

# Involvement of insulin-regulated aminopeptidase in the effects of the renin–angiotensin fragment angiotensin IV: a review

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**Abstract** For decades, angiotensin (Ang) II was considered as the end product and the only bioactive peptide of the renin–angiotensin system (RAS). However, later studies revealed biological activity for other Ang fragments. Amongst those, Ang IV has drawn a lot of attention since it exerts a wide range of central and peripheral effects including the ability to enhance learning and memory recall, anticonvulsant and anti-epileptogenic properties, protection against cerebral ischemia, activity at the vascular level and an involvement in atherogenesis. Some of these effects are AT<sub>1</sub> receptor dependent but others most likely result from the binding of Ang IV to insulin-regulated aminopeptidase (IRAP) although the exact mechanism(s) of action that mediate the Ang IV-induced effects following this binding are until now not fully known. Nevertheless, three hypotheses have been put forward: since Ang IV is an inhibitor of the catalytic activity of IRAP, its *in vivo* effects might result from a build-up of IRAP's neuropeptide substrates. Second, IRAP is colocalized with the glucose transporter GLUT4 in several tissue types and therefore, Ang IV might interact with the

uptake of glucose. A final and more intriguing hypothesis ascribes a receptor function to IRAP and hence an agonist role to Ang IV. Taken together, it is clear that further work is required to clarify the mechanism of action of Ang IV. On the other hand, a wide range of studies have made it clear that IRAP might become an important target for drug development against different pathologies such as Alzheimer's disease, epilepsy and ischemia.

**Keywords** Angiotensin IV · Insulin-regulated aminopeptidase · Memory · Epilepsy

## Introduction

The renin–angiotensin system (RAS) is widely recognized as the most powerful signalling system for controlling sodium balance, body fluid volumes and arterial blood pressure. The precursor of the RAS is the tetradecapeptide angiotensinogen from which renin, an aspartyl proteinase, forms the biologically inactive Ang I. The octapeptide angiotensin II (Ang II), a major effector peptide of the RAS, is then formed by enzymatic processing of Ang I by the angiotensin converting enzyme (ACE) (Fig. 1) in plasma as well as in tissues such as the brain, kidney and heart [1]. Ang II is well known for its hypertensive effect and its ability to stimulate cardiac re-modeling. Receptors of the AT<sub>1</sub> subtype play a major role in these processes. When stimulated by Ang II, they increase the growth and contractility of cardiac myocytes and vascular smooth muscle cells, enhance sympathetic activity and trigger the release of catecholamines, aldosterone and vasopressin [2–4]. Prevention of the hypertensive and trophic actions of Ang II is proven to be amongst the most successful strategies for the treatment of hypertension and congestive

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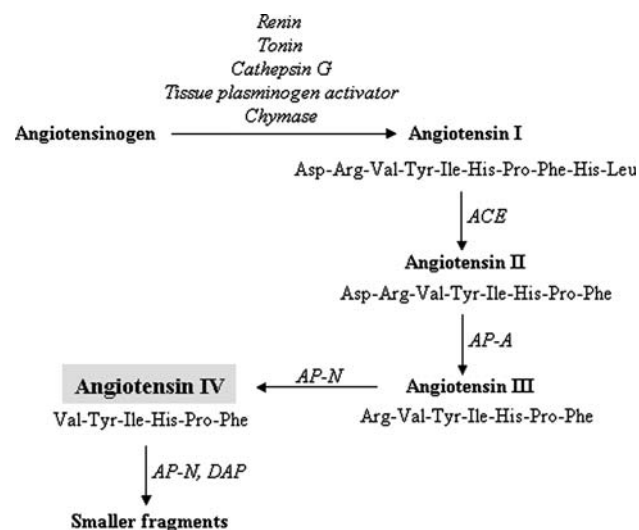
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heart failure. To this end, ACE inhibitors were introduced to decrease the plasma level of Ang II and, in a later stage, non-peptide antagonists were developed to selectively block the AT<sub>1</sub> receptor. AT<sub>2</sub> receptors constitute the other major Ang II receptor subtype. Although they are mainly expressed in foetal tissues, in adulthood, a dramatic upregulation can occur in most tissues after injury. They may be involved in the inhibition of cell proliferation as well as in apoptosis and neuronal differentiation but the underlying intracellular signalling pathways are still poorly defined [4, 5].

Although Ang II has long been assumed to represent the end product of the RAS, more recent studies indicate that shorter Ang peptides, such as Ang IV or Ang IV-(3–8), Ang-III, Ang-(1–7) and Ang-(3–7) also accomplish central, cardiovascular and renal functions [6–14].

### An independent renin–angiotensin system in the brain

Next to the circulating RAS that is a crucial component in blood pressure control, the existence of local RASs in several tissues is evidenced by the fact that many of its components are localized in these tissues as well. A local RAS is for instance present in the heart, the kidney, the liver, the adrenal and in vascular tissues [15]. Moreover, in 1971 Fisher-Ferraro et al. discovered RAS components in the brain [16]. Since, peptides and proteins are unable to pass the blood–brain barrier efficiently, this finding suggests the existence of an independent RAS in the brain [8, 11, 17].



**Fig. 1** Overview of the enzymes involved in the formation and metabolism of Ang IV [7, 44, 45]. ACE: angiotensin-converting enzyme, AP-A: aminopeptidase A, AP-N: aminopeptidase N, DAP: dipeptidyl aminopeptidase

Since 1971, most of the RAS' peptides and enzymes have been localized in brain tissue [18–24]. However, the co-localization of angiotensinogen, renin and Ang I within a single brain cell has failed [25]. Consequently, cross-talk between glia and neurons has been proposed as an element of regulation of the brain RAS [25]. However, since angiotensinogen is present in astrocytes [26, 27] as well as in neurons [28, 29], angiotensins might be produced in both. Another possibility is that angiotensins are synthesized extracellularly [24, 25, 30]. Renin mRNA levels are low or undetectable in the brain [31–39]. Therefore, it has been hypothesized that other enzymes besides renin are producing Ang peptides from angiotensinogen inside the brain. In this respect, biochemical experiments have indicated that tonin [40], cathepsin G [41], tissue plasminogen activator and chymase [42, 43] can fulfill this role.

### Formation and degradation of angiotensin IV

Ang IV is formed *in vivo* from Ang II in two steps: first, Ang II is metabolized into Ang III by aminopeptidase A (AP-A). Next, aminopeptidase N (AP-N) metabolizes Ang III into Ang IV that has the amino acid sequence Val-Tyr-Ile-His-Pro-Phe [44, 45] (Fig. 1). In brain tissue, Ang IV is probably formed and stored mainly intracellularly. Indeed, using the *in vivo* microdialysis technique and LC-MS/MS, we were only able to measure it immediately after probe insertion [46, 47]. After restoration of the tissue integrity, extracellular baseline levels in rat brain were extremely low and estimated to be around 46 pM [47]. The intracellular formation of Ang IV requires the intracellular presence of its precursor Ang II. In this respect, receptor-mediated internalization of Ang II has been demonstrated in neuronal cells [48–51]. Moreover, inside neurons, Ang II is rapidly converted to predominantly Ang IV (80% of all fragments) next to smaller amounts of Ang III, Ang-(1–7) and Ang-(1–6) [48, 52, 53]. These findings suggest that the intracellular conversion of Ang II is not merely a breakdown process but rather a biologically significant conversion to other Ang peptides, of which Ang IV is the most important. How and under which conditions Ang IV is then released remains to be further elucidated. However, we already showed that neuronal depolarization is apparently not able to trigger this since K<sup>+</sup>-stimulation did not increase extracellular basal concentrations of Ang IV *in vivo* [47].

In the periphery, there are until now only sparse data concerning the site of generation of Ang IV and its release. In principle, every organ in which Ang II, AP-A and AP-N are present can generate Ang IV. This is for instance the case for the kidneys which have a high density of AP-A [54] and AP-N [55] and which express AT<sub>1</sub> receptors [56],

implying the presence of Ang II. The same could be true for several other organs such as the heart, the liver, the adrenal and the cardiovascular system. Moreover, Ang IV is most likely generated in human atherosclerotic plaques, monocytes and macrophages [57, 58].

Ang IV is inactivated through enzymatic processing into smaller peptide fragments [44, 59] by predominantly AP-N [7] (Fig. 1). In our most recent study, we further demonstrated the importance of AP-N in the metabolism of Ang IV as we showed that an AP-N inhibitor was able to enhance the Ang IV-induced increase in dopamine release in rat brain [60].

### Angiotensin IV as an active renin–angiotensin fragment

There are numerous studies in which Ang IV was found to produce certain biological functions in the central nervous system suggesting its possible role as a neuropeptide and/or neuromodulator. Initial interest in Ang IV originates from its *ability to increase memory recall and learning* in passive and conditioned avoidance response studies. Braszko and colleagues were the first to demonstrate in 1988 that intracerebroventricular (i.c.v.) injection of Ang IV in rats enhances memory retention in a passive avoidance task [61]. This observation was reproduced independently by Wright et al. and Tchekalarova et al. [62, 63]. Ang IV or an Ang IV analogue such as Norleucine-Ang IV (Nle<sup>1</sup>-Ang IV) was also able to facilitate memory in conditioned avoidance [64], object recognition [65] and Barnes maze experiments [66]. Interestingly, Ang IV and analogues not only improve learning and memory in healthy rodents, they have also been found to prevent memory deficits caused by scopolamine [67–69], mecamlamine [70], alcohol abuse [71, 72], ischemia [73] or bilateral knife cuts of the perforant path [74]. Electrophysiological and biochemical studies revealed that the cognitive effects are at least partially mediated via the hippocampus. In the dentate gyrus and the CA1 field of the hippocampus both *in vitro* [75] and *in vivo* [76], Ang IV and its analogues significantly enhance long-term potentiation (LTP). Ang IV also potentiates potassium-evoked release of acetylcholine from rat hippocampal slices [77]. Since, hippocampal cholinergic–glutamatergic interactions are known to be implied in learning and memory processes, manipulation of the equilibrium between the extracellular acetylcholine and glutamate concentration in the hippocampus was proposed as a mechanism of action of the memory-promoting effects of Ang IV. However, using *in vivo* microdialysis, we were unable to observe changes in the extracellular hippocampal glutamate concentration during i.c.v. administration of Ang IV [14]. In contrast, after i.c.v. injection of Ang IV we observed a tendency to increase followed by a significant

sustained decrease of the extracellular hippocampal acetylcholine concentration [78]. Next to glutamate and acetylcholine,  $\gamma$ -aminobutyric acid (GABA) is also implicated in memory function since patients taking benzodiazepines can suffer from retrograde amnesia and since transgenic mice lacking the  $\alpha 5$  GABA<sub>A</sub> receptor subunit perform significantly better in a water maze paradigm [79]. Moreover, a meta-analysis based on studies of four behavioural tasks of learning and memory (Morris water maze, radial maze, passive avoidance and spontaneous alternation) demonstrated that also the monoaminergic (dopamine, serotonin noradrenalin) systems are involved in cognitive processing [80]. These neurotransmitter systems might thus be involved in the memory-enhancing effect of Ang IV. Indeed, i.c.v. administered peptide produces a decrease of the hippocampal GABA levels next to an increase of the extracellular dopamine and serotonin concentration [14]. Moreover, the cognitive effects of Ang IV such as its facilitation of conditioned avoidance responses, increase of a passive avoidance and improvement of object recognition were blocked by the selective D<sub>2</sub> dopamine receptor antagonist remoxipride [65].

Next to its memory promoting properties, Ang IV also dose-dependently attenuates PTZ-induced seizures [81]. Furthermore, Ang IV shows an *anti-epileptogenic effect* as it not only suppresses the maintenance of the generalization phenomenon during the kindling procedure but also blocks the development of epileptic-like state in mice [82, 83]. We showed that i.c.v. administered Ang IV is anticonvulsant in the acute pilocarpine model for focal epilepsy in rats [14]. This was accompanied by a concomitant increase of the hippocampal extracellular dopamine and serotonin concentration. Possibly, this plays an important role in the anticonvulsant effect of Ang IV. Indeed, several well-known anti-epileptic drugs can elicit a monoaminergic stimulation [84–90]. Moreover, it was shown in our laboratory that intrahippocampally administered dopamine and serotonin protect rats against pilocarpine-induced convulsions via respectively D<sub>2</sub> and 5-HT<sub>1A</sub> receptor activation [91].

Ang IV is also able to influence the dopaminergic neurotransmission in the striatum. We showed that local administration of Ang IV is able to cause an increase of the extracellular dopamine concentration in the rat striatum, a functionally important structure of the basal ganglia implicated in the control of movement but also in the procedural or habit memory system [12]. This can be of importance for the development of new therapies against Parkinson's disease since it is characterized by a depletion of dopamine in the striatum [92].

Several studies have reported a potential role for angiotensin peptides the *protection against cerebral*

*ischemia*-induced neurological damages. It was already stated by some groups that this effect is independent from AT<sub>1</sub> receptors [93–95]. Instead, the AT<sub>2</sub> receptor was put forward as the mediating binding site [96–98]. However, recently the study of Faure et al. points for the first time to Ang IV as the effector of this cerebral protection [99]. An injection in the carotid artery of rats with Ang IV decreased the brain infarct volume, resulting in a marked decrease in mortality. This effect was indeed AT<sub>2</sub> receptor independent but unfortunately, the involvement of AT<sub>1</sub> receptors was not excluded. Pretreatment with the nitric oxide synthase inhibitor L-NAME abolished the protective effect, suggesting that Ang IV triggers a nitric oxide dependent pathway [99]. Ang IV has also been shown to promote cell survival in the hippocampus [100].

Ang IV, as similar to Ang II and Ang III, *increases blood pressure* after central administration in anaesthetized rats [101–103]. Chronic elevation of Ang IV specifically in the brain in a transgenic mouse model was also associated with an increased blood pressure [104]. Intriguingly, these effects were AT<sub>1</sub> dependent as they were blocked with an AT<sub>1</sub> receptor antagonist. In contrast, Faure and colleagues recently observed a vasoconstrictor response to Ang IV in isolated rat basilar arteries, an *ex vivo* preparation that fails to constrict in the presence of Ang II. This effect was unaffected by AT<sub>1</sub> or AT<sub>2</sub> receptor blockade, but instead abolished by removal of endothelium and blockade of endothelin ET<sub>A</sub>/ET<sub>B</sub> receptors [105].

The peripheral role of Ang IV, particularly in the kidney, is unclear since contradictory effects have been reported. Nevertheless, Ang IV is certainly *active at the vascular level* since internal carotid or renal infusion of Ang IV causes changes in cerebral or renal blood flow. Some groups observed an increased cerebral and renal cortical blood flow [106–108] that was independent upon AT<sub>1</sub> receptor activation [108]. Other groups including ours however have observed a reduction in renal blood flow by Ang IV [109–112, Yang et al., personal communication] that arises after renal cortical vasoconstriction through AT<sub>1</sub> receptor activation [113, Yang et al., personal communication]. Ang IV was also found to elicit a natriuretic effect [108], which would result from an increased renal sodium excretion, possibly independent from its renal hemodynamic effects. However, we failed to produce any alteration in the urinary sodium concentration and the urinary volume during intrarenal infusion of Ang IV [Yang et al., personal communication].

As a pathophysiological agent, Ang IV participates in different steps of *atherogenesis* including the initial plaque formation and later stages such as plaque rupture and thrombus formation [114]. In a model of balloon injury, Ang IV binding was increased in media, neointima and re-endothelialized cell layer, suggesting a role for Ang IV in

vascular re-modeling after damage [115]. Ang IV up-regulates several pro-inflammatory factors [116] and could therefore participate in some steps of the inflammatory response. In vascular smooth muscle cells, Ang IV increases the production of monocyte chemoattractant protein-1, the main chemokine involved in monocyte recruitment and up-regulates the expression of the intercellular adhesion molecule-1 that is involved in the attachment and transmigration of circulating cells into the damaged tissue. It also increases cytokines such as interleukin 6 and tumour necrosis factor  $\alpha$  and stimulates the production of prothrombotic factor plasminogen activator inhibitor-1 and could therefore also participate in the perpetuation of the inflammatory response and the thrombus formation [116, 117]. Finally, Ang IV was found to activate the nuclear transcription factor- $\kappa$ B (NF- $\kappa$ B), a pivotal transcription factor involved in inflammatory diseases and immune responses [116]. The mechanism by which Ang IV produces these effects in vascular smooth muscle cells is unclear but the involvement of AT<sub>1</sub> receptors was excluded since the same effects occurred in AT<sub>1</sub> receptor knock-out mice [116].

### The Ang IV binding site(s)

Some of the effects of Ang IV are mediated by its interaction with AT<sub>1</sub> and/or AT<sub>2</sub> receptors. This has indeed been shown for many of its peripheral effects [109–112, Yang et al., personal communication] and for some central effects [61, 118, 104, Yang et al., personal communication]. Indeed, Ang IV is a full agonist for the AT<sub>1</sub> receptors and the corresponding EC<sub>50</sub> value is in the micromolar range [119, 120]. Moreover, Wright et al. showed that Ang IV also binds to AT<sub>2</sub> receptors [62, 121].

### The AT<sub>4</sub> receptor as a novel angiotensin-binding site

Many Ang IV-induced effects are already observed at nanomolar concentrations and, most importantly, are not blocked by classical non-peptide AT<sub>1</sub> and/or AT<sub>2</sub> receptor antagonists such as losartan, candesartan, PD123.177 and PD123.319 [12, 14, 93–95, 99, 105, 108, 116, 122, 123]. Moreover, it was found that certain effects previously attributed to Ang II are in fact induced after its conversion to Ang IV. Indeed, using *in vivo* microdialysis, Mendelsohn et al. [124] and Brown et al. [125] first proposed AT<sub>1</sub> receptor dependency of the increase of dopamine release observed after the local administration of Ang II in the rat striatum. Using a similar experimental set-up, we showed that next to Ang II, local administration of Ang IV also leads to a concentration-dependent increase of the

extracellular striatal dopamine concentration [12]. Interestingly, the effects of both Ang II and Ang IV could neither be blocked by the AT<sub>1</sub> antagonist candesartan nor by the AT<sub>2</sub> antagonist PD123, 319, which is in line with Song et al. who were unable to detect AT<sub>1</sub> or AT<sub>2</sub> receptor binding sites in the striatum [126]. Instead, the effect of Ang II was inhibited by the AP-A inhibitor EC33 as well as the AP-N inhibitor PC18, indicating that the effect of Ang II is mediated via metabolism into Ang IV. In a similar way, Braszko et al. suggested that cognitive effects attributed to Ang II may result from its conversion to Ang IV [65].

Thus the inability of AT<sub>1</sub> and AT<sub>2</sub> antagonists to block several effects induced by Ang IV together with the previous identification of binding sites with high affinity for [<sup>125</sup>I]-Ang IV in different tissue types [106, 127–134] that had only a very low affinity for Ang II and AT<sub>1</sub> and AT<sub>2</sub> receptor antagonists [127, 135–138] provided compelling evidence for the existence of a novel angiotensin receptor subtype which was called the AT<sub>4</sub> receptor [4, 139] of which the pharmacological profile deviates significantly from that of AT<sub>1</sub> and AT<sub>2</sub> receptors. Instead, it is activated by Ang IV and by synthetic peptide analogues like Nle<sup>1</sup>-Ang IV [131, 140, 141] and Norleucinal [140].

Structure-activity studies revealed that the first three amino acid residues of Ang IV are critical for binding to the AT<sub>4</sub> receptor [142]. An N-terminal primary  $\alpha$ -amine and an L-conformation for the first amino acid  $\alpha$ -carbon are requisites for high-affinity binding of the hexapeptide to the AT<sub>4</sub> receptor [143]. An activated aromatic ring in the side chain of amino acid residue 2 and a hydrophobic amino acid in position 3 are important for high-affinity binding [144]. Discrete modifications to the subdomains of the valine residue in position 1, in particular a straight-chain aliphatic moiety containing four carbons, resulted in the 100-fold higher affinity analogue Nle<sup>1</sup>-Ang IV [143]. The putative AT<sub>4</sub> receptor antagonist Divalinal-Ang IV is generated by the replacement of the amide bonds between Val<sup>1</sup> and Tyr<sup>2</sup> and between Val<sup>3</sup> and His<sup>4</sup> with [CH<sub>2</sub>-NH]. Divalinal-Ang IV binds to the AT<sub>4</sub> receptor in bovine adrenal membranes with a K<sub>i</sub> of  $\sim$ 445 nM, which is about 20-fold higher than Ang IV (K<sub>i</sub>  $\sim$ 16.8 nM) [141]. It antagonizes some of the effects of Ang IV such as enhancement of long-term potentiation in rat hippocampus [76], facilitation of K<sup>+</sup>-evoked acetylcholine release [77] and the activation of NF- $\kappa$ B in vascular smooth muscle cells [116]. On the other hand, its antagonistic nature remains controversial since it mimics some effects of Ang IV such as the increase of the extracellular dopamine concentration in the striatum of the rat [12] and the phosphorylation of Erk-1/2 and p38 kinase in human proximal tubule epithelial cells [145].

### LVV-haemorphin-7 as an AT<sub>4</sub> ligand

The undetectable extracellular baseline Ang IV levels and the mismatch between the components of the RAS and the distribution of AT<sub>4</sub> binding sites in the brain have previously led to the hypothesis that the native ligand for the AT<sub>4</sub> receptors may not be Ang IV. Exploring this possibility, an extract of sheep cerebral cortex was screened for Ang IV binding site affinity. Resulting from these experiments, the decapeptide LVV-haemorphin-7 (LVV-H7) with the amino acid sequence Leu-Val-Val-Tyr-Pro-Trp-Thr-Gln-Arg-Phe was proposed as the native AT<sub>4</sub> ligand [146–148], exhibiting a high affinity (K<sub>i</sub>  $\sim$ 73 nM) [141]. LVV-H7 is indeed abundantly present in the brain, namely approximately 2 nmol of peptide per gram of sheep brain tissue [146, 149]. It is formed from  $\beta$ -globin that was identified in embryonic mouse brains [150] by the enzymes pepsin [151] and a high-molecular-weight aspartic proteinase [152]. LVV-H7 mimics most of the biological effects of Ang IV. We showed an LVV-H7-induced increase of the extracellular dopamine levels in the rat striatum as similar to Ang IV [12]. Moreover, it is also able to stimulate spatial learning [66, 153]. Whereas the central actions of LVV-H7 thus seem consistent with its affinity for the Ang IV binding site, it does not mimic the peripheral effects of Ang IV as it has no effect on renal blood flow or blood pressure [110]. This is not surprising as those effects of Ang IV are predominantly induced after activation of AT<sub>1</sub> receptors, whereas we observed a disability of LVV-H7 to bind at AT<sub>1</sub> receptors [Demaegdt et al., personal communication].

### Insulin-regulated aminopeptidase corresponds to the AT<sub>4</sub> receptor

Initially the classification of the AT<sub>4</sub> receptor was based on its distinct pharmacological properties. Later, structural studies also provided evidence that, unlike AT<sub>1</sub> and AT<sub>2</sub> receptors, it does not belong to the family of 7-transmembrane domain receptors. Instead, photoaffinity labelling experiments of Ang IV binding sites from bovine tissue with [<sup>125</sup>I]benzoylphenyl-Ala-Ang IV revealed that AT<sub>4</sub> receptors exist as complexes formed of three different peptides with molecular weights of 165 kDa, 50–60 kDa and 70–80 kDa, respectively [130, 154, 155]. Using [<sup>125</sup>I]Nle<sup>1</sup>-BzPhe<sup>6</sup>-Gly<sup>7</sup>-Ang IV, the 165 kDa peptide was labelled in membranes from SK-N-MC cells, a human neuroblastoma cell line that expresses binding sites with a high affinity for Ang IV [156]. In 2001, Albiston and colleagues identified the 165 kDa peptide as insulin-regulated aminopeptidase (IRAP), a membrane-associated aminopeptidase homologous to AP-A, AP-N and other

Zn<sup>2+</sup>-dependent aminopeptidases included in the large family of gluzincin aminopeptidases [140, 157]. It is an integral membrane protein of 916 amino acid residues, consisting of an acidic intracellular region (109 AA) followed by a hydrophobic transmembrane segment (22 AA  $\alpha$ -helix) and a 785 AA extracellular domain containing its aminopeptidase activity [158–161]. Experiments with IRAP transfected cells reveal that it indeed binds the radiolabelled Ang IV analogue [<sup>125</sup>I]Nle<sup>1</sup>-Ang IV with high affinity and that it can be selectively cross-linked with [<sup>125</sup>I]Nle<sup>1</sup>-BzPhe<sup>6</sup>-Gly<sup>7</sup>-Ang IV. The correspondence between the AT<sub>4</sub> receptor and IRAP was also evidenced by the similar regional distribution of IRAP mRNA (in situ hybridization histochemistry), IRAP positive immunoreactivity (immunohistochemistry) and [<sup>125</sup>I]Nle<sup>1</sup>-Ang IV binding (autoradiography) in mouse brains [140].

IRAP was formerly known as gp160 and vp165 and was used to designate an enzyme in the rat. Cystein aminopeptidase or oxytocinase (Otase) (EC 3.4.11.3) are considered as the human variant of IRAP since there is an 87% homology of their amino acid sequences [157, 162–165]. In turn, Otase was found to be identical to placental leucine aminopeptidase (P-LAP) [166], a major human placental protease. To ensure the clarity of this review, the membrane protein IRAP/Otase/P-LAP will be referred to as IRAP.

### Distribution of insulin-regulated aminopeptidase

Northern blotting and immunoblotting indicate that IRAP has a broad tissue distribution. Besides adipocytes and skeletal muscle, IRAP was also found to be present in the brain, heart, kidney, spleen, lung, testis, bladder, prostate, adrenals and colon [121, 127, 134, 160, 167, 168]. IRAP is also released from the apical membranes of the placental syncytiotrophoblasts [169].

Within the brain, the distribution pattern of IRAP is consistent amongst monkeys [171], rats [122, 134], mice [170] and guinea pigs [135]. In general, IRAP is distributed in most brain areas including cortical regions, hippocampus, amygdala, thalamus, hypothalamus, caudate nucleus, basal nucleus of Meynert, nucleus accumbens, lateral olfactory tract, ventral tegmental area, substantia nigra pars compacta, superior colliculus, periaqueductal gray, granular and molecular layers of the cerebellum, inferior olivary nucleus, lateral vestibular nucleus, locus coeruleus, motor trigeminal and facial nuclei [132, 134, 140, 168]. Within the spinal cord, autoradiography has visualized Ang IV binding sites on somatic and autonomic motor neurons in the lateral horn of thoracic and lumbar segments, in all dorsal root ganglia and in lamina II of the dorsal horn [171].

Sub-cellular fractionation and immunohistochemistry of different neuronal cell lines demonstrated that IRAP is predominantly present intracellularly [172]. IRAP immunoreactivity appeared punctuate throughout the somata and proximal dendrites of neurons [134].

### Mechanism(s) of action of angiotensin IV through interaction with insulin-regulated aminopeptidase

At the moment there is no conclusive evidence with respect to the molecular mechanism of how interaction with IRAP by Ang IV leads to the above-described effects. However, three hypotheses have been put forward to explain the role of IRAP in the physiological effects of Ang IV. They include (i) inhibition of the catalytic activity of IRAP and the consequent build-up of its substrates, (ii) Ang IV mediated modulation of the glucose uptake via interference with the translocation of IRAP containing GLUT4 vesicles and (iii) activation of IRAP acting as a receptor and causing intracellular signalling.

### Ang IV and analogues are inhibitors of the aminopeptidase activity of IRAP

IRAP is able to cleave the N-terminal amino acid from several bioactive peptides in vitro. These peptides include Ang III, but its degradation is relatively slow compared with the other substrates such as oxytocin [157], vasopressin, lys-bradykinin, met-enkephalin, dynorphin A 1–8, neurokinin A, neuromedin B, somatostatin and cholecystokinin-8 [141, 163, 168, 173]. Although N-terminal cystein residues contained in vasopressin and oxytocin peptides were initially thought to be the preferential targets for the enzyme, several peptides that do not contain cystein residues are also hydrolysed by IRAP in vitro (lys-bradykinin, met-enkephalin, dynorphin A, neurokinin A and neuromedin B). However, other peptides that possess N-terminal cystein residues and intramolecular disulfide bonds, such as calcitonin and endothelin are not cleaved by the enzyme [174].

Recently, the first in vivo evidence for the catalytic action of IRAP emerged. Wallis et al. showed that in vivo N-terminal degradation of intravenously injected vasopressin and to a lesser extent oxytocin was indeed partially dependent upon the presence of IRAP [175]. Vice versa, the level of endogenous vasopressin in the blood was increased in IRAP knock-out mice. These data strongly suggest that a normal IRAP activity is important in regulating the concentration of several neuropeptide substrates such as vasopressin and oxytocin. This clearing function is particularly essential in gestation during which IRAP

activity increases, reaching a maximum at near term [169, 176, 177]. This is possibly induced by an excessive concentration of one or more substrates as a negative feedback mechanism. An increase of IRAP activity at the cell surface was indeed observed in renal tubule epithelial cells with vasopressin via the V<sub>2</sub> receptor [178] and in umbilical vascular endothelial cells with oxytocin [179]. Since, oxytocin is the most potent uterotonic peptide hormone, IRAP most likely prevents premature onset of uterine contraction by degrading oxytocin, thus playing a crucial role in the maintenance of a normal pregnancy. A decrease of IRAP activity is indeed observed in sera of women that experienced spontaneous preterm delivery [176, 177].

The AT<sub>4</sub> ligands Ang IV, LVV-H7 and Nle<sup>1</sup>-Ang IV are relatively high affinity competitive inhibitors of the catalytic activity of IRAP in vitro [141]. Therefore, it has been proposed that “AT<sub>4</sub> ligands”-induced physiological effects are related to the reduced processing of these substrates of IRAP [140, 141]. Ang IV and analogues inhibit the enzyme activity of IRAP with Ki values between 113 nM and 2.3 μM (Table 1). They bind to the catalytic site of IRAP, especially to the Gly and Ala residues of the exopeptidase motif GAMEN [180] but also to the characteristic gluzincin aminopeptidase Zn<sup>2+</sup> binding HEXXH(X<sub>18</sub>)E motif [157, 160, 162, 180, 181]. An intriguing phenomenon relates to the observation that Ang IV and other AT<sub>4</sub> ligands display a significantly (up to about 20-fold) lower affinity in the enzyme assays compared to competition [<sup>125</sup>I]-Ang IV binding experiments (Table 1). Recently, this discrepancy could be ascribed to the absence or presence of Zn<sup>2+</sup> chelators. In the binding assay, divalent cation chelators such as EDTA and phenantroline remove the Zn<sup>2+</sup> ion from IRAP. In contrast, these substances are not required for the enzyme activity assay. Whereas high-affinity binding of [<sup>125</sup>I]-Ang IV can only be detected with the apo-enzyme, inhibition of enzyme activity occurs to the native enzyme [182].

Next to Ang IV, Divalinal-Ang IV is also an inhibitor of the aminopeptidase activity of IRAP in vitro [141]. This might explain why this semi-synthetic peptide in certain experimental set-ups mimics the effects of Ang IV [12,

145]. However, the discrepancy with other experiments in which it antagonizes an Ang IV-induced effect [76, 77] remains unsolved.

Previously, the selectivity of AT<sub>4</sub> ligands has been questioned since the catalytic activity of the structurally related aminopeptidase AP-N has also been found to be sensitive to LVV-H7 and Ang IV [8, 148]. In addition, LVV-H7-sensitive high-affinity binding of [<sup>125</sup>I]Ang IV was observed in membranes of rabbit collecting duct cells [148]. As the kidney is a rich source of AP-N, this enzyme may be considered to represent a target for [<sup>125</sup>I]Ang IV in the binding studies. However, when comparing the enzymatic and binding properties of human recombinant IRAP and AP-N, we discovered that both enzymes have a clearly distinct pharmacological profile and that high affinity [<sup>125</sup>I]Ang IV binding is only detectable to IRAP [183].

Until now, the hypothesis that Ang IV and analogues mediate their effects via an inhibition of the catalytic activity of IRAP could not be proven in an in vivo experimental set-up. However, we showed that the anti-convulsant effect and the increase of the extracellular hippocampal dopamine and serotonin levels caused by i.c.v. administered Ang IV could be completely abolished by the concomitant i.c.v. infusion of the somatostatin receptor 2 antagonist cyanamid 154806 [14]. These data suggest that these effects of Ang IV are mediated via an inhibition of IRAP, leading to an enhanced concentration of its substrate somatostatin and eventually the anticonvulsant effect. Taken together, our study is the first to obtain indirect in vivo evidence for the inhibition hypothesis. Next to somatostatin, other IRAP substrates may also play a role in the anticonvulsant effect of Ang IV. Dynorphin is widely accepted to have significant anti-seizure properties and is even seen as an endogenous anticonvulsant [184]. There is also evidence that cholecystokinin-8 and met-enkephalin possess anticonvulsant effects in a variety of animal seizure models [185–187].

Next to the anticonvulsant effect of Ang IV, its memory-enhancing properties might also arise from an inhibition of the aminopeptidase activity of IRAP. Indeed, amongst its substrates, vasopressin is known for its convincing facilitation of memory consolidation and retrieval in the passive avoidance paradigm [188–190]. Moreover, vasopressin reversed memory deficits induced by scopolamine [191] and transient forebrain ischemia [192]. Finally, a gene replacement study revealed that the expression of the vasopressin V<sub>1a</sub> receptor in the lateral septum is a necessity for social recognition memory [193]. Oxytocin might also be involved since it improves reference memory in mice [194]. In social stimulus situations characterized by a mild increase in emotional arousal and a negligible degree of stress, oxytocin produces vasopressin-like facilitatory effects on memory processing [195, 196]. It convincingly

**Table 1** Comparison of the apparent affinities of AT<sub>4</sub> ligands, as assessed by radioligand displacement and fluorescent substrate cleavage inhibition assays [141]

	Ki (binding assay) (nM)	Ki (enzyme assay) (nM)
Nle <sup>1</sup> -Ang IV	1.5	340
Ang IV	16.8	113
LVV-H7	73	845
Divalinal-Ang IV	445	2300

Nle<sup>1</sup>-Ang IV: Norleucine<sup>1</sup>-Ang IV, Ang IV: angiotensin IV, LVV-H7: LVV-haemorphin-7

improved LTP and long-term (but not short-term) spatial memory within the eight-arm radial maze task [194]. However, the role of oxytocin on memory storage and retrieval seems not as clear-cut as for vasopressin since its effect depends highly on the type of learning tasks presented, the dose of oxytocin injected and in the case of central administration, the specific brain site injected [197–202]. Indeed, in aversive learning situations or in learning contexts based on emotionally arousing and stressful situations, oxytocin exerts amnesic actions [200, 202]. As an example of this, it was shown that oxytocin, injected into the nucleus basalis of Meynert, plays an inhibitory role in spatial learning in the stressful Morris water maze task [203]. Next to vasopressin and oxytocin, cholecystokinin-8 also facilitates memory consolidation in the inhibitory avoidance paradigm [204] and the two-trial memory task [205] and reverses performance deficits of spatial learning in aged Fischer rats [206] and spatial recognition impairment induced by stress [207]. It protected against amnesia induced by electroconvulsive shock, scopolamine, NMDA antagonists or protein kinase inhibitors in the passive avoidance test [208–211]. Decreased cholecystokinin within areas such as the prefrontal cortex, amygdala and hippocampus are associated with learning and memory deficits [212]. Cholecystokinin immunoreactivity is decreased in human Alzheimer's disease brain [213] and cholecystokinin analogues prevent cholinergic degeneration in the rat cerebral cortex in an animal model of Alzheimer's disease [214]. Moreover, an age-associated decrease in cortical cholecystokinin concentration may underlie the learning and memory deficits attributable to normal aging in rodents and humans [215, 216].

### Modulation of the translocation of IRAP and GLUT4

As its name already reveals, IRAP is morphologically and possibly also functionally involved in the glucose homeostasis. Keller and colleagues were the first to identify IRAP in intracellular vesicles that carry the insulin-regulated glucose transporter GLUT4 [160, 217]. Deprived from insulin, 85–90% of the GLUT4 vesicles are found intracellularly. This is in line with the recent study of Fernando et al. who could only detect a small amount of IRAP at the plasma membrane of mouse brain neurons [172]. In contrast, they showed that IRAP co-localizes with the vesicular marker VAMP2 [172]. Moreover, electron microscopy identified IRAP specific precipitate associated with secretory vesicles [172].

GLUT4 vesicles migrate to the cell surface after exposure with insulin, thereby enhancing the glucose uptake in the cell [218–224]. Since, IRAP is situated on GLUT4 vesicles, insulin also regulates its trafficking and hence the

amount of IRAP that is present at the cell surface [158, 160, 161, 225–230]. This is well described in several cell lines such as 3T3-L1 adipocytes [161, 225], cardiomyocytes [226] and skeletal muscle cells [228]. The co-translocation of IRAP and GLUT4 is likely to be related to the presence of a similar dileucine trafficking motif in their cytoplasmic domain and to the recognition of these motifs by the same intracellular retention/sorting proteins [223, 231–233], including the Rab GTPase-activating protein AS160, a substrate of the protein kinase Akt [234, 235], p115, a vesicle tethering-factor implicated in endoplasmic reticulum to Golgi apparatus and post Golgi apparatus movements [236], FHOS, a formine homologue overexpressed in spleen [237] and acyl-coenzyme A dehydrogenases such as tankyrase-1 and tankyrase-2 [238–240]. The importance of those interactions is revealed in tankyrase-1 knockdown 3T3-L1 adipocytes in which insulin-stimulated IRAP and GLUT4 translocation as well as glucose uptake were attenuated [240].

Ang IV is a unique ligand because of its ability to bind to IRAP, thereby possibly interfering with its recycling and by thus enhancing or prolonging the exposure of GLUT4 at the cell surface. The consequent increased glucose uptake could then be responsible for the Ang IV-induced effects such as the enhancement of learning and memory. In this context, trials with both rodents and humans have demonstrated that glucose enhances cognitive performance [241]. Interestingly, this is particularly the case in elderly subjects and in patients with Alzheimer's disease [241, 242]. Possibly, the effect of glucose on learning and memory results from an interference with the neurotransmission. In this respect, glucose has been shown to enhance both acetylcholine synthesis and release [243]. Glucose may also account for the higher metabolic rate that arises during learning tasks [244]. Finally, glucose stimulates the tuberous sclerosis complex-mammalian target of rapamycin (TSC-mTOR) pathway [245], a major intracellular cascade that controls the synthesis of several proteins that are involved in cellular growth processes by activation and inactivation of ribosomal S6 kinase and 4E binding protein 1 [246–252]. A disruption of the TSC-mTOR pathway impairs memory formation.

The link between glucose and epilepsy is until now not fully understood. Heterozygote mutation in the gene for GLUT1, the sodium- and insulin-independent glucose transporter, causes impaired glucose transport across the blood–brain barrier, leading to hypometabolism and seizures [253] that are mostly treated with a strict ketogenic diet. On the other hand, inhibition of glycolysis by 2-deoxy-D-glucose has powerful antiepileptic effects in rats and was recently proposed as a scientific basis for the ketogenic diet [254]. This put forward inhibitors of glycolysis as a potentially pharmacological approach for refractory epilepsy.



## IRAP as a receptor for Ang IV

As described above, local administration of Ang IV and LVV-H7 elicited a clear-cut increase of the extracellular dopamine concentration in the rat striatum [60]. Interestingly, this effect could not be reproduced by administration of the aminopeptidase inhibitor 2(S)-benzyl-3-[hydroxy(1'R-aminoethyl)phosphinyl]propanoyl-L-tyrosine (compound 7B) at a concentration at which it is capable of inhibiting the enzymatic activity of IRAP. Since the effect on the dopamine release is AT<sub>1</sub> and AT<sub>2</sub> independent [12] and since no other binding sites of Ang IV have been discovered, a receptor function for IRAP and an agonist role for Ang IV was assumed. This would imply that IRAP is capable of activating certain intracellular signalling pathways. This was indeed observed with Ang IV in several cell lines since it caused an increased DNA synthesis as measured by [<sup>3</sup>H]-thymidine incorporation in mouse neuroblastoma NG-108-15 [115], human neuroblastoma SK-N-MC and in Chinese hamster ovary cells CHO-K1 [Demaegd et al., personal communication]. Ang IV was also found to induce a rapid and transient rise of [Ca<sup>2+</sup>]<sub>i</sub> in Madin Darby bovine kidney cells [255], in human proximal tubule epithelial cells [145] and in opossum kidney cells [256]. In porcine aortic endothelial cells, the increase in [Ca<sup>2+</sup>]<sub>i</sub> was found to trigger the production of nitric oxide and cGMP [11, 257, 258]. Nanomolar concentrations of Ang IV increased the phosphorylation of Erk-1/2 and p38 kinase in human proximal tubule epithelial cells. Similarly, Ang IV caused a concentration-dependent tyrosine phosphorylation of p125 focal adhesion kinase and p68 paxillin in pig proximal tubule cells [259]. Moreover, it stimulated c-Fos expression in the hippocampus and the piriform cortex of the rat [122]. More recently, Ang IV was shown to activate NF-κB in smooth muscle cells [116]. Finally, Ang IV activated signalling molecules involved in lung endothelial cell proliferation such as PI3 kinase, Erk-1/2, PKBα, p70 ribosomal S6 kinase and the eukaryotic RNA translation initiation factor 4EBP1 [260, 261].

As IRAP is likely to be a membrane-bound protein with a single membrane spanning α-helix only, dimerization is required for it to act as a receptor. At first glance, this seems hardly compatible with the classification of IRAP as an enzyme and with its three-dimensional structure comprising a single membrane spanning α-helix. However, homodimer formation is one of the characteristic features of the membrane-bound M1 metallopeptidase family [262] to which IRAP belongs. As a dimer, this enzyme can convey information across the cell membrane in the same way as growth factors and cytokine receptors. In this line, it has already been shown that the structurally related AP-N (EC 3.4.11.2) and dipeptidylpeptidase IV (DP IV, EC 3.4.14.3) have a similar subcellular location and dimeric

structure and that they are able to mediate intracellular signalling [263–267]. Monoclonal antibodies towards AP-N trigger both IP<sub>3</sub> receptor-linked Ca<sup>2+</sup> release and phosphorylation of the mitogen-activated protein kinases (Erk1/2, JNK and p38) in human monocytes [267, 268]. Signalling has also been described for dipeptidylpeptidase IV which is involved in T cell activation by potentiating the proliferative response after stimulation of the TcR/CD3 complex [263, 269]. Despite the dipeptidylpeptidase IV molecule has a short cytoplasmic domain of only six amino acids, the binding of enzyme inhibitors and of certain monoclonal antibodies to the extracellular domain of this enzyme leads to the tyrosine phosphorylation of several signalling proteins and activation of mitogen-activated protein kinases in T lymphocytes (involved in its antitumour effect) as well as human hepatocarcinoma cells [265, 270, 271]. It was also found to mediate early phosphorylation mechanisms in non-haematopoietic cells such as hepatocarcinoma [265].

## General conclusion

Since 1988, several studies examining different biological effects have made it clear that Ang IV is not merely a metabolite of Ang II but that it is an active member of the RAS with central and peripheral effects. Some of its effects such as the increase in blood pressure and cerebral and renal cortical blood flow have turned out to be AT<sub>1</sub> receptor mediated. On the other hand, Ang IV also exerts effects that are not caused by AT<sub>1</sub> or AT<sub>2</sub> receptor activation. Amongst them is the enhancement of learning and memory, the anticonvulsant and anti-epileptogenic effect, the ability to increase the striatal dopamine release and the protection against cerebral ischemia. These findings unequivocally proved that Ang IV has an own pharmacodynamic profile distinct from Ang II. The mechanism by which these effects are caused is up till now not fully understood although three hypotheses have been brought forward, based on the high affinity of Ang IV for IRAP. First, there is in vitro and indirect in vivo evidence that Ang IV is capable of inhibiting the catalytic activity of IRAP, thereby prolonging the half-life of its neuropeptide substrates. Subsequently, these neuropeptides would produce the observed effects of Ang IV. On the other hand, since IRAP is co-localized with the glucose transporter GLUT4, it has been hypothesized that the binding of Ang IV to IRAP would modulate the translocation of GLUT4 resulting in an increased glucose uptake in e.g. neurons. The final and most intriguing hypothesis ascribes receptor properties to IRAP, implying it to form a dimer. In that case, Ang IV would behave as an agonist, eliciting intracellular signalling. Moreover, it cannot be excluded that

Ang IV mediates its effects via a mixture of all three mechanisms, possibly depending upon tissue type. For instance, the existence of IRAP as an enzyme in one tissue and as a receptor in other tissues could explain why Divalinal-Ang IV in some experiments appears to behave as an Ang IV antagonist while in others, it mimics the effects of Ang IV. Taken together, it is clear that further work is required to identify the exact mechanism of action of Ang IV. Nevertheless, the different effects observed with Ang IV points toward IRAP as a potentially interesting target for future drugs that could be used for the treatment of e.g. Alzheimer's dementia or epilepsy.

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