Evidence That the Angiotensin IV (AT₄) Receptor Is the Enzyme Insulin-regulated Aminopeptidase*

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Central infusion of angiotensin IV or its more stable analogues facilitates memory retention and retrieval in normal animals and reverses amnesia induced by scopolamine or by bilateral perforant pathway lesions. These peptides bind with high affinity and specificity to a novel binding site designated the angiotensin AT₄ receptor. Until now, the AT₄ receptor has eluded molecular characterization. Here we identify the AT₄ receptor, by protein purification and peptide sequencing, to be insulin-regulated aminopeptidase (IRAP). HEK 293T cells transfected with IRAP exhibit typical AT₄ receptor binding characteristics; the AT₄ receptor ligands, angiotensin IV and LVV-hemorphin 7, compete for the binding of [¹²⁵I]Nle¹-angiotensin IV with IG₅₀ values of 32 and 140 nM, respectively. The distribution of IRAP and its mRNA in the brain, determined by immunohistochemistry and hybridization histochemistry, parallels that of the AT₄ receptor determined by radioligand binding. We also show that AT₄ receptor ligands dose-dependently inhibit the catalytic activity of IRAP. We have therefore demonstrated that the AT₄ receptor is IRAP and propose that AT₄ receptor ligands may exert their effects by inhibiting the catalytic activity of IRAP thereby extending the half-life of its neuropeptide substrates.

Central infusions of the hexapeptide VYIHPF (angiotensin IV, Ang IV) or its more stable analogues, Nle¹-Ang IV and Norleucinal Ang IV, facilitate memory retention and retrieval in rats in the passive avoidance and Morris water maze paradigms (1–3). In two rat models of amnesia induced by the muscarinic antagonist, scopolamine, or bilateral perforant pathway lesion, the Ang IV analogues reversed the memory deficits detected utilizing the Morris water maze paradigm (3, 4). Enhancement of long term memory by Ang IV has also been demonstrated in species as distant as crabs (5). Angiotensin IV and its analogues enhance long term potentiation in both the dentate gyrus in vitro (6) and the CA1 region of the hippocampus in vitro (7), possibly via actions at the post-synaptic terminal. We have also shown that Ang IV enhances K⁺-evoked acetylcholine release from rat hippocampal slices (8).

The actions of Ang IV and its analogues are mediated by the angiotensin AT₄ receptor, defined by an international nomenclature committee (9) as the high affinity binding site specific for Ang IV (10). The AT₄ receptor has since been shown to bind with nanomolar affinity the decapeptide, LVYYPWTQRF (LVV-H7), isolated from sheep cerebral cortex (19).

Although first identified in bovine adrenal, the receptor is widely distributed throughout the brain and peripheral organs (11). In the central nervous system, its distribution is highly conserved in guinea pig (12), macaque monkey (13), and human (14) brains. AT₄ receptors occur in high levels in the basal nucleus of Meynert, in the CA1 to CA3 regions of Ammon’s horn in the hippocampus, and throughout the neocortex, areas important for cognitive processing. Despite the dramatic central effects of Ang IV and the abundance of the receptor in the central nervous system, the identity of the AT₄ receptor and the mechanism by which its ligands mediate their actions were unknown.

MATERIALS AND METHODS

Protein Purification—AT₄ receptors in bovine adrenal membranes (16 mg of membrane protein) were cross-linked to the photoactivatable analogue of Ang IV, [¹²⁵I]Nle¹-BzPh⁶-Gly²-Ang IV as described previously (15). Cross-linked membranes were solubilized in solubilization buffer (1% CHAPS, 20 mM Tris-HCl, pH 7.5, 5 mM EDTA) with shaking at room temperature for 48 h, and insoluble material was pelleted by centrifugation at 100,000 × g for 1 h at 4°C. Non-cross-linked membranes (48 mg of protein) were solubilized and centrifuged similarly, and the supernatant was combined with that from cross-linked membranes. Solubilized membrane proteins were applied to a 1-ml DEAE fast flow anion exchange column, and the receptor was eluted with solubilization buffer plus 150 mM NaCl. Proteins were resolved by SDS-PAGE, and the gel was stained first with Coomassie Brilliant Blue and then overstained with silver nitrate. Radioactive bands were de-
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Australia) or LVV-H7 (Chiron Mimotopes, Melbourne, Australia) for 2 h at 37 °C in a 50 mM Tris, 5 mM EDTA, 150 mM sodium chloride buffer containing 100 μM phenylmethylsulfonyl fluoride, 20 μM bestatin, 100 μM phanethroline, 0.1% bovine serum albumin (15).

In Vitro Receptor Autoradiography—For the determination of [¹²⁵I]Nle¹-Ang IV binding, the brains were removed and frozen in isopentane chilled to -40 °C on dry ice. 10-μm frozen sections were cut, thaw-mounted onto gelatin-coated slides, and dried for 2 h under reduced pressure. The sections were then rinsed in buffer (pH 7.4) containing 50 mM Tris, 5 mM EDTA, 150 mM sodium chloride for 30 min at room temperature. This was followed by a 2-h incubation in the buffer described above to which had been added 100 μM phenylmethylsulfonyl fluoride, 20 μM bestatin, 100 μM phanethroline, 0.1% bovine serum albumin, and 130 pM [¹²⁵I]Nle¹-Ang IV. The sections were then washed four times in Tris-buffered saline at 0 °C for 2 min each, dried rapidly, and exposed to x-ray film.

In Situ Hybridization Histochemistry—Four oligonucleotides, two antisense and two sense, were prepared from different coding regions of the IRAP gene. The mouse sequence for IRAP was retrieved from the Celera data base and contiged. The predicted amino acid sequence was 84 and 87% homologous with the human and rat sequences, respectively. The oligonucleotides used for in situ hybridization corresponded to nucleotides 621–591 and 2253–2233 of the human sequence (U62768). The oligonucleotides were 3’ end-labeled with [³²P]dATP using alkaline phosphatase and purified on a PD column. 10-μm frozen sections were cut and thaw-mounted onto saline-coated slides. The sections were then hybridized with 1 × 10⁶ counts per min of labeled oligonucleotide in a 100-μl total volume of 50% formamide, 4 × SSC, 1 × Denhardt’s solution, 2% sarcosyl, 20 mM sodium phosphate buffer (pH 7.0), 10% dextran sulfate, 50 μg/ml herring sperm DNA, and 0.2 mM dithiothreitol. After a 16-h hybridization period at 42 °C, the sections were washed four times in 1 × SSC at 55 °C, for 15 min each, rinsed in distilled water, and dehydrated through increasing alcohol. The sections were then exposed to x-ray film.

Immunohistochemical Localization of IRAP in the Mouse Brain—Mice (C57 Black) were anesthetized with an intraperitoneal injection of pentobarbitone and perfused with phosphate-buffered saline (PBS) fol- lowed by 1% paraformaldehyde, 2% glutaraldehyde in phosphate buffer (pH 7.4). The brains were removed, stored in 20% sucrose overnight at 4 °C, and then snap-frozen in isopentane cooled with dry ice. 10-μm thick sections were cut on a cryostat and collected into PBS. The free-floating sections were then incubated for 1 h at room temperature in PBS with normal horse serum (10%) and Triton X-100 (0.3%). The sections were transferred to a blocking solution (PerkinElmer Life Sciences) and then incubated for 48 h at room temperature in anti-rabbit and mouse anti-NeuN (Chemicon) antibodies with the primary antibodies. Both antibodies, the rabbit anti-IRAP (a gift from D. E. James) and mouse anti-NeuN (Chemicon) antibodies were diluted in PBS with 2% normal horse serum and 0.3% Triton X-100. The sections were incubated for 48 h at 4 °C. Following incubation with the primary antibodies, the sections were washed three times in PBS and incubated for 1 h at room temperature in anti-rabbit and anti-mouse IgG secondary antibodies conjugated to Texas Red (anti- rabbit) and fluorescein isothiocyanate (anti-mouse). These antibodies were diluted in PBS with 2% normal horse serum. The sections were washed three times in PBS, mounted onto gelatin-coated slides, and covered with DAKO fluorescent mounting medium.

Enzyme Activity—Membranes were prepared from HEK 293T cells transfected with pCI-IRAP or empty vector as described above except EDTA was omitted from the membrane buffer. The membranes were solubilized in 50 mM Tris- HCl containing 1% Triton X-100 at 4 °C for 16 h. The enzymatic activity of IRAP was determined by the hydrolysis of a synthetic substrate L-leucine-β-naphthylamide monitored by the fluorescence was monitored for 5 min. Either Ang IV or LVV-H7 was added at concentrations of 0.1, 1, or 10 μM, and enzymatic activity was assayed for a further 10 min.

RESULTS

Identification of the AT₄ Receptor as IRAP—The receptor was purified from bovine adrenal membranes, which provided an abundant source (B_max = 3 nmol/mg protein) of this integral membrane protein. Of the three tryptic peptides identified by mass spectrometry, one is 95% identical to residues 978–996 of human insulin regulated aminopeptidase (TrEMBL accession number O00769). When the purification procedure was repeated with a wheat germ lectin-agarose affinity chromatography step included after ion exchange chromatography, once again, one of the tryptic peptides isolated is identical to an internal sequence of human insulin-regulated aminopeptidase (residues 288–300). Of all the peptides identified, IRAP is the most likely candidate, because its size (17) and tissue distribution (17) closely resemble those of the AT₄ receptor, and the enzyme has been shown to bind Ang IV (18).

IRAP-transfected Cells Gain AT₄ Receptors—To confirm that the AT₄ receptor is IRAP, HEK 293T cells were transfected with the expression vector pCI, containing the full-length cDNA for human IRAP (pCI-IRAP), and analyzed for the biochemical and pharmacological properties of the AT₄ receptor. Membranes from transfected cells were cross-linked with [¹²⁵I]Nle¹-BzPhe⁶-Gly⁷-Ang IV and resolved by SDS-PAGE. Specific to cells transfected with pCI-IRAP, but not with empty vector, a major radiolabeled band of 165 kDa and a minor band of >250 kDa were observed under non-reducing conditions, consistent with labeling observed previously for AT₄ receptors in SK-N-MC cells, a human neuroblastoma cell line (15), and in bovine adrenal membranes (Fig. 1A). Both bands were absent when the membranes were incubated in the presence of 10 μM unlabeled Ang IV, confirming the specific interaction of the photofactivatable Ang IV analogue with the AT₄ receptor expressed by the IRAP cDNA.
In competition binding studies, the total binding of \[^{[125}I\]Nle\(^1\)-Ang IV to membranes from transfected cells was 30 to 40-fold higher than binding to membranes from cells transfected with empty vector. Unlabeled Ang IV and LVV-H7 competed for the binding of \[^{[125}I\]Nle\(^1\)-Ang IV with IC\(_{50}\) values of 32 and 140 nM, respectively (Fig. 1B). These results are in close agreement with IC\(_{50}\) values obtained previously for AT\(_4\) receptors in SK-N-MC cells, which were 20 nM for Ang IV and 168 nM for LVV-H7 (15).

**IRAP Distribution in the Brain Parallels AT\(_4\) Receptor Localization**—The distribution of the AT\(_4\) receptor in mouse brain, as visualized by \[^{[125}I\]Nle\(^1\)-Ang IV binding, was compared with that of IRAP mRNA and IRAP-positive immunoreactivity. Using in situ hybridization histochemistry and immunohistochemistry we found that the distribution of IRAP mRNA (Fig. 1C, right panel) and IRAP protein (results not shown) in the mouse brain parallels that of \[^{[125}I\]Nle\(^1\)-Ang IV binding. Similar to the distribution of AT\(_4\) receptors in the brains of other species, \[^{[125}I\]Nle\(^1\)-Ang IV binding sites and IRAP mRNA were widely distributed and occurred in high abundance in the medial septum, in the pyramidal cell layer of CA1 to CA3 region of the hippocampus, and throughout the neocortex (Fig. 1C, left panel), a distribution closely resembling cholinergic neurones and their projections. High levels of binding were also found in brain regions involved in motor control. In the CA1-CA3 region of the hippocampus, IRAP-positive cells were also immunoreactive for NeuN but not for glial fibrillary acidic protein, indicating expression in pyramidal neurones and not glia (Fig. 2).

**AT\(_4\) Receptor Ligands Inhibit IRAP Enzymatic Activity**—The effect of known AT\(_4\) receptor ligands on the enzymatic activity of IRAP was investigated with an assay for the enzyme using the fluorogenic substrate, L-leucine-\[^{[125}I\]naphthylamide (18), and IRAP solubilized from membranes of HEK 293T cells transfected with pCI-IRAP. Both peptides, Ang IV and LVV-H7, known ligands of the AT\(_4\) receptor, inhibited the hydrolysis of the substrate in a dose-dependent manner (Fig. 3). In this system complete inhibition of enzymatic activity of IRAP was observed at concentrations of 1 and 5 \(\mu\)M of Ang IV and LVV-H7, respectively.

**DISCUSSION**

The results presented here identify the AT\(_4\) receptor as the transmembrane aminopeptidase, IRAP, and confirm that IRAP has the same pharmacological and biochemical characteristics as the AT\(_4\) receptor. Cells transfected with the full-length cDNA for human IRAP expressed a high affinity binding site for Ang IV with a pharmacological profile identical to that obtained for the endogenous human AT\(_4\) receptor (15). The localization of both IRAP mRNA and protein in the brain parallels the distribution of the AT\(_4\) receptor determined by in vitro receptor autoradiography. We also demonstrate that the well described peptide ligands of the AT\(_4\) receptor inhibited the catalytic activity of IRAP in vitro.

IRAP was originally identified from GLUT4 vesicles (17). GLUT4 is the insulin-regulated glucose transporter that is abundantly expressed in muscle and adipose cells. The translocation of GLUT4 to the plasma membrane is the primary mechanism of insulin-stimulated glucose uptake in these cells (22) and is responsible for the maintenance of glucose homeostasis. This process involves the formation of vesicles containing both GLUT4 and IRAP, that translocate to the cell surface and fuse with the plasma membrane by a mechanism that in part parallels synaptosomal vesicle trafficking in the regulation of neurotransmitter release in the brain (23).

IRAP and GLUT4 have very similar distribution patterns in the central nervous system. In the rat brain, GLUT4-positive immunoreactivity is associated predominantly with motor areas and with neocortex and hippocampus (20, 21). These are the regions that are enriched with AT\(_4\) receptors as detected by \[^{[125}I\]Nle\(^1\)-Ang IV binding and with IRAP-positive immunoreactivity and IRAP mRNA observed in this study. In the neurones in these brain regions, GLUT4 was localized to rough endoplasmic reticulum, some mitochondria, vesicles, and mitochondria and to dendritic spines as detected by electron microscopy (20).

IRAP is a type II membrane-spanning protein and a member of the M1 family of zinc-dependent metallopeptidases. This enzyme is also known as placental leucine aminopeptidase or oxytocinase, because it was also cloned from a human placental cDNA library as the peptidase involved in the degradation of oxytocin (24). The enzyme specifically cleaves the N-terminal cysteine from oxytocin and vasopressin. Although N-terminal cysteine residues appear to be the preferential targets for the enzyme, a number of peptides that do not contain cysteine residues are also hydrolyzed by IRAP in vitro. These include Lys-bradykinin, angiotensin III, Met-encephalon, dynorphin A, neurokinin A, and neuromedin B (18) (25). However, other peptides that possess N-terminal cysteine residues and intramolecular disulfide bonds, such as calcitonin and endothelin, are not cleaved by the enzyme. The substrates of IRAP in vitro are unknown, and the physiological relevance of the translocation of the enzyme to the cell surface in response to insulin in adipocytes and skeletal muscle remains to be elucidated. The
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AT$_4$ receptor ligands will be useful tools with which to investigate this system.

We have demonstrated that two structurally unrelated AT$_4$ receptor ligands, Ang IV and LVV-H7, are potent inhibitors of the aminopeptidase activity of IRAP. A previous study by Herbst et al. (18) suggested that Ang IV was a potential inhibitor of IRAP activity in adipocytes with an IC$_{50}$ value of 20 nM. We postulate that AT$_4$ receptor ligands mediate their physiologic effects by inhibiting IRAP activity. Thus, we propose that inhibition of IRAP in the central nervous system extends the half-life of endogenous neuropeptides that potentiate memory. The inhibition of IRAP may also modulate the levels of peptides involved in the regulation of GLUT4 trafficking. These findings open a new field of inquiry to unite the positive cognitive effects of AT$_4$ receptor ligands with the properties of IRAP and its potential role in glucose uptake.

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