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European Journal of Pharmacology 337 (1997) 125–136

ejp

Possible role of nitric oxide-cyclic GMP pathway in object recognition memory: Effects of 7-nitroindazole and zaprinast

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Received 23 June 1997; revised 7 August 1997; accepted 26 August 1997

Abstract

The effects of 7-nitroindazole, a putative selective inhibitor of neuronal nitric oxide (NO) synthase and zaprinast, a cGMP-selective phosphodiesterase inhibitor, were evaluated on recognition memory of rats in the object recognition test. This test is based on the differential exploration of a new and a familiar object. Two doses of 7-nitroindazole (10 and 30 mg/kg) and zaprinast (3 and 10 mg/kg) were used. The substances were administered i.p. immediately after the exposure to two identical objects, i.e., at the start of the delay interval. After a delay interval of 1 h, control rats spent more time exploring the new object which demonstrates that they recognized the familiar one. Both doses of 7-nitroindazole impaired the discrimination between the two objects after the 1 h interval. After a 4 h interval, control rats did not discriminate between the objects. The highest dose of zaprinast facilitated object recognition after the 4 h interval. In addition, this dose of zaprinast (10 mg/kg) reversed the recognition memory deficit induced by 7-nitroindazole (10 mg/kg) at the 1 h interval. The highest dose of 7-nitroindazole slightly increased mean arterial blood pressure 1 h after its administration. 4 h after administration of zaprinast (10 mg/kg), mean arterial blood pressure was also slightly increased, but not after 1 h after zaprinast administration. However, these effects on blood pressure do not explain the differential effects on object recognition memory. These results therefore suggest that NO-cGMP signal transduction is involved in object recognition memory independently of its cardiovascular role. Finally, since 7-nitroindazole affected mean arterial blood pressure it can not be regarded as a selective inhibitor of neuronal NO synthase. © 1997 Elsevier Science B.V.

Keywords: Memory; (Rat); Nitric oxide (NO); cGMP; Blood pressure; 7-Nitroindazole; Zaprinast

1. Introduction

Long-term potentiation can be defined as a stable and long-lasting increase in the efficiency of synaptic transmission. This form of synaptic plasticity is believed to be the physiological substrate for learning and memory (Bliss and Collingridge, 1993). It is likely that this increase in synaptic transmission is maintained, at least in part, by an increase in presynaptic neurotransmitter (glutamate) release. The latter implicates presynaptic changes of long-lasting nature. For presynaptic changes to occur after postsynaptic activation, it is necessary to hypothesize a retrograde messenger which is released postsynaptically (Bliss and Collingridge, 1993). The molecule nitric oxide (NO) has many of the properties which are imposed on a retrograde messenger: it is synthesized postsynaptically by a calcium/calmodulin-dependent enzyme, NO synthase, is short-lived and freely diffusible (Bliss and Collingridge, 1993; Garthwaite and Boulton, 1995). Moreover, administration of NO synthase inhibitors can impair spatial learning (Chapman et al., 1992; Estall et al., 1993; Mogensen et al., 1995; Yamada et al., 1995) and can block induction of long-term potentiation in hippocampal slices (O'Dell et al., 1991; Bon et al., 1992; Böhme et al., 1993).

NO is a powerful activator of the cyclic GMP (cGMP)-synthesizing enzyme soluble guanylyl cyclase (Murad et al., 1978). It has been suggested that activation of soluble guanylyl cyclase may be a major pathway for the NO messenger function in the brain (De Vente et al., 1990; Garthwaite, 1991; Southam and Garthwaite, 1993). This is corroborated by studies showing that long-term potentiation induction in hippocampal slices can be blocked with

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soluble guanylyl cyclase inhibitors (Zhuo et al., 1994; Arancio et al., 1995; Boulton et al., 1995).

The present study examines whether the NO-cGMP transduction pathway is involved in memory processes. The effects of 7-nitroindazole, a NO synthase inhibitor, and zaprinast, a highly selective inhibitor of the cGMP-selective phosphodiesterase (phosphodiesterase type V) (Beavo and Reifsnyder, 1990), were evaluated on recognition memory of rats. The used object recognition test is based on spontaneous exploratory activity and the differential exploration of a new and a familiar object. The recognition of the familiar object reflects object recognition memory (Ennaceur and Delacour, 1988). Zaprinast was used because we had found that NO-mediated cGMP accumulation in hippocampal slices in the presence of zaprinast was selectively enhanced in the CA2/CA3 region (De Vente et al., 1996).

There are two isoforms of NO synthase that are activated by calcium: neuronal NO synthase and endothelial NO synthase. Consequently, interference with the NO-cGMP pathway can also induce cardiovascular effects (Dundore et al., 1990, 1991). As there are few data available on the cardiovascular effects in vivo of zaprinast and especially 7-nitroindazole, we studied the effects of both compounds on mean arterial blood pressure in conscious animals. We used 7-nitroindazole because it is assumed to be a selective inhibitor of neuronal NO synthase (Moore et al., 1993) and, therefore, its vascular effects should be minimal. An abstract of this work has been published previously (Prickaerts et al., 1996).

2. Materials and methods

2.1. Object recognition memory measurements

2.1.1. Animals

In the object recognition memory test we used thirty male random-bred Tryon–Maze–Bright rats, which were supplied by our own animal facility. Two weeks before behavioral testing, the animals were housed individually in standard Makrolon cages on sawdust bedding in an air-conditioned room (20°C). They had free access to food and water and were kept under a reversed 12/12 h light/dark cycle (lights on from 19.00 to 07.00 h). At the start of behavioral testing, the rats were three months old and weighing 342 ± 4 g (mean $\pm$ S.E.M.). At the last behavioral test, the rats were six months old and weighing 465 ± 5 g (mean $\pm$ S.E.M.).

2.1.2. Procedures

The object recognition test was performed as described elsewhere (Ennaceur and Delacour, 1988). The apparatus consisted of a circular arena, 83 cm in diameter. Half of the 40 cm high wall was made of grey polyvinyl chloride, the other half of transparent polyvinyl chloride. The light intensity was equal in the different parts of the apparatus. Two objects were placed in a symmetrical position about 10 cm away from the grey wall. Each object was available in triplicate. We used three different sets of objects. The different objects were: (1) a cone consisted of a grey polyvinyl chloride base (maximal diameter 18 cm) with a collar on top made of brass (total height 16 cm), (2) a standard 1 l brown glass bottle (diameter 10 cm, height 22 cm) filled with water and (3) a massive metal cube ($10.0 \times 5.0 \times 7.5$ cm) with two holes (diameter 1.9 cm). The objects could not be displaced by a rat.

In the week preceding testing, the animals were handled daily and were finally adapted to the procedure in two days, i.e., they were allowed to explore the apparatus (without any objects) twice for 5 min each day. Also the rats were then adapted to an i.p. injection by a puncture of the injection needle after the first exploration period. Two days after the last adaptation session, testing began. Testing took place in the same room as where the animals were housed. A radio, which was playing softly, provided background noise in the room. Fluorescent red tubes and a light bulb, which was switched on during testing only, provided a constant illumination of about 20 lx on the floor of the apparatus. All testing was done between 10.00 and 17.00 h.

A testing session comprised two trials. The duration of each trial was 3 min. During the first trial (T1) the apparatus contained two identical objects (samples). A rat was always placed in the apparatus facing the wall at the middle of the front (transparent) segment. After the first exploration period the rat was put back in its home cage. Subsequently, after a predetermined delay interval, the rat was put back in the apparatus for the second trial (T2), but now with two dissimilar objects, a familiar one (the sample) and a new one. The times spent in exploring each object during T1 and T2 were recorded manually with a personal computer.

Exploration was defined as follows: directing the nose to the object at a distance of no more than 2 cm and/or touching the object with the nose. Sitting on the object was not considered as exploratory behavior. In order to avoid the presence of olfactory trails the objects were always thoroughly cleaned. All combinations and locations of objects were used in a balanced manner to reduce potential biases due to preferences for particular locations or objects.

In a pilot study we had found that after a delay interval of 1 h, rats discriminated between the two objects in T2, i.e., they spent more time in exploring the new object than the familiar one. However, they did not discriminate between the two objects after a 4 h interval. Therefore, 7-nitroindazole, which was expected to impair memory performance, was tested at a delay interval of 1 h. On the other hand, zaprinast was expected to improve memory performance and was therefore tested at the 4 h interval. The effects of a combined treatment, that is zaprinast and

7-nitroindazole, were tested at a 1 h interval with doses based on the results of the first experiments of this study.

2.1.3. Treatment

7-nitroindazole (Research Biochemicals International) was successively suspended in dimethyl sulfoxide and arachis oil (1 and 3% dimethyl sulfoxide for doses of 10 and 30 mg/kg, respectively) and taken into solution with shaking and warming. Zaprinast (Sigma) was dissolved in 0.05 M NaOH (in 0.9% NaCl). This solution was slowly titrated with 1 M HCl to bring a neutral pH (about 7.5). All solutions were made just before use. Injections of 7-nitroindazole (injection volume 4 ml/kg), zaprinast (injection volume 1 ml/kg) and their vehicles, arachis oil and saline (0.9% NaCl), respectively, were given i.p. immediately after T1 of each session.

The experimental protocol is summarized in Table 1. The rats were matched for body weights and assigned to two experimental groups of 15 animals each. In each group one dose of 7-nitroindazole and zaprinast was tested. The rationale for the doses used were based on data from the literature. For instance, 30 mg/kg 7-nitroindazole was used because this dose can already inhibit NO synthase activity maximally within 0.5 h (e.g., MacKenzie et al., 1994). A dose of 10 mg/kg zaprinast was chosen to start with because this dose apparently has no or only a minimal effect on blood pressure (e.g., Dundore et al., 1992). One group was first treated with 7-nitroindazole (30 mg/kg) and four weeks later with zaprinast (3 mg/kg). The other group was first treated with zaprinast (10 mg/kg) and three weeks later with 7-nitroindazole (10 mg/kg). Finally, that is after a wash-out period of at least seven weeks after their last treatment, all rats were used for a combined zaprinast (10 mg/kg) and 7-nitroindazole (10 mg/kg) treatment. During this wash-out period one rat died for unknown reasons. Another rat was excluded from the entire experiment because of its extreme bias toward one location of the objects within each trial.

Table 1
Experimental protocol

Group	*n*	Week	Treatment	Dose (mg/kg)	Delay (h)
Group 1	15	1	7-NI	30	1
Group 2	14	3	ZAP	10	4
Group 1	15	5	ZAP	3	1
Group 2	14	6	7-NI	10	4
Group 1+2	28	13	ZAP/7-NI	10/10	1

Rats were assigned to two groups of 15 rats each and were submitted to the object recognition test. In each group, one dose of 7-nitroindazole (7-NI) and zaprinast (ZAP) was tested at a delay interval of 1 and 4 h, respectively. The combined treatment was tested in all rats at a 1 h delay. Except for the combined treatment experiment, each treatment experiment was done twice in a week. One rat was excluded because of a bias in its exploratory behavior and another rat died in the period before the combined treatment experiment.

Table 2
Measures involved in the object recognition test

Exploration	Habituation	Discrimination
$e1 = a1 + a2$	$h1 = e1 - e2$	$d1 = b - a$
$e2 = a + b$		$d2 = d1/e2$

$e1$ is the measure of the time spent in exploring both identical objects ($a1$ and $a2$) in the first trial and $e2$ is the measure of the time spent in exploring both the familiar (a) and new object (b) in the second trial; $h1$ is the measure of global habituation from trial 1 to trial 2; $d1$ and $d2$ are the measures of discrimination between the new and familiar objects.

Each rat served as its own control. Therefore, for each treatment experiment, rats were first submitted to a control session (vehicle) and 24 h later to a drug session (7-nitroindazole or zaprinast). This procedure, that is a control session followed 24 h later by a drug session, was repeated 48 h later. Thus, each treatment experiment was performed twice in a rat. The combined treatment experiment, to which all rats were subjected, was performed only once in a rat. In the control session, both injections of vehicle (saline and arachis oil) were given. In the drugs session, 24 h later, the rats received an injection of zaprinast and immediately thereafter an injection of 7-nitroindazole.

2.1.4. Statistical analysis

The basic measures were the times spent by rats in exploring an object during T1 and T2. The time spent in exploring the two identical samples will be represented by '$a1$' and '$a2$'. The time spent in T2 in exploring the sample and new object will be represented by 'a' and 'b', respectively. The following variables were calculated: $e1 = a1 + a2$, $e2 = a + b$, $h1 = e1 - e2$, $d1 = b - a$ and $d2 = d1/e2$ (see Table 2). $e1$ and $e2$ are measures of the total exploration time of both objects during T1 and T2, respectively. $h1$ was considered as an index measure of global habituation of exploratory behavior from T1 to T2. $d1$ and $d2$ were considered as index measures of discrimination between the new and the familiar objects. In fact, $d2$ is a relative measure of discrimination which corrects $d1$ for exploration activity ($e2$). Thus, there should be no differences in $d2$ indices between experiments with similar treatments at similar intervals. However, this need not be the case for $d1$ because of differences in exploration activity.

Except for the combined treatment experiment, each treatment experiment was comprised of two control and two drug sessions. The results of both the two control sessions and the two drug sessions were averaged.

Comparisons within a session were based on a paired-comparisons *t*-test (SAS, *t*-test procedure) which tests the significance of a variable for a given session by comparing the mean value of this variable with zero. This test, which is essentially the same as a paired *t*-test, was applied to the h and d variables. Because each rat served as its own

control, comparisons of variables between control and drug sessions were based on a paired *t*-test.

Object preferences were tested within sessions by comparing *e*1 according to the nature of the objects (one-factorial analysis of variance) and, if apparent, evaluated in more detail with Duncan's post hoc multiple range test. Moreover, location preferences were tested by comparing *a*1 and *a*2 for each object (paired *t*-test).

2.2. Mean arterial blood pressure measurements

2.2.1. Animals

For the blood pressure measurements we used ten male random-bred Tryon–Maze–Bright rats. One week before testing, the animals were housed individually. Housing conditions were the same as in the behavioral study as mentioned above, except that now the rats were kept under a normal 12/12 h light/dark cycle (lights on from 07.00 to 19.00 h). At the start of testing, the rats were three months old and weighing 340 ± 7 g (mean ± S.E.M.).

2.2.2. Procedures and treatment

In the week preceding testing, the animals were handled daily and were finally adapted to an i.p. injection in two days by a puncture of the injection needle on each day. Two days later, animals were anesthetized with sodium pentobarbital (60 mg/kg, i.p.) and a catheter (PE-10) was inserted into the abdominal aorta through a femoral artery for measurement of mean arterial blood pressure. The catheter was exteriorized in the neck, filled with heparinized saline (50 IU/ml) and closed with a metal plug. After the operation, animals were allowed one day for recovery.

Mean arterial blood pressure measurements were performed at three subsequent days. On each day the catheters were connected to low-volume displacement pressure transducers (CP 01; Century Technology, Inglewood, CA). Signals were fed into a computer for on-line derivation of mean arterial blood pressure.

After an adaptation period of about 30 min, four measurements of mean arterial blood pressure were taken at 5 min intervals. The average of these four measurements represented the baseline condition. On the first day, rats received an i.p. injection of saline (0.9% NaCl). On the second and third day, zaprinast (10 mg/kg, i.p.) and 7-nitroindazole (10 or 30 mg/kg, i.p.) were administered, respectively. Solutions and injection volumes were the same as mentioned in the behavioral experiments. After administration of a solution, the mean arterial blood pressure response was taken at 5 min intervals for a maximum period of 5 h (after saline and zaprinast administration), or for at least a 3 h period (after 7-nitroindazole administration). After the daily mean arterial blood pressure measurements, the catheters were flushed with heparinized saline and closed again with a metal plug.

Not all measurements were or could be done in all rats. One rat was excluded from the experiment because its catheter let loose. The mean arterial blood pressure responses after zaprinast administration were measured in all rats for a period of 4 h. Moreover, in three rats the measurements were continued till 5 h had elapsed. After 7-nitroindazole administration, the mean arterial blood pressure measurements of three rats could not be used due to technical problems. Hence, of both 7-nitroindazole treatments there were only three mean arterial blood pressure recordings used.

2.2.3. Statistical analysis

During the interval after administration of a solution, each mean arterial blood pressure response was expressed as the change in mean arterial blood pressure (in mm Hg) from pretreatment baseline mean arterial blood pressure value. In addition, the mean arterial blood pressure response over an entire interval was expressed as the average change in mean arterial blood pressure response from the baseline. Treatment effects on mean arterial blood pressure responses between the control (saline) and drug (zaprinast

Table 3
Results of treatment with 7-nitroindazole (7-NI) on the measures of the object recognition test

	Control	7-NI 10	*t*	*P* <	Control	7-NI 30	*t*	*P* <
(A) Mean values (± S.E.M.) of total exploration time (s) during the first (*e*1) and second trial (*e*2)								
*e*1	13.01 (1.61)	14.10 (1.32)	0.88	n.s.	21.36 (1.22)	17.35 (1.49)	−1.85	n.s.
*e*2	14.66 (1.82)	15.09 (1.58)	0.34	n.s.	21.68 (1.23)	23.12 (1.67)	0.79	n.s.
(B) Mean values (± S.E.M.) of the index of global habituation (*h*1) from the first to the second trial								
*h*1	−1.66 (1.08)	−0.99 (0.73)	0.60	n.s.	−0.32 (1.55)	−5.77 (2.43) [a]	−1.75	n.s.
(C) Mean values (± S.E.M.) of the index of discrimination (*d*1) between the new and familiar objects								
*d*1	4.38 (0.70) [b]	1.29 (0.95)	−2.23	0.05	8.57 (1.65) [b]	2.76 (1.58)	−2.59	0.05

Rats received an i.p injection of 7-nitroindazole at a dose of 10 ($n = 14$) or 30 ($n = 15$) mg/kg after the first trial. Each rat served as its own control (arachis oil treatment). For both the control and 7-nitroindazole treatment the averaged data of two sessions are presented. The delay interval between the first and second trial was 1 h. Between sessions effects are depicted with corresponding *t* and *P* values. Within session effects on the *h* and *d* measures are depicted with [a] and [b] ([a] $P < 0.05$; [b] $P < 0.01$).

or 7-nitroindazole) measurements were analyzed with a paired *t*-test.

3. Results

3.1. Effects of 7-nitroindazole in the object recognition test

Rats were submitted to a 7-nitroindazole experiment with a dose of either 10 or 30 mg/kg 7-nitroindazole. The results of the 7-nitroindazole experiments are summarized in Table 3. When comparing the total exploration time according to the nature of the objects, no object preferences were found in both 7-nitroindazole experiments. In addition, when comparing the exploration time according to the nature of the objects and their location in the apparatus, it was found that rats had a preference for the location of one of the objects in the 7-nitroindazole 10 session.

In both 7-nitroindazole experiments, the total time spent in exploring the objects in T1 ($e1$) as well as in T2 ($e2$) did not differ between the control and 7-nitroindazole sessions (see Table 3A). The index measure of habituation of exploratory behavior ($h1$) showed that the exploration time did not change from T1 to T2 in both the control and 7-nitroindazole session of the 7-nitroindazole 10 experiment, i.e., in both sessions no within session effect was found (see Table 3B). The significance of the $h1$ measure of the 7-nitroindazole 30 session indicated that the exploration time was increased from T1 to T2. However, the level of this $h1$ measure was not different from its control level, i.e., no between session effect was found (see Table 3B).

The index measure of discrimination ($d1$) between the new and familiar objects of both 7-nitroindazole experiments showed that in the control sessions the familiar object was less explored than the new object in T2 after the 1 h delay interval (within session effect on $d1$; see Table 3C). In contrast, the $d1$ measures of the 7-nitroindazole sessions indicated that after administration of 7-nitroindazole at a dose of both 10 and 30 mg/kg, the rats did not discriminate anymore between the objects.

3.2. Effects of zaprinast in the object recognition test

Rats were submitted to a zaprinast experiment with a dose of either 3 or 10 mg/kg zaprinast. The results of the zaprinast experiments are summarized in Table 4. When comparing the exploration time according to the nature of the objects and their location in the apparatus, two object preferences (one in the control session of the zaprinast 3 experiment and one in the zaprinast 10 session) and a location preference of one object (in the zaprinast 3 session) were found.

In the zaprinast 3 experiment, the level of exploration in T1 ($e1$) of the zaprinast session was higher than that of the control session (see Table 4A). The level of exploration in T2 ($e2$) was not different between the zaprinast 3 and the control session. In the control session, the within session effect on $h1$ indicated that the exploration time was increased from T1 to T2. However, there was no difference in $h1$ between the control and zaprinast 3 session (see Table 4B).

The level of exploration in T1 ($e1$) was similar in the zaprinast and the control session of the zaprinast 10 experiment. After treatment with 10 mg/kg zaprinast, the level of exploration in T2 ($e2$) was increased compared with the control treatment (see Table 4A). However, $h1$ showed that in both the zaprinast and control session the exploration time did not change from T1 to T2 and neither was there a difference in $h1$ between both sessions (see Table 4B).

After the 4 h interval, the rats did not discriminate between the new and the familiar objects in the control sessions. Administration of zaprinast at a dose of 3 mg/kg had no effect on discrimination. On the other hand, after administration of zaprinast at a dose of 10 mg/kg the rats spent more time exploring the new object than the familiar one indicating that they discriminated between the objects (within session effect on $d1$; see Table 4C).

Table 4
Results of treatment with zaprinast (ZAP) on the measures of the object recognition test

	Control	ZAP 3	*t*	*P* <	Control	ZAP 10	*t*	*P* <
(A) Mean values (±S.E.M.) of total exploration time (s) during the first ($e1$) and second trial ($e2$)								
$e1$	14.45 (1.20)	19.77 (1.56)	2.90	0.05	23.48 (2.42)	23.78 (2.02)	0.13	n.s.
$e2$	19.05 (1.37)	20.68 (1.16)	0.96	n.s.	20.23 (2.18)	24.53 (2.31)	2.24	0.05
(B) Mean values (±S.E.M.) of the global index of habituation ($h1$) from the first to the second trial								
$h1$	−4.61 (1.99) [a]	−0.92 (1.29)	1.47	n.s.	3.25 (1.68)	−0.75 (1.92)	−1.62	n.s.
(C) Mean values (±S.E.M.) of the index of discrimination ($d1$) between the new and familiar objects								
$d1$	0.40 (0.70)	0.85 (0.89)	0.31	n.s.	1.04 (1.22)	6.33 (0.69) [b]	4.21	0.01

Rats received an i.p injection of zaprinast at a dose of 3 ($n = 15$) or 10 ($n = 14$) mg/kg after the first trial. Each rat served as its own control (saline treatment). For both the control and zaprinast treatment the averaged data of two sessions are presented. The delay interval between the first and second trial was 4 h. Between sessions effects are depicted with corresponding *t* and *P* values. Within session effects on the *h* and *d* measures are depicted with [a] and [b] ([a] $P < 0.05$; [b] $P < 0.01$).

Table 5
Results of combined treatment with zaprinast (ZAP) and 7-nitroindazole (7-NI) on the measures of the object recognition test

	Control	ZAP/7-NI	*t*	*P* <
(A) Mean values (±S.E.M.) of total exploration time (s) during the first (*e*1) and second trial (*e*2)				
*e*1	15.60 (1.39)	19.19 (1.48)	2.47	0.05
*e*2	13.31 (1.29)	8.84 (1.09)	−2.79	0.01
(B) Mean values (±S.E.M.) of the index of global habituation (*h*1) from the first to the second trial				
*h*1	2.29 (1.65)	10.36 (1.83) [b]	3.25	0.01
(C) Mean values (±S.E.M.) of the index of discrimination (*d*1) between the new and familiar objects				
*d*1	5.23 (0.87) [b]	4.09 (0.93) [b]	−2.59	n.s.

Rats ($n = 28$) received i.p injections of zaprinast (10 mg/kg) and 7-nitroindazole (10 mg/kg) after the first trial. Each rat served as its own control (arachis oil and saline treatment). The delay interval between the first and second trial was 1 h. Between sessions effects are depicted with corresponding *t* and *P* values. Within session effects on the *h* and *d* measures are depicted with [b] ([b] $P < 0.01$).

3.3. Effects of combined zaprinast and 7-nitroindazole treatment in the object recognition test

Rats were submitted to a combined treatment experiment with doses of 10 mg/kg zaprinast and 10 mg/kg 7-nitroindazole. The results of the combined zaprinast/7-nitroindazole experiment are summarized in Table 5. There were neither preferences for objects nor for their locations within the apparatus.

The level of exploration in T1 (*e*1) of the zaprinast/7-nitroindazole session was higher than that of the control session (see Table 5A). In T2 there was a decrease in the level of exploration (*e*2) after the zaprinast/7-nitroindazole treatment compared with the control treatment. The exploration time did not change from T1 to T2 in the control session, while it decreased from T1 to T2 in the zaprinast/7-nitroindazole session (within and between session effect on *h*1; see Table 5B).

The index of discrimination (*d*1) showed that after administration of 7-nitroindazole together with zaprinast, the rats still discriminated between the objects at a level which was not different from that in the control session after the 1 h interval (see Table 5C).

3.4. Effects of zaprinast and 7-nitroindazole on d2 in the object recognition test

For an overall view on discrimination, the effects of the different treatments on the relative discrimination index *d*2, which is constituted by the index of discrimination d1 divided by the exploration activity (*e*2), are presented in Fig. 1.

Compared within sessions, *d*2 was statistically significant in both control sessions of the 7-nitroindazole experiment (both $t > 5.10$, $P < 0.01$), which indicated that rats discriminated between the new and familiar objects after a 1 h interval. After treatment with both doses of 7-nitroindazole, no more discrimination within each session was found (both $t < 2.08$, n.s.). This finding is supported by the between session effect in both 7-nitroindazole experiments (see Fig. 1).

After a 4 h interval, *d*2 revealed that there was no

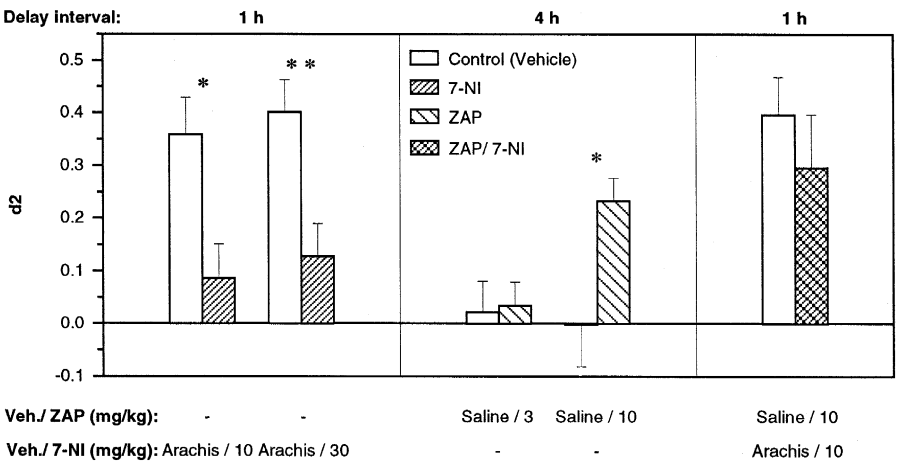

Fig. 1. Effects of the different treatments on the index of discrimination (*d*2) in the object recognition test (mean values ± S.E.M.). In the control sessions, rats were treated with vehicle (saline and/or arachis oil). In the drug sessions, rats were treated with zaprinast (ZAP) and/or 7-nitroindazole (7-NI). Between sessions effect: * $P < 0.05$; * * $P < 0.01$

discrimination in the control sessions and the zaprinast 3 session (all $t < 0.75$, n.s.). However, after treatment with the highest dose of zaprinast (10 mg/kg), rats were able to discriminate between the objects. This was indicated by the within session effect ($t = 5.35$, $P < 0.01$) and between session effect (see Fig. 1).

When 7-nitroindazole (10 mg/kg) was administered together with zaprinast (10 mg/kg), it was found that the discrimination index $d2$ was not affected after the 1 h interval (see Fig. 1). In both the control and zaprinast/7-nitroindazole session, the rats discriminated between the objects (both $t > 2.94$, $P < 0.01$) and there was no difference in the level of discrimination between the control and zaprinast/7-nitroindazole session ($t = -0.96$, n.s.)

Therefore, the results of the index of discrimination $d2$ were not different from that of $d1$. As expected, it was found that the level of discrimination, as defined by $d2$, was not different between the control sessions of the 7-nitroindazole experiments and the combined treatment experiment at the 1 h interval (both $t < -0.09$, n.s.). Neither did $d2$ differ between the control sessions of the zaprinast experiments at the 4 h delay interval ($t = 0.24$, n.s.; see also Fig. 1).

3.5. Effects of zaprinast and 7-nitroindazole on mean arterial blood pressure

Mean arterial blood pressure responses were measured at three subsequent days to assess respectively the effects of saline control, zaprinast (10 mg/kg) and 7-nitroindazole (10 or 30 mg/kg) treatment. The profile of mean arterial blood pressure responses to the different treatments is presented in Fig. 2. There were no differences in baseline mean arterial blood pressure values between the days, which had an average value of 123 ± 1 mm Hg (mean ± S.E.M.).

Administration of 7-nitroindazole at a dose of 10 mg/kg had only incidentally a statistical effect on the individual mean arterial blood pressures compared with the saline treatment. At the higher dose of 30 mg/kg an elevation in mean arterial blood pressures was generally observed from 40 min to more than 2 h after administration of 7-nitroindazole (see Fig. 2A). After administration of zaprinast, mean arterial blood pressures were generally increased from 1 to more than 4 h compared with the saline treatment (see Fig. 2B). For further statistical analysis, the average change in mean arterial blood pressure response over an interval (of whole hours) was calculated. After administration of 7-nitroindazole at the low dose, the average change in mean arterial blood pressure over the entire 3 h interval was, even then, not different from that of the saline treatment ($t = 0.66$, n.s.). For the high dose of 7-nitroindazole it was found that over the interval of the first hour the average mean arterial blood pressure was not different from the saline treatment ($t = 2.75$, n.s.). But from the 2 h interval on, this dose of 7-nitroindazole treatment produced an increase in the average mean arterial blood pressure (both $t > 4.67$, $P < 0.05$). Further, it was found that the average change in mean arterial blood pressure over the interval of the first hour was not different between the zaprinast and saline treatment ($t = 1.17$, n.s). From the 2 h interval on, zaprinast treatment resulted in an elevation in the average mean arterial blood pressure at each interval (all $t > 2.91$, $P < 0.05$).

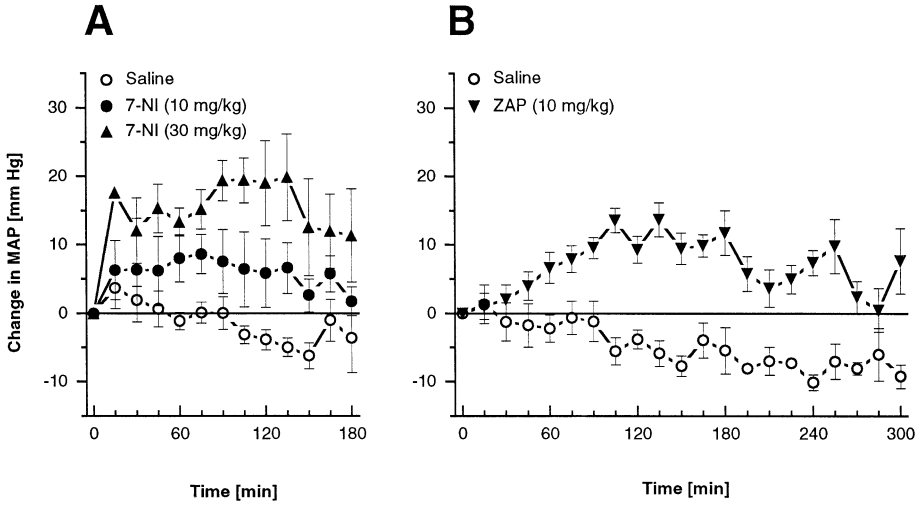

Fig. 2. Treatment effects on the mean arterial blood pressure (MAP). On day 1 the rats received an i.p. injection of saline and on day 2 they received an i.p. injection of zaprinast (ZAP) at a dose of 10 mg/kg. On day 3 the rats received an i.p. injection of 7-nitroindazole (7-NI) at a dose of either 10 or 30 mg/kg. Data are presented as changes in mean arterial blood pressure (mean ± S.E.M.) from pretreatment baseline values. (A) Effects of the two 7-nitroindazole treatments and the corresponding saline control treatments, which have been averaged for clearness sake, on mean arterial blood pressure (each dose of 7-nitroindazole, $n = 3$). (B) Effects of the zaprinast treatment and the corresponding controls on mean arterial blood pressure (0–240 min, $n = 9$; 240–300 min, remaining $n = 3$).

4. Discussion

4.1. The NO-cGMP transduction pathway and memory formation

The results of the present study showed that both doses of 7-nitroindazole (10 and 30 mg/kg) impaired the discrimination between the two objects after the 1 h interval. On the other hand the highest dose of zaprinast facilitated object recognition after the 4 h interval. In addition, this dose of zaprinast (10 mg/kg) reversed the recognition deficit induced by 7-nitroindazole (10 mg/kg) at the 1 h interval.

The inhibition of NO synthase in the brain after systemic administration of 30 mg/kg 7-nitroindazole is probably already maximal 30 min after injection (Connop et al., 1994; MacKenzie et al., 1994). Administration of 10 mg/kg 7-nitroindazole probably only results in a partial inhibition of NO synthase activity in the brain (Babbedge et al., 1993). The inhibition of NO synthase is transient with complete recovery after 24 hours (MacKenzie et al., 1994). The decline in memory performance after systemic administration of both doses of 7-nitroindazole in our study is in agreement with the generally found impaired acquisition, as found in spatial memory tasks, after systemic administration of several nonselective NO synthase inhibitors (Chapman et al., 1992; Estall et al., 1993; Mogensen et al., 1995; Yamada et al., 1995). Moreover, it has recently been reported that i.p. administration of 7-nitroindazole (25–65 mg/kg) impaired spatial acquisition as well (Meyer et al., 1996; Hölscher et al., 1996). Likewise, in studies using local administration of NO synthase inhibitors it was observed that NO synthase blockade in the hippocampus affected working memory, as found in the three-panel runway (Ohno et al., 1993) and processes generated during or shortly after training, as found in passive avoidance learning (Fin et al., 1995). At the same time it has been reported that NO levels in the hippocampus increased immediately after passive avoidance training (Bernabeu et al., 1995). Thus, it has been argued that NO affects mechanisms in the hippocampus that are important for memory consolidation (Bernabeu et al., 1995; Fin et al., 1995).

Biochemical and immunocytochemical studies have provided evidence that activation of soluble guanylyl cyclase and consequently cGMP formation, in the brain may be one of the major effects of NO (De Vente et al., 1990; Garthwaite, 1991; Southam and Garthwaite, 1993). cGMP degradation in the brain is very fast and appears to involve several isoforms of phosphodiesterase. Zaprinast is an inhibitor of the cGMP-selective phosphodiesterase (phosphodiesterase type V) and has been reported to increase cGMP levels in hippocampal slices (Boulton et al., 1994). Recently, we have demonstrated an increase in NO-stimulated cGMP content in axonal fibers in the CA2/CA3 area of the hippocampus of the rat in the presence of zaprinast using hippocampal slices (De Vente et al., 1996). So far the presence of phosphodiesterase-V in the brain has not been reported. Our results showed that there is a very restricted localization of the effects of zaprinast on NO-mediated cGMP accumulation, i.e., no effects of zaprinast were found in the cortex, the caudate putamen and the cerebellum (unpublished data). I.p. administration of zaprinast improved memory performance in our present study and completely attenuated the memory deficits of the additionally administered NO synthase inhibitor 7-nitroindazole. Based on these results, an effect of zaprinast on object recognition memory is likely to involve NO mediated cGMP synthesis in the CA2/CA3 area of the hippocampus. Although zaprinast is regarded to be well able of penetration into the brain (cf., Svenningsson et al., 1995), our assumption needs further and more direct verification in future research by measuring cGMP levels or phosphodiesterase activity in several brain areas after zaprinast treatment.

Recently, studies have appeared in the literature evaluating the role of cGMP in memory processes. In one study using the passive avoidance task with rats, it was found that the level of cGMP in the hippocampus increased immediately after training and that administration of an analogue of cGMP into the hippocampus immediately after the training enhanced memory performance (Bernabeu et al., 1996). In addition, it was found that infusion of a soluble guanylyl cyclase inhibitor immediately after training caused full amnesia (Bernabeu et al., 1997). Furthermore, next to an increase in hippocampal cGMP levels also cGMP-dependent protein kinase activity increased immediately after training (Bernabeu et al., 1997). Thus, it was argued that cGMP is involved in memory consolidation (Bernabeu et al., 1996, 1997). In another study using mice there was an indication that NO synthase inhibition due to systemic 7-nitroindazole administration decreased cGMP levels in the hippocampus and with a spontaneous alternation task it was demonstrated that an analogue of cGMP (i.c.v. administered) attenuated the effects of another systemically administered (nonselective) NO synthase inhibitor on spatial working memory (Yamada et al., 1996). Conceptually our results are in agreement with these recent findings. Because in our studies 7-nitroindazole and/or zaprinast were administered immediately after training, a strong argument has been found to advocate a role of NO-cGMP transduction in the consolidation of information. Taken together it can be argued that the NO-cGMP transduction pathway is involved in processes of memory formation.

4.2. Behavioral side effects

Two object preferences and two location preferences for an object were found. Due to the incidental character of these biases and the difference in objects between these biases, it may be assumed that these biases did not influ-

ence the behavioral data. Normally, locomotor activity decreases after systemic administration of a NO synthase inhibitor (e.g., Sandi et al., 1995) and based on an earlier study it could be assumed that after administration of 30 mg/kg 7-nitroindazole there is a decrease in locomotor activity (Connop et al., 1994). In our study the index measure of habituation of exploratory behavior ($h1$) was not different between the 7-nitroindazole sessions and its control sessions, that is there appears to be no difference in exploratory activity 1 h after control or 7-nitroindazole treatments. Hence, it may be assumed that 7-nitroindazole does not affect behavioral activity. The same accounts for zaprinast as 4 h after its treatment $h1$ is not different between the zaprinast sessions and its control sessions. However, $h1$ showed that 1 h after combined administration of zaprinast and 7-nitroindazole exploratory behavior decreased. There is no adequate explanation for this decrease in exploration. One possible explanation is that the combined treatment of zaprinast and 7-nitroindazole have synergistic effects on blood pressure. Moreover, the index measures of discrimination $d1$ and $d2$, where $d1$ is corrected for exploratory activity, show both the same effects and indicate that zaprinast attenuated the memory deficit after 7-nitroindazole treatment. Thus, this memory performance is apparently not influenced by a decreased exploratory behavior induced by the combined treatment of zaprinast and 7-nitroindazole.

4.3. Cardiovascular effects

It is well established that NO plays a pivotal role in cardiovascular control (Iadecola et al., 1994). In fact the first known function of NO was endothelial cell dependent vasodilatation, explaining its earlier name as endothelium-derived relaxing factor (Palmer et al., 1987). There are two constitutive isoforms of NO synthase that are activated by calcium: neuronal NO synthase and endothelial NO synthase, although the latter can also be found in neurons (Dinerman et al., 1994). The produced NO can exert its vasodilator action both through a peripheral mechanism via its action on the vasculature, or through a central mechanism via perivascular nerves (Dawson et al., 1992; Iadecola et al., 1994). It has been found that systemic administration of nonselective NO synthase inhibitors (e.g., Sandi et al., 1995) increased blood pressure. In the present study we have found that systemic administration of 30 mg/kg 7-nitroindazole increased mean arterial blood pressure. Normally there is a lack of a pressor response in rodents after i.p. administration of 7-nitroindazole, which led to the general assumption that 7-nitroindazole is selective for neuronal NO synthase (Moore et al., 1993; Connop et al., 1994; Kelly et al., 1995), although there is some in vitro evidence that 7-nitroindazole may inhibit endothelial NO synthase (Babbedge et al., 1993). However, closer examination of the in vivo data from rodents suggests that 7-nitroindazole is not totally devoid of cardiovascular effects. For example, in conscious rats i.p. administration of 7-nitroindazole (25–50 mg/kg) had no effect on mean arterial blood pressure, although heart rate decreased immediately (Kelly et al., 1995). In addition, despite the apparent lack of peripheral vasoconstriction, a decrease in cerebral blood flow was measured, indicating a cerebrovascular effect. Thus, based on the putative selectivity of 7-nitroindazole for neuronal NO synthase, it was initially speculated that NO, synthesized and released from neuronal NO synthase (rather than endothelial NO synthase) in the brain, might play an important role in central cardiovascular control by perivascular nerves (Kelly et al., 1995). However, the same results can also be used to argue in the first place that 7-nitroindazole is not totally devoid of vascular effects because, for instance, the decrease in heart rate may be due to the baroreceptor reflex mechanism. Furthermore, the decrease in cerebral blood flow may indicate a direct effect upon endothelial NO synthase to induce vasoconstriction (cf., Kelly et al., 1994). In another study it was reported that 7-nitroindazole (80 mg/kg, i.p.) did not increase mean arterial blood pressure over a 48 h period in conscious rats, although also in this study closer inspection of the data shows that there are at least indications that 7-nitroindazole influences blood pressure transiently (Connop et al., 1994). Moreover, in a recent study it has been demonstrated that 7-nitroindazole (50 mg/kg, i.p.) immediately increased mean arterial blood pressure and decreased heart rate in conscious rats, thus further adding evidence to the assumption that 7-nitroindazole affects directly endothelial NO formation in vivo (Zagvazdin et al., 1996).

It has been found that systemic administration of cGMP-selective phosphodiesterase inhibitors like zaprinast (and also other phosphodiesterase inhibitors) decreased the blood pressure probably by lowering the total peripheral resistance in conscious rats, while other hemodynamic effects are only adventitious, e.g., an increase in heart rate is due to a reflexive mechanism (Dundore et al., 1992, 1993). Surprisingly, a slight increase in mean arterial blood pressure after administration of 10 mg/kg zaprinast has been found in the present study. Normally, a depressor response after systemic administration of zaprinast is found, but at doses that are above 10 mg/kg zaprinast (Dundore et al., 1991, 1992, 1993). Closer examination of the available data revealed that a slight pressor response after administration of low doses of zaprinast (≤ 10 mg/kg) has also been observed (Dundore et al., 1991, 1992, 1993). The mechanism by which zaprinast elevates mean arterial blood pressure is not clear, but it might be due to ancillary pharmacological effects of zaprinast itself rather than reflexive mechanisms opposing an initial vasodilatation (Dundore et al., 1991, 1992, 1993).

It could be assumed that the vascular effects may have influenced behavioral and memory performance (cf., Sandi et al., 1995). Administration of 10 mg/kg 7-nitroindazole had no real effect on mean arterial blood pressure, while

30 mg/kg 7-nitroindazole increased mean arterial blood pressure. However, both doses of 7-nitroindazole affected memory performance similarly. Administration of 10 mg/kg zaprinast induced an increase in mean arterial blood pressure, while this dose of zaprinast improved memory performance. On basis of these data it is unlikely that effects on blood pressure after zaprinast treatment contributed to the memory improvement of zaprinast.

4.4. Conclusions

Recent studies have shown that 7-nitroindazole impaired the acquisition of spatial tasks (Meyer et al., 1996; Hölscher et al., 1996). Also, 7-nitroindazole prevented the induction of hippocampal long-term potentiation in vivo (Doyle et al., 1996). Assuming that the synaptic plasticity of the hippocampus underlies memory formation it was, therefore, suggested that neuronal NO synthase plays a role in memory processes. However, because of the increase in mean arterial blood pressure after 7-nitroindazole administration in the present study, the selectivity of 7-nitroindazole for neuronal NO synthase can be questioned. In a study using mutant mice lacking neuronal NO synthase, it was found that hippocampal long-term potentiation could still be induced (O'Dell et al., 1994). In addition, it has been found that a functional (membrane-targeted) endothelial NO synthase is required for long-term potentiation (Kantor et al., 1996). These findings suggested that endothelial NO synthase, rather than neuronal NO synthase, generates NO within the postsynaptic cell during long-term potentiation. Moreover, using (immuno)histochemical methods it has been demonstrated that endothelial NO synthase is highly concentrated in hippocampal neurons in contrast to neuronal NO synthase which was only sporadically found in interneurons (Dinerman et al., 1994). Hence, it can be argued that endothelial NO synthase is involved in memory processes. However, one has to realize that the maximum inhibition of NO synthase in brain structures including the hippocampus, is never fully complete after systemic administration of 7-nitroindazole (maximally about 80% inhibition: Babbedge et al., 1993; Connop et al., 1994; MacKenzie et al., 1994; Tobin et al., 1995). Possibly the remaining NO synthase activity could still be sufficient for long-term potentiation induction and memory formation. The question which constitutive isoform of NO synthase, neuronal or endothelial, is involved in memory processes should be addressed in future research using more specific types of NO synthase inhibitors. Recently, it has been reported in a study using knock-out mice for endothelial NO synthase and/or neuronal NO synthase that only long-term potentiation was reduced in the double knock-out mice (Son et al., 1996). This indicates that both isoforms of constitutive NO synthase can compensate for each other in the single knock-outs. Moreover, it suggests that both isoforms can be involved in memory processes, though it has very recently been argued that the contribution of neuronal NO synthase to long-term potentiation would be minimal (Wilson et al., 1997).

Our results suggest that cGMP is also involved in processes of memory formation. However, it is not clear how cGMP exerts its action in this respect. Several mechanism of action of cGMP have been suggested to explain the role of cGMP in synaptic plasticity (Schuman and Madison, 1994; Garthwaite and Boulton, 1995). For example, cGMP is thought to act through regulation of cGMP-gated ion channels, regulation of cAMP-phosphodiesterases or activation of cGMP-dependent protein kinases (Schmidt et al., 1993; Zhuo et al., 1994; Arancio et al., 1995; Zufall, 1995). The latter has recently been corroborated by the finding that cGMP levels and cGMP-dependent protein kinase activity were increased in the hippocampus after passive avoidance training (Bernabeu et al., 1997). Whether and how the possible actions of cGMP could result in synthesis and/or release of neurotransmitter, and thereby explain the maintenance of long-term potentiation, is still not clear.

Our behavioral results are in agreement with the generally found results on memory performance after systemic administration of NO synthase inhibitors. However, the possible role of NO and cGMP in memory formation has also been questioned, because it has sometimes been found that systemic administration of NO synthase inhibitors did not affect acquisition in spatial memory tasks (Bannerman et al., 1994a; Tobin et al., 1995). It was concluded that the behavioral effects after NO synthase inhibition may be a result of or be affected by nonspecific (physiological) effects (Bannerman et al., 1994a; Tobin et al., 1995). Nonspecific effects could be related to a decrease in locomotor activity and/or an increase in blood pressure. However, in the present study the behavioral data appeared not to be influenced by these effects. Nevertheless, in future behavioral studies focussed on learning and memory processes, it is better to use local instead of systemic administration to minimize effects on for instance locomotor and/or blood pressure (cf., Sandi et al., 1995). But even data obtained after administration of NO synthase inhibitors into the brain data can be controversial as also no or only a moderate effect was found, when comparing several studies with each other, even with relation to the same behavioral task (e.g., passive avoidance task Fin et al., 1995; Huang and Lee, 1995; Toyoda et al., 1996). This could be due to differences in animal (rat versus mouse), differences in location of administration, type (selectivity) or dose of NO synthase inhibitor. In addition, it has been found that hippocampal long-term potentiation could still be induced after inhibition of NO synthase (Bannerman et al., 1994b; Bannerman et al., 1995) or soluble guanylyl cyclase (Boulton et al., 1995), although long-term potentiation was not maximal anymore. This can be interpreted as long-term potentiation has a component that involves neither NO nor cGMP (cf., Boulton et al., 1995; Garthwaite

and Boulton, 1995). Based on these findings it can be argued that the function of the NO-cGMP transduction pathway in synaptic plasticity is probably only a modulating one.

In conclusion, NO synthase and cGMP are possibly involved in recognition memory processes independently of their cardiovascular effects. The present study also demonstrated that 7-nitroindazole is not a selective inhibitor of neuronal NO synthase. Future research is needed to demonstrate which isoform of constitutive NO synthase, neuronal or endothelial, is involved in memory formation, and how cGMP is involved.

Acknowledgements

The authors would like to thank A.M. Teuben and W. Honig for carrying out a part of the experimental work and C.M. Eerdmans from the Department of Pharmacology for helping with the blood pressure measurements. Furthermore, we thank Dr. W. Raaijmakers for his contribution to the initial experimental set up. Finally, in particular we thank Dr. A. Blokland for his helpful and inspiring comments.

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