A Screening Concept Based on a Hypothesis Led to the Development of a Putative Cognitive Enhancer That Stimulates Penile Erection

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ABSTRACT—Starting from the hypothesis that drugs which specifically activate the hippocampal cholinergic nerve activity may ameliorate memory impairments, we carried out a series of evaluations for a novel cognitive enhancer using enhancement of penile erection as a sign of cholinergic activation, and found FR64822. The compound facilitated penile erection in naive rats, and it ameliorated scopolamine-induced amnesia of rats in passive avoidance tasks with bell-shaped dose-response curves, while it dose-dependently reduced body weight gain in Zucker fatty rats. Pretreatment with sulpiride (32 mg/kg, p.o.) hardly affected the former two activities, but significantly reduced the anorectic activity in Zucker rats. Further evaluation of FR64822 derivatives characterized a second compound, FR121196, which induces penile erection and memory enhancement, but not body weight reduction. Memory enhancing and erection stimulating activities of FR121196 were abolished in rats treated with either cysteamine (200 mg/kg, s.c.), a somatostatin depletor, or lesioning of the serotonergic raphe nuclei. Thus, classic whole animal studies based on a hypothesis proved to be efficient for reaching our objective, the discovery of a new drug. They also gave us insight into the common somatostatinergic and serotonergic mechanisms underlying penile erection and memory improvement.

Keywords: FR121196, Memory, Penile erection, Hippocampus, Somatostatin

It could be interpreted that the discovery of H_2-receptor antagonists (1) is based upon two hypotheses: that gastric acid causes ulcerations (no acid-no ulcer) and that histamine is a final common mediator of gastric secretion (2). Widely known at that time but still to be established, these hypotheses were substantiated by the discovery of H_2-antagonists. Thus began a new era of drug discovery, which until then totally depended on accidental findings. Hypotheses are no doubt strong weapons for the discovery of novel drugs.

It has been widely accepted that the hippocampal cholinergic nerves are degenerated in patients with Alzheimer's disease (AD) and senile dementia of the Alzheimer's type (SDAT), and the degree of the change parallels that of the dementing disorder (3, 4). Clinical evidence as well as experimental observations in animals support the hypothesis that the hippocampal cholinergic nerve activity plays a crucial role in memory formation (5-7). Thus we speculate that drugs which selectively activate the hippocampal cholinergic nerves may relieve the symptom in such patients.

The above-mentioned hypothesis led us to carry out a series of evaluations for a novel cognitive enhancer that resulted in the discovery of FR121196, a cognitive enhancer that also possesses stimulating activity on penile erection. In the present paper, we characterize both the methods of discovery and properties of this novel putative cognitive enhancer.

MATERIALS AND METHODS

Animals

Eight-week-old male Wistar strain rats (body weight of 250-320 g) were employed in the passive avoidance task. In the penile erection studies, 10-week-old male Fischer-344 strain rats (200-250 g) were used. All rats were obtained from Charles River Japan, Inc. (Atsugi). Effects of drugs on the body weight were investigated using 7-week-old male Zucker obese (fa/fa) and lean (Fa/-) rats obtained from Charles River, Inc. (Boston, MA, USA). All the animals were purchased at least one week before the experiments, housed five animals to a stainless mesh cage, and given food and water ad libitum in a temperature-controlled environment (22±1℃) under a 12:12 hr light-

/dark cycle with lights on at 8:00 a.m.

Dorsal- and median-raphe lesion

The animals were anesthetized with sodium pentobarbital (50 mg/kg, i.p.) and fixed on a stereotaxic apparatus with the incisor bar set 2.3 mm below the intra-aural line. In all lesions, stereotaxic coordinates were referred to the atlas of König and Klippel (8).

Specific lesions of serotonergic neurons were made by stereotaxically placed local injections of 5,7-dihydroxytryptamine (5,7-DHT, in 1 μl of 0.1% ascorbic acid-saline solution, 0.2 μl/min) through a 26-gauge cannula connected by polyethylene tubing to a gear-driven Hamilton syringe. Sham animals were injected with the vehicle. Injections into the median raphe nucleus were made at the coordinates 8.3 mm posterior to the bregma, on the midline, and 8.0 mm ventral from the skull surface; injections into the dorsal raphe nucleus were made at the coordinates 8.7 mm posterior to the bregma, on the midline, 6.3 mm ventral from the skull surface. Both nuclei were injected with 2 μg of 5,7-DHT in a volume of 1 μl, and the rats were pretreated with desipramine (25 mg/kg, i.p.) at 30–45 min prior to the local injections in order to protect noradrenergic neurons from the lesion (9). The behavioral test was started 14 days after the surgery.

Passive avoidance

The apparatus and experimental procedure employed in the passive avoidance task were similar to those described previously (10). In brief, a two-compartment step-through passive avoidance apparatus made of black perspex was used. The apparatus consisted of an illuminated cylindrical compartment (50-cm diameter, height of 42 cm) that was attached to a dark compartment (15 × 18, height of 42 cm) equipped with a grid floor. These compartments were separated by a guillotine door. The rat was placed in the illuminated compartment; and 10 sec thereafter, the door was raised. After entering the dark compartment, the rat was returned to its home cage (habituation trial). Rats were given either an s.c.-injection of cysteamine or i.p.-injection of scopolamine 30 min after the habituation trial. Sixty minutes after the habituation trial, the rat was again placed in the illuminated chamber (acquisition trial). When the rat entered the dark chamber, the guillotine door was closed and 4-mA scrambled electrical foot-shocks were delivered for 3 sec through the grid floor using a shock generator/scrambler (Muromachi Kikai, Tokyo; model SGS-001). In the test trial that was given 24 hr after the acquisition trial, the rat was placed again in the illuminated compartment, and the response latency to enter the dark compartment was measured to a maximum of 300 sec (retention trial). The drug was administered i.p. immediately after the acquisition trial. The results were recorded as the average of latency for each group of rats.

Penile erection

The experiments were carried out between 13:30 and 19:00 in the same room as where the rats were housed. All animals were handled 3 min a day for 3 successive days before the behavioral tests. The rats were tested in groups of six and various doses of drugs were given in semi-randomized order. Immediately after the drug injection, each rat was placed in a perspex box (27 × 17 × 13 cm) and its behavior was observed for 60 min, during which time the number of penile erections was counted. A mirror was situated behind each box to facilitate observation of the animal. Antagonists were administered 15 min before the behavioral observation.

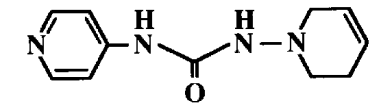
Lesioned and sham-operated rats were allocated so as to receive all doses throughout a series of test sessions. Briefly, each group of six rats was administered with vehicle or doses of a drug in the first session. In the next session, each of them received a dose of the drug in ascending order, and the rats receiving the largest dose were given the vehicle. In this way, the test sessions were repeated the same number of times as the total number of vehicle and drug doses. At least a 3-day intervening interval was provided between each session, and a series of tests was finished within 4 weeks at the longest.

Effects of drugs on body weight and food intake in obese and lean rats

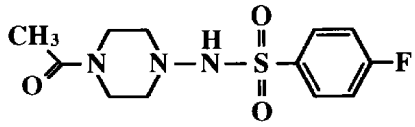
Effect of drugs on body weight were assessed in Zucker fatty and lean rats. Purchased rats were allocated to 4 groups each in a randomized fashion. Each group of rats was treated orally with vehicle or a dose of drugs. The treatment was started on Thursday and performed daily at 17:00, except on Saturdays and Sundays. Body weight was determined at the same time. Effects of the antagonists on anorexia induced by FR64822 were assessed in obese Zucker rats fasted for 16 hr. Thirty minutes after the antagonist, the rats were given FR64822 or the vehicle, transferred to individual cages, and observed for food intake for the subsequent 2 hr.

Drugs

FR64822 (*N*-[4-pyridylcarbamoyl]amino 1,2,3,6-tetrahydropyridine) and FR121196 (*N*-[4-acetyl-1-piperazinyl]-4-fluorobenzensulfonamide) (Fig. 1) were synthesized in the Exploratory Research Laboratory of Fujisawa Pharmaceutical Co., Ltd. and suspended in 0.5% methylcellulose. Scopolamine hydrobromide was purchased from Nacalai Tesque (Kyoto) and dissolved in saline. Cysteamine hydrochloride (2-mercaptoethylamine) was purchased from Sigma Chemical Co. (St. Louis, MO,



FR64822



FR121196

Fig. 1. Chemical structure of FR64822 and FR121196.

USA) and dissolved in saline with pH adjusted to 7.0. It was administered in a volume of 2 ml/kg at 30 min before the acquisition trial of passive avoidance or 2 hr before the penile erection experiment. Other drugs were administered in a volume of 1 ml/kg.

Statistics

All results were expressed as the mean±S.E.M. In behavioral experiments, the statistical significance between groups was calculated by non-parametric analysis of the Mann-Whitney *U*-test (two-tailed) for the retention latency and the number of penile erections. Statistical significance of changes in body weight was calculated by one way ANOVA followed by Student's *t*-test (two-tailed).

RESULTS

Effects of FR64822 on scopolamine-induced passive avoidance deficits, penile erection and body weight gain in rats

As shown in Fig. 2A, the latency in entering the dark compartment decreased significantly ($P<0.001$) in the scopolamine-treated rats compared with the control (284.0±11.0 vs. 102.9±34.1 sec). The shortened latencies that represent impaired memory in scopolamine-treated rats were attenuated by graded doses (0.1–10 mg/kg) of FR64822, with significance obtained at 3.2 mg/kg; the dose-response curve was bell-shaped.

A spontaneous self grooming behavior, penile erection, was observed in naive rats (0.33±0.21/hr); and this was

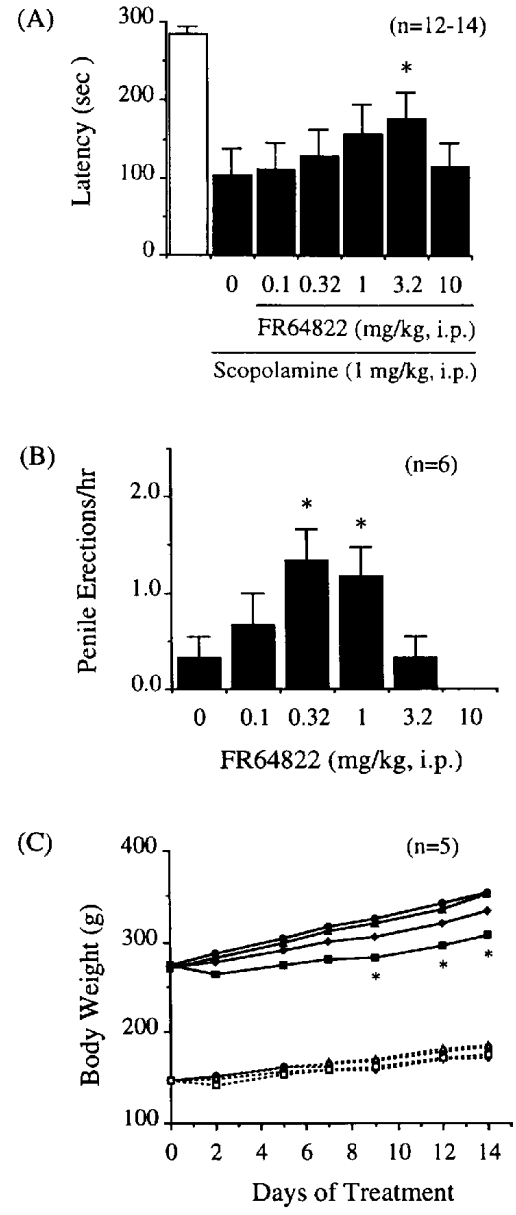


Fig. 2. Effect of FR64822 on scopolamine-induced memory impairment (A), penile erection (B) and body weight gain in obese rats (C). Each experimental procedure was conducted as described in Methods. The number of rats tested are given in parentheses. (A): FR64822 was given i.p. to the scopolamine-treated rats immediately after the acquisition trial. The ordinate represents the median retention latencies in the 24-hr retention test for rats. Each column and bar represents the mean±S.E.M. Scopolamine (1 mg/kg, i.p.) was administered 30 min before the acquisition trial. Open and closed columns represent the mean latencies of the saline-vehicle- and scopolamine-drug-treated groups of rats, respectively. *$P<0.05$, statistically significant compared with the vehicle control (scopolamine alone treated) group (by the Mann-Whitney *U*-test). (B): The ordinate represents the mean number of penile erections that were observed in 1 hr following i.p. administration of FR64822 or vehicle. Each column and bar represents the mean±S.E.M. *$P<0.05$, statistically significant compared with the vehicle-treated group (by the Mann-Whitney *U*-test). (C): Each group of rats was daily treated orally with vehicle (●○ 0.5% methylcellulose) or a dose of FR64822 (▲△ 1 mg/kg, ◆◇ 10 mg/kg, ■□ 100 mg/kg). The ordinates represent the mean value of body weights in obese Zucker (closed symbol) and lean (open symbol) rats. Asterisks represent a statistically significant difference (*$P<0.05$ by Student's *t*-test) from the vehicle-treated control.

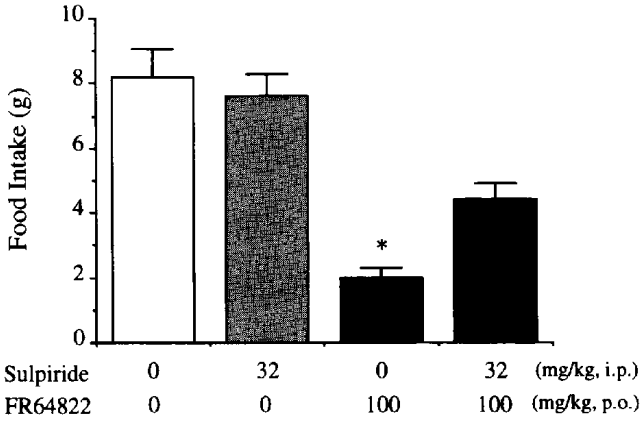


Fig. 3. Effects of sulpiride pretreatment on anorexia induced by FR64822. The ordinate represents the mean value of food intake for 1 hr in 16-hr-food-deprived obese Zucker rats. Each column and bar represents the mean ± S.E.M. *$P < 0.05$, statistically significant compared with the vehicle-treated control (by Student's *t*-test).

enhanced by FR64822, with significance obtained at 0.32 and 1 mg/kg (Fig. 2B). The dose-response curve was bell-shaped.

Daily treatment with FR64822 for 2 weeks dose-dependently reduced body weight gain in male Zucker obese rats while minimally affecting that in lean rats (Fig. 2C).

Effects of sulpiride pretreatment on anorexia induced by FR64822 in Zucker rats

As shown in Fig. 3, oral administration of FR64822 (100 mg/kg) significantly reduced food intake in fasted Zucker rats compared with rats given the vehicle. Sulpiride pretreatment (32 mg/kg, i.p.) hardly affected the food intake, but countered the anorectic effect of FR64822. On the other hand, sulpiride pretreatment did not attenuate the activities of the drug on penile erection or memory in the passive avoidance tests (data not shown).

Effects of FR121196 on scopolamine-induced passive avoidance deficits, penile erection and body weight gain in rats

The penile erection in naive rats was enhanced by FR121196, with significance obtained at 1 – 10 mg/kg (Fig. 4B). In addition, the shortened latency of the scopolamine-pretreated rats in the passive avoidance tests was attenuated by graded doses (0.1 – 10 mg/kg, i.p.) of FR121196, with statistically significance obtained at 3.2 mg/kg (Fig. 4A). The two dose-response curves were almost identical, each being bell-shaped with a maximal response at 3.2 mg/kg.

Daily oral administration of FR121196 (1 – 100 mg/kg) hardly affected body weight gain in obese Zucker rats as well as in lean rats (data not shown).

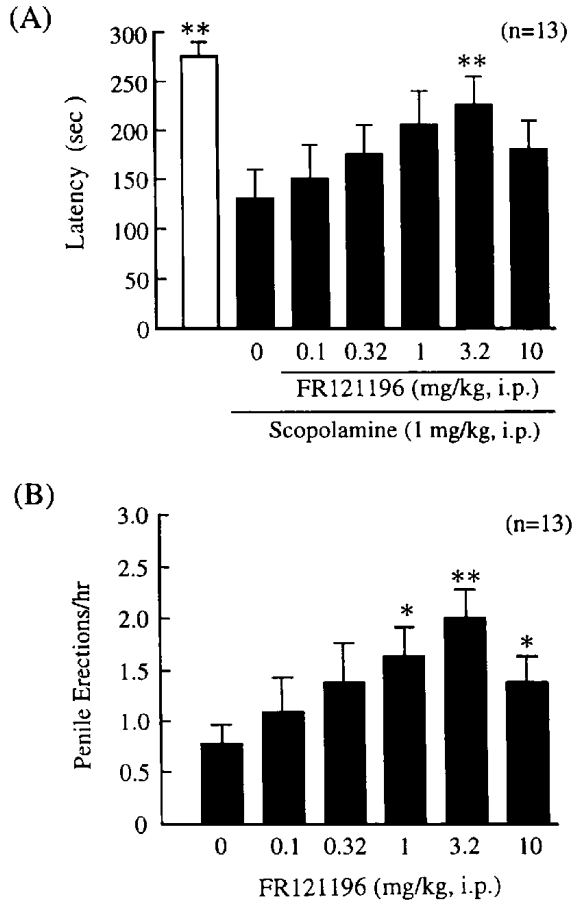


Fig. 4. Effects of FR121196 on scopolamine-induced memory impairments (A) and penile erection (B). Experimental procedures were the same as in Fig. 2. FR121196 was suspended in 0.5% methylcellulose and given i.p. The number of rats tested are given in parentheses. (A): The ordinate represents the median retention latencies in the 24-hr retention test. Each column and bar represents the mean ± S.E.M. **$P < 0.01$, statistically significant compared with the vehicle control (scopolamine alone-treated) group (by the Mann-Whitney *U*-test). (B): The ordinate represents the mean number of penile erections that were observed in 1 hr. Each column and bar represents the mean ± S.E.M. *$P < 0.05$, **$P < 0.01$, statistically significant compared with the vehicle-treated control (by the Mann-Whitney *U*-test).

Effects of FR121196 on memory impairment and penile erection in cysteamine-pretreated rats

As shown in Fig. 5A, the cysteamine-treated rats showed a shortened latency in our memory test, which resembled that in scopolamine-treated rats (Figs. 2A and 4A). However, the shortened latency in the cysteamine-treated rats was not significantly ameliorated by FR121196 (0.1 – 10 mg/kg).

In addition, the same doses of FR121196 did not enhance penile erection in the cysteamine-treated rats (Fig. 5B).

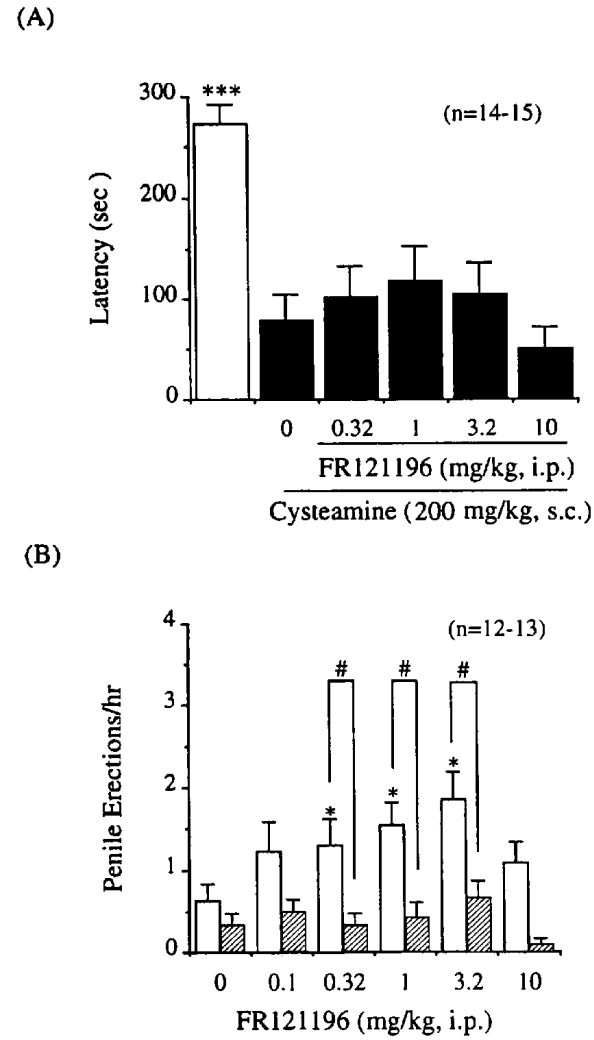


Fig. 5. Effect of FR121196 on memory impairment (A) and penile erection (B) in cysteamine-pretreated rats. The number of rats tested are given in parentheses. (A): Experimental procedures were the same as in Fig. 2, except that memory impairment was induced by cysteamine instead of scopolamine. Cysteamine was dissolved in saline with the pH adjusted to 7.0 and injected s.c. 30 min before the acquisition trial. The ordinate represents the median retention latencies in the 24-hr retention test. Each column and bar represents the mean±S.E.M. ***$P<0.001$, statistically significant compared with the vehicle control (cysteamine alone-treated) group (by the Mann-Whitney *U*-test). (B): Experimental procedures were the same as in Fig. 2, except that the rats were pretreated with cysteamine 120 min before the test. The ordinate represents the mean number of penile erections that were observed in 1 hr. Each column and bar represents the mean±S.E.M. *$P<0.05$, statistically significant compared with the vehicle-treated group in saline-pretreated rats. #$P<0.05$, compared with each corresponding dosed group of saline- and cysteamine-pretreated rats (by the Mann-Whitney *U*-test). □ Saline, ▨ Cysteamine.

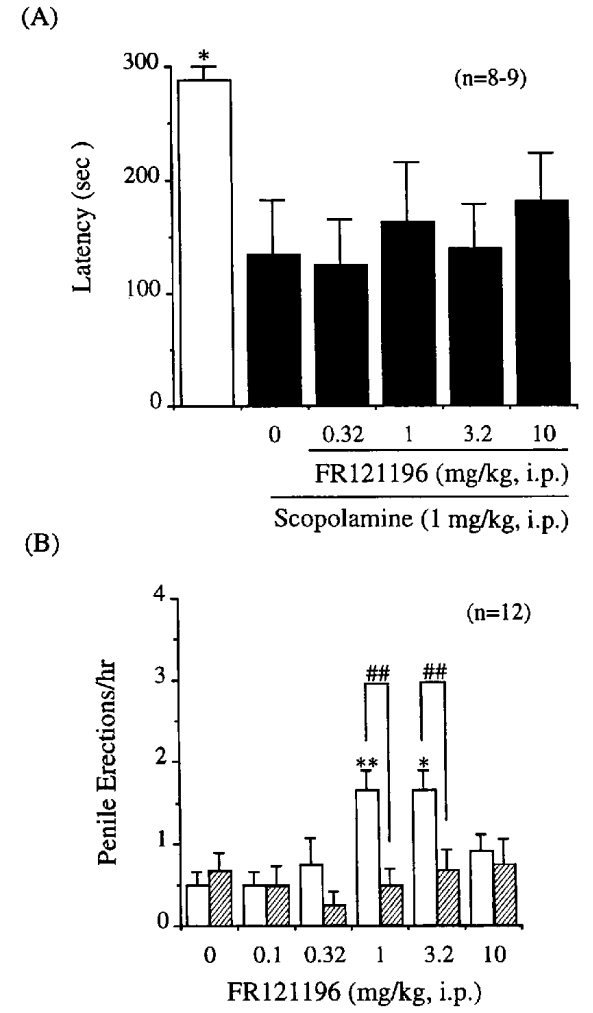


Fig. 6. Effects of FR121196 on scopolamine-induced memory impairments (A) and penile erection (B) in raphe-lesioned rats. Neurotoxic lesion of median- and dorsal-raphe nuclei was performed 2 weeks before the behavioral experiments as described in Methods. The number of rats tested are given in parentheses. Experimental procedures were the same as in Fig. 2. (A): The ordinate represents the median retention latencies in the 24-hr retention test. Each column and bar represents the mean±S.E.M. *$P<0.05$, statistically significant compared with the vehicle control (scopolamine alone-treated) group (by the Mann-Whitney's *U*-test). (B): The ordinate represents the mean number of penile erections that were observed in 1 hr. Each column and bar represents the mean±S.E.M. *$P<0.05$, **$P<0.01$, statistically significant compared with the vehicle-treated group of sham-operated rats. ##$P<0.01$, compared with the corresponding dosed group of sham-operated and lesioned rats (by the Mann-Whitney's *U*-test). □ Sham, ▨ Raphe lesion.

Effects of FR121196 on scopolamine-induced passive avoidance deficits and penile erection in raphe-lesioned rats

Figure 6A illustrates the effects of FR121196 on scopolamine-induced passive avoidance deficit in raphe-lesioned rats. Median- and dorsal-raphe lesioning itself had little effect on acquisition of avoidance response (data not shown). In the raphe-lesioned rats, scopolamine pretreatment significantly reduced memory retention, but

FR121196 (0.32–10 mg/kg) hardly countered the amnesic action of scopolamine. As shown in Fig. 6B, raphe-lesioning also canceled the erectile response induced by FR121196.

DISCUSSION

There are two major cholinergic systems within the basal forebrain: one derives from the nucleus basalis of Magnocellulalis and mainly innervates the neocortex, and the other originates from the medial-septum/diagonal band of Broca and projects primarily to the hippocampus via the fimbria-fornix complex (11, 12). These cholinergic nerves are degenerated in patients with dementing disorders such as AD and SDAT (13, 14), and the degree of change parallels that of the disorder. These observations consist of the hypothesis that the etiology of dementia involves cholinergic nerve dysfunction, and it is supported by the fact that their activation by cholinergic drugs relieve the symptom in the patients (15–17). While there have been continuing efforts to find a brain-specific cholinergic stimulant, we took a different strategy on our attempt to find a specific activator of the hippocampal cholinergic nerves. We have previously shown that amantadine and apomorphine, which activate the central dopaminergic system, indirectly activate the septohippocampal cholinergic pathway to induce penile erections in naive rats (18). Thus we studied the effects of amantadine and apomorphine on the scopolamine-induced amnesia of rats in passive avoidance tasks, and we found that they effectively ameliorated the amnesia (unpublished observation). The results justified our strategy for finding a novel cognitive enhancer among drugs inducing penile erections. However, the animals treated with amantadine and apomorphine showed behavioral excitation due to dopaminergic stimulation, which was enough to make us to give up further evaluation along these lines.

A further search for less toxic and more selective compounds using enhancement of penile erection as the first screen led us to the serendipitous discovery of our compound FR64822, which exhibits an antinociceptive activity through a novel mechanism of action, indirect stimulation of dopamine D_2-receptors (19). In addition to the penile erection, FR64822 ameliorated scopolamine-induced passive avoidance deficits and suppressed body weight gain in obese rats (Fig. 2). This unique combination of actions led us to suppose that FR64822 may interact with some other endogenous substance that has erectile response stimulating, memory improving and body weight reducing activities. In support of this assumption, several lines of evidence suggest that a number of endogenous substances such as acetylcholine, serotonin, vasopressin and adrenocorticotropin play an important role in the control of memory formation (20, 21) as well as self grooming behaviors (18, 22–24). It has also been reported that some of these substances may be involved in the control of appetite (25, 26).

The present study demonstrated that sulpiride treatment attenuated the anorectic but not erection stimulating or memory enhancing activities of FR64822. The results suggest that the anorectic activity could be ascribed to its dopamine D_2-receptor stimulation, and could be differentiated from the other two activities. Thus we further evaluated many compounds in search for a novel cognitive enhancer devoid of anorectic activity, and we successfully characterized the novel cognitive enhancer FR121196. The present study demonstrated that FR121196 induces memory enhancement and penile erection in normal rats but not in those treated with cysteamine, which is reportedly a specific depletor of somatostatin (27, 28) as well as in rats with selectively degenerated limbic serotonergic nerves produced by lesioning of the median- and dorsal-raphe nuclei. These results suggest that both memory enhancing and erection stimulating activities of FR121196 are mediated by activation of the hippocampal somatostatinergic and serotonergic nerves. Good correlation of dose-response curves of FR121196 in memory enhancement and penile erection further supports its selective action on a common mechanism between the two. Discussion on the detailed mechanism of action is out of the scope of the present paper, and will be described elsewhere (29). Interestingly, somatostatin involvement in cognitive dysfunction has been postulated in post-mortem analysis of brains of individuals with Alzheimer's disease that revealed a reduction of cortical and hippocampal somatostatin-like immunoreactivity (30, 31), and positive regulation of raphe serotonergic nerves on the septo-hippocampal cholinergic nerves in rodent's memory has also been suggested (32, 33).

In conclusion, whole animal observations based on a hypothesis and serendipity led to our discovery of a novel drug that has a wide range of activity as a putative cognitive enhancer (10). In addition, endogenous somatostatinergic and serotonergic mechanisms are suggested to play important roles in memory formation and sexual behavior.

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