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Effect of angiotensin IV on the acquisition of the water maze task and ryanodine channel function

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Abstract

In the present study, we investigated the effect of angiotensin IV (Ang IV) on the acquisition of spatial task by rats, expression and function of ryanodine receptors (RyRs) and on Ca^{2+} transport in microsomal membranes isolated from rat hippocampus, the brain structure essential for spatial memory.

Wistar rats, injected intracerebroventricularly with 1 nmol of Ang IV or saline were subjected to the water maze training using hidden (learning) or visible (nonlearning) escape platform. Rats showed overall good acquisition of the task and mean escape latency decreased from 55 s to less than 10 s during the 5-day training. Learning significantly increased [^{3}H]-ryanodine binding to microsomal RyRs and markedly decreased both receptor affinity constant for the ligand and microsomal Ca^{2+} uptake.

Ang IV was without effect on the rate of acquisition of the spatial task but increased (by 47%) maximal ryanodine binding in hippocampal microsomes of the trained rats. The peptide, however, did not affect decreased net Ca^{2+} uptake in rats subject to learning procedure. Since microsomal Ca^{2+}-ATPase activity was similar in all tested groups, the lower net Ca^{2+} uptake in the trained rats could be attributed to the elevated expression of RyRs and resulting to increased Ca^{2+} release.
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Keywords: Angiotensin IV; Spatial memory; Ryanodine receptors; Calcium; Hippocampus; Rats

1. Introduction

The brain rennin–angiotensin system regulates numerous important physiological functions, including cognitive processing (Braszko, 1996; Wright and Harding, 1997). The proteolytic degradation of angiotensin I resulting in formation of angiotensin II and III is mostly known to influence water balance, blood pressure and sexual behavior, while the hexapeptide angiotensin IV (Ang IV), acting via AT_4 receptor, facilitates learning (Braszko et al., 1988; Von Bohlen Und Halbach, 2003), reverses scopolamine-induced memory deficits (Pederson et al., 2001) and is associated with long-term potentiation (LTP) (Wayner et al., 2001). AT_4 receptors are distributed mostly in the mammalian hippocampus, neocortex and cerebellum (Chai et al., 2000), the brain structures engaged in consolidation of transient sensory experiences into long-term memory (Curtis et al., 2000). It was shown that AT_4 receptor antagonists block spatial memory acquisition in water maze (Wright et al., 1999), but the molecular mechanism of AT_4 receptor activation is not known. Short stimulation of AT_4 receptors does not affect classical second messengers (Briand et al., 1998). Other data show that Ang IV may alter Ca^{2+} homeostasis in cultured nonneuronal cells (Chen et al., 2000; Handa, 2001).

An increase in cytoplasmic Ca^{2+} in hippocampal neurons plays an important role in the regulation of neuronal processes, including synaptic transmission and synaptic plasticity such as LTP and long-term depression (Balkowiec and Katz, 2002; Nishiyama et al., 2000). Neural cells have at least two mechanisms of intracellular Ca^{2+} release: stimulation of inositol 1-,4-,5-triphosphate receptors and activation of ryanodine receptors (RyRs). It was shown that hippocampal RyRs, particularly microsomal type-2 ryanodine receptor (RyR_2) isoform, is up-regulated in the hippocampus of rats intensely trained in a water maze (Zhao et al., 2000). Moreover, various associative learning forms corre-

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late with increased expression of the Ca^{2+}-releasing RyR (Alkon et al., 1998; Blackwell and Alkon, 1999). RyRs-mediated calcium release participates in LTP, spatial learning and changes in synaptic plasticity (Balschun et al., 1999) and increases after activation of *N*-methyl-D-aspartate receptors and the acetylcholine muscarinic receptors (Power and Sah, 2002; Raymond and Redman, 2002). RyR type 3 (RyR_3)-deficient mice exhibited impairments of performance in the contextual fear conditioning test, passive avoidance test and Y-maze learning test (Kouzu et al., 2000), and it was suggested that RyR_3-mediated intracellular Ca^{2+} release from endoplasmic reticulum (ER) may inhibit hippocampal LTP and spatial learning (Futatsugi et al., 1999).

In the present study, we investigated spatial learning and Ca^{2+} transport mechanisms in the ER of rat hippocampus after intracerebroventricular Ang IV administration to rats.

2. Materials and methods

2.1. Subjects

The experimental procedures were carried out in accordance with the National Institutes of Health Guide for Care and Use of Laboratory Animals (Publication No. 85-23, revised 1985) and were approved by the local Ethics Commission for Animal Experimentation. Male Wistar rats (160–180 g) were housed in groups of eight in the plastic cages at 22 °C with a 12:12-h light–dark cycle. All animals were provided ad libitum access to water and laboratory rat chow. The experiments were conducted between 10:00 a.m. and 3:00 p.m. A 30-min habituation period to the experimental room preceded all the behavioral experiments. There were four experimental groups, 12 rats each. Fifteen minutes before the first water maze learning or nonlearning session on Day 1, 24 animals were injected intracerebroventricularly (right side) with saline and another 24 rats (two groups with Ang IV) were injected with Ang IV (1 nmol in 2 μl of saline).

2.2. Surgery

Under light ether anesthesia, a round piece of skin, 7 mm in diameter, was cut off the rat's head and the underlying skull surface was cleaned from soft tissue. A burr hole, 0.5 mm in diameter, was drilled in the skull 2.5 mm laterally and 1 mm caudally from the point of intersection of the bregma and the superior sagittal suture on the right side of the head. The operation took about 2 min, and after a 48-h recovery, the wound was completely dry and the animal behaved normally. On the following day (i.e., 3 days after surgery), the intracerebroventricular injections were made freehand into the right lateral cerebral ventricle with a 10-μl Hamilton syringe using a removable KF 730 needle cut 40.5 mm from its base. This procedure allowed lowering the tip of the needle about 0.5 mm below the ceiling of the cerebral ventricle. It was relatively nontraumatic as the animal, gently fixed by the bare hand of the experimenter, was usually quiet and no vocalization occurred. The injection volume was 2 μl administered over 3 s. Upon completion of each experiment, all rats were sacrificed and the sites of injections were verified microscopically after brain sectioning.

2.3. Water maze procedure

The water maze procedure allows assessment of spatial learning and memory in rats using their natural aversion for water (Morris, 1984). The rat should find and climb a circular platform (9 cm in diameter) that is submerged in water and its escape latency is recorded. Our water maze was a circular tank 180 cm in diameter, filled with 36-cm deep warm (26 °C) water that was made opaque by the addition of brown color stain (grain instant coffee). The maze was sectioned into four equal quadrants of SW, SE, NW and NS. There were four experimental groups of 12 rats each. Two of them, called learning groups, underwent the procedure whereby each rat was placed in the maze at the point S and allowed to swim for 60 s or until it found the hidden escape platform positioned 1 cm below the water surface. The rat could rest on the platform for 15 s. Such trial was repeated six times with each rat and the position of the platform was alternated between NW and NE quadrants. Each rat had the platform kept in the same quadrant for the all five daily six-trial sessions. During the intertrial (10–15 min) intervals, the animals were towel-dried and moved to a heated recovery cage. Fifteen minutes before the first session on Day 1, the animals were injected with saline (Group III) or Ang IV (Group IV) as described. The remaining two unlearning groups (Groups I and II) underwent the same procedure except for the platform, which was made visible and placed in another quadrant (of four) in every trial. In such a way, rats of the nonlearning groups had exactly the same swimming conditions except for they did not have to learn anything. The animals of the nonlearning groups that could not find the platform within the period of time, which was the mean of the swimming times found in the learning groups run in parallel, were removed from the water and placed on the platform for 15 s. Escape latencies were expressed as the mean time of the six trials per animal per day.

2.4. Hippocampal dissection

One hour after the last water maze session, rats were anesthetized with halothane and were decapitated. After the brain was removed, it was placed immediately into chilled glass plate and a midsagittal section was made using a Teflon-coated spatula to gently remove the hippocampus. Isolated rat hippocampi were frozen on dry ice and stored at − 80 °C before use.

2.5. Isolation of microsomal membranes

Rat hippocampi were individually briefly sonicated in ice in 5 mM HEPES buffer, pH 7.4 containing 0.32 M sucrose and protease inhibitors. Microsomal membranes were isolated by differential centrifugation and resuspended in homogenization buffer (Zhao et al., 2000).

2.6. [^{3}H]-ryanodine binding

[^{3}H]-ryanodine equilibrium binding was determined using a mixture containing NaCl (0.5 M), HEPES (20 mM), EGTA (1 mM), [^{3}H]-ryanodine (5–500 nM; specific activity 56.0 Ci/mmol; NEN Life Sci. Products), microsomal suspension (0.5 mg protein per ml) and 0.2 mM [^{1}H]-ryanodine to determine nonspecific binding (Martinez-Merlos et al., 1997). Samples were incubated in triplicate for 60 min in the dark at 37 °C, vacuum filtrated onto polyethylenimine-treated Whatman GF/B glass fiber filters and washed with 4 ml of ice-cold wash buffer (5 mM Tris/HCl pH 7.0, 0.5 M KCl, 250 μM $CaCl_2$). The radioactivity remaining on the filters was quantified in a scintillation counter (Beckman 6500), the binding data were shown to follow Michaelis–Menten kinetics, and were fitted to a one-site model with Origin 6.1 software (Microcal, Northampton, MA, USA) using nonlinear regression and Scatchard–Rosenthal analysis. The affinity constant of the [^{3}H]-ryanodine-RyR complex (K_d) and the maximal density of binding sites (B_{max}) values were obtained from saturation binding curves.

2.7. Measurement of Ca^{2+} transport

Ca^{2+} uptake and release by microsomes was monitored using a double beam spectrophotometer (Perkin Elmer EZ201) through the differential absorption changes of antipyrylazo III at 720–790 nm (Gilchrist et al., 1997). In a typical experiment, Ca^{2+} fluxes were measured in microsomal suspension (0.2 mg protein per ml) in 0.1 M phosphate buffer (pH = 7.0) containing KCl (0.1 M), HEPES (20 mM), Mg^{2+} (0.5 mM), ATP (0.5 mM) and an ATP-regenerating system consisting of creatine phosphokinase (30 U/ml) and creatine phosphate (8 mM). Experiments were initiated by the addition of $CaCl_2$ (final Ca^{2+} concentration of 0.06 mM) and changes in absorbance of antipyrylazo III were plotted against time.

2.8. Ca^{2+}-ATPase activity

The Ca^{2+}-ATPase activity was determined by measuring the accumulation of inorganic phosphate during the hydrolysis of ATP (Srivastava et al., 1990). Microsomes (0.1 mg/ml) were incubated for 20 min at 37 °C in HEPES (0.05 M, pH = 7.0), containing ATP (1 mM), ouabain (0.2 mM), $CaCl_2$ (0.06 mM), alamethacin (0.001 mg/ml) and $MgCl_2$ (0.5 mM). The reaction was stopped by addition of 0.3 ml of 20% (w/v) TCA and, after centrifugation (3 min, 5000 × *g*), an aliquot of 0.1 ml was transferred into a solution of 0.034% malachite green (0.8 ml, in 1 M HCl). The color development was fixed by addition of 0.1 ml of 1% ammonium molybdate and absorbance was determined at 640 nm.

2.9. Statistical analyses

Statistical analyses were performed with statistical package Statistica (Statsoft, Cracov, Poland) using one- and two-way ANOVAs and regression analysis. For equilibrium binding data, nonlinear regression (Origin 6.1) was used and the goodness of fit of the predicted value from different equations to the data set was determined by chi-square analysis. *P* values less than .05 were considered statistically significant.

3. Results

Rats of Groups III and IV showed overall good acquisition of the escape platform location irrespectively on Ang IV administration, and there were no observable differences in swimming speed in both groups of animals. In both groups of the learning rats, the mean escape latencies decreased from about 55 s on the Day 1 to less than 10 s on Day 5, indicating that rats effectively memorized the location of the hidden escape platform. The escape latency slopes decreased with trial length fitting first order process and regression analysis of semilog plots (Figs. 1 and 2) followed by statistical comparison of slopes and intercepts (two-way ANOVA) indicated that Ang IV administration was without effect on the memory acquisition (P>.05).

Activation of RyRs can be tested with [^{3}H]-ryanodine since the ligand binds the receptor only when the channel is in open conformation. [^{3}H]-ryanodine bound to the hippo-

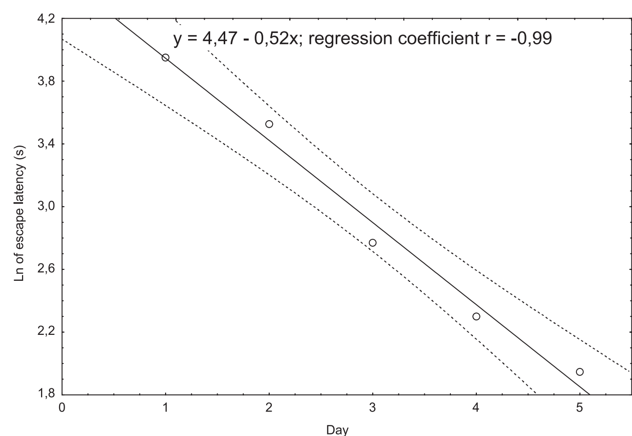

Fig. 1. Regression analysis of escape latencies of rats (Group III) tested in the water maze during five consecutive days of learning using the hidden platform. Rats received NaCl intracerebroventricularly as described in the Materials and methods section.

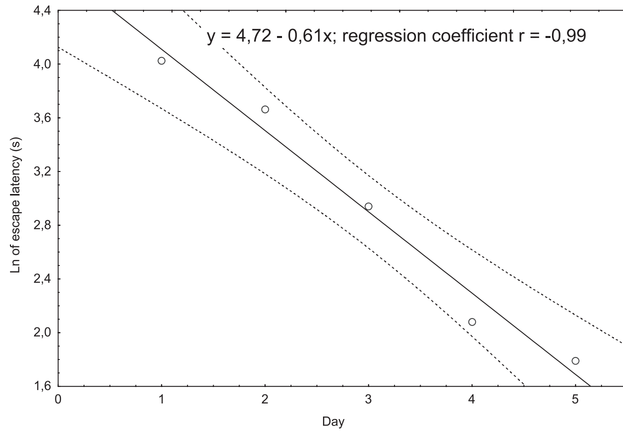

Fig. 2. Regression analysis of escape latencies of rats (Group IV) tested in the water maze during five consecutive days of learning using the hidden platform. Rats received 1 nmol Ang IV intracerebroventricularly as described in the Materials and methods section.

campal microsomes isolated from control rats (Group I) with estimated $K_d = 103$ nM and $B_{max} = 655$ fmol/mg of protein, and Ang IV administration did not significantly affect both saturable binding parameters (Group II $B_{max} = 730$ fmol/mg protein, $K_d = 122$ nM; Fig. 3). Straight regression lines (insets) of Rosenthal plots (regression coefficients: −.94; −.98; −.88; −.72 for data from Groups I, II, III and IV, respectively) suggest single site binding for the ligand. Training significantly increased (by 80%, $P < .05$) maximal receptor occupancy (Group III; $B_{max} = 1121$ fmol/mg protein) and decreased (by 47%, $P < .05$) its affinity constant (Group III; $K_d = 55$ nM) comparing to values of Group I. In rats subjected to spatial learning that received Ang IV (Group IV), B_{max} increased by about 1.5-fold (1611 fmol/mg protein, $P < .05$) comparing to control rats (Group I) with slightly increased affinity to RyR (K_d decreased by 23%). [^{3}H]-ryanodine affinity to its receptor increased with learning by 46% ($P < .05$) in groups receiving saline (Group I vs. Group III) and by 35% in groups receiving Ang IV (Group II vs. Group IV, $P < .05$). B_{max} in Group IV was significantly higher ($P < .05$) than in Group III.

Hippocampal microsome Ca^{2+} fluxes were measured under conditions of active vesicle loading in the presence of ATP-regenerating system (Fig. 4). The addition of $CaCl_2$

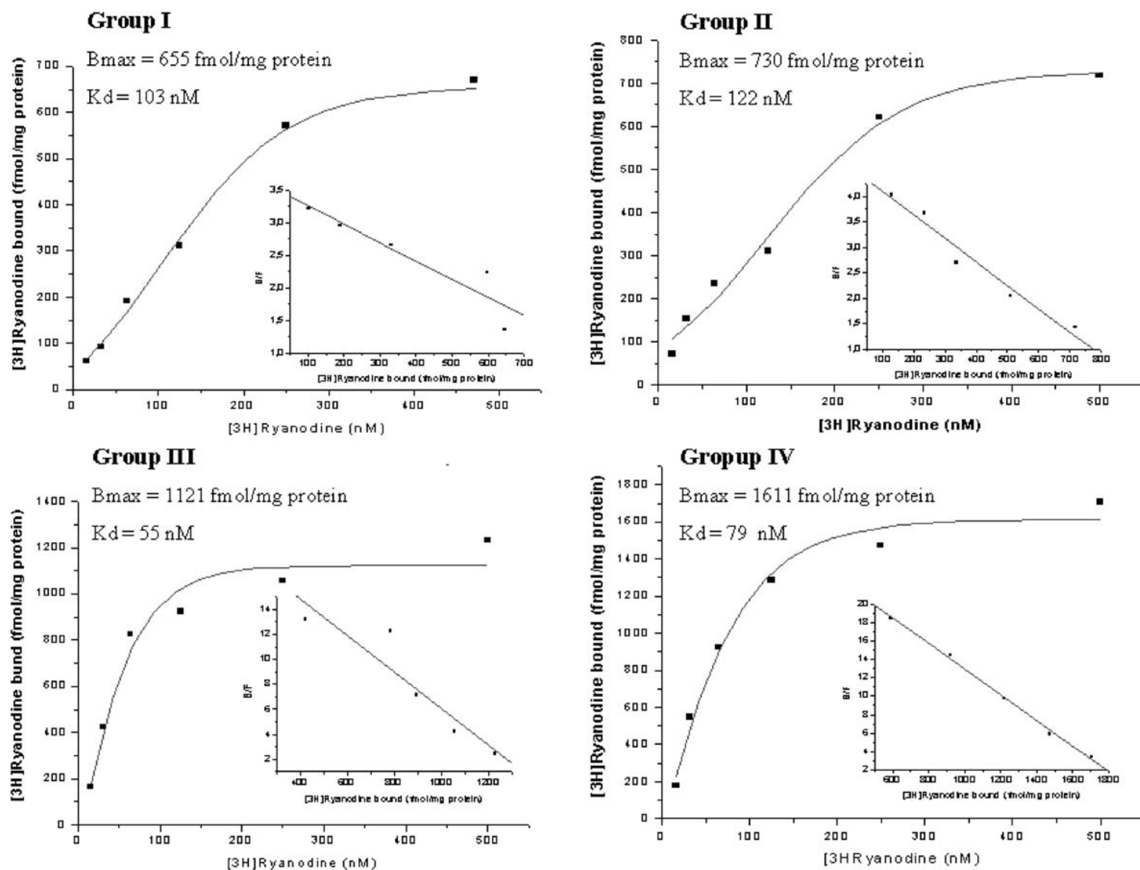

Fig. 3. Representative isotherms of [^{3}H]-ryanodine binding to microsomal fractions isolated from hippocampi of control rats (Group I), animals receiving 1 nmol icv Ang IV (Group II), rats subjected to spatial learning in the water maze (Group III), injected with saline (Group III) and animals trained as Group III but received 1 nmol icv Ang IV (Group IV). The equilibrium binding constants were obtained with nonlinear regression analysis. Statistically significant differences ($P < .05$) in maximal ligand binding and affinity constant were found between Groups I–III; I–IV; II–III; II–IV and in maximal ligand binding between Groups III–IV.

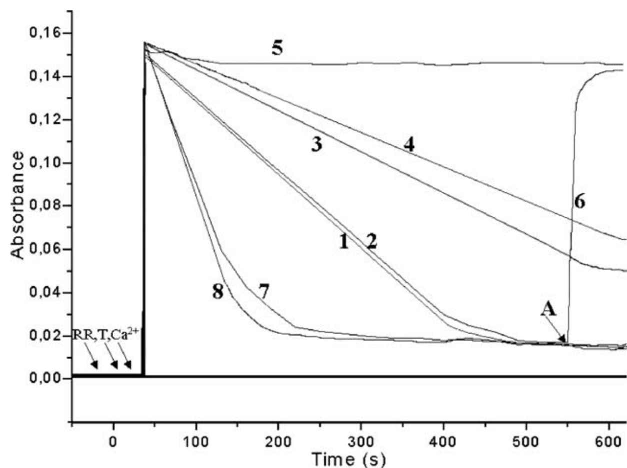

Fig. 4. Representative plots of Ca^{2+} uptake in hippocampal microsomes isolated from control rats (Group I, Trace 1), animals receiving 1 nmol icv Ang IV (Group II, Trace 2), rats subjected to 5-day space learning trials in the water maze (Group III, Trace 3) and trained animals receiving Ang IV (Group IV, Trace 4). Ca^{2+} addition instantly increased absorbance of Ca-antipyrylazo III and then absorbance decreased with time due to Ca^{2+} uptake by microsomal vehicles. Calcium fluxes were governed by Ca^{2+}, Mg^{2+}-ATPase [Trace 5 shows enzyme inhibition by 1 μM T, a specific ATPase inhibitor] and by Ca^{2+}-releasing ryanodine channel [Traces 7 and 8 show the effect of 2 μM RR, a compound blocking RyRs]. Ca^{2+} ionophore-A23187 (3 μg/ml, Trace 6) was used to release accumulated Ca^{2+} to assess maximal microsomal Ca^{2+} load in each assay. Experiments were repeated three times with similar results. Statistically significant differences ($P<.05$) in calcium fluxes were found between Groups I–III; I–IV; II–III; and II–IV.

(100 μM) to microsomal suspension resulted in a rapid increase of the absorbance due to formation of a Ca^{2+}-AP-III complex. Then, the absorbance decreased to its initial value as a result of the Ca^{2+}-ATPase-driven transport of Ca^{2+} into the microsomal vehicles. The transport equilibrium was driven by Ca^{2+}, Mg^{2+}-ATPase-mediated uptake and RyR-mediated release since no decrease in the absorbance was observed in the absence of ATP (data not shown). One micromolar tapsigargin (T), a specific ATPase inhibitor that completely blocked microsomal vesicle loading when added just before Ca^{2+} addition [Trace 5. Ca^{2+} ionophore-A23187 (A, 3 μg/ml)], was added at the end of each pumping experiment to release accumulated calcium and to compare microsomal membrane Ca^{2+} loadings (Trace 6). Hippocampal microsomes derived from rats subjected to spatial learning (Group III; Trace 3) significantly differ ($P<.05$) in calcium fluxes from those isolated from non-trained rats (Group I; Trace 1). Ang IV administration was without significant effect on the net calcium uptake dynamics in naïve rats (Trace 1-Group I vs. Trace 2-Group II) and only slightly decreased net calcium uptake in space learning rats (Trace 3-Group III vs. Trace 4-Group IV, about 15% decrease). Two micromolars of ruthenium red (RR), a compound blocking RyRs, significantly affected calcium uptake dynamics [decreased net uptake by 30% and 54% for Group II (Trace 7; $P<.05$) and Group IV (Trace 8; $P<.05$)].

Microsomal Ca^{2+}-ATPase activity was similar in all microsomal fractions (results not shown).

4. Discussion

Angiotensin and its proteolytic degradation products are acting via diverse angiotensin receptors on cerebral blood flow and angiogenesis, but also on memory acquisition and retrieval (Wright and Harding, 1997). We have previously shown that single intracerebroventricular injection of angiotensin II or different angiotensin-derived peptides, including the angiotensin II C-terminal (3-8) degradation fragment-Ang IV, affects cognitive performance of rats (Braszko et al., 1988, 1995; Braszko, 1996). These results were confirmed and further extended by other studies (Wright et al., 1993; Tchekalarova et al., 2001; Pederson et al., 2001), and there is now considerable evidence for an important role of Ang IV in mediating learning and memory in mammalian brain.

In the present study, quite unexpectedly, we were unable to observe any major influence of Ang IV on the rate of learning of a water maze task. This is in contrary to the other studies (Pederson et al., 1998, 2001) wherein 1 nmol of norleucine[1]-Ang IV, a stable analog of Ang IV infused into the lateral brain ventricle, significantly decreased mean swim latency and shortened the mean path distance to the submerged platform thus showing improvement of spatial memory in rats. The close analysis of the procedures used by these authors revealed some differences that may account for different results obtained in their studies. First, working (Pederson et al., 1998, 2001) versus reference (this study) memories were tested as indicated by changing, respectively, from trial-to-trial versus stable starting points for swimming rats. Second, 30-s intracerebroventricular infusion by Pederson et al. (1998, 2001) versus 3-s intracerebroventricular injection (this study) of the peptide was employed possibly, making its action more effective in the former studies. In addition, the norleucine[1]-Ang IV is certainly more stable than native Ang IV. Finally, Sprague–Dawley rats used by Pederson et al. (1998, 2001) might be far more sensitive than our Wistar rats to the procognitive action of Ang IV as strain-dependent differences in the expression of angiotensin receptors were found (Braszko et al., 2000). Also, a test-specific involvement of different angiotensin receptors in memory reflecting possibly differences in their distribution in various brain areas was already described (Braszko et al., 1995; Braszko, 2002).

Published data indicate that Ang IV increases intracellular calcium concentrations in cultured epithelial cells (Chen et al., 2000; Handa, 2001) and AT_4-mediated calcium signaling appear to operate also in neural cells. Elevation of intracellular Ca^{2+} in postsynaptic neurons is achieved through Ca^{2+} channels localized on the plasma membrane and via Ca^{2+} release from intracellular stores. This increase in intracellular Ca^{2+} is associated with induction of LTP, widely recognized neuronal substrate of learning and mem-

ory (Balkowiec and Katz, 2002) and with altered gene expression necessary for long-term memory formation (Cavallaro et al., 2002). It is suggested that AT_4 agonists facilitate LTP (Wayner et al., 2001); thus, Ca^{2+} signaling appear to make a link between memory and the hippocampal synaptic transmission.

The RyR, one of the two major intracellular Ca^{2+} channels highly expressed in subregions of the hippocampus, has been an important target for studying Ca^{2+} signaling in learning and memory. Immunohistochemistry, hybridization and agonist binding data show that rat hippocampal RyR_2 mRNA and RyR_2 protein significantly increase after spatial learning (Zhao et al., 2000). Our present study is aimed at assessing the influence of spatial learning and/or Ang IV on RyRs density, affinity and Ca^{2+}-ATPase/RyR-mediated Ca^{2+} transport in rat hippocampal ER.

Under resting conditions, the concentration of Ca^{2+} in the ER lumen is considerably higher than the Ca^{2+} concentration in the cytoplasm and this gradient is maintained by an ATP-dependent Ca^{2+} pump. This pump was functional in isolated microsomal fractions as evidenced using specific ATPase inhibitor—tapsigargin, which completely blocked Ca^{2+} uptake by isolated hippocampal microsomes. The net Ca^{2+} uptake was driven also by Ca^{2+}-releasing channels because RR, an inhibitor of RyRs, significantly increased microsomal Ca^{2+} uptake. Significantly lower Ca^{2+} uptake was observed in microsomes isolated from rats subjected to spatial learning than in microsomal preparations from the control rats. At the same time, microsomal Ca^{2+}-ATPase activity was unchanged and it may indicate that reduced net Ca^{2+} uptake may be a consequence of increased Ca^{2+} release via RyRs. It is known that spatial learning increases RyRs density (Cavallaro et al., 1997; Zhao et al., 2000). Our ryanodine binding data also showed increased RyRs expression and increased RyRs affinity for the ligand in rats subjected to spatial learning. In brain tissue, there are three isoforms of RyRs with RyR_2—the most frequently found in different brain areas and RyR_3—highly expressed in the hippocampus (Giannini et al., 1995). It is known that deletion of RyR_3 results in a reduced flexibility in relearning a new target in the water maze, even if learning performance in the acquisition phase and during probe trial did not differ between the mutants and their wild-type littermates (Balschun et al., 1999). Other data indicate that RyRs function changes in passive avoidance conditioning (associative memory) (Alkon et al., 1998; Blackwell & Alkon, 1999) and in formation of long-term memory (Sun et al., 1999). Increased affinity for the ligand may be related to altered expression of diverse isotype RyRs and/or by conformational changes of RyRs. Since RyRs affinity for their ligand increases with rising intraluminal Ca^{2+} (Shmigol et al., 1995), it is possible that calcium-dependent activation of RyRs in hippocampal ER may play a role in spatial memory formation.

Our results showed that single intracerebroventricular administration of Ang IV to rats before the first learning trial was without effect both on initial acquisition of the escape response and on the terminal rat performance since animals treated with Ang IV learned the task at a rate comparable to controls. This is in contrast with other data where Ang IV or its more stable analog—Norleucine(1)-Ang IV—was chronically infused via an osmotic pump (Wright et al., 1999). In this study, prolonged delivery of Norleucine(l)-Ang IV facilitated acquisition of the circular water maze task but only during the initial 2 days of training. Due to short half-life of Ang IV, it is possible that effects of single peptide administration are weak and hardly detectable. On the other hand, in our previous studies, single administration of 1 nmol Ang IV significantly increased acquisition of conditioned avoidance responses (Braszko et al., 1988). Other data indicate that even lower (10 pmol) doses of Norleucine(1)-Ang IV normalize acquisition of the circular water maze tasks altered by scopolamine (Pederson et al., 2001). Both bolus (Braszko et al., 1988; Tchekalarova et al., 2001) and prolonged infusion of Ang IV (Wright et al., 1993) have been reported to improve retention in a passive avoidance situation. Our results indicate that in contrast to the increased maximal ryanodine binding after Ang IV, behavioral effects of the peptide in a water maze are rather subtle. These results neither exclude nor support the possibility of Ang IV involvement in the mechanisms controlling spatial memory. Cognitive processes, which can be modulated by several neurotransmitters, appear to depend on the complex harmonious activity of the angiotensin peptides acting at the different receptor subpopulations including AT_1, AT_2 and AT_4.

Acknowledgements

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