www.elsevier.com/locate/neuropharm

Potentiation of cholinergic transmission in the rat hippocampus by angiotensin IV and LVV-hemorphin-7

Joohyung Lee a, b, Siew-Yeen Chai a, Frederick A.O. Mendelsohn b, Margaret J. Morris b, Andrew M. Allen a,*

a The Howard Florey Institute of Experimental Physiology and Medicine, University of Melbourne, Melbourne, Victoria 3010, Australia
b Department of Pharmacology, University of Melbourne, Melbourne, Victoria 3010, Australia

Received 29 June 2000; received in revised form 10 October 2000; accepted 18 October 2000

Abstract

Recent evidence demonstrates that the fragment of angiotensin II, angiotensin II (3–8) termed angiotensin IV, binds with high affinity to a specific binding site, the AT4 receptor. Intracerebroventricular injection of AT4 receptor agonists improves the performance of rats in passive avoidance and spatial learning paradigms. AT4 receptors and cholinergic neurons are closely associated in regions involved in cognitive processing, such as the hippocampus and neocortex. We therefore postulated that AT4 receptors affect cognitive processing by modulating cholinergic neurotransmission. To test this, we examined the effect of AT4 receptor ligands, angiotensin IV and LVV-hemorphin-7, on potassium-evoked [3H]acetylcholine ([3H]ACh) release from rat hippocampal slices. Hippocampal slices from male Sprague–Dawley rats were incubated with [3H]choline chloride, perfused with Krebs–Henseleit solution and [3H]ACh release was determined. Angiotensin IV and LVV-hemorphin-7 both potentiated depolarisation-induced [3H]ACh release from rat hippocampal slices. Potentiation of release by both agonists was attenuated by the AT4 receptor antagonist, divalinal-Ang IV. Angiotensin IV-induced potentiation was not affected by AT1 and AT2 receptor antagonists. These results indicate that stimulation of AT4 receptors can potentiate depolarisation-induced release of ACh from hippocampal slices and suggest that potentiation of cholinergic transmission may be a mechanism by which AT4 receptor ligands enhance cognition. © 2001 Elsevier Science Ltd. All rights reserved.

Keywords: AT4 receptor; Ang IV; LVV-hemorphin-7; Acetylcholine; Memory

1. Introduction

Recent evidence suggests that angiotensin II (3–8), termed angiotensin IV (Ang IV), plays a role in memory and learning: intracerebroventricular administration of Ang IV and its analogues potentiate learning in associative and spatial tasks (Braszko and Wisniewski, 1988; Wright et al., 1993, 1999; Pederson et al., 1998). Furthermore, ivc injections of Ang IV stimulate c-fos expression in the CA2–CA3 fields of the rat hippocampus, an area thought to be involved in memory formation (Roberts et al., 1995).

Ang IV binds with high affinity to a pharmacologically distinct binding site which was initially characterized in the bovine adrenal (Swanson et al., 1992). This site has been termed the AT4 receptor (de Gasparo et al., 1995). In addition to Ang IV, the AT4 receptor also binds the decapptide, LVV-hemorphin-7, which we isolated from the sheep brain (Moeller et al., 1997). LVV-hemorphin-7 binds to the AT4 receptor with high affinity (IC50=4.15 nM), and is present in high concentrations in the central nervous system (Moeller et al., 1997). Hence, LVV-hemorphin-7 may be an endogenous ligand for the AT4 receptor in the brain.

The AT4 receptor is widely distributed in the central nervous system (Miller-Wing et al., 1993; Moeller et al., 1996), being abundant in motor nuclei and in areas associated with cognitive and sensorimotor functions, such as the hippocampus, neocortex and cerebellum (Miller-
Wing et al., 1993; Moeller et al., 1996). Of particular interest is the occurrence of high AT₄ receptor densities in areas of basal forebrain cholinergic nuclei and their terminal fields, including the medial septal complex, the basal nucleus of Meynert, the CA1 and CA3 fields of the hippocampus, the dentate gyrus and the neocortex (Miller-Wing et al., 1993; Moeller et al., 1996).

Considerable evidence implicates cholinergic neurons in cognition (for review see Woolf, 1998). Cholinergic neurons, located in the medial septal nucleus and in the nuclei of the diagonal band, project to the hippocampal formation (Woolf et al., 1984). This septo-hippocampal cholinergic projection has a critical role in the generation and maintenance of theta rhythms, which are functionally important in learning and memory (Vinogradova, 1995). Many studies have demonstrated that anticholinergic agents such as scopolamine and atropine can disrupt both the acquisition and performance of a variety of learned behaviours (Bammer, 1982; Molchan et al., 1992). In line with this, loss of basal forebrain cholinergic neurons has been associated with cognitive disorders, such as Alzheimer’s disease (Coyle et al., 1983).

Given the abundance of AT₄ receptors in regions containing cholinergic cell bodies or their terminal fields and the demonstration that the scopolamine-induced spatial learning impairments could be attenuated by Ang IV analogues (Pederson et al., 1998), we postulated that the activation of AT₄ receptors potentiate cognitive processes via modulation of cholinergic transmission. Thus, the aim of this study was to examine the effect of AT₄ receptor stimulation on acetylcholine (ACh) release in the rat hippocampus.

2. Materials and methods

All experiments were performed in accordance with the Australian National Health and Medical Research Council code of practice for the care and use of animals for scientific purposes and were approved by the Animal Experimentation Ethics Committee of the Howard Florey Institute.

2.1. [³H]Acetylcholine release experiment

Adult male Sprague–Dawley rats (250–330 g) were deeply anaesthetised with isoflurane and killed by decapitation. The hippocampus was rapidly removed and placed in ice-cold carbogen-saturated Krebs–Henseleit solution (composition in mM: NaCl 125; KCl 3; NaHCO₃ 22; NaH₂PO₄ 1.0; MgSO₄ 1.2; CaCl₂ 2.5; glucose 10). All the solutions used in the experiment were carbogen-saturated. Transverse hippocampal slices (400 μm) were prepared using a McIlwain tissue chopper. The slices were incubated in 3 ml of Krebs–Henseleit solution containing 0.1 μM [³H]choline chloride solution (NEN, Boston, USA; specific activity 75 Ci/mmol), at 37°C for 20 min. Following incubation, the slices were transferred to six superfusion chambers (SF-06 superfusion system, Brandel, Gaithersberg, USA), two of which were used as controls. The slices were superfused with 37°C Krebs–Henseleit buffer, containing 10 μM hemicholinium (Sigma, NSW, Australia), at a flow rate of 500 μl/min.

After a 60-min washout period, the superfusate samples were collected at 5 min intervals for the duration of the experiment. At the 26th (S1) and 60th min (S2), the release of [³H]ACh was evoked by superfusing with 30 mM KCl–Krebs buffer (composition in mM: NaCl 100; KCl 30; NaHCO₃ 22; NaH₂PO₄ 1.0; MgSO₄ 1.2; CaCl₂ 2.5; glucose 10) for 4 min. Previous reports have demonstrated that the tritium radioactivity collected during evoked release reflects [³H]ACh (Szerb and Somogyi, 1973; Pohorecki et al., 1988). This assumption was supported by our demonstration that evoked release was Ca²⁺-dependent (data not shown).

To test the effect of peptides on the evoked release of [³H]ACh, the tissues were exposed to each peptide for 15 min prior to and during S2. The effects of the antagonists were tested by co-administration of the antagonist with the peptides. The superfusate samples were collected for a total of 80 min.

Acetylcholine release was estimated from the outflow of tritium from the tissue. One millilitre aliquots of effluent samples were added to 10 ml scintillant fluid (Ultima Gold, Canberra Packard, Vic, Australia) and the tritium radioactivity was determined using a liquid scintillation analyzer (Tri-Carb® 1900-CA, Packard, CT, USA). On completion of the experiment, the slices were removed from the chambers and dissolved in 1 ml of tissue solvent (Soluene®, Canberra Packard, Vic, Australia) overnight. The solubilized tissue was added to 10 ml Pico-Fluor 40 (Canberra Packard, Vic, Australia) and the tritium radioactivity was determined. To convert the values for each collection period to a percentage of fractional release, the counts were divided by the calculated total amount of radioactivity present in the tissue at the end of each collection period. The evoked release of radioactivity (S1 and S2) was that produced by potassium depolarisation after subtraction of the estimated basal release. To quantify the effect of drug on evoked release, the ratio of S2/S1 was calculated for each group.

2.2. Statistics

Data were expressed as the mean±SEM of S2/S1. From the six superfusion chambers, two chambers served as vehicle controls. The control value was taken as the mean of the two channels. The differences between the means for the drug-treated groups and their
corresponding controls were determined by one-way analysis of variance (ANOVA) (Graphpad Prism, Graphpad Prism Software Inc., San Diego, USA). Where there was a significant effect between groups \( (P<0.05) \) on ANOVA, Dunnet’s post-hoc test was used to determine the significance of differences between the control and particular drug-treatment groups and Bonferroni’s post-hoc test was used to determine the significance of difference between each groups.

2.3. Materials

Angiotensin IV (Val–Tyr–Ile–His–Pro–Phe) (Auspep, Parkville, Australia), LVV-hemorphin-7 (Leu–Val–Val–Tyr–Pro–Trp–Thr–Gln–Arg–Phe) (Chiron, Parkville, Australia) and divalinal Ang IV (H-Val–(R)-Tyr–Val–(R)-His–Pro–Phe–OH) (Auspep, Parkville, Australia) were dissolved in 0.05 M acetic acid and stored as 1 mM stock solutions at \(-20\)°C. The AT\(_1\) receptor antagonist, candesartan (kind gift from Astra Hassle, Molndal, Sweden), AT\(_2\) receptor antagonist, PD123319 (Parke-Davis, Sydney, Australia) and non-selective opioid antagonist, naloxone (Sigma, NSW, Australia) were dissolved in distilled water and stored as 1 mM stock solutions at \(-20\)°C. Stock solutions were thawed and diluted with vehicle on the day of the experiment.

3. Results

3.1. Potassium-evoked release of \([\text{H}]\text{ACH}\) from rat hippocampal slices

Basal release of ACh was linear and proceeded on average at a rate of 0.2% per 5 min. Depolarisation of rat hippocampal slices by 30 mM KCl evoked \([\text{H}]\text{ACH}\) release, which was at least twice basal release. Evoked release was 
\(\text{Ca}^{2+}\)-dependent since it was blocked by the omission of \(\text{Ca}^{2+}\) and inclusion of 0.1 mM EGTA in the superfusion buffer (data not shown).

3.2. Effect of Ang IV on evoked release of \([\text{H}]\text{ACH}\) from rat hippocampal slices

The addition of Ang IV \( (10^{-9}–10^{-6}\text{M}) \) to the superfusion buffer potentiated the evoked release of \([\text{H}]\text{ACH}\) in a concentration-dependent manner (Fig. 1A). The maximal effect of Ang IV was observed at \(10^{-7}\text{M}\), which was 45.5±7.5% above control \( (P<0.01) \). The potentiation of \([\text{H}]\text{ACH}\) release induced by \(10^{-7}\text{M}\) Ang IV \( (S2/S1: 1.213±0.198) \) was attenuated in the presence of the AT\(_4\) receptor antagonist, divalinal-Ang IV \( (S2/S1: 0.899±0.131) \) (Fig. 2A) and was not significantly different from control. Ang IV-induced potentiation of \([\text{H}]\text{ACH}\) release was not affected by addition of a combination of the selective AT\(_1\) (candesartan) and AT\(_2\) (PD123319) antagonists (Fig. 3).

3.3. Effect of LVV-hemorphin-7 on evoked release of \([\text{H}]\text{ACH}\) from rat hippocampal slices

The addition of LVV-hemorphin-7 \( (10^{-9}–10^{-6}\text{M}) \) to the superfusion buffer also potentiated the evoked release of \([\text{H}]\text{ACH}\) in a concentration-dependent manner (Fig. 1B). The maximal effect of LVV-hemorphin-7 was observed at \(10^{-7}\text{M}\), which was 95.8±19% above control \( (P<0.01) \). In contrast to Ang IV, a decline in response was observed at the highest concentration of LVV-hemorphin-7 \( (10^{-6}\text{M}) \) with respect to \(10^{-7}\text{M}\) LVV-hemorphin-7 \( (P<0.001) \). LVV-hemorphin-7-induced potentiation of \([\text{H}]\text{ACH}\) release \( (S2/S1: 0.986±0.06) \) was significantly attenuated in the presence of the AT\(_4\) receptor antagonist, divalinal-Ang IV \( (S2/S1: 0.726±0.03) \) \( (P<0.001) \) (Fig. 2B).

3.4. Effect of naloxone on LVV-hemorphin-7-induced release of \([\text{H}]\text{ACH}\) release

We postulated that the decrease in evoked release observed with \(10^{-6}\text{M}\) LVV-hemorphin-7 might be due
Fig. 2. Effect of the AT$_4$ receptor antagonist, divalinal-Ang IV, on (A) Ang IV-induced or (B) LVV-hemorphin-7-induced potentiation of potassium-evoked [³H]ACh release from rat hippocampal slices. The control group (Cont) received only 30 mM potassium stimulation. Ang IV (A) and LVV-hemorphin-7 (B) were superfused alone at 10⁻⁷ M (j) or in combination with divalinal-Ang IV (10⁻⁶ M) ( ■). Divalinal-Ang IV (10⁻⁶ M) alone ( □) had no effect on depolarisation-evoked [³H]ACh release. Results are expressed as mean±SEM of S2/S1 ratios (n=6 in each group). Significance of the differences was determined by ANOVA followed by Dunnet’s and Bonferroni’s post-hoc test. *Significantly different from control (P<0.05). **Significantly different from control (P<0.01). ‘Significantly different from LVV-hemorphin-7 (P<0.001).

4. Discussion

The present study demonstrates that stimulation of the AT$_4$ receptor, by either Ang IV or LVV-hemorphin-7, can potentiate depolarisation-evoked release of ACh in rat hippocampal slices. The potentiating effects of Ang IV and LVV-hemorphin-7 on ACh release appear to be mediated via the AT$_4$ receptor, since these effects were significantly attenuated in the presence of the AT$_4$ receptor antagonist, divalinal-Ang IV. Divalinal-Ang IV, given alone, did not modify evoked or basal ACh release, supporting its role as a specific antagonist of AT$_4$ receptor stimulation.

Previous studies have demonstrated that Ang IV interacts with AT$_1$ receptors with low affinity (EC$_{50}$=10 μM, Li et al., 1995) and that Ang II can inhibit depolaris-
tation-induced ACh release in the temporal cortex (Barnes et al., 1990). As expected, Ang IV-induced release of ACh, in the doses used in this study, is not affected by the AT₁ or AT₂ receptor antagonists, supporting the specificity of this effect for the AT₄ receptor.

An interesting feature of our findings was the bell-shaped nature of the LVV-hemorphin-7 concentration–response curve. Previous studies have shown that LVV-hemorphin-7 acts as a weak opioid agonist in a guinea pig ileum assay (IC₅₀=29.1 µM, Piot et al., 1992), and weakly competes for [³H]naloxone binding (IC₅₀=30.7 µM) in the rat brain (Garreau et al., 1995). Furthermore, Jackisch et al. (1986) have reported that opioid agonists inhibit the evoked release of [³H]ACh from rabbit hippocampal slices. Thus, we tested whether the bell-shaped dose–response to LVV-hemorphin-7 is due to an interaction with opioid receptors at higher (µM) concentrations. Superfusion with the non-selective opioid antagonist, naloxone, augmented the response of 10⁻⁶M LVV-hemorphin-7 to levels comparable to those observed with 10⁻⁷M LVV-hemorphin-7. This suggests that LVV-hemorphin-7 can also interact with opioid receptors at higher concentrations to inhibit ACh release in the rat hippocampus.

The current study has shown that both Ang IV and LVV-hemorphin-7 can potentiate ACh release in the rat hippocampus. This study did not address the issue of the precise site of action of AT₄ receptor ligands to enhance [³H]ACh release. However, high densities of AT₄ receptors occur in the region of the cholinergic nuclei (medial septal complex), which project to the hippocampus, as well as in the region of the terminal fields of these neurons (hippocampus, neocortex) (Wright et al., 1993; Moeller et al., 1996). On the basis of this anatomical evidence, it is possible that the receptors are produced in the cell bodies and transported to a pre-synaptic location. Thus the potentiation observed with AT₄ receptor stimulation may involve a direct action on pre-synaptic receptors located on cholinergic neurons. Alternatively, these AT₄ receptors may reside post-synaptically on the pyramidal cells of the hippocampus, and activate a retrograde messenger, such as nitric oxide, to modulate ACh release at pre-synaptic terminals.

Although the exact mechanism by which AT₄ receptor stimulation leads to enhancement of cognition is not fully elucidated, our current data, in concert with previous anatomical findings (Wright et al., 1993; Moeller et al., 1996) supports the possibility of an interaction with cholinergic neurotransmission. Based on a wide array of behavioural and pharmacological studies in animals and humans, ACh is thought to play a critical role in memory formation (see Woolf, 1998). Anti-muscarinic agents, such as scopolamine and atropine, disrupt both the acquisition and performance of a variety of learned behaviours (Bammer, 1982; Molchan et al., 1992). Furthermore, the release of ACh in the hippocampus can initiate a cascade of events leading to memory formation, such as enhancement of long-term potentiation (Auerbach and Segal, 1994; Segal and Auerbach, 1997) and changes in dendritic structure (Woolf, 1998). We thus propose that AT₄ receptor stimulation, and consequent potentiation of cholinergic transmission in the hippocampus (and possibly neocortex), may underlie the facilitation of cognitive processes observed following icv administration of Ang IV and analogues (Wright et al. 1993, 1999; Pederson et al., 1998). This proposal is also supported by the observation that icv injection of the Ang IV analogue, norleucine²⁰-Ang IV, restores deficits in spatial learning induced by the muscarinic antagonist, scopolamine (Pederson et al., 1998).

In conclusion, the results described demonstrate that AT₄ receptor stimulation, by Ang IV or LVV-hemorphin-7, enhances depolarisation-evoked release of ACh from the rat hippocampus. This may represent an important mechanism by which activation of AT₄ receptors leads to improved cognitive performance.

Acknowledgements

This study was supported by the Australian National Health and Medical Research Council No. 983001. The authors thank Ms A. Gibson for technical assistance.

References


