Ci/mmol, Amersham International plc., Little Chalfont, Buckinghamshire, England) for 20 min at 37°C. The incubated punches (3 punches/chamber) were then transferred to superfusion chambers (Brandel, Biomedical Research & Development Laboratories, Inc., Gaithersburg, MD, USA) and continuously super-fused with oxygenated Krebs Ringer bicarbonate buffer containing 10 nM hemicholinium-3, at a flow rate of 1 ml/min.

Following a washout period of 35 min, fractions were collected every 2.5 min. The ACh release from the micro-punches was elicited at 40 min after the washout period by perfusion with Krebs Ringer bicarbonate buffer containing 40 mM KCl for 5 min. The K+ evoked outflow of tritium from the tissue pre-incubated with 0.1 nM [3H]-choline chloride is a good measure of [3H]-ACh release (24, 25). Fractions were collected for an additional 15 min following stimulation. At the end of the experiment, the micro-punches were solubilized by means of tissue solubilizer (Soluene-350, Packard Instrument Company, Inc., Meriden, CT, USA). The radioactivity in each collected fraction and solubilized tissue was measured by a liquid scintillation counter. The fractional release of transmitter over each 2.5-min period was calculated by means of a microcomputer program, which calculated the evoked release of radioactivity by subtracting the basal release. In the figures, results are given as values of the mean±S.E.M. For statistical analysis, Student’s t-test was used.

Cholineacetyltransferase activity

ChAT activity was determined according to the method of Fonnum (26). Three tissue punches were homogenized by an ultrasonicicator (30% power, Handy Sonic®, 6 sec, Tomy Seiko Co., Ltd., Tokyo) in 100 µl of 50 mM Tris-HCl buffer (pH 7.4). The protein concentration of each aliquot was measured by a BIO-RAD protein assay kit (Bio-Rad Laboratories, Richmond, CA, USA). The homogenate was resuspended in 2/5 volume of 2.5% Triton-X 100 plus an equal volume of 10 mM EDTA. Eight microliters of suspension and 20 µl of substrate mixture, consisting of 50 mM sodium phosphate buffer (pH 7.4), 0.2 mM [3H]-Acetyl CoA (1 µCi/mm, DuPont/NEN Research Products, Wilmington, DE, USA), 20 mM EDTA, 300 mM NaCl, 8 mM choline chloride and 10 µM physostigmine salicylate, were mixed in a mini scintillation vial. Following a 15-min incubation at 37°C, the vials were placed into the ice bath, and the reaction was stopped by adding 1 ml of ice cold 10 mM sodium phosphate buffer (pH 7.4). Then 0.4 ml of acetonitrile containing 2 mg Kalignost (tetraphenylboron sodium salt) was added. The [3H]-ACh was extracted into 2 ml toluene scintillation cocktail (4 g/l PPO and 0.1 g/l POPOP), and its radioactivity was measured by a scintillation counter.

Receptor binding assay

The mice were sacrificed by decapitation. The cerebral cortex was removed and homogenized in 10 vol. of 50 mM Tris-HCl buffer (pH 7.4) with a polytron. The homogenate was centrifuged at 1,000 x g for 10 min (4°C). The supernatant was recentrifuged at 43,000 x g for 15 min (4°C). The pellet was resuspended in 10 vol. of 50 mM Tris-HCl buffer (pH 7.4) by a polytron and recentrifuged at 1,000 x g for 10 min at 4°C (3 times). The pellets were stored at −80°C until the receptor binding assay.

Guinea pigs were sacrificed by decapitation. The pancreases were removed and homogenized in 25 vol. of 10 mM PIPES (piperazine-N,N'-bis (2-ethanesulfonic acid)) buffer containing 30 mM MgCl2 (pH 6.5) by a polytron. The homogenate was filtered through gauze and centrifuged at 1,000 x g for 10 min (4°C). The supernatant was recentrifuged at 43,000 x g for 15 min (4°C). The pellet was resuspended in 10 vol. of PIPES buffer by using a polytron and recentrifuged at the same setting as used before. The pellets were stored at −80°C until the receptor binding assay.

The membrane was resuspended in 10 vol. of 10 mM HEPES (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid) buffer (pH 7.4) containing 1 mM EGTA, 0.5 g/l bacitracin, 118 mM NaCl, 4.7 mM KCl and 5 mM MgCl2 for the central receptor binding assay or 10 mM PIPES buffer (pH 7.4) containing 30 mM MgCl2, 0.2 g/l bacitracin and 0.2 g/l soybean trypsin inhibitor for the peripheral receptor binding assay. The protein concentration was measured by a BIO-RAD protein assay kit (Bio-Rad Laboratories) and adjusted to 2 mg/ml (for the central receptor binding assay) or 1 mg/ml (for the peripheral receptor binding assay). CCK8 was dissolved in HEPES buffer (4 x 10^-6 M - 4 x 10^-12 M) and SUT-8701 was dissolved in the buffer for each receptor binding assay (4 x 10^-3 M - 4 x 10^-11 M), each one also containing 5% DMSO. A 100-µl aliquot of the tissue homogenate, 50 µl of [125I]-CCK8 (DuPont/NEN Research Products, 0.1 nM, for the central receptor binding assay or 0.08 nM, for the peripheral receptor binding assay) and a 50-µl aliquot of displacer, at different concentrations, was added to a 96-well micro assay plate. Non-specific binding was determined in the presence of 1 µM unlabeled CCK8. After a 1-hr incubation at room temperature, radioactivity was isolated by filtration through a presoaked Whatman GF/B glass filter using a cell harvester and washed 5 times with 300 µl of the appropriate ice cold incubation buffer. The radioactivity on the filter was counted by an autowell gamma-counter.
Measurement of the food intake

Measurement of the food intake in mice was performed according to the method reported by Kubota et al. (27). Liquid food (7% protein, 7.7% fat, 10.2% sugar, 1.6% ash, 73.5% water and 135 kcal/100 g) was used in order to accurately measure the amount of food. The mice had free access to water throughout the experiments and were previously fed with liquid food for a week so they would become accustomed to liquid food. Food was withdrawn for 24 hr, and the mice were put into the wire framed isolation cage. CCK8 and SUT-8701 were intraperitoneally injected to the fasted mice 20 min before starting the measurement of liquid food intake. The experiments were started at 5 PM, and the cumulative food intakes of mice were measured by the decrease of the liquid food.

RESULTS

Basal release of \([^3H]-ACh\) from cortical micropunches and the level of \([^3H]-\)choline uptake in the micropunches were not altered following nbm lesion. As shown in the Fig. 4, there was no significant differences among the regions in K⁺-evoked \([^3H]-ACh\) release following the lesion. K⁺-evoked \([^3H]-ACh\) release was significantly reduced to about 50% at two weeks following the lesion. CCK8 at 1 μg/animal/day, s.c. significantly preserved K⁺-evoked \([^3H]-ACh\) release in the dissection of CC1 and CC2 after the lesion. The CCK8 preservation effect was less in the rostral part of the cerebral cortex (CC3 to CC5). SUT-8701 at 1 μg/animal/day, s.c. significantly preserved the K⁺-evoked \([^3H]-ACh\) release after the le-

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**Fig. 4.** Effect of SUT-8701 and CCK8 infusions on the decrease of K⁺-evoked ACh release from CC micropunches 2 weeks after nbm lesion. The columns show the mean of % ACh basal release ± S.E.M. Percent ACh basal release was calculated as a total % increase from the baseline at the peak. ■: sham-operated, □: lesioned, △: lesioned and 0.1 μg/animal/day (s.c.) SUT-8701 treated, ○: lesioned and 0.5 μg/animal/day (s.c.) SUT-8701 treated, ▲: lesioned and 1 μg/animal/day (s.c.) SUT-8701 treated. ■: lesioned and 1 μg/animal/day (s.c.) CCK8 treated. Student’s t-test was used for comparisons; n=6 to 8 animals per group; **(P<0.01), *(P<0.05), significant difference between the untreated group and each treated group.

**Table 1.** Effect of CCK8 and SUT-8701 infusions on the cholinergic markers in the cerebral cortex of sham-operated rats

| Region | ACh release (%) | ChAT activity (nmol/mg protein/min) |
|--------|----------------|----------------------------------|
|        | control | CCK8 | SUT-8701 | control | CCK8 | SUT-8701 |
| CC1    | 374.4 ± 48.2 | 417.4 ± 34.1 | 384.5 ± 99.0 | 1.38 ± 0.05 | 1.41 ± 0.08 | 1.33 ± 0.10 |
| CC2    | 430.7 ± 54.2 | 476.3 ± 43.9 | 470.5 ± 80.7 | 1.35 ± 0.06 | 1.41 ± 0.06 | 1.42 ± 0.07 |
| CC3    | 361.1 ± 47.0 | 388.0 ± 46.1 | 382.3 ± 50.4 | 1.32 ± 0.07 | 1.33 ± 0.08 | 1.28 ± 0.14 |
| CC4    | 412.0 ± 50.5 | 470.6 ± 60.0 | 428.7 ± 51.0 | 1.34 ± 0.05 | 1.28 ± 0.11 | 1.27 ± 0.19 |
| CC5    | 386.0 ± 47.1 | 366.8 ± 43.1 | 385.4 ± 37.7 | 1.37 ± 0.05 | 1.35 ± 0.10 | 1.31 ± 0.15 |

The values are means ± S.E.M. CCK8 and SUT-8701 were administered at the dose of 1 μg/animal/day (s.c.).
lion in all parts of the cerebral cortex (CC 1 to CC5). This preservation was more effective than that of the same dose of CCK8. CCK8 or SUT-8701 did not have any effect on the K+-evoked [3H]-ACh release from the micropunches of the sham-operated site (Table 1).

ChAT activity was significantly reduced to about 50% at two weeks following the lesion (Fig. 5). There was no regional variation in ChAT activity at two weeks post-lesion in either the sham-operated or lesioned hemisphere (Fig. 5). SUT-8701 at 1 pg/animal/day, s.c. significantly preserved ChAT activity in all parts of the cerebral cortex (CC1 to CC5) after the lesion (Fig. 5). SUT-8701 at 0.5 pg/animal/day, s.c. significantly preserved ChAT activity after the lesion in the dissection of CC1 and CC2. CCK8 at 1 pg/animal/day, s.c. significantly preserved ChAT activity in CC3 to CC5 after the lesion (Fig. 5). CCK8 or SUT-8701 did not have any effect on the ChAT activity in the micropunches of the sham-operated site.
The displacement curves for SUT-8701 and CCK8 paralleled each other in the mouse cerebral cortex and the guinea pig pancreas (Figs. 6 and 7). IC50 values of CCK8 and SUT-8701 for the CCK receptor in the mouse cerebral cortex homogenate were 7.41 x 10^{-10} M and 1.23 x 10^{-9} M, respectively (Fig. 6). IC50 values of CCK8 and SUT-8701 for the CCK receptor in the guinea pig pancreas homogenate were 2.88 x 10^{-10} M and 3.09 x 10^{-8} M, respectively (Fig. 7).

CCK8 dose-dependently suppressed the food intake in mice (Fig. 8). In the first 20-min experimental period, 0.1 mg/kg and 1 mg/kg CCK8, i.p. significantly suppressed the food intake (Fig. 8). In the first 60-min experimental period, 1 mg/kg CCK8, i.p. significantly suppressed the food intake (Fig. 8). SUT-8701 did not have any significant effect on the food intake of mice up to 1 mg/kg (Fig. 9). Even mg/kg 61-SIT1 SUT1, i.p. significantly suppressed the food intake in both experimental time periods (Fig. 9).

**DISCUSSION**

In the symptomatic therapy of AD, the ACh content in the brain is increased by the use of AChE inhibitor or a cholinergic agonist in order to increase cholinergic transmissions. However, many trials have failed to obtain dramatic changes in memory impairment of AD patients, unlike the effectiveness of drugs used in Parkinson’s disease. Such failure may be due to the fact that the memory function is more complex than the coordinate function of movement. Unlike the destruction of the cholinergic system damaged in AD, the CCK system still survives even in the advanced stage of AD (28, 29). We reported that CCK8 at 10 ng/day/animal, i.c.v. could prevent the cholinergic degeneration in rat cerebral cortex following nbm-lesion; and from these results, we argued that CCK8 could be a candidate for an anti-dementia drug for AD (7). In this kind of therapy, the patients would require a long period of drug treatment. From this point of view, it is better for these drugs to be administered systemically and preferable that they have a long activity in the body.

In this report, systemically administered CCK8 prevented the cholinergic degeneration in the cerebral cortex after nbm lesion. Even when 100 times the i.c.v.-dose was systemically administered, the effect was still less than that of i.c.v.-administered CCK8 (7). Systemically administered SUT-8701 also prevented cholinergic degeneration in the cerebral cortex after nbm-lesion. SUT-8701 was more potent than CCK8 at the same dose. On the other hand, SUT-8701 had a lower satiety action than CCK8. In addition, the satiety effect may have a relation to CCKA receptors, since it was blocked by L-364,718 (CCKA antagonist) (K. Kojima et al., unpublished data). SUT-8701 is considered to be more lipophilic than CCK8 and to pass the blood brain barrier more easily than CCK8. When SUT-8701 and CCK8 are peripherally administered, CCK8 is likely to produce its central action via mainly acting on the vagus nerve, while SUT-8701 acts more directly on the central sites. This difference may be reflected in their inconsistent regional effects in the cortex.

In the binding experiment, we used mice for the binding
assay on the central type CCK receptors (CCK\textsubscript{B} receptor) and guinea pigs for the assay on the peripheral type of receptors (CCK\textsubscript{A} receptor) since no species difference has been demonstrated for these two subtypes of CCK receptor. SUT-8701 and CCK8 had similar affinities to mouse cerebral cortex membrane preparation (IC\textsubscript{50}SUT-8701/IC\textsubscript{50}CCK8 = 1.6), and the former had much lower affinity to the guinea pig pancreas membrane preparation than the latter (IC\textsubscript{50}SUT-8701/IC\textsubscript{50}CCK8 = 107). The results of the receptor binding assay suggest that SUT-8701 is a more selective compound for the CCK\textsubscript{B} receptor than CCK8. The results of these three types of experiments are in good agreement with each other, and they indicate that the anti-degenerative effect of SUT-8701 may be mainly concerned with CCK\textsubscript{B} receptors.

Several possible mechanisms of the anti-degenerative action by SUT-8701 have been proposed: 1) SUT-8701, a CCK8 analog, might act directly on the cholinergic system in the cerebral cortex as a neurotrophic drug, because the concentration of CCK receptor is the highest in the cerebral cortex (2, 3), and CCK8 increases the ACh concentration in the rat cerebral cortex (5). 2) SUT-8701 might protect the cholinergic neurons in the cerebral cortex by increasing the cerebral blood flow. 3) The other possibility is a direct protective effect on the cholinergic neurons in the nbm, because CCK blocks some effects of kainic acid in the CA3 region of hippocampal slices (30).

Even this nbm-lesion model is not a real AD, but it mimics the destruction of the cholinergic system in AD cerebral cortex. Systemically injected SUT-8701 prevented the destruction of the cholinergic system in this animal model and had less peripheral effect (satiety) as compared with CCK8. These results suggest the potential of SUT-8701 as an anti-dementia drug for AD.

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