Enhancement of learning behaviour by a potent nitric oxide-guanylate cyclase activator YC-1

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Abstract

Memory is one of the most fundamental mental processes, and various approaches have been used to understand the mechanisms underlying this process. Nitric oxide (NO), cGMP and protein kinase G (PKG) are involved in the modulation of synaptic plasticity in various brain regions. YC-1, which is a benzylindazole derivative, greatly potentiated the response of soluble guanylate cyclase to NO (up to several hundreds fold). We have previously shown that YC-1 markedly enhances long-term potentiation in hippocampal and amygdala slices via NO-cGMP-PKG-dependent pathway. We here further investigated whether YC-1 promotes learning behaviour in Morris water maze and avoidance tests. It was found that YC-1 shortened the escape latency in the task of water maze, increased and decreased the retention scores in passive and active avoidance task, respectively. Administration of YC-1 30 min after foot-shock stimulation did not significantly affect retention scores in response to passive avoidance test. Administration of scopolamine, a muscarinic antagonist, markedly impaired the memory acquisition. Pretreatment of YC-1 inhibited the scopolamine-induced learning deficit. The enhancement of learning behaviour by YC-1 was antagonized by intracerebroventricular injection of NOS inhibitor L-NAME and PKG inhibitors of KT5823 and Rp-8-Br-PET-cGMPs, indicating that NO-cGMP-PKG pathway is also involved in the learning enhancement action of YC-1. YC-1 is thus a good drug candidate for the improvement of learning and memory.

Introduction

Learning and memory is one of the most intensively studied subjects in neuroscience. Various approaches have been used to understand the underlying cellular and molecular mechanisms (Abel & Lattal, 2001; Schafe *et al.*, 2001). Long-term potentiation (LTP) has been identified as a potential synaptic mechanism in several regions of the brain involved in learning and memory. Schaffer collateral inputs to pyramidal neurons in the hippocampus CA1 region exhibit a form of LTP that critically depends on *N*-methyl-D-aspartate (NMDA) receptor-mediated Ca^{2+} influx into the postsynaptic cell (Parker *et al.*, 1993; Tsien *et al.*, 1996). Nitric oxide (NO) is widely used in neural circuits giving rise to learning and memory. NO is an unusual neurotransmitter in its modes of release and action. Nitric oxide participates in several types of synaptic plasticity, including long-term depression (LTD) in the cerebellum and striatum and LTP in the hippocampus and cerebral cortex (Daniel *et al.*, 1998; Hawkins *et al.*, 1998; Prast & Philippu, 2001; Bon & Garthwaite, 2003). It has been suggested that NO, generated postsynaptically by Ca^{2+} -calmodulin-dependent NO synthase (NOS), acts as a retrograde messenger (Son *et al.*, 1996; Wilson *et al.*, 1997). A major target of NO is soluble guanylate cyclase (sGC), which generates the intracellular second messenger cGMP. Consistent with a functional role of cGMP in the expression of LTP, sGC inhibitors suppress LTP (Zhuo *et al.*, 1994; Boulton *et al.*, 1995) and membrane-permeable dibutyryl-cGMP

partially reverses the reduction of LTP by an NOS inhibitor (Haley *et al.*, 1992). It is well known that cGMP regulates the activity of diverse proteins, including protein kinase G (PKG). The critical role of PKG was suggested by the finding that PKG inhibitors block LTP, and PKG activators facilitate LTP in response to rather weak tetanic stimuli (Zhuo *et al.*, 1994).

A novel synthetic compound YC-1 [3-(5-hydroxymethyl-2-furyl)-1-benzyl-indazole] has been shown to activate purified sGC and sensitizes the enzyme for NO *in vitro*, in human platelets, and in smooth muscle cells (Ko *et al.*, 1994; Mulsch *et al.*, 1997; Friebe *et al.*, 1998). YC-1 produced an enormous stimulation of the sensitivity of the purified enzyme to NO up to several hundredfold (Friebe *et al.*, 1996). We previously found that YC-1 enhanced LTP in hippocampal Schaffer collateral–CA1 synapse via a NO-cGMP-PKG-dependent pathway (Chien *et al.*, 2003). In addition, YC-1 also potentiated LTP induction in the amygdala (Chien *et al.*, 2003). Here we found that YC-1 greatly improved rodent learning behaviour when examined with the Morris water maze and avoidance tests.

It is known that the central cholinergic systems play an important role in learning and memory. The cholinergic deficit is a prominent hallmark of Alzheimer's disease (Palmer, 1996). It was found that the decrease of acetylcholine release may be responsible for memory disturbances (Aprikyan & Gekchyan, 1988). Here we further investigated the effects of YC-1 on memory disturbances induced by the antagonist of cholinergic receptors, scopolamine. It was demonstrated that YC-1 is also able to inhibit the scopolamine-induced memory deficits. These *in vivo* findings suggest a therapeutic potential of YC-1 as a drug candidate for improving learning and memory.

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Materials and methods

Electrophysiology

Transverse slices of hippocampus (400- μ m thick) were prepared from adult Wistar rats (150–250 g). The rats were killed by cervical dislocation and decapitated. Slices were immediately placed in ice-cold cutting buffer containing (in mM): Slices were immediately placed in ice-cold cutting buffer containing (in mM): NaCl, 124; KCl, 3; Na₂HPO₄, 1; NaHCO₃, 25; CaCl₂, 0.5; MgSO₄, 5; glucose, 10; saturated with 95% O₂ and 5% CO₂. Slices were then maintained in an interface chamber at room temperature, where they were subfused with artificial cerebrospinal fluid (ACSF) containing (in mM): NaCl, 124; KCl, 4.4; Na₂HPO₄, 1; NaHCO₃, 25; CaCl₂, 2; MgSO₄, 1; glucose, 10; bubbled with 95% O₂ and 5% CO₂. A bipolar tungsten-stimulating electrode was placed in the middle of the stratum radiatum in the CA1 region, and extracellular field potentials were recorded using a glass microelectrode (5–10 M Ω , filled with 2 M NaCl solution). The pulse duration was 100 μ s, and test responses were elicited at 0.05 Hz. The perfusion rate of ACSF was 1–2 mL/min.

Behavioural tasks

Morris water maze

Wistar rats (200–300 g) were used. Animals were housed individually and maintained on a 12 h/12 light/darkness cycle. All rats were allowed free access to food and water. The Morris water maze was performed in a circular pool (224 cm in diameter, 46 cm in height) located in a room with distinctive visual cues. Water was filled to a depth of 36 cm and a transparent plastic platform (25 $\times$ 25 cm, 32 cm in height) was located at the centre of a fixed quadrant. Training started by acclimating the rat to the task environment with 2 days of free-swimming in the pool with no platform. Each session lasted for 2 min and the rat was picked up from the pool by the experimenter. Rats received four consecutive daily training trials for the following 4 days. In each trial, the rat was placed into the water randomly from one of the quadrants. The rat had to swim until it climbed onto the platform submerged underneath the water. The time duration from entering the water to climbing onto the platform was recorded and defined as escape latency. If the rat failed to find the platform by 120 s, it placed onto the platform by the experimenter. The rat stayed on the platform for 60 s, which also served as the interval of trial. At the termination of each day's training, the rat was dried with towels and an electric heater before being placed into its home cage. On the seventh day, the probe test was performed. The platform was not placed in the tank and the rats were allowed to swim freely for 120 s. The total time that subjects spent in each quadrant of the tank was recorded.

YC-1 (1 mg/kg) or vehicle was injected (i.p.) 10 min before the first trial. L-NAME (1 μ mol in 10 μ L/100 mM) or PKG inhibitors of KT5823 (0.2 nmol in 10 μ L/20 μ M) and Rp-8-Br-PET-cGMPs (1 nmol in 10 μ L/0.1 mM) were administered by intracerebroventricular (i.c.v.) injections 20 min before the first trial. The cannula was positioned under anaesthesia (200 mg/kg trichloroacetaldehyde) 1.0 mm lateral to the midline, 1.5 mm caudal to bregma, and 3.5 mm below the surface of the skull, according to the atlas of Paxinos & Watson (1997). Cannulae were made of 23-gauge stainless steel tubing, with a 0.33-mm inner diameter and a 0.63-mm outer diameter, to a length of 10 mm. Dental cement was used to secure the cannula to two screws inserted in the skull. Rats were kept warm until recovery from anaesthesia. They recuperated for at least 1 week before being subjected to behavioural testing.

Passive avoidance and active avoidance

Rats were trained and tested on a one-trial step-through passive inhibitory avoidance task with a procedure modified from that described by Liang *et al.* (1998). Briefly, the apparatus was a trough-shape alley divided by a sliding door into a well-lit safe compartment and a dark shock compartment. On day 0 designated as context acclimation, the rat was placed into the lit side facing away from the door. As the rat turned around, the door was opened. It was allowed to enter the dark compartment for three times without receiving any foot-shock. The rat was then retrieved and returned to its home cage. On day 1 designated as foot-shock training, the rat was placed into the lit side and allowed to enter the dark site as in the previous day. After the rat stepped into the dark site, the door was closed and the rat received an inescapable foot-shock via a constant current shocker controlled by a timer (Lafayette Instruments, Model 80240 and Model 58010, IN, USA). After administration of the foot-shock (0.6 mA, 0.6 s), the rat was removed from the alley and returned to its home cage. In a retention test given on days 2, 3 or 11, the rat was reintroduced into the alley and the latency to enter the shock compartment was taken as the retention score. No foot-shock was administered on the retention test, and testing was terminated at 600 s.

For the active avoidance task, the rat was placed facing the door in the dark compartment of the alley described above. The door was lowered halfway to form a hurdle such that the rat could leap over the door into the lit compartment. Any rat that spontaneously crossed into the lit side within 10 s was excluded from this experiment. The foot-shock was turned on 10 s after the door was lowered; the rat had to escape into the lit compartment and the door was closed. The retention test was administered 24 h later. The latency to leave the dark compartment was measured as the retention score.

Startle test

Startle response to stimulus was determined using a standard startle chamber (San Diego Instruments, San Diego, CA, USA). The animal was held in a Plexiglas cylinder tube (length 20 cm, diameter 10 cm) with a vibration sensor attached to the base. Each tube was encoded in a ventilated sound-attenuating cabinet (length 38 cm, width 38 cm, and height 55 cm) that was dark, ventilated and sound attenuated. The acoustic startle stimuli were high-intensity white noise bursts delivered by a speaker 30 cm above the animal. The shock stimuli were square-wave direct currents generated from a programmable shocker (TI 30; Coulbourn Instruments, San Diego, CA, USA). Scrambled shocks were delivered to a grid floor consisting eight metal rods inserted inside the cylinder. Motion of the animal within the cylinder was detected via vibration sensor for a period of 200 ms after initiation of the stimulus. The vibration was transduced into voltage, then the output of the transducer was led to a computer for sampling. The startle amplitude of each trial is defined as the maximal vibration within the 200 ms.

After receiving injections of 0.1% dimethylsulphoxide (DMSO) in saline or YC-1, rats were held in their cage for 15 min and then placed into the startle apparatus with a continuous 55 dB background noise. After a 5-min acclimation period, 45 trials were presented with a 30-s intertrial interval (ITI). Two types of stimuli were dispensed to elicit startle: One type of stimuli contained nine different intensities of electric shocks that were ranging from 0 to 1.6 mA with increments of 0.2 mA (0.1 s). The other type of stimuli was white noise bursts that were composed by 10 noise bursts (40 ms in duration) with 30-s ITIs of each of three intensities (95, 105 and 115 dB).

Each session contained 3 blocks of trials, and each block consisted of two different series. Each series was composed of six acoustic trials

(two trials at each level.) followed by nine shock trials (one trial at each shock intensity). Different intensities for each stimuli modality were presented in a semi-random order within the separated phases of series. The total time elapsed for a test session was 28 min including the acclimation periods. At the end of the startle session, the rats were returned to their home cages. Average transducer output just prior to noise burst was saved as a baseline (VStart). In addition, the computer found the maximum startle amplitude within each of the samples (VPeak) and this value was also saved. Peak startle amplitude was expressed as VPeak – VStart for analysis.

The water maze and startle test were analysed using two-way analysis of variance (ANOVA) followed by the *post hoc* Scheffe test. The avoidance responses were analysed by the nonparametric Kruskal–Wallis test followed by the Mann–Whitney *U*-test. All protocols complied with institutional guidelines and were approved by Animal Care Committees of Medical College, National Taiwan University. This study adhered to guidelines for care and use of Experimental Animals of the National Science Council, R.O.C.

Drugs used

L-NAME, scopolamine, KT5823 and Rp-8-Br-PET-cGMPS were obtained from Sigma-Aldrich (St. Louis MO, USA); YC-1 was provided by Yung-Shin Pharmaceutical Industry Co. Ltd. (Taichung, Taiwan).

Results

Concentration-dependent enhancement of LTP by YC-1

We first examined the effect of YC-1 on the induction of LTP in Schaffer collateral–CA1 pathway of rat hippocampal slices. As shown in Fig. 1A, two trains of extracellular stimulation of Schaffer collaterals at 100 Hz for 1 μ s induced LTP in the synaptic inputs to CA1 pyramidal cells. Perfusion of YC-1 (5.3 μ M) for 6 min (3 min before and 3 min after weak tetanus) markedly increased the extent of LTP. YC-1 potentiated LTP in a concentration-dependent manner (Fig. 1B).

Effects of YC-1 on learning behaviour

Morris water maze

Substantial evidence implicates the hippocampus in acquisition and retention of spatial memory (Barnes, 1988). We then examined the effect of YC-1 on the acquisition of spatial memory in rats, using the Morris water maze. The rats received four training trials each day. YC-1 was injected (i.p., 1 mg/kg) 10 min before the first trial of each daily training session. As shown in Fig. 2A, YC-1 shortened the escape latency of trials 2–4 on the first training day, suggesting that YC-1 promotes spatial memory. The escape latencies of trials 2–4 were 93.8 ± 8.7 s, 79.3 ± 12.3 s and 57.6 ± 12.5 s for control rats and 50.4 ± 10.2 s, 47.3 ± 6.8 s and 30.3 ± 10.3 s for the YC-1-treated group, respectively ($n = 10$). The escape latencies of the first trial on the following three training days were also markedly shortened by YC-1. The escape latencies were 91.1 ± 9.8 s, 47.1 ± 3.9 s and 35.3 ± 6.1 s for control rats and 55.4 ± 12.8 s, 15.3 ± 2.1 s, and 13.4 ± 1.7 s for the YC-1-treated group, respectively ($n = 10$). These results demonstrate that long-term memory is markedly improved by YC-1 administration. The summarized results are shown in Fig. 2B. The escape latency of the first trial is shown in this figure. The escape latency is persistently shorter in YC-1-treated group throughout the

FIG. 1. Concentration-dependent enhancement of LTP by YC-1 in hippocampus. (A) Hippocampal slices were stimulated in the Schaffer collateral–CA1 pathway at a basal stimulating rate at 0.05 Hz. LTP was induced by strong tetanus (100 Hz for 1 s, each separated by periods of 20 s). YC-1 (5.3 μ M) given 3 min before and 3 min after strong tetanus potentiated the induction of LTP. The concentration-dependent effect of YC-1 is shown in B ($n = 4$ –8, mean $\pm$ SEM).

whole experimental period up to 21 days, although YC-1 was only administered for initial 4 consecutive days. The mean escape latencies on day 21 were 13.5 ± 1.8 s and 6.0 ± 0.5 s ($n = 10$ each) for the control and YC-1-treated rats, respectively. We further examined the effect of oral intake of YC-1 on the performance of the rats in the water maze task. The rats were given YC-1 mixed with 1.25% carboxymethylcellulose (10 mg/kg) orally 30 min before the first trial for six consecutive days. Similar to intraperitoneal administration of YC-1, oral intake of YC-1 also enhanced the spatial memory, as shown by the escape latency of the first trial in each daily training session (Fig. 2C).

As the NO–cGMP–PKG pathway is involved in the LTP-potentiating action of YC-1 (Chien *et al.*, 2003), we then investigated the *in vivo* effect of L-NAME, KT5823 and Rp-8-Br-PET-cGMPS on the improvement of learning and memory by YC-1. In these experiments, L-NAME (1 μ mol), KT5823 (0.2 nmol) or Rp-8-Br-PET-cGMPS (1 nmol) was administered i.c.v. 10 min before YC-1 administration (i.p., 1 mg/kg). As shown in Fig. 3, the NOS inhibitor L-NAME significantly blocked the enhancing effect of YC-1 on learning and memory. Intracerebroventricular administration of the PKG inhibitors

FIG. 2. Enhancement of water maze task by YC-1. YC-1 was injected (i.p., 1 mg/kg) 10 min before the first trial of each daily training session. Note that YC-1 shortened the escape latencies of trials 2–4 on the first day and of the first trial on the following 3 training days (A). (B) Each point represents the escape latency of the first trial of each daily training session. Note that YC-1 shortened the escape latency throughout the whole experimental period until day 21 although YC-1 was only given on the initial four consecutive days. ($n = 9$ and 10 for control and YC-1, respectively). (C) YC-1 was mixed with CMC (1.25%) and administered orally (10 mg/kg) 30 min before the first trial of daily training. Note that oral intake of YC-1 still enhanced water maze learning. Each point represents the escape latency of the first trial of each daily training session ($n = 9$ and 15 for control and YC-1, respectively). * $P < 0.05$ compared with control using two-way ANOVA followed by the *post hoc* Scheffé test. Open circles show control; closed circles show YC-1.

KT5823 and Rp-8-Br-PET-cGMPS also attenuated the memory enhancement caused by YC-1 (Fig. 3, ANOVA test among three groups: $F_{3,41} = 49.2$, $P < 0.001$ for Fig. 3A and $F_{3,41} = 29.2$, $P < 0.001$ for Fig. 3B). It seems that L-NAME and KT5823 exert greater inhibition approximately throughout training on days 2–5. These results suggest that the potentiation of spatial memory by YC-1 is sensitive to inhibition by NOS and PKG inhibitors. Intracerebroventricular administration of L-NAME, KT5823 or Rp-8-Br-PET-cGMPS alone also slightly exerted inhibition of learning behaviours as shown in the escape latency of first trial (Fig. 4A) or the mean escape latency of four trials (Fig. 4B) in the daily training sessions.

Passive and active avoidance tasks

Fear memory requires the participation of neurons in the amygdala (Cahill & McGaugh, 1990; Gilbert *et al.*, 1991). There is also evidence that NO is involved in activity-induced synaptic plasticity in some amygdaloid nuclei (Bernabeu *et al.*, 1995; Watanabe *et al.*, 1995; Telegdy & Kokavszky, 1997). Because we found that YC-1 also enhanced LTP induction in the amygdala (Chien *et al.*, 2003), we therefore investigated the effect of YC-1 on the fear memory using passive avoidance test. As shown in Fig. 5A, rats received YC-1 (i.p., 1 mg/kg) 10 min before foot-shock training exhibited prolonged retention latencies in the safe compartment. The retention scores remained at a high level even if the rats were tested on day 11. More rats had retention latencies > 10 min in the YC-1-treated group (5 out

of 27 in the control group and 20 out of 24 in the YC-1-treated group, respectively) tested on both day 2 and day 11. When YC-1 was mixed with 1.25% CMC and delivered orally, the drug also greatly improved retention in the task. The mean retention scores on the day 2 test were 157.1 ± 64.3 s and 369.2 ± 70.2 s ($n = 10$ for each), respectively, for the control (2 out of 10 were longer than 5 min) and YC-1-treated group (8 out of 10 were longer than 5 min). A similar treatment with YC-1 was also effective in improving learning and memory in aged rats (1 year old) although the effect was much less pronounced than that in younger rats (data not shown).

The results shown above indicate that YC-1 can prolong the retention of a passive avoidance response in rats. In this task, YC-1 preferentially affects the formation of new memory, a process conceived to be related to the induction phase of LTP. The fear memory of passive avoidance relies on a brief experience of foot-shock. Consistent with the idea that NO, if generated by this training experience, is quickly metabolized, we found that YC-1 did not exert any effect on rat avoidance behaviour when it was injected 30 min after foot-shock (Fig. 5B). In addition, the enhancement effect of YC-1 could be antagonized significantly by L-NAME and KT5823 (i.c.v., 1 μ mol and 0.2 nmol, respectively; Fig. 5C). These results suggest that YC-1 can improve learning behaviours only when it was administered shortly before or during the learning experience. More interestingly, YC-1 given 10 min before the context acclimation phase in which no foot-shock was administered also prolonged the retention

FIG. 3. Antagonism of L-NAME, KT5823 and Rp-8-Br-PET-cGMPS on the enhancement of learning by YC-1. YC-1 (i.p., 1 mg/kg) was simultaneously injected with L-NAME (i.c.v., 1 μ mol), KT5823 (i.c.v., 0.2 nmol), Rp-8-Br-PET-cGMPS (i.c.v., 1 nmol) or saline 10 min before the first trial of daily training. Note that L-NAME, KT5823 and Rp-8-Br-PET-cGMPS impaired the enhancing effects of YC-1 on the first trial (A) and the mean escape latency of four trials (B) of each daily training session. The number of animals tested per group was indicated in parenthesis. * $P < 0.05$ compared with control using two-way ANOVA followed by the *post hoc* Scheffe test.

latencies in the test if foot-shock was administered on the subsequent day (Fig. 5D), suggesting that YC-1 is capable of enhancing an otherwise latent memory for environmental cues.

For the active avoidance task, rats were put into the dark compartment. Foot-shock was administered 10 s later in motivating the rat to shuttle into the lit side. Retention was tested 2 days later. Injection of YC-1 10 min before foot-shock caused a marked improvement in retention performance by decreasing the escape latency from the shock-compartment to the safe compartment (Fig. 6). Furthermore, the memory enhancing effect of YC-1 can also be effectively inhibited by L-NAME and KT5823 (i.c.v., 1 μ mol and 0.2 nmol, respectively). Results above show that YC-1 significantly improves learning and memory of both passive and active avoidance responses through a NO-cGMP-PKG signalling pathway in rats. We observed no change in startle response after injection of YC-1 as assessed by foot-shock as well as acoustic stimulations (Fig. 7). Oral

FIG. 4. Inhibition of water maze task by intracerebroventricular administration of L-NAME, KT5823 and Rp-8-Br-PET-cGMPS. L-NAME (1 μ mol), KT5823 (0.2 nmol), Rp-8-Br-PET-cGMPS (1 nmol) or saline was i.c.v. applied 10 min before the first trial of daily training. Each point represents the escape latency of the first trial (A) or the mean escape latency of 4 trials (B) of each daily training session. Note that L-NAME, KT5823 or Rp-8-Br-PET-cGMPS administration slightly impaired the learning behaviour. The number of animals tested per group was indicated in parenthesis. * $P < 0.05$ compared with control using two-way ANOVA followed by the *post hoc* Scheffe test.

administration of YC-1 (10 mg/kg per day) for 3 weeks also did not affect the withdrawal latency on the hot-plate thermal (55 °C) test (control, 14.3 ± 2.3 s, $n = 9$; YC-1, 12.9 ± 3.8 s, $n = 10$). These results suggest that the enhancement of fear memory by YC-1 does not result from the increase of shock sensitivity.

Effect of YC-1 on scopolamine-induced impairment in learning behaviour

In the Morris water maze test, administration of scopolamine (0.2 mg/kg, i.p. 20 min before the first trial of each daily training session) impaired learning abilities significantly. YC-1 (1 mg/kg, i.p.) given concomitantly, antagonized the scopolamine-induced impairment of learning abilities on the performance in the Morris water maze. The escape latencies of the first trial (Fig. 8A) and the mean value of the four trials (Fig. 8B) during the six training days were markedly prolonged by the scopolamine treatment. YC-1 treatment significantly improved the learning. Figure 8C shows the probe test on day 7. The swimming paths were recorded by a camera and the data

FIG. 5. Effects of YC-1 on passive avoidance tasks. Passive avoidance was assessed on day 2 and day 11 after training by measuring the latency to re-enter a chamber previously paired with foot-shock (0.6 μ A, 0.6 s). Administration of YC-1 (1 mg/kg, i.p.) 10 min before (A) but not 30 min after (B) foot-shock markedly prolonged the retention latency tested on both day 2 and day 11. (C) L-NAME (i.c.v., 1 μ mol) and KT5823 (0.2 nmol) antagonized the memory enhancing effect of YC-1 (i.p.) in the passive avoidance task when the rats were tested 48 h after the shock. (D) Rats received YC-1 (1 mg/kg, i.p.) 10 min before the context acclimation phase also prolonged the retention latencies in the passive avoidance task. Controls, open bars (A, B, D); filled bars, YC-1 treatment. Data are presented as mean $\pm$ SEM (*n*). **P* < 0.05 compared with control using nonparametric Kruskal–Wallis followed by Mann–Whitney *U*-test. #*P* < 0.05 compared with YC-1 + saline group.

were analysed on computer. All three groups showed bias towards the plate probe, and scopolamine significantly reduced the time on the plate probe. Concomitant administration of YC-1 reversed the scopolamine-induced impairment on the probe test. The upper panels show the representative swimming path of the three groups.

We also examined the effect of YC-1 on scopolamine-induced impairment in passive avoidance test. As shown in Fig. 9, scopolamine given 20 min before the foot-shock training markedly decreased the retention scores of passive avoidance test. Concomitant administration of YC-1 significantly antagonized the scopolamine-induced impairment.

Discussion

Nitric oxide synthase is widespread in the nervous system and NO is thought to play important roles in activity-dependent synaptic

plasticity and is involved in several types of learning and memory (Hawkins, 1996; Prast & Philippu, 2001). In the present study, we have demonstrated that YC-1 improves learning and memory of rodents in a variety of behavioural tests through a NO-dependent pathway. We previously found that weak tetanic stimulation at 50 Hz for 0.5 s did not induce LTP unless YC-1 is present (Chien *et al.*, 2003). In neurons, NO synthesis is stimulated by Ca^{2+} influx, which is induced by activation of glutamate receptors, from both NMDA and metabotropic receptors (Chien *et al.*, 2003). Nitric oxide operates primarily as an intercellular messenger. It diffuses rapidly and influences NO-responsive target cells probably within spatial limits of approximately 0.3–0.4 mm (Lancaster, 1997). This property of NO allows YC-1 to enhance LTP in an input-specific manner and its influence on behaviour is therefore experience-dependent. It seems that many effects of NO are mediated by cGMP. Activation of the sGC, increase of cGMP formation and activation of PKG has been

FIG. 6. Effects of YC-1 on active avoidance tasks. Active avoidance was assessed 24 h after training by measuring the latency of escaping from a compartment previously paired with foot-shock (0.6 mA, 0.6 s). Note that YC-1 shortened the retention latency in the active avoidance task. L-NAME (i.c.v., 1 μ mol) and KT5823 (0.2 nmol) also antagonized the memory enhancing effect of YC-1 (i.p.) in the active avoidance task. Data are presented as mean $\pm$ SEM (n). * P < 0.05 compared with control using nonparametric Kruskal–Wallis followed by the Mann–Whitney U -test. # P < 0.05 compared with YC-1 + saline group.

suggested as the main signal transduction pathway of NO. Stimulation of neuronal cGMP synthesis might modulate synaptic functions and lead to the early phase of LTP (Hawkins *et al.*, 1993, 1998). The NO–cGMP–PKG pathway might also contribute to the late phase of LTP by causing induction of immediate early genes through phosphorylation of CREB (Gudi *et al.*, 1996). CREB phosphorylation and gene induction are thought to contribute to the late, protein synthesis-dependent phase of hippocampal LTP (Bourtchouladze *et al.*, 1994; Carew & Sutton, 2001), which may involve presynaptic as well as postsynaptic changes (Bernabeu *et al.*, 1995; Bolshakov *et al.*, 1997).

The relation between LTP and memory is a focus of intensive investigation (Shors & Matzel, 1997; Malenka & Nicoll, 1999). In the present study, administration of YC-1 greatly improved learning and memory in several tasks involving different brain structures including hippocampus, cerebral cortex and amygdala. We found that YC-1 did

not affect memory retention when administered 30 min after foot-shock training, suggesting the action of YC-1 occurs during or immediately following the training. The present study also showed that YC-1 consistently improves memory in tasks with opposite motor demands (passive and active avoidance) and in tasks relying on different sorts of sensory and motivational stimuli. The startle scores in response to foot-shock or acoustic stimuli are not affected by YC-1 treatment and the ability of YC-1 to enhance learning in the passive avoidance task when given in the context acclimation phase, suggesting that the memory improvement by YC-1 might not result from altering shock sensitivity.

NOS inhibitors reduce the capability of treated animals to acquire or retain information in several learning tasks (Prast & Philippu, 2001). Here we reported that intracerebroventricular injection of L-NAME, KT5823 and Rp-8-Br-PET-cGMPs results in amnesia on later tests and severely impaired the enhancing effects of YC-1 on long-term memory formation. NO is known to be required for consolidation of new memory into long-term storage in different behavioural tasks (Li *et al.*, 1995). It is also required for LTD induction in cerebellar slices because NOS inhibitors block LTD (Ito, 2000). Furthermore, cGMP and PKG have also been shown to produce activity-dependent long-lasting effects in cerebellum and sensory-motor cortex (Ito & Karachot, 1990; Shibuki & Okada, 1991). Therefore, our results provide further evidence that NO–cGMP–PKG is an important signalling pathway for synaptic plasticity and the consolidation of new memory. YC-1 potentiated the response of sGC to NO and enhanced LTP induction and memory acquisition in various kinds of learning tasks. It has been reported that YC-1 at higher concentrations might inhibit phosphodiesterase (Friebe *et al.*, 1998; Galle *et al.*, 1999). Therefore, the slight inhibition of phosphodiesterase by lower concentrations of YC-1 used in the present study can not be excluded and may partially contribute to the increase of cGMP. The downstream effectors or transcription factors regulated by PKG are likely to be involved in memory consolidation, and their identities remain to be elucidated. We previously found that incubation of un-stimulated hippocampal slices with YC-1 (1.6 μ M) plus the NO donor nitroprusside increased the immunofluorescence and protein expression of pERK and pCREB in pyramidal neurons, which were inhibited by the PKG inhibitor KT5823 (Chien *et al.*, 2003). It appears that improvement of memory by YC-1 could result from the direct effect of YC-1 on synaptic plasticity but not through cerebral vasodilation. In addition, it needs higher concentrations of YC-1 to cause vasodilation (Galle *et al.*, 1999).

FIG. 7. The lack of effect of YC-1 on shock startle (left panel) and acoustic startle (right panel).

FIG. 8. Effect of YC-1 on scopolamine-induced impairment on Morris water maze task. Both scopolamine (i.p., 0.2 mg/kg) and YC-1 were injected (i.p., 1 mg/kg) 20 min before the first trial of each daily training session. Note that scopolamine impaired the learning behaviour and YC-1 reversed the impairment induced by scopolamine both on the first trial (A) and the mean value of the four trials (B). The probe test was examined on day 7 and YC-1 antagonized the decrease of the time on plate zone caused by scopolamine (C). Data are presented as mean $\pm$ SEM ($n = 15$ for each). * $P < 0.05$ compared with control using two-way ANOVA followed by the *post hoc* Scheffé test.

Scopolamine is a drug commonly used in the study of memory. It blocks the muscarinic cholinergic receptors, which are involved in learning and memory. In animal studies, scopolamine administration has been used as a simple method to induce cholinergic deficit, which

produces impairments on a wide range of learning and memory tasks (Sitaram *et al.*, 1928; Rubaj *et al.*, 2003). Our studies show that YC-1 ameliorates scopolamine-induced impairment of learning behaviours in both Morris water maze performance and passive avoidance task.

FIG. 9. Effect of YC-1 on scopolamine-induced impairment on passive avoidance task. Passive avoidance was assessed on day 2 after training by measuring the latency for re-entering a chamber previously paired with foot-shock. Scopolamine decreased the retention scores and rats received both scopolamine (0.2 mg/kg, i.p.) and YC-1 (1 mg/kg, i.p.) 20 min before foot-shock markedly prolonged the retention latency tested on day 2. Data are presented as mean $\pm$ SEM (n). * P < 0.05 compared with control using nonparametric Kruskal–Wallis followed by the Mann–Whitney U -test. # P < 0.05 compared with scopolamine-treated group.

These results suggest that YC-1 may be effective for therapy in patients with a dementia such as Alzheimer's disease.

In conclusion, YC-1-like substances are intriguing candidates to act as potent modulators of cGMP-mediated effects within the central nervous system. Our results show that YC-1 improves the formation of new memory and reverses the scopolamine-induced memory deficits in different learning tasks. The remarkable characteristics of YC-1 in potentiating NO-stimulated guanylate cyclase activity during the induction of activity-dependent synaptic plasticity and the high spatial and temporal specificity of NO-induced cellular actions warrant that YC-1 will enhance acquisition of new memory and suggest that YC-1 might be a good candidate for new therapeutic drugs aimed at improving learning and memory in humans.

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Abbreviations

ACSF, artificial cerebrospinal fluid; CMC, carboxymethylcellulose; KT5823, (9S, 10R, 12R)-2,3,9,10,11,12, hexahydro-10-methoxy-2,9-dimethyl-1-oxo-9,12-epoxy-1H-diindolo[1,2,3-fg:3',2',1'-kl]pyrrolo[3,4-l]1,6]benzodiazocine-10-carboxylic acid methyl ester; i.c.v., intracerebroventricular; i.p., intraperitoneal; L-NAME, N^G-nitro-L-arginine-methylester; LTD, long-term depression; LTP, long-term potentiation; NMDA, N-methyl-D-aspartate; NO, nitric oxide; NOS, NO synthase; ODQ, 1 H-[1,2,4]-oxadiazolo(4,3-a)-quinoxalin-1-one; PKG, cGMP-dependent protein kinase; Rp-8-Br-PET-cGMPS, β -phenyl-1,N2-etheno-guanosine3',5'-monophosphorothioate; sGC, soluble guanylate cyclase; YC-1, [3-(5-hydroxymethyl-2-furyl)-1-benzyl-indazole].

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