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Metabolism of angiotensin II is required for its *in vivo* effect on dopamine release in the striatum of the rat

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Abstract

The effect of angiotensin (Ang) IV, an inhibitor of insulin-regulated aminopeptidase (IRAP), on extracellular dopamine levels in the striatum of freely moving rats was examined using *in vivo* microdialysis. The Ang IV was administered locally in the striatum through the microdialysis probe. A concentration-dependent (10–100 μM) increase in extracellular striatal dopamine was observed. The effect of Ang II (10–100 μM), which has only a weak affinity for IRAP, was similar to that observed for Ang IV. The effects of both peptides could not be blocked by the AT_1 antagonist candesartan (10 nM and 1 μM) nor by the AT_2 antagonist S-(+)-1-([4-(dimethylamino)-3-methylphenyl]methyl)-5-(diphenyl-acetyl)-4,5,6,7-tetrahydro-1H-amidazo(4,5-c) pyridine-6-carboxylic acid (1 μM), suggesting that the observed effects are both AT_1 and AT_2 independent. The effect of Ang II could be blocked by the aminopeptidase-A inhibitor (S)-3-amino-4-mercaptobutylsulphonic acid as well as the aminopeptidase-N inhibitor 2-amino-4-methylsulphonylbutane thiol, indicating that the effect of Ang II is mediated via metabolism into Ang IV. Other IRAP inhibitors, such as Divalinal-Ang IV and LVV-haemorphin-7, had similar effects on extracellular dopamine levels as compared with Ang IV. We propose a role for IRAP as mediator for the effects of Ang IV and related peptides on extracellular dopamine levels in the striatum of the rat.

Keywords: aminopeptidase-A and -N, angiotensin II and IV, extracellular dopamine, insulin-regulated aminopeptidase, striatum.

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The renin–angiotensin (Ang) system is a signalling system which makes use of a number of Ang peptides. Angiotensin II is well known for its hypertensive effect and its ability to stimulate cardiac remodelling. AT_1 receptors play a major role in these processes (Saavedra 1999; de Gasparo *et al*. 2000). AT_2 receptors constitute the other major Ang II receptor subtype but their role is not as well defined as that of the AT_1 receptors (de Gasparo *et al*. 2000).

Although Ang II was long considered as the end product of the renin–Ang system, there is accumulating evidence that shorter peptide fragments appear to be effectors of this system because they exert diverse biological effects. Among them, the Ang II-(3–8) fragment (Ang IV) is reported to have central as well as peripheral effects (Ardaillou and Chansel 1997; Chansel and Ardaillou 1998; Mustafa *et al*. 2001). Angiotensin IV is formed *in vivo* from Ang II in two steps: first, Ang II is metabolized into Ang III by aminopeptidase (AP)-A and second, AP-N metabolizes Ang III into Ang IV. Angiotensin IV is then further cleaved into smaller peptide fragments. The degradation of Ang II can be blocked with selective AP-A and AP-N inhibitors (Zini *et al*. 1996; Reaux *et al*. 1999) (Fig. 1).

Intracerebroventricular administration of Ang IV facilitates memory acquisition and retrieval and enhances

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Abbreviations used: Ang, angiotensin; AP, aminopeptidase; DA, dopamine; EC33, (S)-3-amino-4-mercaptobutylsulphonic acid; IRAP, insulin-regulated AP; LVV-H7, LVV-haemorphin-7; PC18, 2-amino-4-methylsulphonylbutane thiol; PD123,319, S-(+)-1-([4-(dimethylamino)-3-methylphenyl]methyl)-5-(diphenyl-acetyl)-4,5,6,7-tetrahydro-1H-amidazo(4,5-c) pyridine-6-carboxylic acid.

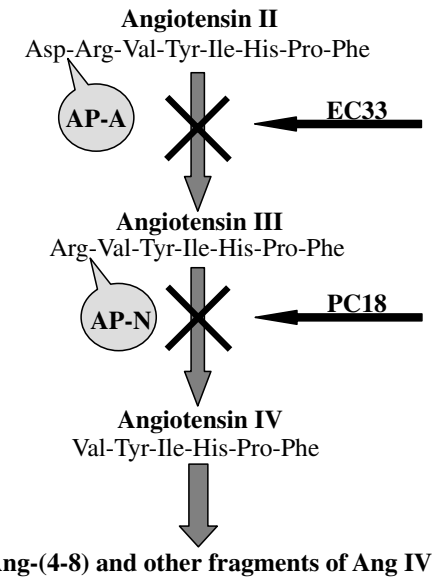

Fig. 1 The metabolism of angiotensin (Ang) II into Ang IV. First, Ang II is metabolized into Ang III by aminopeptidase (AP)-A. This conversion can be blocked with (S)-3-amino-4-mercaptobutylsulphonic acid (EC33). Second, Ang III is converted into Ang IV by AP-N. This can be blocked with 2-amino-4-methylsulphonylbutane thiol (PC18). Finally, Ang IV is metabolized into smaller peptide fragments.

potassium-evoked release of acetylcholine. Interestingly, these effects were not blocked by classical non-peptide AT_1 and AT_2 receptor antagonists (Pederson *et al*. 1998; Wright *et al*. 1999; Belcheva *et al*. 2000; Lee *et al*. 2001). Moreover, the occurrence of binding sites with high affinity (nanomolar range) for [^{125}I]-Ang IV in the central nervous and also in the vascular and renal systems (Swanson *et al*. 1992; Harding *et al*. 1994) led to the concept of a novel Ang receptor subtype, 'the AT_4 receptor' (de Gasparo *et al*. 1995, 2000). The decapeptide LVV-haemorphin-7 (LVV-H7), that was identified in the sheep brain, exhibits high affinity (nanomolar range) for binding sites which display a regional distribution in sheep brain that is identical to that of Ang IV (Moeller *et al*. 1997; Albiston *et al*. 2001). Therefore, this abundant peptide (approx. 2 nmol of peptide/g of sheep brain tissue) was suggested as the endogenous ligand of the AT_4 binding site (Mustafa *et al*. 2001).

The 'receptor' nature of the AT_4 binding site is controversial. Whereas Ang IV is clearly able to trigger intracellular cascades in certain cell types (Vauquelin *et al*. 2002), recent experiments have provided strong evidence that the AT_4 binding site corresponds to a membrane-associated AP formerly known as insulin-regulated AP (IRAP)/oxytocinase/placental leucine AP (Albiston *et al*. 2001). Recent *in vitro* experiments pointed out that Ang IV, LVV-H7 and also Divalinal-Ang IV, which was initially proposed as an AT_4 antagonist (Krebs *et al*. 1996), are inhibitors of IRAP with IC_{50} values between 0.2 and 4 μM (Lew *et al*. 2003). *In vitro* IRAP mediates the degradation of a number of neuropeptides, such as vasopressin, oxytocin and somatostatin (Herbst *et al*. 1997). However, the *in vivo* substrates of IRAP have not yet been determined.

The present study was conceived to evaluate and compare the effects of Ang II and IV on dopamine (DA) release from dopaminergic neurones projecting to the striatum. This brain structure plays an important role in the control of movement. It has already been shown in two *in vivo* microdialysis studies in adult rats that Ang II, locally administered in the striatum, is able to increase the release of DA (Mendelsohn *et al*. 1993; Brown *et al*. 1996). It was suggested that this effect was AT_1 dependent because it could be blocked with the AT_1 antagonist losartan at a concentration of 1 μM. However, a low density of AT_1 and AT_2 receptors and a high density of Ang IV binding sites has been found in the striatum of adult rats (Wright and Harding 1997). Moreover, there are no data about the effect on extracellular DA levels of Ang IV. As no experimental data exist we have investigated, in the present study, the effect of local administration of Ang IV on extracellular DA levels in the striatum as measured by *in vivo* microdialysis and compared it with the effect caused by Ang II. To assess the involvement of AT_1 or AT_2 receptors in the observed effects, candesartan was used as an AT_1 antagonist and S-(+)-1-([4-(dimethylamino)-3-methylphenyl]methyl)-5-(diphenyl-acetyl)-4,5,6,7-tetrahydro-1H-amidazo(4,5-c) pyridine-6-carboxylic acid (PD123,319) as an AT_2 antagonist. The AP-A inhibitor (S)-3-amino-4-mercaptobutylsulphonic acid (EC33) and the AP-N inhibitor 2-amino-4-methylsulphonylbutane thiol (PC18) were used to determine whether Ang II exerts its effect on extracellular DA levels in the striatum by itself or through metabolism into Ang IV or a metabolite of Ang IV.

In a second part, the effect of Ang IV was compared with those elicited by other IRAP inhibitors, such as Divalinal-Ang IV and LVV-H7. Naltrexone was used as a non-selective opioid antagonist to exclude the possible opioid effects of LVV-H7 (Glamsta *et al*. 1992). Angiotensin-(4–8), which has weaker affinity for 'the AT_4 binding site' (Swanson *et al*. 1992), was also tested (Fig. 1).

Materials and methods

Animals

All experiments were carried out on freely moving rats according to the national guidelines on animal experimentation and were approved by the Ethical Committee for Animal Experiments of the Faculty of Medicine and Pharmacy of the Vrije Universiteit Brussel. All efforts were made to minimize pain or discomfort of the animals.

The experiments were performed on male Wistar rats (Iffa Credo, Brussels, Belgium) weighing 250–300 g. The rats were given free access to food and water.

Stereotaxic implantation of microdialysis probe

The rats were first anaesthetized with a mixture of ketamine/diazepam (60/4.5 mg/kg i.p.) and placed on a stereotaxic frame. The skull was exposed and a burr hole was drilled to implant a guide cannula (CMA Microdialysis, Stockholm, Sweden) positioned 3 mm above the left dorsal striatum according to the atlas of Paxinos and Watson (1986) (coordinates relative to bregma: L, −2.4; A, +1.2 and V, +2.8). The rats received 4 mg/kg ketoprofen i.p. as analgesic. After surgery, a CMA12 (CMA Microdialysis) probe with a membrane length of 3 mm was introduced via the cannula. The probe was perfused with modified Ringer's solution containing 147 mM NaCl, 4 mM KCl and 1.2 mM $CaCl_2$ at a constant flow-rate of 2 μL/min using a microdialysis pump (CMA 100; CMA Microdialysis).

Animals were allowed to recover from surgery overnight and dialysate collection was started the day after the surgery.

In vivo microdialysis experiments

Samples were collected every 20 min, yielding 40 μL dialysates. Filtered antioxidant mixture (10 μL; 0.1 M acetic acid, 3.3 mM L-cystein, 0.27 mM Na_2EDTA, 12.5 μM ascorbic acid) was added to prevent oxidation of DA. Four to six dialysate samples were collected before any pharmacological manipulation was performed. The mean of these neurotransmitter dialysate concentrations was taken as baseline value at time zero.

In experiments where the effect of Angs or proposed IRAP ligands on extracellular DA levels in the striatum was tested, six baseline values were taken followed by a perfusion of the drug for 1 h. A dose–response relationship was determined for Ang II and IV (1 nM–100 μM). Divalinal-Ang IV (10 and 100 μM), Ang-(4–8) (10 and 100 μM) and LVV-H7 (10 μM) were also tested.

The metabolism of Ang II into Ang IV was examined in two steps. First, the effect of an AP-A inhibitor on the Ang II-induced DA release was tested. EC33 was used at a concentration of 6 μM (Reaux *et al*. 2000) and was dissolved in modified Ringer's solution together with 12.5 μM ascorbic acid to prevent oxidation. In these experiments, four baseline values were taken, followed by the perfusion for 40 min of EC33. Second, Ang II (10 μM) was perfused together with EC33 (6 μM) for 1 h. Control experiments with EC33 were also performed in which six baseline values were taken followed by a perfusion of EC33 (6 μM) for 2 h. Similarly, the effect of the AP-N inhibitor PC18 on the Ang II-induced DA release was tested. PC18, dissolved in modified Ringer's solution together with 12.5 μM ascorbic acid, was used at a concentration of 0.16 μM (Reaux *et al*. 2000).

In another series of experiments, the effect of AT_1 and AT_2 receptor antagonists on the Ang II- and IV-induced DA release was tested. Candesartan (10 nM and 1 μM) was used as an AT_1 receptor antagonist and PD123,319 (1 μM) as an AT_2 receptor antagonist. The effect of naltrexone (100 nM), a non-selective opioid antagonist, on the LVV-H7 (10 μM)-induced effect was also examined. Four baseline values were taken, followed by the perfusion of the antagonist for 40 min. Hereafter, Ang II, IV or LVV-H7 were perfused together with this antagonist for 1 h. Control experiments with the antagonists were also performed in which six baseline values were taken followed by a perfusion of the antagonist for 2 h.

In all experiments, the drugs were dissolved in the modified Ringer's solution and were locally administered via the probe. The pharmacological manipulation was always followed by a perfusion with the modified Ringer's solution for another 100 min. At the end of the experiment, the rats were killed with an overdose of pentobarbital (Nembutal®).

Chromatographic assay

For the determination of the DA concentration in the dialysates, a microbore liquid chromatography (LC) assay was used. The system consisted of an isocratic pump (BAS, Indianapolis, IN, USA) delivering the mobile phase to the analytical column at a rate of 100 μL/min. The mobile phase contained 0.1 mM sodium acetate, 20 mM citric acid, 2 mM decanesulphonic acid, 0.5 mM Na_2EDTA adjusted to pH 5.5. Acetonitrile (28 mL) was added to 200 mL of this buffer solution.

A microbore column (100 × 1.0 mm i.d. with C8, 5 μm packing material) (Unijet; BAS) was used coupled to an amperometric detector (Antec, Leiden, the Netherlands) with a glassy carbon working electrode. The operating potential was set at 450 mV versus an Ag/AgCl reference electrode.

The samples were injected via a high precision auto-injector equipped with a cooling system (Kontron, San Diego, CA, USA). The injection volume was 10 μL and the limit of detection was 50–100 pM (Sarre *et al*. 1997).

The integration of the chromatograms was done with the software programme Kroma 2000 (Kontron).

Materials

Angiotensin II and IV were supplied by Neosystem (Strasbourg, France). Naltrexone HCl and PD123,319 were purchased from Sigma (St Louis, MO, USA) and Ang-(4–8) from Bachem (Bubendorf, Switzerland). Candesartan was a gift from Astra Zeneca (Mölndal, Sweden). LVV-H7 was synthesized by ResGen (Huntsville, AL, USA) and Divalinal-Ang IV by Dr G. Muske (Washington State University, Pullman, WA, USA). EC33 and PC18 were gifts from Prof. M.C. Fournié-Zaluski (Université Rene Descartes, Paris, France) (Reaux *et al*. 2000). All other chemicals were analytical grade or better and supplied by Merck (Darmstadt, Germany). All aqueous solutions were prepared in fresh water purified by a Seralpur Pro 90 CN system (Merck Belgolabo, Overijse, Belgium) and filtered through a membrane filter with pore size 0.2 μm.

Data analysis

No corrections were made for probe recovery across the dialysis membrane. Therefore, the reported extracellular concentrations are actually dialysate concentrations. The extracellular concentrations of DA in baseline conditions were calculated in nM (mean ± SEM). Extracellular DA levels after the pharmacological manipulation were expressed as percentages (mean ± SEM) of the mean baseline value, expressed as 100%. For statistical significance of differences in DA levels after administration of drugs compared with baseline values, a one-way ANOVA for repeated measures was used. If the ANOVA was significant, a paired-samples *t*-test was carried out comparing the extracellular DA levels after the pharmacological manipulation with the baseline value.

To compare maximal effects between different treatments, a Mann–Whitney test was carried out. For all statistical analyses $\alpha = 0.05$.

Results

Effect of the local perfusion of angiotensin II and IV

The mean (± SEM) basal output of DA in the striatum of freely moving Wistar rats is 2.06 ± 0.14 nM ($n = 99$).

Angiotensin II and IV were perfused locally in the striatum of the freely moving rat in a concentration range of 1 nM–100 μM. Figure 2 only depicts the effect of Ang II and IV in concentrations of 10 and 100 μM as the perfusion of lower concentrations was without effect.

At a concentration of 10 μM, both peptides provoked a significant increase in the extracellular DA concentration (ANOVA, $p < 0.0001$). The increase was maximal after 60 min of perfusion and was approximately 150% of baseline. The DA concentration at the end of the experiment was still slightly increased but not significantly different from the baseline level ($p = 0.07$).

Administered at a dose of 100 μM, Ang IV as well as Ang II significantly elevated the extracellular DA concentration (ANOVA, $p < 0.0001$). The effect of Ang IV was maximal after 80 min of perfusion and was about 190% of the baseline value. The effect of Ang II was maximal after 80 min of perfusion and was about 225% of baseline. The DA concentration at the end of the experiment was still slightly increased but not significantly different from the baseline level ($p = 0.06$). There was no significant difference between the maximal effects of Ang II and IV.

Effect of AT_1 and AT_2 receptor antagonists on angiotensin II- and IV-induced dopamine release

The effect of Ang II (10 μM) could not be blocked by candesartan (10 nM and 1 μM) or by PD123,319 (1 μM) (Fig. 3) ($p < 0.05$). The antagonists themselves had no effect on the extracellular DA concentration (data not shown).

Similar to the effects with Ang II, the effect of Ang IV was not blocked by candesartan (1 μM) or by PD123,319 (1 μM) ($p < 0.05$) (Fig. 4).

Effect of aminopeptidase-A and -N inhibitors on angiotensin II-induced dopamine release

The effect of Ang II (10 μM) was blocked by EC33 (6 μM) as well as by PC18 (0.16 μM) (Fig. 3). The perfusion of EC33 (6 μM) or PC18 (0.16 μM), dissolved in modified Ringer's solution together with ascorbic acid (12.5 μM), had no effect on the extracellular DA concentration (data not shown).

Effect of Divalinal-angiotensin IV, LVV-H7 and angiotensin-(4–8)

Divalinal-Ang IV (10 μM) caused a significant increase in the extracellular DA levels in the striatum (ANOVA, $p < 0.0001$). The maximal effect was reached after 60 min perfusion and was about 150% of baseline. The effect of LVV-H7 (10 μM) was similar. With both peptides, the extracellular DA levels remained significantly elevated until the end of the experiment. The perfusion of Ang-(4–8) at a concentration of 10 μM was without effect (Fig. 5).

Divalinal-Ang IV (100 μM) caused a significant increase of the extracellular DA levels in the striatum (ANOVA,

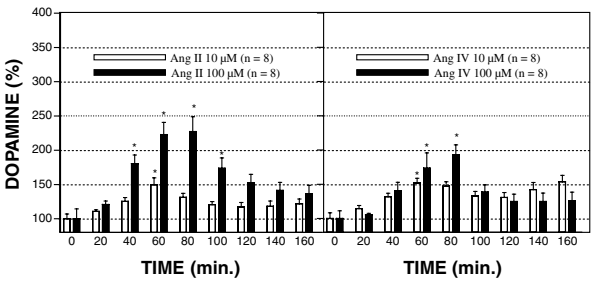

Fig. 2 Effect of the local perfusion of angiotensin (Ang) II and IV (10–100 μM) on the extracellular dopamine (DA) concentration in the striatum of the rat. Baseline levels are set to 100% (mean of six values). The values at all other points are expressed as a percentage of the baseline value. Each value is the mean ± SEM. The peptides are perfused for 1 h (shown by the horizontal bars). Data were analysed by ANOVA for repeated measures followed by a paired-samples *t*-test comparing the extracellular DA levels after the pharmacological manipulation and the baseline value. *Statistical significance ($p < 0.05$).

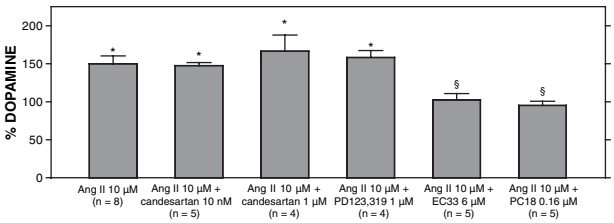

Fig. 3 Effect of AT_1 and AT_2 receptor antagonists and aminopeptidase-A and -N inhibitors on the angiotensin (Ang) II-induced dopamine release in the striatum of the rat (mean ± SEM). Within each group of experiments, the data were analysed as in Fig. 2 but only the maximal effect is shown. This occurred in all experiments after 60 min. *Statistical significance of the maximal effect compared with the baseline level which is 100% ± SEM in each experiment ($p < 0.05$). A Mann–Whitney test was used to determine differences between the maximal effects. §Statistical significance ($p < 0.05$). PD123,319, S-(+)-1-([4-(dimethylamino)-3-methylphenyl]methyl)-5-(diphenyl-acetyl)-4,5,6,7-tetrahydro-1H-amidazo(4,5-c) pyridine-6-carboxylic acid; EC33, (S)-3-amino-4-mercaptobutylsulphonic acid; PC18, 2-amino-4-methylsulphonylbutane thiol.

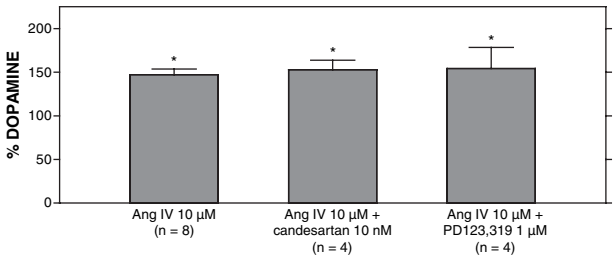

Fig. 4 Effect of AT_1 and AT_2 receptor antagonists on the angiotensin (Ang) IV-induced dopamine release in the striatum of the rat (mean ± SEM). Within each group of experiments, the data were analysed as in Fig. 2 but only the maximal effect is shown. This occurred in all experiments after 60 min. *Statistical significance of the maximal effect compared with the baseline level which is 100% ± SEM in each experiment ($p < 0.05$). A Mann–Whitney test was used to determine differences between the maximal effects. There was no statistical significance ($p < 0.05$). PD123,319, S-(+)-1-([4-(dimethylamino)-3-methylphenyl]methyl)-5-(diphenyl-acetyl)-4,5,6,7-tetrahydro-1H-amidazo(4,5-c) pyridine-6-carboxylic acid.

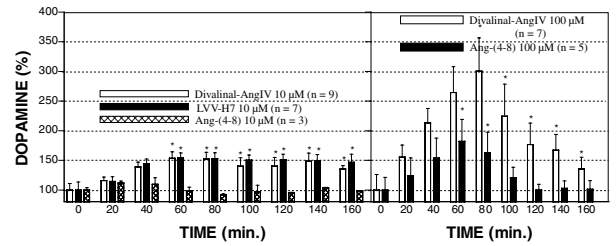

Fig. 5 Effect of Divalinal-angiotensin (Ang) IV, Ang-(4–8) (10–100 μM) and LVV-H7 (10 μM) on the extracellular dopamine (DA) level in the striatum of the rat. Baseline levels are set to 100% (mean of four to six values). The values at all other points are expressed as a percentage of the baseline value. Each value is the mean ± SEM. The peptides were perfused for 1 h (shown by the horizontal bars). Data were analysed by ANOVA for repeated measures followed by a paired-samples *t*-test comparing the extracellular DA levels after the pharmacological manipulation and baseline value. *Statistical significance ($p < 0.05$).

$p < 0.0001$). This increase was maximal after 80 min and was about 300% of baseline. The extracellular DA levels remained significantly elevated until the end of the experiment. Only at a concentration of 100 μM did Ang-(4–8) cause a significant increase of the extracellular DA concentration in the striatum of the rat (ANOVA, $p = 0.002$). The increase was maximal after 60 min and about 180% of baseline. After the increase, the extracellular DA levels returned to baseline (Fig. 5).

Figure 6 shows that the effect of LVV-H7 (10 μM) could not be blocked with naltrexone (100 nM). The local administration of naltrexone at a concentration of 100 nM had no effect on extracellular DA levels in the striatum of the rat.

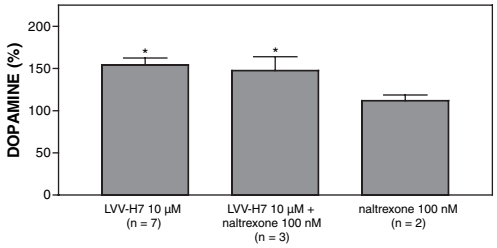

Fig. 6 Effect of naltrexone (100 nM) on the LVV-H7 (10 μM)-induced dopamine release in the striatum of the rat (mean ± SEM). Within each group of experiments, the data were analysed as in Fig. 5 but only the maximal effect is shown. This occurred in all experiments after 60 min. *Statistical significance of the maximal effect compared with the baseline level which is 100% ± SEM in each experiment ($p < 0.05$).

Discussion

This study shows that Ang IV dose dependently affects the dopaminergic system in the striatum of the freely moving rat. As both the AT_1 receptor antagonist candesartan and the AT_2 receptor antagonist PD 123,319 are unable to block the effect produced by Ang IV, it is tempting to suggest the involvement of the proposed 'AT_4 receptor'; binding sites with a high affinity (nanomolar range) for Ang IV have been found in the brain (Swanson *et al*. 1992; Harding *et al*. 1994). A high density of Ang IV binding sites was detected in the striatum (Wright and Harding 1997). Recently, it has been shown that this 'AT_4 receptor' is a membrane-associated AP, IRAP (Albiston *et al*. 2001). Insulin-regulated AP is a member of the M1 family of zinc metallopeptidases which also includes AP-A and -N. However, in contrast with other APs, like AP-N, IRAP is unable to metabolize Ang IV (Lew *et al*. 2003). Instead, Ang IV is a potent and competitive inhibitor of IRAP ($IC_{50} = 0.2$ μM) (Lew *et al*. 2003) and, as such, is able to prevent the degradation of neuropeptides like vasopressin, oxytocin and somatostatin *in vitro* (Herbst *et al*. 1997). Therefore, the biological effects of IRAP inhibitors like Ang IV may be the consequence of the inhibition of the cleavage by IRAP of one or more of these bioactive peptides. Indeed, previous *in vivo* microdialysis studies have shown that vasopressin (Van Heuven-Nolsen and Versteeg 1985) and somatostatin (Thermos *et al*. 1996) are able to increase striatal DA release in rats.

The effect of Ang IV and, surprisingly, also the effect of Ang II are AT_1 and AT_2 receptor independent. Indeed, the effect could not be blocked with candesartan or by PD123,319. However, there appears to be a discrepancy between the effects of candesartan and losartan on the Ang II-induced DA release. Indeed, Mendelsohn *et al*. (1993) and Brown *et al*. (1996) were able to block the effect of Ang II with losartan at a concentration of 1 μM. However, Grove and Speth (1993) showed that losartan, in addition to binding at the AT_1 receptor, also has an affinity for a non-Ang II displaceable binding site. Thus, this compound appears to

have additional actions that are unrelated to Ang receptor antagonism. Furthermore, Song *et al*. (1992) were not able to visualize AT_1 or AT_2 receptor binding sites in the rat striatum and candesartan causes a longer lasting blockage of the AT_1 receptor than losartan (Fierens *et al*. 2001). Taken together, it seems unlikely that Ang II exerts its effect on striatal DA release via AT_1 or AT_2 receptors.

A high density of Ang IV binding sites has been found in the striatum (Wright and Harding 1997). It is unlikely that Ang II elicits its effect via direct binding to the Ang IV binding site as it has only a very low affinity for it (Harding *et al*. 1994). Handa (2000) showed that low-affinity Ang II binding to the 'AT_4 receptor' was shifted towards high-affinity binding following renal metabolism of the peptide. Taken together, it may be hypothesized that Ang II exerts its effect in the striatum after its metabolism. Indeed, Ang II is rapidly converted *in vivo* into Ang III by AP-A. Angiotensin III is then metabolized into Ang IV by AP-N (Zini *et al*. 1996; Reaux *et al*. 1999). In agreement with this, the present study shows that the effect of Ang II was blocked by EC33, a selective AP-A inhibitor, or by PC18, a selective AP-N inhibitor. Other experiments with amastatin, a non-selective AP-A inhibitor, led to the same findings (data not shown). This shows that the effect of Ang II is indeed mediated via metabolism into Ang IV or possibly a metabolite of Ang IV.

In line with the involvement of the 'AT_4 receptor', LVV-H7 and Divalinal-Ang IV have similar effects to Ang IV. This suggests a common binding site in the striatum, namely IRAP. Indeed, LVV-H7 elicits similar effects as Ang IV and has already been shown to be an inhibitor of IRAP (IC_{50} 1.5 μM) (Albiston *et al*. 2001; Lew *et al*. 2003). LVV-H7 also exhibits a low affinity for μ- and δ-opioid receptors (Glamsta *et al*. 1992). However, the LVV-H7-induced effect on extracellular DA levels in our study is not mediated via opioid receptors as its effect was not blocked by naltrexone. Whereas Divalinal-Ang IV was originally considered as an AT_4 antagonist (Krebs *et al*. 1996), in a more recent study (Handa 2001) it was shown to cause similar effects to Ang IV in kidney proximal tubular cells. Indeed, recent *in vitro* experiments revealed that Divalinal-Ang IV is an inhibitor of IRAP (IC_{50} 4 μM) (Lew *et al*. 2003). Although Divalinal-Ang IV has a higher IC_{50} than Ang IV (IC_{50} 0.2 μM) for inhibiting IRAP enzyme activity (Lew *et al*. 2003), Divalinal-Ang IV appears to exert a slightly higher DA release than Ang IV in our study. Moreover, after the maximal effect, the extracellular DA levels remain significantly elevated in contrast with the Ang IV effect. LVV-H7 also causes a long-lasting increase in extracellular DA. This is possibly due to a higher resistance towards degradation. Angiotensin-(4–8) has a lower affinity for IRAP than Ang IV and LVV-H7 (Swanson *et al*. 1992). In agreement with this, Ang-(4–8) only causes an increase of the extracellular DA levels at a concentration of 100 μM.

Taking into account a probe recovery of about 10% (CMA Microdialysis), the concentrations of Ang IV, LVV-H7 and Divalinal-Ang IV necessary to elicit an effect on extracellular DA levels are in the lower micromolar range (1–10 μM). In agreement with this, Ang IV, LVV-H7 and Divalinal-Ang IV are found to inhibit the enzyme activity of IRAP in the same concentration range (Lee *et al*. 2003). However, the concentrations used in radioligand binding assays with IRAP can be up to 100-fold lower compared with enzyme inhibition data (Lee *et al*. 2003). A possible explanation for this discrepancy is the difference between the methods used in a radioligand binding assay and an enzyme inhibition assay. In the binding assay, chelators, like EDTA and/or phenanthroline, are present that remove the zinc in the catalytic site of IRAP. In an enzyme inhibition assay and *in vivo*, IRAP is present with a bound zinc. It is possible that the presence or absence of this ion changes the affinity of the inhibitors for IRAP (Lew *et al*. 2003). It is, therefore, more relevant to compare *in vivo* data with enzyme inhibition assays than with radioligand binding assays.

In conclusion, this study shows that the effects of the renin–Ang system peptides in the striatum of the rat are not mediated via AT_1 or AT_2 receptors. In recent years, it has become obvious that Ang II is not the only active Ang peptide. Increasing attention has been focused on the physiological role of Ang IV, especially on its effects in memory retrieval and cognition. The effect of Ang IV had never been examined in the striatum. Our results show that Ang IV or one of its metabolites is able to affect the DA release in the dorsal striatum, suggesting a role in the control of movement.

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