EFFECTS OF ANGIOTENSIN II AND ITS RECEPTOR ANTAGONISTS ON MOTOR ACTIVITY AND ANXIETY IN RATS

The neuropeptide angiotensin II (Ang II) has been recently found to be involved in cognitive processes. Both AT₁ and AT₂ angiotensin receptors seem to mediate this action. However, unspecific behavioural effects of the peptide, particularly motor and emotional, appear to influence the interpretation of cognition-oriented tests and contribute to considerable differences in opinions of various authors on the subject.

In this study, aimed specifically at the assessment of these effects, we found small and insignificant changes in motor performance measured in open field after intracebroventricular injections of Ang II and its receptor subtype-specific antagonists; losartan (AT₁) and PD 123319 (AT₂). However, Ang II was found to increase substantially anxiety measured in elevated 'plus' maze and impair motor coordination measured in 'chimney test'. Interestingly, both antagonists abolished Ang II generated anxiety and only losartan counteracted impaired motor coordination caused by the peptide. The AT₂ receptor antagonist PD 123319 impairing motor coordination on its own, nonetheless partly diminished that caused by Ang II. Therefore it appears safe to conclude that mood but not motor effects of AT₁ and AT₂ receptor affecting drugs may significantly bias interpretation of the cognition - oriented tests on these drugs.

Key words: angiotensin II, losartan, PD 123319, memory, motor activity, anxiety

INTRODUCTION

Although involvement of the neuronal angiotensin II (Ang II) in cognitive processes appears to be well established (1-3) its nature is still equivocal. Most authors support cognition - enhancing action of the peptide (4-10) but some claim opposite. For example, in an early study (11) impaired recall of information and in
more recent one (12) impaired retention were found after intracerebral Ang II. As expected, work denying any influence of Ang II on learning can also be found (13).

Even in our own hands Ang II given to the lateral cerebral ventricle (i.c.v.) produced different cognitive effects keeping though within a wide frame of learning facilitation and improvement of recall (6,14-17). Considering possible causes of such a variety of the outcomes of cognition-oriented tests applied in the Ang II research one has to take into account several "unspecific" behavioural effects of the peptide making the interpretation of the cognitive tests difficult. Two such effects, especially pertinent to Ang II, are motor disturbances (this study) and anxiety (18) caused by the peptide.

Since most, if not all, cognitive tests use rats motility as means for expressing memorised 'knowledge' any change in their motor performance unrelated to memory processing can obviously distort experimental outcome and lead to false conclusions.

Although these issues in general context of the drug-related memory changes have been raised by many authors (19-22) they have never been considered as possible causes of discrepancies regarding cognitive effects of Ang II.

Therefore, in this study we attempted to examine motor and emotional effects of Ang II and its subtype receptor selective antagonists.

The possibility of occurrence of such effects is well substantiated by anatomical and physiological studies. Walters and Speth (23) found specific Ang II binding sites in rat inferior olivary nuclei whose lesion caused motor deficits. Recently, Jöhren et al. (24) characterized these receptors as being of AT$_2$ subtype present also in molecular layer of cerebellar cortex involved in motor coordination. The majority (65%) of Ang II receptors found in the same part of human cerebellum were also of AT$_2$ subtype, the remaining 35% being of AT$_1$ (25).

Obviously, administration of agonists or antagonists of Ang II receptors or changing levels of endogenous Ang II by for example angiotensin - converting enzyme inhibitors (26) can affect motor performance and the interpretation of memory tests. Such notion is supported by motor and learning deficits in AT$_2$ gene knock-out mice found by Ichiki et al. (27), the results confirmed by Sakagawa et al. (28) and also by Okuyama et al. (29).

Emotional aspects of Ang II activity have been raised by several authors. Kaiser et al. (18) described anxiolytic properties of the AT$_1$ receptor antagonist losartan suggesting anxiogenic potency of the AT$_1$ stimulation. This was supported by our studies showing reversal of anxiogenic action of i.c.v. Ang II by an equimolar (low) dose (2 nmol) of i.c.v. losartan (9). Also, 'anxiogenic' profile of transgenic (mREN2)27 rats characterised by increased level of brain angiotensin and fulminant hypertension (30) points to the increased emotionality caused by Ang II. The neuroanatomic site of these anxiogenic effects of Ang II may be amygdala AT$_1$ and AT$_2$ receptors (31) and possibly AT$_2$ receptors in locus coeruleus (29), both structures being involved in producing emotional behaviour (32-33).
Notably, a reciprocal inhibitory regulation between AT<sub>1</sub> and AT<sub>2</sub> receptors (34, 35) assures modification of effects mediated from both receptor subtypes. For example, increased anxiety found in the AT<sub>2</sub> receptor deficient mice (36) might have been caused by unopposed AT<sub>1</sub> receptor rather than by lack of the AT<sub>2</sub> receptors.

To be able to assess better possible contribution of unspecific behavioural effects of Ang II and its subtype selective receptor antagonists, in this study effects of Ang II, losartan (AT<sub>1</sub> selective) and PD 123319 (AT<sub>2</sub> selective) on motor activity in open field (37), anxiety in elevated 'plus' maze (38, 39) and motor coordination and anxiety in 'chimney' test (40) were examined.

**MATERIALS AND METHODS**

**Subjects**

Male Wistar rats weighing 160-180 g at the time of testing, i.e. about 8 weeks old, were used. The animals were housed in groups of eight in the standard laboratory cages (43 x 34 x 26 cm) (length x width x height) in a temperature controlled room with a constant 12 h light/dark cycle (lights on at 7:00 am) and with free access to the lab chow and tap water up to the time of experimentation. The experiments were conducted between 10:00 and 15:00 hours. A 30 min habituation period to the experimental room preceded the behavioural testing.

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 the Animal Experimentation.

**Surgery**

Under light ether anaesthesia, a round piece of skin, 7 mm in diameter, was cut off the rat's head and the underlying skull surface was cleaned from the soft tissue. A burr holes, 0.5 mm in diameter, were 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 and left side of the head. The operation took about 2 min and, after 48 h recovery, the wound was completely dry and the animal behaved normally. On the following day (i.e. 3 days after surgery) the i.c.v. injections were made freehand into the lateral cerebral ventricles with a 10 µl Hamilton syringe, using a removable KF 730 needle cut 4.5 mm from its base. This procedure allowed lowering the tip of the needle about 0.5 mm below the ceiling of the lateral cerebral ventricle. It was relatively nontraumatic as the animal, gently fixed by the left 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. One rat in twenty was usually found to be injected incorrectly.

**Experimental design**

There were six groups of animals injected i.c.v. (left + right): Control (Saline + Saline), Losartan (Los + Saline), PD 123319 (PD + Saline), Ang II (Saline + Ang II), Losartan + Ang II (Los + Ang II), PD 123319 + Ang II (PD + Ang II).
There was only one injection made to each lateral cerebral ventricle. Injections (left - right) were separated by a 5 min period. All tests begun 15 min after the second injection.

Open field

Locomotor exploratory activity was measured in an open field which was a square 100 cm x 100 cm white floor divided by eight lines into 25 equal squares and surrounded by a 47 cm high wall (37). Four plastic bars, 20 cm high, were designed as objects of possible animal's interest and fixed perpendicularly, parallel to each other, in four line crossings in the central area of the floor. Fifteen min after the i.c.v. injection the rat was placed in the center of the floor and following 1 min of adaptation, crossings, rearings and bar approaches were counted manually for 5 min.

Elevated 'plus' maze

Anxiety was evaluated in an elevated 'plus' maze (constructed of gray coloured wooden planks) consisted of two open arms, 50 cm (length) x 10 cm (width) and two enclosed arms, 50 cm (length) x 10 cm (width) x 40 cm (height), covered with a removable lid, such that the open or closed arms were opposite to each other. The maze was elevated to a height of 50 cm from the floor. Ten minutes after the i.c.v. injection rat was placed for a 5 min in a pretest arena (60 cm x 60 cm x 35 cm, constructed from the same material) prior to exposure to the maze. This step allows the facilitation of exploratory behaviour. The experimental procedure was similar to that described by Pellow et al. (38). Immediately after the pretest exposure rats were placed in the center of the elevated 'plus' maze facing one of the open arms. During the 5 min test period the following measurements were taken: the number of entries into the open and closed arms and the time spent in the open and closed arms. An entry was defined as all four feet into one arm. An increase in open arms entries and increase in time spent in open arms were interpreted as indicative of potential anxiolytic activity.

'Chimney' test

Motor coordination was evaluated using 'chimney' test described originally for mice (40). Fifteen min after an i.c.v. injection rat was allowed to enter a glass laboratory cylinder 452 mm long and 57 mm in diameter laid on its side. Upon reaching its bottom by the animal position of the cylinder was rapidly changed from horizontal to vertical and a timer started. The animal immediately begun to move backwards. The timer was stopped after the rat left the cylinder and assumed a sitting posture on the top of the vessel. The time of exit from the cylinder was accepted as a measure of motor coordination and, possibly, anxiety (40, 41).

Drugs

Ang II (Asp-Arg-Val-Tyr-Ile-His-Pro-Phe) was obtained from Sigma-Aldrich (Steinheim, Germany).

Losartan (2-n-butyl-4-chloro-5-hydroksymethyl-1-[2'-(1H-tetrazol-5-yl) biphhenyl-4-yl) methyl] imidazole, potassium salt) was a gift from dr Ronald Smith of Du Pont Merck (New Jersey, USA).

PD 123319 (1-[[4-(dimethylamino)-3-methylphenyl][methyl]-5-(diphenylacetyl)-4,5,6,7-tetrahydro-1H-imidazo[4,5-c]pyridine-6-carboxylic acid, ditrifluoroacetate, monohydrate) was purchased from RBI (Natick, USA).

All drugs were dissolved in saline and given intracerebroventricularly (i.c.v.) at the volume of 2 µl per one lateral cerebral ventricle.
Statistics

All results were analysed with one-way ANOVA followed by Bonferroni test for chosen comparisons. Levels were deemed significant at $p<0.05$.

RESULTS

Effect of angiotensin II, losartan and PD 123319 on locomotor exploratory activity of rats in open field

No statistically significant differences were found in the numbers of crossings, rearings, and bar approaches counted in the open field after pretreatment of rats with Ang II, preceded or not by the $\text{AT}_1$ or $\text{AT}_2$ receptor blocker, losartan or PD 123319, respectively (Fig. 1). Both angiotensin receptor antagonists alone were also ineffective in this test.

Fig. 1. Effect of angiotensin II (Ang II) receptor blockers; losartan (Los, 2 nmol, $\text{AT}_1$) or PD 123319 (1.5 nmol, $\text{AT}_2$), given to the left cerebral ventricle 20 min before testing, followed 5 min later by 1 nmol of Ang II given to the right cerebral ventricle, on the number of crossings, rearings, and bar approaches in the open field. Columns represent means ± SE of the values obtained from $n$ rats indicated at the bottom of the figure. The control injections were made of 0.9% NaCl (Saline) at the appropriate times and routes.
Effect of angiotensin II, losartan and PD 123319 on the behaviour of rats in the elevated 'plus' maze

One way ANOVA of the times spent by rats in open arms of the 'plus' maze yielded $F_{(5, 96)} = 6.109$ showing thus significant ($p<0.0001$) differences between the groups (Fig.2). Post-hoc comparisons of preselected groups with Bonferroni test revealed that Ang II treated rats spent significantly less time in open arms of the maze than control ($p<0.01$) and losartan ($p<0.01$) or PD 123319 ($p<0.05$) pretreated and then Ang II treated animals.

One way ANOVA of the numbers of open arms entries yielded $F_{(5, 96)} = 8.55$ ($p<0.0001$) revealing significant differences between the groups. Further pairwise comparisons with Bonferroni test showed that Ang II treated rats entered the open arms of the maze significantly less often than control ($p<0.01$) and losartan ($p<0.01$) or PD 123319 ($p<0.05$) pretreated and then Ang II treated animals.

Effect of angiotensin II, losartan and PD 123319 on the rats' exit from the 'chimney'

ANOVA of times of the animals' exit from the glass cylinder yielded $F_{(5, 43)} = 12.69$ ($p<0.0001$) revealing statistically significant differences (Fig.3). Pairwise
comparisons with Bonferroni test showed statistically significant increase of the mean exit time of rats treated with PD 123319 (\(p<0.001\)) and Ang II (\(p<0.01\)) in comparison with control. Moreover, mean exit time in the former group was statistically significantly (\(p<0.001\)) longer than that of the rats treated with Ang II after PD 12319 and in the latter one with Ang II after losartan (\(p<0.05\)).

DISCUSSION

The concept of this study was based on the assumption that unspecific, particularly motor and emotional aspects of a drug action can make interpretation of cognitive tests on this drug difficult if not impossible. While this matter has already been discussed from various points of view (19-22, 42, 43) it has only marginally been raised in respect to the cognitive effects of Ang II and its receptor antagonists (6, 44).

Taking into account increasing importance of AT\(_1\) angiotensin receptor antagonists in the treatment of various cardiovascular disorders (45-47) the suggestions about some memory attenuating effects of these drugs resulting from certain recent animal studies (6) seem to be worthy of careful assessment.

One single behavioural aspect of cognitive studies on rats critical for the proper evaluation of results is how the examined substance affects animal's motor
performance (20, 22). If, for instance Ang II, repeatedly shown to improve acquisition of conditioned avoidance responses (CARs) i.e. learning, increased rats' undirected ambulation, the number of 'positive' CARs might also increase simply by chance, without any true improvement of learning. Incidentally, open field test, often used to control for noncognitive motor effects of drugs, revealed occasionally significant psychomotor stimulation after Ang II administration (16, 17).

Also, passive avoidance situation (48) which makes agitated rats more prone to exhibit weaker memory (by increasing possibility of chance entering the dark chamber wherein the rat experienced footshock 24 h earlier) can not entirely solve the problem because the rat may be, after Ang II, more afraid to enter unknown (i.e. forgotten) dark part of the apparatus then the control one showing thus (untruly) better "retention" of memory. Also, the animal might be more afraid to fall down from the narrow elevated platform of the classic passive avoidance setting (48) used by same investigators (37-49) an element non existing in the two compartment box used by the others (50-51) where there is no possibility of falling down.

This line of reasoning finds a support in the present results whereby Ang II increased animals' anxiety in the elevated 'plus' maze, and decreased their motor coordination in the 'chimney' test (40). Interestingly, similar to Ang II effect on motor coordination was caused by PD 123319, selective AT2 angiotensin receptors antagonist. The impairment of motor coordination by both PD 123319 and Ang II was unexpected and is difficult to explain. Whereas action of the AT2 antagonist (PD 123319) is well in line with the role of cerebellar AT2 angiotensin receptors in motor control (24), causes of similar action of Ang II remain to be determined. One possible explanation could be the well known decreasing performance effect of high arousal (52) possibly caused by Ang II. The open field test in which we did not observe any significant influence of Ang II on motor activity might not be enough sensitive. Both behavioural changes, potentially contributing to the delay in exit from the 'chimney' i.e. anxiety and motor impairment have been described in the AT2 knockout animals (29).

In conclusion, the present results confirm the necessity of careful consideration of "unspecific" motor and emotional aspects of drugs while examining their potential cognitive effects. In the particular issue of improving memory and facilitating recall action of Ang II antagonised by its AT1 and AT2 receptor blockers the present results make it possible to explain some discrepancies found in the literature (6, 51) and also confirm the previous hypotheses about beneficial cognitive effects of Ang II and about its anxiogenic action effectively abolished by AT1, and less by AT2 angiotensin receptor antagonists i.e. losartan and PD 123319. Further animal research concerning the impaired motor coordination caused by Ang II as well as the human studies aimed at searching for anticognitive potential of the AT1 receptor antagonists are presently carried out in our laboratories.
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