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FK962, a novel enhancer of somatostatin release, exerts cognitive-enhancing actions in rats

Kenichi Tokita *, Tatsuo Inoue, Shunji Yamazaki, Feng Wang, Takayuki Yamaji, Nobuya Matsuoka, Seitaro Mutoh

Pharmacology Research Labs., Astellas Pharma Inc., 21 Miyukigaoka, Tsukuba, Ibaraki 305-8585, Japan

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Abstract

FK962 (*N*-(1-acetylpiperidin-4-yl)-4-fluorobenzamide) is a derivative of FK960 (*N*-(4-acetyl-1-piperazinyl)-*p*-fluorobenzamide monohydrate), with putative anti-dementia properties. Here, we wanted to determine whether FK962 retained the ability of the parent compound to both facilitate somatostatinergic nerve activity in hippocampal neurons and to ameliorate cognitive dysfunction in rat models. FK962 (10^{-9}–10^{-6} M) significantly enhanced high K^+-evoked somatostatin release from rat hippocampal slices. FK962 also significantly reduced somatostatin-induced inhibition of Ca^{2+} channels at 10^{-9}–10^{-7} M in single rat hippocampal neurons using whole-cell patch-clamp. Furthermore, administration of FK962 (0.032–3.2 mg/kg, i.p.) significantly ameliorated memory deficits in passive avoidance task in animal models: scopolamine-treated rats, nucleus basalis magnocellularis-lesioned rats and aged rats. FK962 (0.01–1 mg/kg, i.p.) significantly improved spatial memory deficits induced by nucleus basalis magnocellularis-lesion in water maze task. These results suggest that FK962 ameliorates cognitive impairment in rats via activation of the somatostatinergic nervous system in the hippocampus, indicating that FK962 could be a potent cognitive enhancer and therefore might be of therapeutic value for cognitive disorders such as Alzheimer's disease.

Keywords: FK962; Memory; Passive avoidance; Somatostatin; Water maze; Whole-cell patch-clamp

1. Introduction

Brain cholinergic neurons degenerate in patients with Alzheimer's disease and senile dementia of the Alzheimer's type, and the degree of degeneration parallels functional loss in these disorders (Davies and Maloney, 1976; Perry et al., 1978; Coyle et al., 1983). This clinical evidence, as well as experimental observations in animals, supports the hypothesis that cholinergic nerve activity plays a crucial role in various forms of cognitive function (Bartus et al., 1982; Hepler et al., 1985; Hagan and Morris, 1988; Matsuoka et al., 1991b). Based on this "cholinergic hypothesis", many attempts have been made to reverse cognitive deficits by increasing brain cholinergic activity through the use of cholinomimetics such as acetylcholinesterase inhibitors (Brinkman and Gershon, 1983; Rogers et al., 1998). However, these compounds have shown modest efficacy and frequent unfavorable side-effects due to parasympathetic over-stimulation, thereby limiting their long-term treatment (Benzi and Moretti, 1998; Nordberg and Svensson, 1998). Additional highly tolerable therapies for Alzheimer's disease with alternative mechanisms of action need to be developed (Doody, 2003).

Somatostatin is a neuropeptide distributed mainly in the cerebral cortex, hippocampus and hypothalamus (Chan-Palay, 1987; Johansson et al., 1984). Several lines of evidence have suggested that brain somatostatin plays an important role in learning and memory (Schettini, 1991; Ohno et al., 1993; McNamara and Skelton, 1993; Epelbaum et al., 1994; Matsuoka et al., 1994, 1995; Vécsei et al., 1983). Clinical studies have also revealed that impairments of somatostatin-mediated neurotransmission in the brain are associated with dementia in Alzheimer's disease (Davies et al., 1980; Bissette and Myers, 1992). A novel anti-dementia compound, FK960 (*N*-(4-acetyl-

* Corresponding author. Tel.: +81 29 863 7173; fax: +81 29 854 1616.
E-mail address: kenichi.tokita@jp.astellas.com (K. Tokita).

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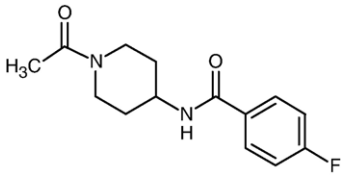

Fig. 1. Chemical structure of FK962, *N*-(1-acetylpiperidin-4-yl)-4-fluorobenzamide.

1-piperazinyl)-*p*-fluorobenzamide monohydrate), has been shown to improve memory deficits in various experimental amnesia models in rats as well as in nonhuman primates (Yamazaki et al., 1996; Matsuoka and Aigner, 1997), to facilitate high K^+-evoked release of somatostatin from the hippocampus (Inoue et al., 2001) and to block somatostatin-induced inhibition of Ca^{2+} currents in hippocampal neurons (Wang et al., 2004). Many neurotransmitters are known to inhibit neuronal Ca^{2+} channels (Tsien et al., 1988; Anwyl, 1991; Hille, 1992, 1994), and this inhibition serves as an autoreceptor-mediated negative feedback regulation of neurotransmitter release in presynaptic nerve terminals. Thus, the positive modulatory effect of FK960 on voltage-activated Ca^{2+} channels provides a putative mechanistic explanation for how changing somatostatinergic neurotransmission leads to cognitive facilitating effects in animal models (Yamazaki et al., 1996; Matsuoka and Aigner, 1997).

Through further chemical optimization to improve the potency of pharmacological action as well as pharmacokinetic properties, we have recently identified FK962 (*N*-(1-acetylpiperidin-4-yl)-4-fluorobenzamide) (Fig. 1) as a promising back-up compound of FK960. In the present study, we investigated the effects of FK962 on somatostatin release from rat hippocampal slices, and used whole-cell patch-clamping to determine whether FK962 could regulate somatostatin-induced modulation of voltage-activated Ca^{2+} channels in rat hippocampal neurons. Furthermore, we investigated the effects of FK962 on memory formation in young rats treated with scopolamine, a muscarinic antagonist; in nucleus basalis magnocellularis-lesioned rats, which decreases the activity of the cortical choline acetyltransferase (Matsuoka et al., 1995), an indicator for monitoring the functional state of cholinergic neurons (Oda, 1999); as well as in aged rats in the passive avoidance task. We also assessed the activity of FK962 on the mnemonic process of spatial memory in nucleus basalis magnocellularis-lesioned rats using the Morris water maze task.

2. Materials and methods

2.1. Animals

Male Fischer-344 rats (7–9 weeks old, $n=13–15$) were used to evaluate somatostatin release from hippocampal slices. Male Wistar rats (7–11 days postnatal, $n=5–9$) were used for current recording in hippocampal neurons. Male Wistar rats (8 weeks old, $n=12–13$) and male Fischer-344 rats (14 weeks old, $n=11–12$) were used in scopolamine-induced amnesia and in nucleus basalis magnocellularis-lesioned model, respectively, in the passive avoidance task. For the study on memory deficit of aged rats, aged Fischer-344 rats (26–27 months old) and young Fischer-344 rats (11 weeks old) were used in passive avoidance task ($n=13–14$). Male Fischer-344 rats (14 weeks old, $n=10–15$) were used for water maze task. All animals were obtained from Charles River Inc. (Atsugi, Japan) and were given food and water ad libitum. They were housed in a temperature-controlled environment (22±2 °C) under a 12:12-h light/dark cycle with lights on at 7:00 a.m. When the behavioral testing began, rats were used at ages specified above. Rats were obtained at least 1 week before the experiments for behavioral tests and handled 3 days prior to the start of the behavioral experiments. All animal experimental procedures were performed under the guidelines of the Animal Experiment Committee of Fujisawa Pharmaceutical Co., Ltd. (now Astellas Pharma Inc.).

2.2. Somatostatin release from hippocampal slices

Rats were sacrificed by decapitation, and the brain quickly removed and placed on an ice-cold dissecting tray. Hippocampi were excised and 350 μm thick transverse slices prepared with a McIlwain tissue chopper. Somatostatin release experiments were carried out using a batch incubation technique as described by Vezzani et al. (1992) with minor modification. Krebs-bicarbonate medium containing 118 mM NaCl, 4.8 mM KCl, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 25 mM $NaHCO_3$, 10 mM Glucose, 2.5 mM $CaCl_2$ and, additionally, 0.1% bovine serum albumin (BSA) and 0.01 mg/ml aprotinin (Sigma, St. Louis, MO) was used. The mixture was saturated with 95% O_2:5% CO_2. Thirty hippocampal slices were suspended in the medium (300 μl) and put into a chamber made of spin columns (φ8×50 mm) with nylon fiber at the bottom. Three hundred microliters of the medium was then collected every 10 min by drainage from a bottom pin hole, and the same volume of fresh medium was added each time until the last 15th fraction. High K^+ medium consisting of 50 mM K^+ and 99 mM Na^+ (to maintain isotonicity), sufficient to cause moderate release of somatostatin, was applied at fraction 9. FK962 was added from fraction 7 to the end of the experiment. Fractions were collected at 37 °C and immediately placed on ice. At the end of the experiment, hippocampal slices were quickly frozen on dry ice and stored at −20 °C. All the fraction samples were centrifuged at 1700×*g* for 5 min and 250 μl aliquots of the supernatants were transferred into test tubes. Both samples and the same volume of somatostatin (1–14) standard peptide solution for radioimmunoassay were lyophilized overnight and stored at −20 °C until assay. To extract somatostatin from the frozen hippocampal slices, slices were dropped into 2 ml of boiled 2 M acetic acid and then boiled for a further 5 min. They were homogenized after cooling and centrifuged at 27,000×*g* for 15 min at 4 °C. One milliliter of supernatant was then lyophilized overnight and stored at −20 °C until assay. Somatostatin-like immunoreactivity was determined using a commercially available radioimmunoassay kit (Peninsula Laboratories Inc., San Carlos, CA). The amount of released somatostatin-like immunoreactivity in each fraction is shown as a percentage

of the total somatostatin content remaining in the slices. The amount of released somatostatin was calculated as the sum of the positive values serially after high K^+ stimulation, obtained by subtracting basal release (the value of the prestimulated fraction). FK962 was dissolved in distilled water and diluted in medium before use.

2.3. Current recording by conventional whole-cell patch-clamp techniques

Hippocampal neurons were acutely prepared according to a modified version of the method described by Kay and Wong (1986). In brief, transverse hippocampal slices from rats were prepared in ice-cold oxygenated (100% O_2) piperazine *N*,*N*′-bis-[2-ethanesulfonic acid], 1,4-piperazinediethanesulfonic acid (PIPES) solution containing 120 mM NaCl, 5 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 20 mM PIPES and 25 mM glucose (pH 7.0). After incubation in trypsin (Sigma, St. Louis, MO) at 0.6–0.8 mg/ml containing solution at 30–32 °C for 60 min, slices were rinsed with trypsin-free solution and left at room temperature in an O_2 atmosphere. Before use, cells were triturated mechanically into individual cells with fire-polished Pasteur pipettes in Dulbecco's Modified Eagle Medium. Cells were then transferred to a recording chamber and allowed to settle for about 10 min before recording.

Whole-cell recordings were performed using conventional patch-clamp techniques (Hamill et al., 1981) with an Axopatch 200A amplifier (Axon Instruments, Union City, CA). The voltage-activated Ca^{2+} currents of hippocampal neuron were obtained in the internal solution containing 100 mM CsCl, 5 mM $MgCl_2$, 10 mM ethylene-bis(oxyethylenenitrilo)tetraacetic acid (EGTA), 40 mM 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES), 4 mM ATP–Tris, 0.2 mM GTP–Tris (pH 7.3) and the external perfusion solution consisted of 135 mM tetraethylammonium (TEA)–Cl, 10 mM $BaCl_2$, 10 mM HEPES (pH 7.3). The patch pipette was fire-polished and had a tip resistance of 2–4 MΩ. The recording chamber was continuously perfused with the external solution with or without test drugs through gravity-fed flow pipes at a constant flow rate. Whole-cell inward currents were elicited every 10 s by a depolarization to 0 mV from a holding potential of −80 mV. Currents were four-pole Bessel-filtered at 2 kHz and digitized at 10 kHz with DiGiData 1200 Interface (Axon Instruments, Union City, CA). Data were acquired and leak subtracted using the P/4 protocol under the control of the pCLAMP (Ver. 6) software (Axon Instruments, Union City, CA). All experiments were carried out at room temperature (23±1 °C). A 10 mM stock solution of FK962 was prepared daily that was then diluted to the desired final concentrations in the external solution before use. Somatostatin was purchased from Sigma Chemical Co. (St. Louis, MO).

2.4. Passive avoidance task

The apparatus and experimental procedure used in the passive avoidance task were similar to those described previously (Matsuoka et al., 1992). In brief, a two-compartment step-through passive avoidance apparatus made of black perspex was used. The apparatus consisted of illuminated and dark compartments attached to a grid floor and were separated by a guillotine door. The rat was placed in the illuminated compartment and the door was raised. After entering the dark compartment, the rat was returned to its home cage (habituation trial). In the scopolamine-treated model, rats were given an i.p. injection of scopolamine (Nacalai Tesque, Kyoto, Japan) at 1 mg/kg 30 min after the habituation trial and, after a further 30 min, the rat was again placed in the illuminated compartment (acquisition trial). When the rat entered the dark compartment, the guillotine door was closed. Scrambled electrical foot shocks by 0.4 mA in the scopolamine-treated and the nucleus basalis magnocellularis-lesioned model and 0.3 mA in the aged rats were delivered for 4 s through the grid floor using a shock generator (NS-SG01, Neuroscience Co., Tokyo, Japan). FK962 was administered i.p. immediately after the acquisition trial (60 min after the habituation trial). In the test trial made 24 h after the acquisition trial, the rat was placed again in the illuminated compartment and the response latency to enter the dark compartment was measured up to a maximum of 300 s (retention trial). The results were recorded as average latency for each group of rats. The percentage of rats reaching criterion (300 s) was also calculated. FK962 and scopolamine were dissolved in physiological saline just before the tests and administered i.p. in a volume of 1 ml/kg.

2.5. Water maze task

The apparatus and experimental procedure were similar to those described previously (Yamazaki et al., 1995). In brief, all animals were trained in the Morris water maze using a two-trial per day regimen. The water maze apparatus (Neuroscience Co., Tokyo, Japan) consisted of a circular pool 150 cm in diameter that was filled with water to a depth of 30 cm. A clear circular platform (16 cm in diameter), made of plastic and supported by a base resting on the bottom of the pool, was placed 1–1.5 cm below the surface of the water. Water temperature was 19–20 °C. For descriptive data collection, the pool was subdivided into four equal quadrants by imaging lines that interested in the center of the pool at right angles called north, south, east and west. The platform always resided in the center of the southwest quadrant. At the start of a trial, the rat was placed at one of the two cardinal starting locations. When the rat found the platform, they were allowed to remain on it for 15 s. If the rat did not locate the platform within 90 s, they were removed from the water and placed on the platform for 15 s. The second daily trial began 4 min after conclusion of the first. Starting locations were ordered in a semi-random manner. The first trial was started from one of the two locations farthest from the platform (north or east) but never the same first location on 2 consecutive days. The starting location of the second trial was a random choice of one of the two remaining locations (south or west). After the trial, the rat was dried thoroughly with a towel and placed in a drying cage equipped with a hairdryer before it was returned to its home cage. The animals were

trained in the water maze for 4 consecutive days. Data collection was automated by an on-line video device designed to track the object in its field with the highest contrast, which was always the white rat on the black background. Tracking was achieved by a system consisting of a black and white video camera directly over the center of the pool. The tracker's digitized coordinate values were sampled in turn using a personal computer. Escape latency (the time to find the platform) was recorded for each trial with a behavioral tracing analyzer (BTA-2, Neuroscience Co., Tokyo, Japan). The daily latency was obtained from the average latencies of two trials each day in each treatment group. The average latencies of consecutive 4-day trainings were also calculated in each treatment group. FK962 was dissolved in physiological saline just before the tests and administered i.p. in a volume of 1 ml/kg 30 min before the first trial for 4 consecutive days. Nucleus basalis magnocellularis-lesioned control group and the sham-operated group were administered physiological saline.

2.6. Neurosurgery for nucleus basalis magnocellularis-lesion

The surgical procedure used in the nucleus basalis magnocellularis-lesion was similar to that described previously (Matsuoka et al., 1992). Briefly, bilateral neurotoxic lesions of the nucleus basalis magnocellularis were produced by injection of ibotenic acid. The animals were anesthetized with sodium pentobarbital (50 mg/kg, i.p.) and fixed on a stereotaxic apparatus (SR-6, Narishige Co., Tokyo, Japan) with the incisor bar set 2.3 mm below the intra-aural line. Positioning of the coordinates followed the stereotaxic atlas of König and Klippel (1963). A stainless injection needle (diameter 0.25 mm), connected via polyethylene tubing to a 10 μl microsyringe mounted on a microdrive injector (KDS100, KD Scientific Inc., Holliston, MA), was inserted into the nucleus basalis magnocellularis. The coordinates were 1.6 mm posterior to the bregma, 2.8 mm lateral to the midline and 7.8 mm ventral from the skull. Ibotenic acid (Sigma Chemical Co., St. Louis, MO) was dissolved in sterile physiological saline at a concentration of 8 μg/μl and then infused directly into the area of one nucleus basalis magnocellularis in a volume of 1 μl for 6 min. After the injection, the cannula was left in place for 5 min to ensure that the drug had diffused away from the needle tip. Since the lesions in both sides of nucleus basalis magnocellularis at the same time cause severe morbidity, a second lesion was made only after recovery from surgery was complete and the weight of the rats had stabilized. The sham-operated rats were placed on the stereotaxic apparatus under anesthesia and infused with saline into the brain area for 6 min. Three weeks after the last operation, the rats were subjected to behavioral tests.

2.7. Drug

FK962 (*N*-(1-acetylpiperidin-4-yl)-4-fluorobenzamide; chemical structure is shown in Fig. 1) was synthesized in Fujisawa Pharmaceutical Co., Ltd. (Osaka, Japan, now Astellas Pharma Inc.).

2.8. Statistical analysis

All results are expressed as the mean±S.E.M. Statistical analysis was performed by Dunnett's multiple comparison test in the experiments of somatostatin release and current recording. The statistical significance between scopolamine-treated groups, nucleus basalis magnocellularis-lesioned groups or aged groups was calculated by non-parametric analysis with the Kruskal–Wallis test followed by Dunnett's multiple comparison test for the retention latency and chi-square test followed by Dunnett's multiple comparison test for percentage of rats reaching criteria in passive avoidance task. The statistical significance between the scopolamine-alone control group and the normal group, the nucleus basalis magnocellularis-lesioned vehicle control group and the sham-operated group or the aged vehicle control group and the young group was calculated by Wilcoxon ranking test for the retention latency and Fisher's exact probability test for percentage of rats reaching criteria in passive avoidance task. Statistical analysis of daily latency and average latency for drug treatment vs. control in the water maze task was

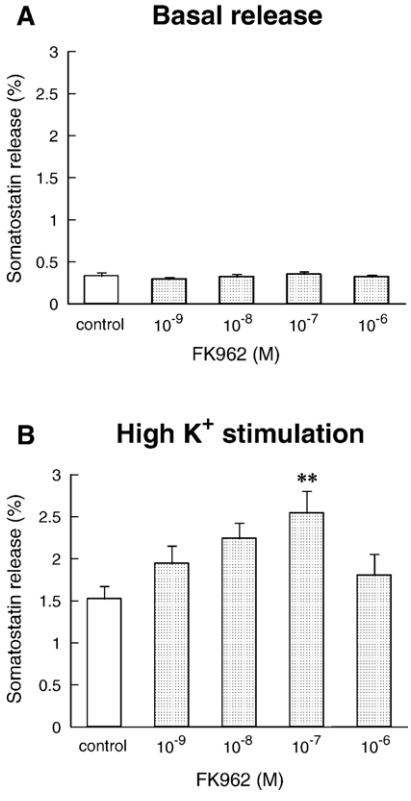

Fig. 2. Effects of FK962 on somatostatin release from hippocampal slices (A) without stimulation (the value of the prestimulated fraction) and (B) evoked by high K^+ stimulation (the sum of released somatostatin obtained by subtracting basal release). Each column represents the mean±S.E.M. (n=13–15). **P<0.01; statistically significant compared with the control (Dunnett's multiple comparison test).

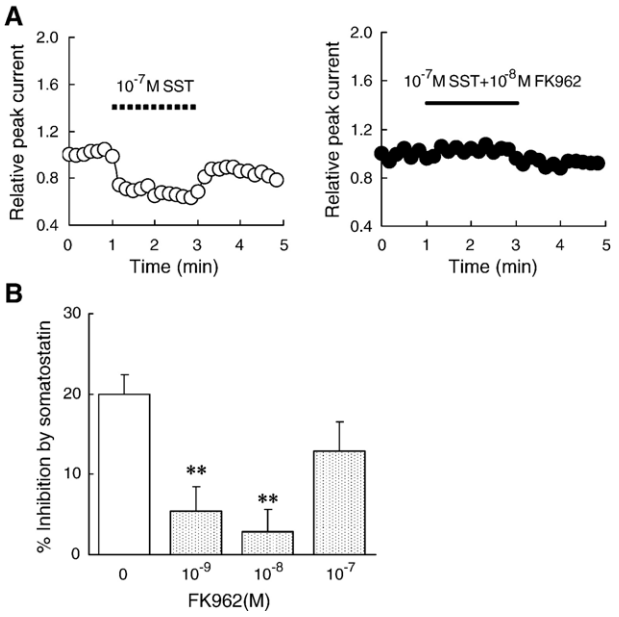

Fig. 3. Effects of FK962 on somatostatin-induced inhibition of Ca^{2+} channel currents in hippocampal neurons. (A) Relative peak current versus time. Inward currents were obtained after application of 10^{-7} M somatostatin with or without 10^{-8} M FK962. (B) Dose–response relationship for the effect of FK962. Pooled results show the percentage reduction of peak currents by somatostatin in the presence of FK962 at the indicated concentration. Data are the mean±S.E.M. (n=5–9). **P<0.01; statistically significant compared to somatostatin alone group (Dunnett's multiple comparison test). SST: somatostatin.

conducted using one-way analysis of variance (ANOVA) followed by post hoc Dunnett's multiple comparison test. For comparison of sham-operated group with nucleus basalis magnocellularis-lesioned control group, daily latency and average latency were analyzed by Student's t-test in water maze task. $P<0.05$ was considered statistically significant.

3. Results

3.1. Somatostatin release from hippocampal slices

Basal release of somatostatin reached high levels immediately after hippocampal slices were transferred from ice-cold buffer to 37 °C incubation buffer (data not shown). One hour later, basal release of somatostatin declined gradually and remained stable at a low level (data not shown). A depolarization stimulus with 50 mM K^+ resulted in robust and prolonged increases in somatostatin release. Basal release of somatostatin without stimulation was about 0.3% of total somatostatin content in the slices and the amount of released somatostatin with 50 mM high K^+ stimulation was 1.52% (Fig. 2).

FK962 at concentrations of 10^{-9} M to 10^{-6} M had no effects on basal release, but at 10^{-7} M significantly enhanced somatostatin release evoked by 50 mM K^+ stimulation, as shown in Fig. 2 ($P<0.01$ by Dunnett's multiple comparison test). The amount of released somatostatin evoked by 50 mM K^+ stimulation with FK962 at 10^{-7} M was 2.54%. This value was 67% greater than somatostatin released by 50 mM K^+ stimulation without FK962.

3.2. Current recording in hippocampal neurons

Ba^{2+} was used as the charge carrier for the recording of Ca^{2+} currents in hippocampal neurons. Under whole-cell patch-clamp configuration, high-voltage-activated inward Ba^{2+} currents were completely blocked by external application of Cd^{2+}, indicating that they pass through Ca^{2+} channels (Wang et al., 2004). Application of somatostatin at 10^{-7} M to a hippocampal neuron rapidly reduced the peak Ca^{2+} currents (Fig. 3A, left), in agreement with our previous study (Wang et al., 2004). Current reduction was recovered when changing the perfusion to the control external solution. FK962 (10^{-8} M) co-treatment with somatostatin blocked this somatostatin-induced inhibition of Ca^{2+} currents (Fig. 3A, right). Somatostatin-mediated inhibition of Ca^{2+} currents recovered after washout of FK962, suggesting that the effect of FK962 was reversible (data not shown). The activity of FK962 showed a bell-shaped dose-dependency with a maximal effect observed at 10^{-8} M. While application of somatostatin alone reduced the peak current by 19.99±2.43% (n=8), the mean inhibition was significantly changed in the presence of 10^{-8} M (2.87±2.76%, n=9) as

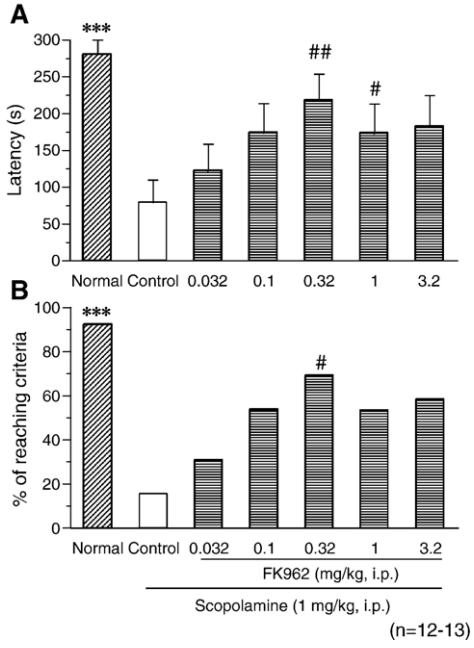

Fig. 4. Effects of FK962 on memory deficits produced by scopolamine in rats in passive avoidance task. (A) Retention latencies. Ordinate represents the mean retention latencies in a 24-h retention test. #$P<0.05$, ##$P<0.01$; statistically significant compared with scopolamine-alone controls (open columns) (by Kruskal–Wallis test followed by Dunnett's multiple comparison test). ***$P<0.001$; statistically significant compared with scopolamine-alone controls (open columns) (by Wilcoxon ranking test). Each column and bar represents the mean± S.E.M. (B) Percent of rats reaching criteria. Ordinate represents the percent of rats reaching criteria (300 s) in a 24-h retention test. #$P<0.05$; statistically significant compared with scopolamine-alone controls controls (open columns) (by chi-square test followed by Dunnett's multiple comparison test). ***$P<0.001$; statistically significant compared with scopolamine-alone controls (open columns) (by Fisher's exact probability test). Numbers of rats are shown in parentheses. Scopolamine was administered i.p. at 1 mg/kg 30 min before the acquisition trial. FK962 was administered i.p. immediately after the acquisition trial.

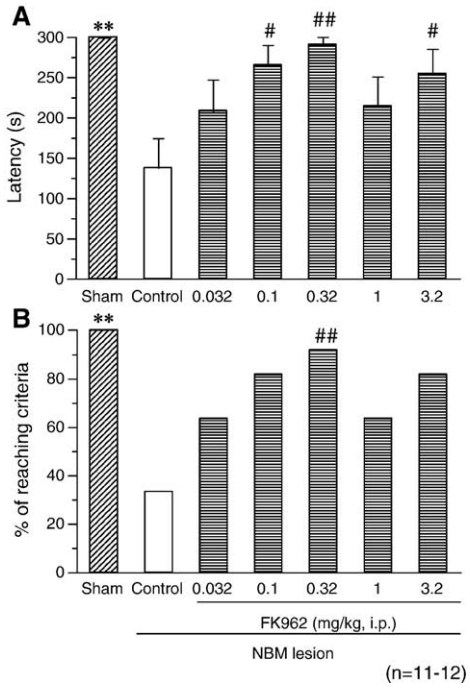

Fig. 5. Effects of FK962 on the memory deficits in nucleus basalis magnocellularis-lesioned rats in passive avoidance task. (A) Retention latencies. Ordinate represents the mean retention latencies in a 24-h retention test. $^{\#}P<0.05$, $^{\#\#}P<0.01$; statistically significant compared with vehicle controls (open columns) (by Kruskal–Wallis test followed by Dunnett's multiple comparison test). $^{**}P<0.01$; statistically significant compared with vehicle controls (open columns) (by Wilcoxon ranking test). Each column and bar represents the mean ±S.E.M. (B) Percent of rats reaching criteria. Ordinate represents the percent of rats reaching criteria (300 s) in a 24-h retention test. $^{\#\#}P<0.01$; statistically significant compared with vehicle controls (open columns) (by chi-square test followed by Dunnett's multiple comparison test). $^{**}P<0.01$; statistically significant compared with vehicle controls (open columns) (by Fisher's exact probability test). Numbers of rats are shown in parentheses. FK962 was administered i.p. immediately after the acquisition trial. NBM: nucleus basalis magnocellularis.

well as 10^{-9} M (5.44±3.06%, $n=5$) of FK962 ($P<0.01$ by Dunnett's multiple comparison test).

3.3. Passive avoidance task in scopolamine-induced amnesia rats

Scopolamine at 1 mg/kg given 30 min before the acquisition trial significantly ($P<0.001$ by Wilcoxon ranking test) reduced the latency in the retention trial determined 24 h thereafter (Fig. 4A). FK962 (0.032 to 3.2 mg/kg) administration immediately after the acquisition trial prolonged the latency in scopolamine-treated rats with a bell-shaped dose-dependency and a maximal and statistically significant effect ($P<0.01$ by Kruskal–Wallis test followed by Dunnett's multiple comparison test) at 0.32 mg/kg (Fig. 4A). Another index of memory, the percentage of rats reaching criteria (Fig. 4B) was significantly reduced by scopolamine ($P<0.001$ by Fisher's exact probability test). FK962 had a similar effect on this parameter, with a bell-shaped dose–response curve, and a maximal and statistically significant effect at 0.32 mg/kg ($P<0.05$ by chi-square test followed by Dunnett's multiple comparison test) (Fig. 4B).

3.4. Passive avoidance task in nucleus basalis magnocellularis-lesioned rats

Nucleus basalis magnocellularis-lesioned rats showed a significantly shorter latency in the retention trial than sham rats in the passive avoidance task ($P<0.01$, Fig. 5A, by Wilcoxon ranking test). FK962 at 0.032 to 3.2 mg/kg completely restored the retention deficit in the nucleus basalis magnocellularis-lesioned rats. The dose–response curve was bell-shaped with the maximal and statistically significant change at 0.32 mg/kg ($P<0.01$ by Kruskal–Wallis test followed by Dunnett's multiple comparison test) (Fig. 5A). The percent of rats reaching criteria paralleled the latency. The percent of rats reaching criteria was significantly reduced in the nucleus basalis magnocellularis-lesioned rats ($P<0.01$, Fig. 5B, by Fisher's exact probability test). FK962 increased the percent of rats reaching criteria with a bell-shaped dose–response curve with the maximal and statistically significant change at 0.32 mg/kg ($P<0.01$ by chi-square test followed by Dunnett's multiple comparison test) (Fig. 5B).

3.5. Passive avoidance task in aged rats

Aged rats (26–27 months old) showed significantly ($P<0.001$ by Wilcoxon ranking test) shorter latency compared

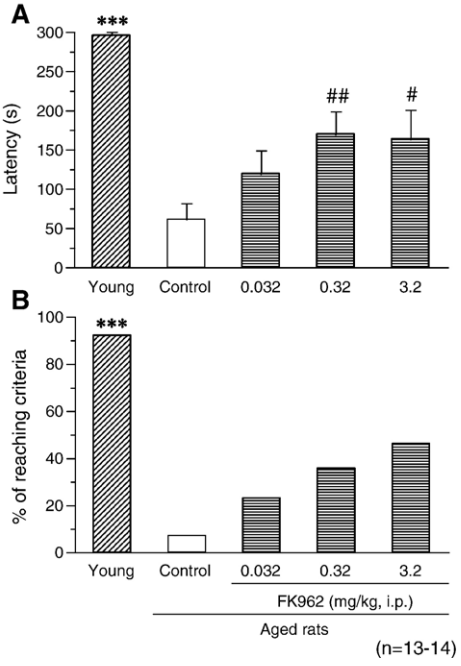

Fig. 6. Effects of FK962 on the memory deficits in aged rats in passive avoidance task. (A) Retention latencies. Ordinate represents the mean retention latencies in a 24-h retention test. $^{\#}P<0.05$, $^{\#\#}P<0.01$; statistically significant compared with vehicle controls (open columns) (by Kruskal–Wallis test followed by Dunnett's multiple comparison test). $^{***}P<0.001$; statistically significant compared with vehicle controls (open columns) (by Wilcoxon ranking test). Each column and bar represents the mean±S.E.M. (B) Percent of rats reaching criteria. Ordinate represents the percent of rats reaching criteria (300 s) in a 24-h retention test. $^{***}P<0.001$; statistically significant compared with vehicle controls (open columns) (by Fisher's exact probability test). Numbers of rats are shown in parentheses. FK962 was administered i.p. immediately after the acquisition trial.

with the young control (11 weeks old) animals (Fig. 6A). FK962 at 0.032 to 3.2 mg/kg prolonged the latency in the aged rats (Fig. 6A). The maximal and statistically significant effect was obtained at 0.32 mg/kg ($P<0.01$ by Kruskal–Wallis test followed by Dunnett's multiple comparison test). The percent of rats reaching criteria paralleled the latency. The percent of rats reaching criteria was significantly reduced in the aged rats ($P<0.001$ by Fisher's exact probability test) (Fig. 6B). FK962 at 0.032 to 3.2 mg/kg increased the percent of rats reaching criteria, although not significantly.

3.6. Water maze task in nucleus basalis magnocellularis-lesioned rats

Fig. 7 represents the results on the effects of FK962 on the spatial memory deficits in nucleus basalis magnocellularis-lesioned rats in the Morris water maze task. In sham rats, the daily latency in finding the platform declined progressively during the training period of 4 consecutive days (Fig. 7A).

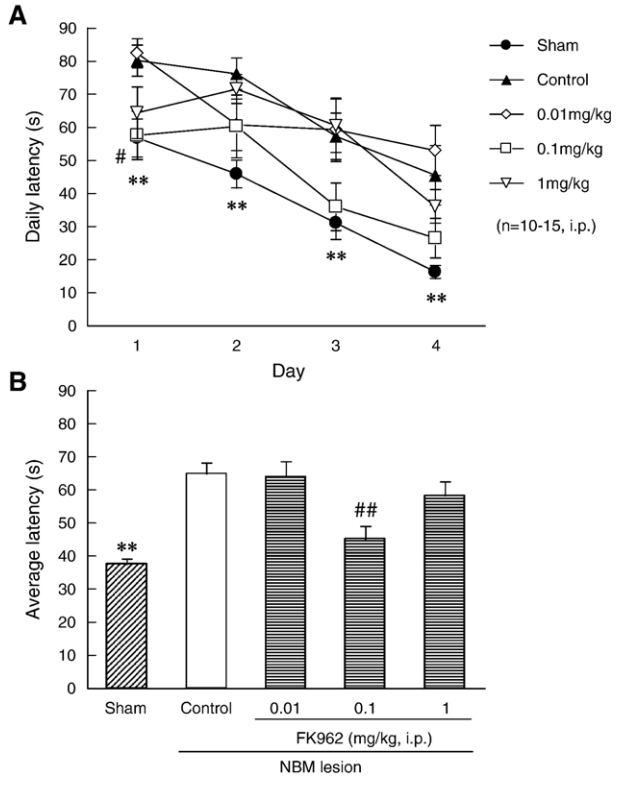

Fig. 7. Effects of FK962 on spatial memory deficits in nucleus basalis magnocellularis-lesioned rats in the Morris water maze task. (A) Daily latencies. Each point and bar represents the mean±S.E.M. $^{\#}P<0.05$; statistically significant compared with nucleus basalis magnocellularis-lesioned control (closed triangle) (by ANOVA followed by Dunnett's multiple comparison test). $^{**}P<0.01$; statistically significant compared with nucleus basalis magnocellularis-lesioned control (by Student's *t*-test). (B) Average latencies. Each column and bar represents the mean±S.E.M. $^{\#\#}P<0.01$; statistically significant compared with nucleus basalis magnocellularis-lesioned control (open column) (by ANOVA followed by Dunnett's multiple comparison test). $^{**}P<0.01$; statistically significant compared with nucleus basalis magnocellularis-lesioned control (by Student's *t*-test). Numbers of rats are shown in parentheses. FK962 was administered i.p. 30 min before the first trial on each day. NBM: nucleus basalis magnocellularis.

The daily latency was prolonged by nucleus basalis magnocellularis-lesioning, and the differences in the daily latency between the sham rats and control rats were statistically significant at all 4 testing days ($P<0.01$ by Student's *t*-test). FK962 at 0.01 to 1 mg/kg shortened the daily latency in nucleus basalis magnocellularis-lesioned rats, with 0.1 mg/kg inducing a statistically significant improvement on testing day 1 ($P<0.05$ by Dunnett's multiple comparison test). When the performances of the animals of each group were averaged for 4 days, the average latency paralleled the daily latency as shown in Fig. 7B. The difference in the average latency between the sham rats and control rats were statistically significant ($P<0.01$ by Student's *t*-test). Daily treatment with FK962 (0.01 to 1 mg/kg) attenuated the average latency increased by nucleus basalis magnocellularis-lesioning, with statistical significance at 0.1 mg/kg ($P<0.01$ by Dunnett's multiple comparison test), but did not change swimming speed (data not shown).

4. Discussion

The present study showed that FK962 increased high K^+-evoked somatostatin release from rat hippocampal slices without affecting basal release, with a bell-shaped dose–response relationship. The results clearly suggest that FK962 exerts facilitatory actions on the mechanism required for the depolarization-dependent release of somatostatin from nerve terminals in the hippocampus. Somatostatin is released by depolarization with KCl or veratridine from hippocampal slices in a concentration-dependent manner and lowering Na^+ concentration evokes somatostatin release independent of the Ca^{2+} concentration (Bonanno et al., 1991; Vezzani et al., 1992). Thus, modulation of Ca^{2+} entry through ion channels might underlie the selective enhancement by FK962 on depolarization-dependent but not basal release of somatostatin.

Five somatostatin receptor subtypes have been cloned and designated somatostatin receptors 1–5, and transcripts for all five have been detected in rat hippocampus (Thoss et al., 1995). It is reported that presynaptic somatostatin receptor, which closely resembles the cloned somatostatin receptor-2, inhibits the release of excitatory transmitters through at least two separate mechanisms in rat hippocampal synapses, the modulation of Ca^{2+} channels and the inhibition of downstream Ca^{2+} entry (Akopian et al., 2000; Boehm and Betz, 1997). In hippocampal neurons, somatostatin inhibits predominately N-type voltage-activated Ca^{2+} channels in a membrane-delimited pathway, via direct G protein/channel interaction (Ikeda and Schofield, 1989; Wang et al., 1990; Hille, 1994; Ishibashi and Akaike, 1995; Viana and Hille, 1996). We have previously demonstrated that somatostatin reduced Ca^{2+} currents associated with kinetic changes in channel activation in hippocampal neurons (Wang et al., 2004). Thus, presynaptic somatostatin receptors are postulated to regulate their own release of somatostatin via the modulation of Ca^{2+} channels. In the present study, FK962 reduced somatostatin-induced inhibition of Ca^{2+} currents. Since inhibition of voltage-activated Ca^{2+} channels appears to be the mechanism underlying presynaptic inhibition on

somatostatin release, the present results suggest that FK962 may modulate the interaction of somatostatin autoreceptors and presynaptic (N-type Ca^{2+}) channels, leading to facilitation of somatostatin release.

Somatostatin is known to play a vital role in modulating mnemonic functions. Matsuoka et al. (1991a) demonstrated that somatostatin augments long-term potentiation, which is considered to be an elementary process of learning and memory (Teyler and Discenna, 1984; Morris et al., 1986), of the mossy fiber-CA3 system in guinea pig hippocampal slices via cholinergic neurons, suggesting that somatostatin stimulates cholinergic mechanisms in the production of long-term potentiation. Morphological and functional evidence indicates a bidirectional neuronal communication between cholinergic and somatostatinergic neurons in the mammalian hippocampus and cortex (Epelbaum et al., 1986). In the rat hippocampus, cholinergic terminals make synaptic contacts on somatostatin-immunoreactive cells (Léránth and Frotscher, 1987). Somatostatin selectively enhances acetylcholine-induced firing of CA1 and CA3 neurons in rat hippocampal and cortical neurons (Mancillas et al., 1986). In addition, neurochemical studies reveal the intraventricular injection of somatostatin increases the acetylcholine turn over rate in hippocampus of rats (Marthe-Sørenssen et al., 1978; Wood et al., 1979) and somatostatin enhances the K^+-evoked release of endogenous acetylcholine from rat hippocampal slices presynaptically (Araujo et al., 1990). Thus, the interaction between somatostatinergic and cholinergic neurons in hippocampus is postulated to be involved in the memory function. Intracerebral or intrahippocampal injections of somatostatin improve memory deficits produced by cholinergic hypofunction in rats (Matsuoka et al., 1994; Ohno et al., 1994). On the other hand, depletion of somatostatin by cysteamine impairs memory formation in the passive avoidance task and water maze task (Fitzgerald and Dokla, 1989; Matsuoka et al., 1994, 1995).

In the present study, FK962 significantly improved memory impairments in scopolamine-treated rats and in nucleus basalis magnocellularis-lesioned rats in passive avoidance task, suggesting that FK962 is a potent cognitive enhancer. FK962 was shown to be efficacious on the memory deficits produced by nucleus basalis magnocellularis-lesion not only in the passive avoidance task that accesses long-term memory but also in the water maze task, which requires the integrity of mnemonic function of more complexed and spatial component, further strengthening the view that FK962 could be potential anti-dementia drug. FK962 penetrates the blood–brain barrier very well. Our preliminary pharmacokinetic data shows that the concentration in rat brain 30 min after FK962 was administered at 0.1 mg/kg; the dose that improved the memory deficits in nucleus basalis magnocellularis-lesioned rats in water maze tasks was approximately 160 nM (unpublished data). The brain concentration of FK962 was comparable with the concentration, which enhanced the somatostatin release from rat hippocampal slices at 10^{-7} M (Fig. 2), suggesting that an activation of somatostatinergic system could be involved in the anti-amnesic actions of FK962. FK960, the predecessor of F962, was previously demonstrated to ameliorate the memory deficits in scopolamine-treated rats, nucleus basalis magnocellularis-lesioned rats and aged rats in passive avoidance task and in nucleus basalis magnocellularis-lesioned rats in water maze task and the maximal efficacy was observed with 1–3.2 mg/kg (Yamazaki et al., 1996), while FK962 also ameliorated the memory deficits in those models at 0.1–0.32 mg/kg in the present study, suggesting that FK962 is more potent than FK960. FK962 also blocked somatostatin-induced inhibition of Ca^{2+} channel current in hippocampal neuron at lower concentrations compared to FK960 (Wang et al., 2004) (10^{-8} M and 10^{-7} M, respectively). The potency of FK962 and FK960 in animal models might parallel with their potencies in modulating the inhibitory effect of somatostatin in vitro.

Another important finding of the present study was that FK962 ameliorated impaired memory in aged rats. We have previously demonstrated that physostigmine and donepezil, both cholinesterase inhibitors, failed to ameliorate memory deficits in aged rats in a passive avoidance task (Matsuoka et al., 1992; Tokita et al., 2002). The lack of efficacy of cholinesterase inhibitors on memory deficits associated with aging suggest that presynaptic cholinergic hypofunction might be partly involved in the memory deficits seen in aged rats. A number of studies on aged rats have observed age-related changes in the brain aminergic system, as well as neuropeptides, that may play a role in memory disturbance in aged animals (Kubanis and Zornetzer, 1981; Ponzio et al., 1982; Luine et al., 1990; Zhang et al., 1991; Wang et al., 1993). Although further studies are needed, we suggest that FK962 ameliorates the memory deficits produced by brain cholinergic hypofunction through indirect stimulation of cholinergic neurons via activation of hippocampal somatostatinergic neurotransmission. FK962 may exert the memory-improving effects via the different mechanisms than existing cholinesterase inhibitors on the memory deficits caused not only by the cholinergic hypofunction but also by other physiological changes as seen in aged rats.

In conclusion, FK962 improved the memory impairment in rat models examined here through a possible potentiation of hippocampal somatostatinergic neurotransmission. The pharmacological profile of FK962 provides a potential alternative to cholinesterase inhibitors, which have proven to be less than optimal due to their possible side-effects of peripheral cholinergic stimulation. These results taken together strengthen the view that FK962 might be of therapeutic value against dementing disorders such as Alzheimer's disease or senile dementia of the Alzheimer's type.

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