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Agonist-Dependent AT_4 Receptor Internalization in Bovine Aortic Endothelial Cells

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Abstract Recent studies have characterized a specific binding site for the C-terminal 3-8 fragment of angiotensin II (Ang IV). In the present study we looked at the internalization process of this receptor on bovine aortic endothelial cells (BAEC). Under normal culture conditions, BAEC efficiently internalized ^{125}I-Ang IV as assessed by acid-resistant binding. Internalization of ^{125}I-Ang IV was considerably decreased after pretreatment of cells with hyperosmolar sucrose or after pretreatment of BAEC with inhibitors of endosomal acidification such as monensin or NH_4Cl. About 50% of internalized ^{125}I-Ang IV recycled back to the extracellular medium during a 2 h incubation at 37°C. ^{125}I-Ang IV remained mostly intact during the whole process of internalization and recycling as assessed by thin layer chromatography. As expected, internalization of ^{125}I-Ang IV was completely abolished by divalinal-Ang IV, a known AT_4 receptor antagonist. Interestingly, ^{125}I-divalinal-Ang IV did not internalize into BAEC. These results suggest that AT_4 receptor undergoes an agonist-dependent internalization and recycling process commonly observed upon activation of functional receptors. J. Cell. Biochem. 75:587–597, 1999.

Key words: AT_4 receptor; angiotensin IV; renin-angiotensin system; internalization; endocytosis; endothelial cells

Angiotensin II (Ang II) is an octapeptide hormone (Asp-Arg-Val-Tyr-Ile-His-Pro-Phe) that plays an important role in the regulation of the cardiovascular system. Ang II produces its effects by activating two distinct classes of receptors designated AT_1 and AT_2 [Timmermans et al., 1993]. Until recently, fragments of Ang II smaller than the heptapeptide (2-8) fragment (Ang III) were thought to be biologically inactive and of little physiological importance. The C-terminal 3-8 hexapeptide fragment of Ang II, with the amino-acid sequence Val-Tyr-Ile-His-Pro-Phe (Ang IV), is formed from Ang II and Ang III by the action of amino peptidases [Haberl et al., 1991; Kerins et al., 1995] and potentially from Ang I (3-10) by an angiotensin-converting enzyme-dependent pathway [Champion et al., 1996; Garrison et al., 1996]. Ang IV was recently shown to exert activities in the central nervous system through interaction with a unique binding site [Harding et al., 1992]. These observations have now been extended and high-affinity binding sites for Ang IV have been described in many different tissues from various species [Bernier et al., 1995, 1994; Hanesworth et al., 1993; Riva and Galzin, 1996; Swanson et al., 1992].

Several biological activities have been attributed to Ang IV. In the central nervous system Ang IV is a modulator of memory acquisition and of exploratory behavior in rats, mice, and crabs [Brasko et al., 1988; Delorenzi et al., 1997; Wright et al., 1995]. Ang IV was also shown to antagonize Ang II-induced hypertrophy of chick myocytes [Baker and Aceto, 1990]. This effect was independent of any interaction with AT_1 or AT_2 receptors for Ang II. Although there are presently some controversies, Ang IV was also implicated in the regulation of blood flow. On one hand, when infused into the renal artery, Ang IV increased renal cortical blood flow [Swanson et al., 1992], and when topically applied in the brain, Ang IV potentiated L-arginine-dependent vasodilation of rabbit

Abbreviations used: Ang II, angiotensin II; Ang III, angiotensin III; Ang IV, angiotensin IV; BAEC, bovine aortic endothelial cells; PBS, phosphate-buffered saline.

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cerebral arterioles [Haberl et al., 1991]. On the other hand, Ang IV caused renal and mesenteric vasoconstrictor effects that were inhibited by losartan [Gardiner et al., 1993] and pulmonary vasoconstrictor effects that were also inhibited by an AT_1 receptor antagonist [Cheng et al., 1994], suggesting that Ang IV is a weak agonist of AT_1 receptor. A recent study showed that Ang IV regulates Na^+ transport in kidney tubules [Handa et al., 1998]. These observations suggest the existence of a specific receptor for Ang IV which has now been designated the AT_4 receptor [Wright et al., 1995]. The intracellular signaling mechanism associated with AT_4 receptor is presently unknown. Intracellular levels of classical second messengers (cAMP, inositoltrisphosphate, Ca^{2+}, arachidonic acid) are not modified during acute stimulation of bovine aortic endothelial cells (BAEC) with Ang IV [Briand et al., 1998]. Taking into account the reported effects of Ang IV on stimulation of DNA and RNA synthesis in cultured rabbit cardiac fibroblasts [Wang et al., 1995], on expression of plasminogen activator inhibitor in BAEC [Kerins et al., 1995], and on enhancement of thymidine incorporation in BAEC [Hall et al., 1995], it is tempting to speculate that AT_4 receptor belongs to a family of growth factor or cytokine receptor. This contention is supported by our recent study describing some molecular properties of the photoaffinity-labeled AT_4 receptor of BAEC [Bernier et al., 1998]. In the present work, in order to indirectly assess the functionality of AT_4 receptor, we evaluated its internalization and recycling processes. These processes are considered part of a desensitization mechanism commonly occurring as a consequence of receptor activation by an agonist ligand.

MATERIALS AND METHODS

Materials

Angiotensin IV (Val-Tyr-Ile-His-Pro-Phe) was purchased from Bachem (Torrance, CA). Monensin was obtained from Sigma (St. Louis, MO). Divalinyl pseudohexapeptide-Ang IV [$Val^{1,3}$ $\psi(CH_2NH)^{1-2,3-4}$] Ang IV (Divalinal-Ang IV) was synthesized on the Merrifield resin in the standard Boc strategy manner. The CH_2NH peptide bonds isostere at valines were introduced by the reductive alkylation reaction between a tert-butyloxycarbonyl-valine aldehyde (Peninsula Laboratories Inc., Belmont, CA) and an amine on the resin-bound peptide employing sodium cyanoborohydride ($NaBH_3CN$) in acidified dimethylformamide (DMF) solution. Boc-valinal (2.5 equiv.) was incubated in DMF containing 1% AcOH in the presence of $NaBH_3CN$ (2.5 equiv.) until free amine test showed positive. This coupling step was repeated twice to reach completion. The peptide was cleaved with HF and purified on reverse phase HPLC. Peptide identification was performed by electrospray mass spectrometry. ^{125}I-Ang IV and ^{125}I-Divalinal-Ang IV (~1,000 Ci/mmol) were iodinated with Iodogen by the method of Fraker and Speck [1978]. They were purified by reverse phase HPLC.

Cell Culture

Bovine thoracic aortas were excised and washed immediately with ice-cold sterile phosphate-buffered saline (pH 7.4: 3.5 mM NaH_2PO_4, 16.5 mM Na_2HPO_4, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 3.5 mM KCl, and 135 mM NaCl). The aortas were cleaned of connective tissue under sterile conditions, opened longitudinally, and fixed on a plate with the intimal face upward. The endothelial surface was carefully scraped and then digested for 15 min at 37°C in 30 ml (for four to six aortas) M199 medium (pH 7.4) containing: 25 mM HEPES, 27 mM $NaHCO_3$, 100 U/ml penicillin, 0.1 mg/ml streptomycin, 2.5 µg/ml fungizone, and 1 mg/ml collagenase. After centrifugation at 500*g* for 10 min, the cells were resuspended in 6 ml of M199 medium supplemented with 10% fetal bovine serum (FBS), 50 U/ml penicillin, 60 µg/ml streptomycin, 2 mM L-glutamine, and 7.5% $NaHCO_3$. Endothelial cells were plated in a cell culture flask (25 cm^2, Falcon) and maintained in a 95% air-5% CO_2 humidified incubator at 37°C. The medium was changed the day after seeding. Results presented in these studies were obtained with cells between the 5th and 25th passages. Cultured cells were identified as endothelial cells, based on their ability to form a typical cobblestone appearance when grown to confluence and by their ability to take up fluorescently labeled acetylated low-density lipoprotein (data not shown).

Preparation of Bovine Endothelial Cell Membranes

BAEC were scraped with a rubber policeman and homogenized with six strokes of a Dounce homogenizer (tight pestle) in a medium containing 100 mM NaCl, 5 mM $MgCl_2$, and 25 mM

Tris-HCl, pH 7.4. After centrifugation at 35,000*g* for 30 min, at 4°C, the pellet was resuspended in the same medium at a concentration of 5–10 mg protein/ml, and then heated at 60°C for 20 min. These preparations were stored at −70°C. The protein content was determined by the method of Lowry et al. [1951].

Angiotensin IV Binding Assay

BAEC membranes (50 µg of protein) were incubated in a medium containing 50 mM Tris-HCl pH 7.4, 150 mM NaCl, 0.5 mM EDTA, and 1 mg/ml bovine serum albumin (BSA). Incubations were performed for 2 h at 37°C in a final volume of 500 µl with ^{125}I-Ang IV (0.6 nM). Non-specific binding was determined in the presence of 10 µM Ang IV. Incubations were terminated by rinsing with 6 ml of cold medium and vaccum filtration through glass fibre filters (Whatman GF/C) that had been presoaked for 2 h in the binding medium. The receptor-bound radioactivity was determined by γ spectrometry.

For binding studies on intact cells, BAEC were grown to confluence in 24-multiwell culture plates. They were washed with 1 ml phosphate-buffered saline (PBS) and incubated for 2 h at 37°C in 500 µl of a medium containing 50 mM Tris-HCl pH 7.2, 150 mM NaCl, 0.5 mM EDTA, 1 mM $CaCl_2$, 5.5 mM dextrose, 0.1% BSA, and 0.6 nM ^{125}I-Ang IV. Incubations were terminated by immersion of the whole culture plates into three successive baths containing ice-cold PBS-glucose. Cells were then solubilized in 500 µl NaOH (0.1 N) and the associated radioactivity was determined by γ spectrometry.

Internalization Studies

Cells were incubated with ^{125}I-Ang IV (0.6 nM) for 2 h at 37°C. Incubations were terminated by immersions of the whole culture plates into three successive baths containing ice-cold PBS-glucose. Cells were then incubated for 15 min in an ice-cold acid buffer (10 mM $MgCl_2$, 50 mM Na citrate and 0.2 mM NaH_2PO_4, pH 4.0) to remove surface-bound ligand. Cells were solubilized in 500 µl NaOH (0.1 N) and their radioactive content was evaluated by γ spectrometry.

Externalization Studies

Cells were incubated with ^{125}I-Ang IV (0.6 nM) for 2 h at 37°C and then washed under acidic condition to remove surface-bound ligand. Cells were then incubated at 37°C with fresh incubation buffer for different periods of time. Recycled ligand (recovered in the ambient medium) and internalized ligand (trapped within the cells) were evaluated by γ spectrometry as previously described.

Thin Layer Chromatography

^{125}I-Ang IV and metabolites were applied on a silica gel thin layer chromatography (TLC) plate and developed in a solvent system consisting of butanol/pyridine/acetic acid/water (30/20/6/24). Radioactive peptides were visualized by autoradiography on Kodak Biomax MS film with an intensifying screen. Exposures lasted for periods ranging from 1 h to 7 days at −70°C.

Statistical Analysis

All the data are expressed as mean ± standard deviation of the mean (SD) of at least three experimental values. Data were analyzed by Student's *t*-test for unpaired data. $P < 0.05$ was considered to be significantly different from control value.

RESULTS

Binding and Internalization of ^{125}I-Ang IV

When ^{125}I-Ang IV was incubated with BAEC for different periods of time at 37°C, specific binding increased as a function of time (Fig. 1A). A steady state was reached within about 1 h. ^{125}I-Ang IV acid-resistant binding also increased with a similar time kinetic without any lag time. This is generally interpreted as agonist-dependent receptor internalization. When the ratio of acid-resistant binding over total binding was plotted as a function of the time, half maximal internalization time was faster than 5 min. Internalization reached a maximum at 1 h and remained stable at least until 2 h. Acid-resistant binding accounted for about 50% of the total cell-associated ligand at steady state. As shown in Figure 1B, the proportion of acid-resistant binding did not vary over a wide range of radioligand concentrations (from 0.12 to 1.2 nM). These results show that internalization of ^{125}I-Ang IV occurs independently of ligand concentration but always represents about 50% of total specific binding.

Effect of Sucrose on Internalization of ^{125}I-Ang IV

To explore the mechanism by which Ang IV internalizes into BAEC, the cells were sub-

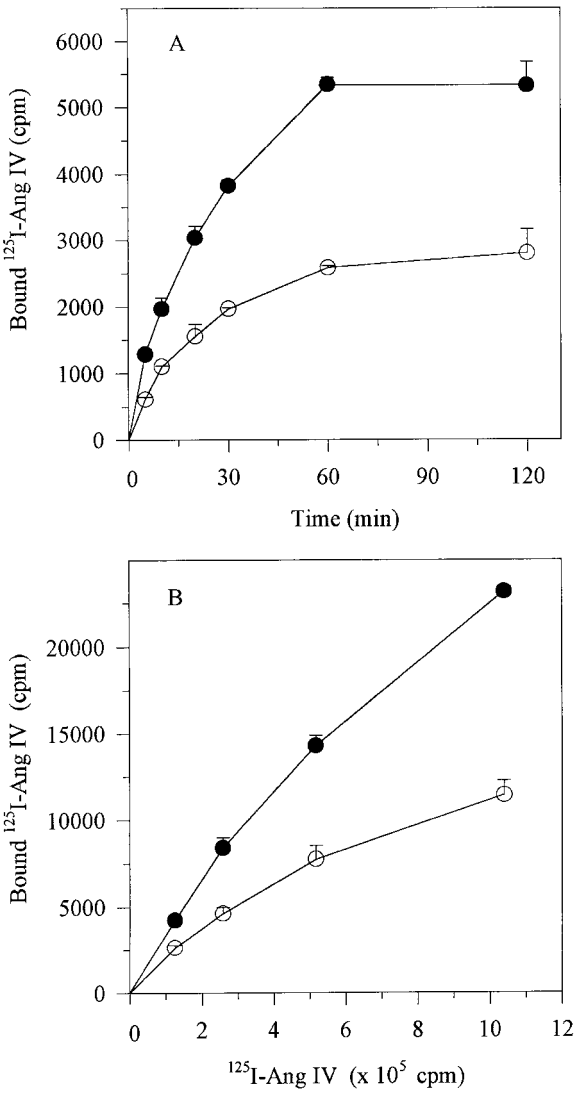

Fig. 1. Binding and internalization of ^{125}I-Ang IV in BAEC. **A:** Cells were incubated with ^{125}I-Ang IV (0.6 nM) for different periods of time at 37°C. **B:** Cells were incubated with different concentrations of ^{125}I-Ang IV for 2 h at 37°C. Total specific binding (●) and acid-resistant binding (○) were evaluated as indicated in Materials and Methods. Non-specific binding was evaluated in the presence of 10 μM Ang IV. Each point represents the mean ± SD of triplicate values. Similar results were obtained in three independent experiments.

jected to hypertonic sucrose shock, a treatment known to inhibit the clathrin-mediated pathway [Hansen et al., 1993; Sandvig et al., 1987]. An hyperosmotic medium perturbs the interaction of clathrin with adaptor proteins [Hansen et al., 1993]. When BAEC were exposed to ^{125}I-Ang IV in a medium containing increased concentrations of sucrose, acid-resistant binding was reduced in a concentration-dependent manner (Fig. 2). Internalization of ^{125}I-Ang IV was reduced by more than 85% in the presence of 0.6 M sucrose. This high sucrose concentration (0.6 M) did not modify significantly the binding of ^{125}I-Ang IV to BAEC membranes (Fig. 2, inset).

Effect of Inhibitor of Endosomal Acidification on Internalization of ^{125}I-Ang IV

Monensin and NH_4Cl are known to neutralize the acidic pH of lysosomes and endosomes which is crucial for the budding of vesicles from the Golgi apparatus [Basu et al., 1981]. Pretreatment of BAEC with NH_4Cl or monensin decreased by about 50% ^{125}I-Ang IV internalization (Fig. 3). These compounds had no direct effect on the binding of ^{125}I-Ang IV to BAEC membranes (Fig. 3, insets).

Peptide Externalization

To directly assess the recycling process, BAEC were incubated with ^{125}I-Ang IV for 2 h at 37°C to allow ligand internalization. Cells were then acid-washed and reincubated in a fresh medium at 37°C for different periods of time. Under these conditions, ^{125}I-Ang IV recycled back to the extracellular medium, in a time-dependent fashion (Fig. 4). After 2 h, approximately 50% of the internalized ligand was recovered in the extracellular medium.

Effect of Pretreatment With Ang IV on ^{125}I-Ang IV Binding Sites

During short incubations (2 h) with BAEC, ^{125}I-AngIV is thus rapidly internalized and also recycled back to the extracellular medium. To determine whether these processes modify the net amount of AT_4 receptors at the cell surface, we performed binding studies on membranes of BAEC that had been pretreated for 2 h with 1 μM Ang IV. Figure 5A shows a binding isotherm on membranes of control BAEC that were not pretreated with Ang IV. Scatchard analysis of these binding data (Fig. 5, inset) was consistent with a single class of high affinity sites with a K_d of 0.85 ± 0.26 nM and a Bmax of 683 ± 69 fmol/mg of protein (mean ± SD of three experiments). Figure 5B shows a binding isotherm on membranes of BAEC that were pretreated for 2 h with 1 μM Ang IV. Scatchard analysis also reveals a high affinity binding of 0.72 ± 0.17 nM and a Bmax of 646 ± 173 fmol/mg of protein (mean ± SD of three experiments). Under both

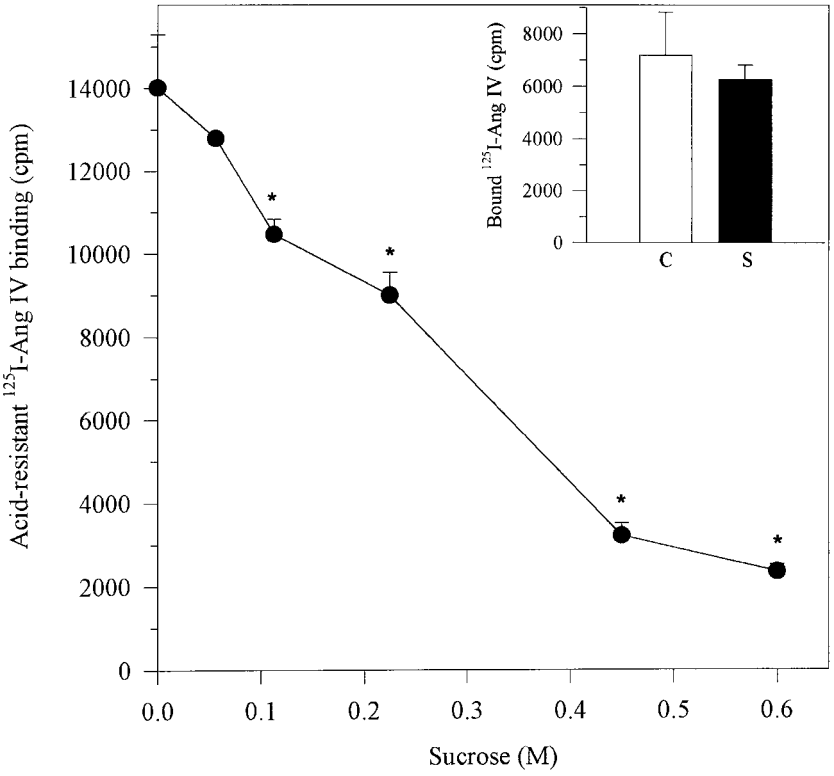

Fig. 2. Effect of sucrose on internalization of ^{125}I-Ang IV in BAEC. Cells were pretreated for 30 min with different concentrations of sucrose. Cells were then incubated with ^{125}I-Ang IV (0.6 nM) for 2 h at 37°C and washed with an acid buffer. Acid-resistant binding was then evaluated as indicated in Materials and Methods. **Inset:** Binding of ^{125}I-Ang IV to BAEC membranes (50 µg of protein) in the absence (C) or the presence (S) of 0.6 M sucrose. Nonspecific binding was evaluated in the presence of 10 µM Ang IV. Each point represents the mean ± SD of triplicate values. $*P < 0.05$, compared to the control value (in the absence of sucrose) according to Student's *t*-test for unpaired data. Similar results were obtained in three independent experiments.

conditions, the total amount of binding sites and their affinity were not significantly different. The recycling mechanism appears to be efficient enough to compensate for the loss of receptors caused by the internalization pathway.

Fate of Internalized Ang IV

We studied the integrity of ^{125}I-Ang IV during its routing across BAEC. After incubation of BAEC with ^{125}I-Ang IV, the released radioactivity can represent ligand-derived small peptides or amino acids that have transited through the lysosome or intact ligand that bypassed lysosomal degradation and is shuttled back to the cell surface where endosomal fusion with the plasma membrane can result in the release of intact ligand, along with the recycling receptor. Delivery of ligand to the lysosome results in degradation followed by the release of fragments into the surrounding medium [Robinson, 1994]. Figure 6A shows that extracellular ^{125}I-Ang IV was slowly degraded during incubations with BAEC, suggesting the presence of ectopic proteases at the surface of BAEC. Despite this proteolytic activity, the major proportion of radioactive material recovered in the extracellular medium after a 2 h incubation with BAEC migrated however as intact Ang IV. Similarly the radioactive material recovered from within the cells also migrated mostly as intact Ang IV (Fig. 6B). Externalized radioactive material was also migrating as intact Ang IV (Fig. 6C). These results clearly demonstrate that in BAEC, Ang IV may undergo a complete cycle of internalization and recycling to the extracellular medium without being degraded during the process.

Binding and Internalization of a AT_4 Receptor Antagonist

Figure 7A shows that the internalization of ^{125}I-Ang IV was completely abolished by divalinal-Ang IV, a known AT_4-receptor antagonist (Fig. 7A). When ^{125}I-divalinal-Ang IV was incubated with BAEC for different periods of time at 37°C, although an important increase in specific binding was observed, no internalization (acid-resistant binding) occurred (Fig. 7B).

DISCUSSION

In the present work, we studied the cellular trafficking of ^{125}I-Ang IV in BAEC. We provided the first evidence that the agonist ^{125}I-Ang IV but not the antagonist ^{125}I-divalinal-Ang IV undergoes an internalization and recycling pro-

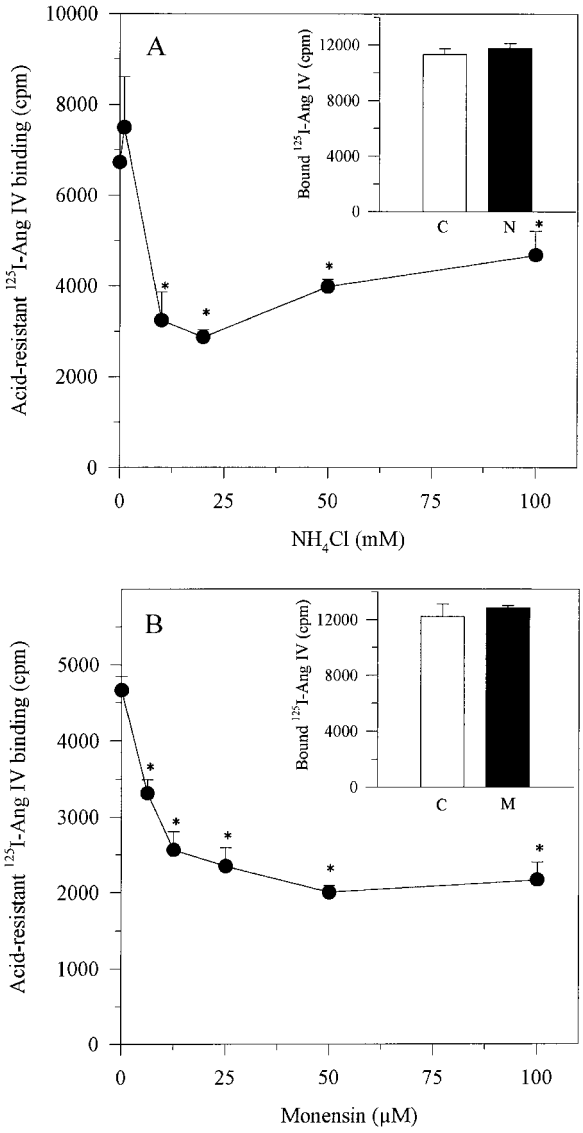

Fig. 3. Effect of NH_4Cl (**A**) and monensin (**B**) on internalization of ^{125}I-Ang IV in BAEC. Cells were pretreated for 30 min with different concentrations of NH_4Cl or monensin. Cells were then incubated with ^{125}I-Ang IV (0.6 nM) for 2 h at 37°C and washed with an acid buffer. Acid-resistant binding was then evaluated as indicated in Materials and Methods. **Insets:** Binding of ^{125}I-Ang IV to BAEC membranes (50 μg of protein) in the absence (C) or the presence of 100 mM NH_4Cl (N) or 100 μM monensin (M). Non-specific binding was evaluated in the presence of 10 μM Ang IV. Each point represents the mean ± SD of triplicate values. $^*P < 0.05$, compared to the control (in the absence of NH_4Cl and monensin) according to Student's *t*-test for unpaired data. Similar results were obtained in three independent experiments.

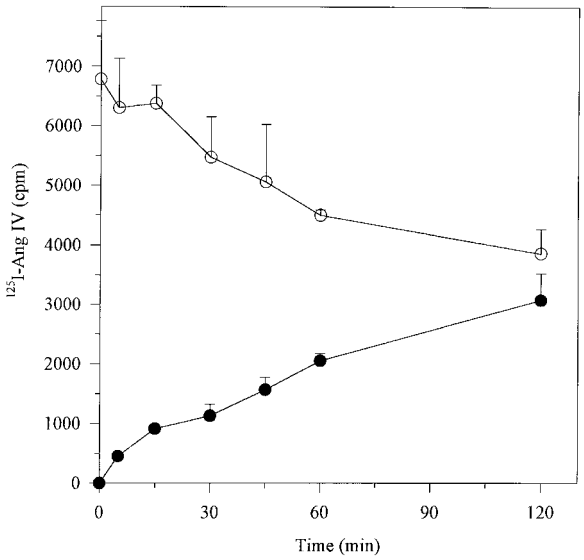

Fig. 4. Peptide externalization studies on BAEC. Cells were incubated with ^{125}I-Ang IV (0.6 nM) for 2 h at 37°C. After an acid wash cells were incubated in 500 μl of fresh medium at 37°C to allow radioactive peptide externalization. After different periods of time the medium was collected to evaluate externalized radioactivity (●) and the cell content was also estimated to evaluate internalized radioactivity (○). Non-specific binding was evaluated in the presence of 10 μM Ang IV. Each point represents the mean ± SD of triplicate values. Similar results were obtained in three independent experiments.

cess. We have shown that about 50% of bound ^{125}I-Ang IV was internalized by BAEC after a 1 h incubation at 37°C. This phenomenon was time-dependent, with a t½ faster than 5 min. The 50% ratio of internalized to bound ligand is comparable with that of many ligands in different cell types including substance P [Garland et al., 1994], vasopressine [Jans et al., 1989, 1990], Ang II [Conchon et al., 1994; Thomas et al., 1995], and neurotensin [Chabry et al., 1995, 1993]. The kinetics of ^{125}I-Ang IV internalization are also comparable with those reported for V1 receptor for vasopressin [Jans et al., 1990; Lutz et al., 1990], AT_{1a} and AT_{1b} receptors for Ang II [Conchon et al., 1994], NK-1 receptor for substance P [Garland et al., 1994], gastrin-releasing peptide receptor [Benya et al., 1995], and neurotensin receptor [Chabry et al., 1995, 1993], with t½ values ranging between 3 and 5 min. The comparable internalization kinetics for these different peptide-receptor complexes suggest that they may be controlled by a common mechanism [Gaudriault et al., 1997]. The ratio of internalized to bound ligand was not sensitive to ligand concentration. These results indicate that the internalization pathway is directly related to the level of occupation of AT_4 receptor, suggesting that internalization is a direct consequence of the activation of the receptor.

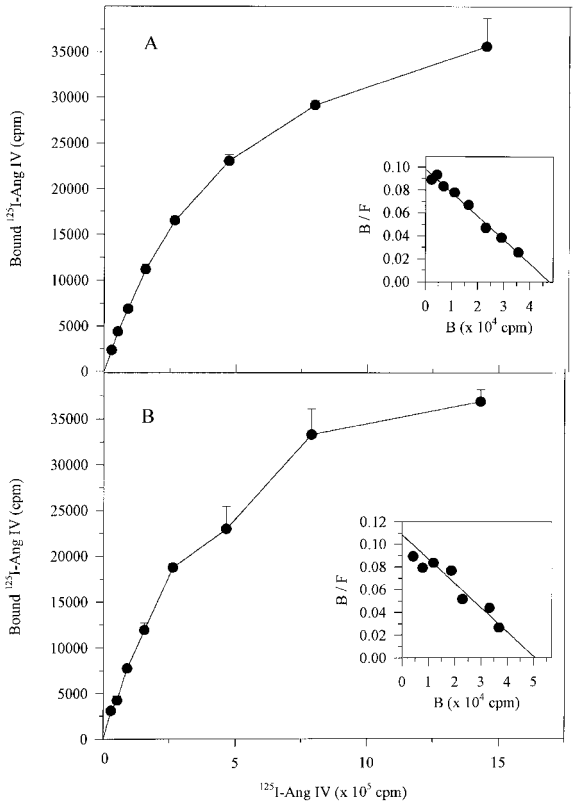

Fig. 5. Effect of pretreatment of BAEC with 1 μM Ang IV for 2 h on ^{125}I-Ang IV binding sites. **A:** Saturation of ^{125}I-Ang IV binding sites on BAEC membranes. **B:** Saturation of ^{125}I-Ang IV binding sites on membranes of BAEC that had been pretreated with 1 μM Ang IV for 2 h and then washed with an acid buffer. Membranes (50 μg of protein) were incubated at 37°C for 2 h in the presence of increasing concentration of ^{125}I-Ang IV. Non-specific binding was determinated in the presence of 10 μM Ang IV. Incubations were stopped by rapid filtration through GF/C filters. **Insets:** Scatchard transformation of the same data. Each point represents the mean ± SD of triplicate values. Similar data were obtained in three independent experiments.

A well known receptor-mediated endocytosis process is the clathrin-coated pit pathway which implies noncovalent binding of agonist ligands to cell-surface receptors, thus inducing the clustering of these complexes into clathrin-coated pits [Beiseigel et al., 1981; Fine and Ockleford, 1984; Watts, 1985]. The ligand-receptor complexes are then internalized as coated pits which invaginate and pinch off to form small intracellular coated vesicles. Clathrin is rapidly removed from the vesicles giving rise to uncoated endosomes [Brown et al., 1983; Helenius et al., 1983]. Within these structures, acidification takes place, followed by a sorting step which establishes the destination of receptors and ligands. Receptors and ligands can be recycled to the cell surface [Brown et al., 1982; Stein and Sussman, 1986], degraded by lysosomal enzymes or sequestered in an intracellular compartment.

We have shown that internalization of ^{125}I-Ang IV is drastically reduced when cells were pretreated with hyperosmolar sucrose, a treatment known to disrupt the formation of functional clathrin lattices [Heuser and Anderson, 1989]. These results suggest that ^{125}I-Ang IV internalization is mediated mostly via clathrin-coated pits. This pathway is used by growth factor receptors such as PDGF receptor and EGF receptor upon activation with their respective ligands [Robinson, 1994; Robinson et al., 1996; Sorkin and Carpenter, 1993]. Since hyperosmolar sucrose did not completely abolish ^{125}I-Ang IV internalization, our results cannot exclude the possibility that an alternative mechanism exists and could account for the internalization of a small proportion of AT_4 receptors. This has been observed for the EGF receptor [Sandvig et al., 1987] and for the cholecystokinin receptor, which both are internalized by the clathrin-coated pit pathway and also by a non-clathrin-coated pit-mediated pathway [Roettger et al., 1995; Wang et al., 1998].

It was suggested that the main purpose of endosomal acidification was to allow the sorting of various ligands and receptors [Mellman et al., 1986; VanRenswoude et al., 1982; Yamashiro, Maxfield, 1987]. Several studies have demonstrated that a pH gradient exists in endosomes, with the earliest endosomes and recycling vesicles having a more neutral interior, whereas multivesicular bodies further along the endocytic pathway may have pH values approaching those of lysosomes as their internal acidity is increased through the action of proton pumps in the vesicle membrane [VanDyke et al., 1985]. Sorting requires acidification of the endosomes, a process that is inhibited by NH_4Cl and by the ionophore monensin [Mollenhauer et al., 1990]. By abolishing the ability of endosomes to acidify their interior, monensin is known to prevent the recycling of receptors [Tartakoff, 1983]. Monensin was shown to inhibit the recycling of several receptors including those for LDL [Basu et al., 1981], insulin [Whittaker et al., 1986], transferrin [Stein and Sussman, 1986], asialoglycoprotein [Berg et al., 1983], and EGF [Gladhaug and Christoffersen, 1988]. We have shown that internalization of ^{125}I-Ang IV was decreased by monensin and by NH_4Cl. These results indirectly

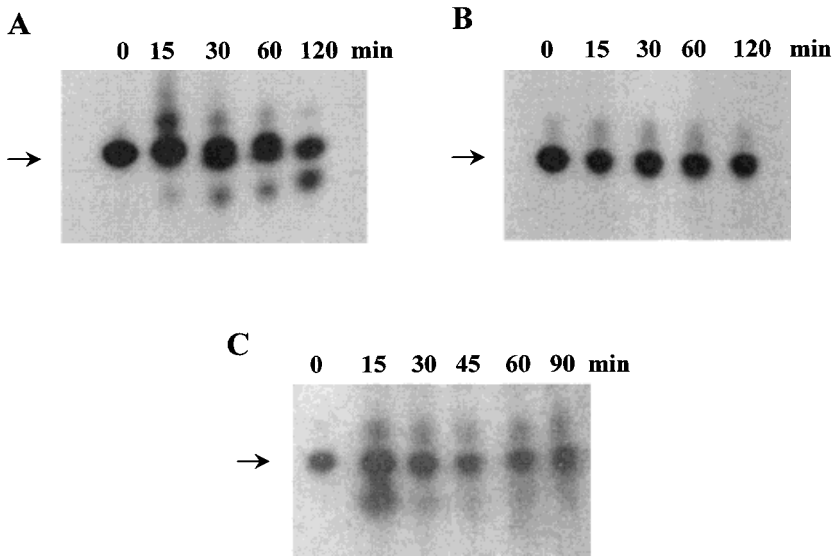

Fig. 6. Fate of internalized Ang IV. **A:** Cells were incubated with ^{125}I-Ang IV (1.2 nM) for different periods of time at 37°C. The radioactive content of the external medium after each incubation period was subjected to TLC. **B:** Cells were incubated with ^{125}I-Ang IV (1.2 nM) for different periods of time at 37°C. After an acid wash, the cells were then solubilized in 0.1 N NaOH. The radioactive cell content after each incubation period was subjected to TLC. **C:** Cells were incubated with ^{125}I-Ang IV (1.2 nM) for 2 h at 37°C. Following an acid wash, cells were incubated at 37°C in 500 µl of fresh medium. Every 15 min the medium was removed and fresh medium was added again for another 15 min, and so on for 90 min. The radioactivity released into the external medium after each incubation period was subjected to TLC. The arrow indicates the migration profile of the authentic form of the peptide.

suggest that internalized ^{125}I-Ang IV undergo recycling in BAEC. When recycling is blocked by monensin or NH_4Cl, the whole trafficking mechanism, including the proximal events (internalization), is slowed down.

This externalization process was directly demonstrated by results showing that as much as 50% of internalized ^{125}I-Ang IV recycled back to the extracellular medium during a 2 h incubation at 37°C. Many hormones have been shown to recycle back to the cell surface after internalization. For example, TGFα rapidly dissociates from its receptor after internalization and both the receptor and the ligand recycle back to the cell surface [Ebner and Derynck, 1991; Sorkin and Waters, 1993]. Receptors can be used more than once as a result of recycling. Isotherm binding studies revealed no change in the amount of cell surface receptors after a 2 h pretreatment of BAEC with 1 µM Ang IV at 37°C. If as observed for TGFα receptor, Ang IV cycles with its AT_4 receptor in BAEC, our results suggest that the recycling mechanism is efficient enough to compensate for the loss of cell surface receptors occuring upon internalization. It is also possible that the renewal of cell-surface AT_4 receptors is due in part to de novo synthesis or recruitment from an intracellular pool.

It is known that after internalization, a ligand may dissociate from its receptor and either be targeted for degradation in the lysosomes or returned to the cell surface within the same vesicle carrying the recycling receptor. We have shown by TLC that internalized ^{125}I-Ang IV was recovered mostly intact after a 2 h stay within BAEC. Moreover, externalized ^{125}I-Ang IV was recovered mostly intact into the medium. Many hormones and growth factors were also recovered intact in the extracellular medium after internalization in their target cells. For example, 80% of IGF-1 was recovered intact after a complete internalization/externalization cycle within rats fibroblasts [Zapf et al., 1994]. TRH was also recovered mostly intact with its receptor [Gershengorn and Osman, 1996]. Less than 10% of internalized ^{125}I-NGF was degraded by 2 h following endocytosis and this endocytosed ^{125}I-NGF was released intact by Chinese hamster ovary (CHO-K1) fibroblasts [Zapf-Colby and Olefsky, 1998]. Also, more than half of the internalized EGF was returned intact to the medium by NIH 3T3 cells [Felder et al., 1990]. Intriguingly, the results shown in Figure 6C indicate that a substantial degradation of Ang IV seems to occur at early externalization period. These results could suggest the existence of an alternative and shorter

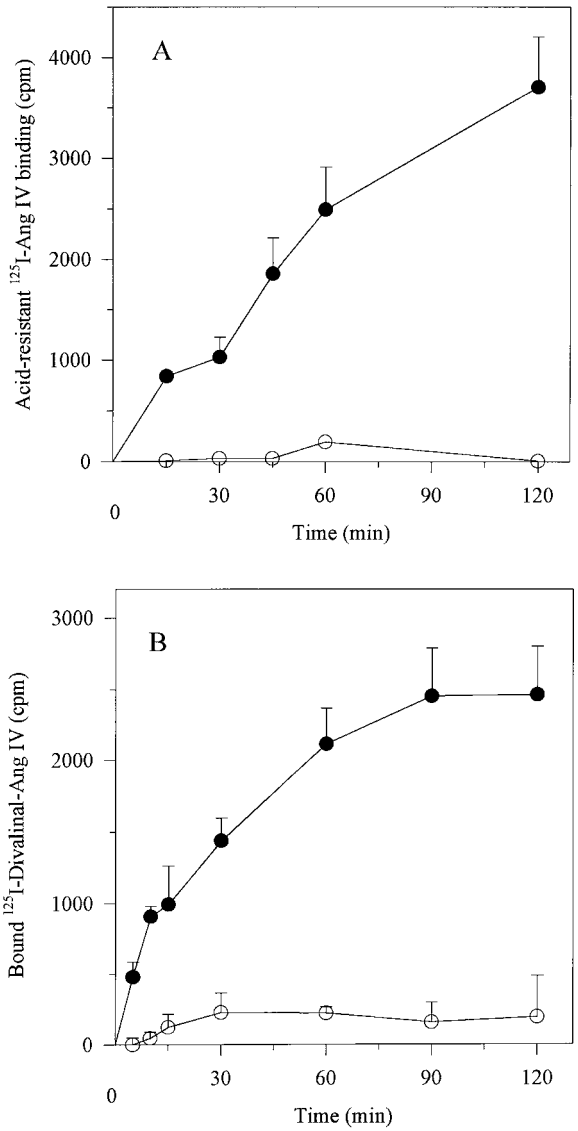

Fig. 7. Lack of divalinal-Ang IV internalization in BAEC. **A:** Cells were pretreated (○) or not (●) with divalinal-Ang IV (10 μM) for 30 min. Cells were then incubated with ^{125}I-Ang IV (0.6 nM) for different periods of time at 37°C and washed with acid buffer. Their radioactive content was then evaluated as previously described. Non-specific binding was evaluated in the presence of 10 μM Ang IV. **B:** Cells were incubated with ^{125}I-Divalinal-Ang IV (0.6 nM) for different periods of time at 37°C. Total specific binding (●) and acid-resistant binding (○) are represented. Non-specific binding was evaluated in the presence of 10 μM Ang IV. Each point represents the mean ± SD of triplicate values. Similar results were obtained in three independent experiments.

cycling pathway through which internalized Ang IV is degraded. Nonetheless, the fact that at later periods ^{125}I-Ang IV recycles back mostly intact argues against the possibility that the internalization process is simply a component of a clearance pathway. We showed that internalization of ^{125}I-Ang IV was completely abolished by divalinal-Ang IV, a compound identified as an AT_4 receptor antagonist [Krebs et al., 1996]. Divalinal-Ang IV is a pseudo-peptide analog of Ang IV with a valine substituted for isoleucine in position 3 and with isostere bonds incorporated between the 1–2 and 3–4 amino acids. These modifications provide both stability and metabolic resistance to the peptide as well as receptor antagonist activity [Robinson et al., 1996]. Interestingly, ^{125}I-divalinal-Ang IV was not internalized by BAEC. These results demonstrate that the internalization pathway is dependent on the occupation of the AT_4 receptor by an agonist and thus, although no direct evidence of AT_4 receptor cycling was provided in the present study, it is tempting to propose that as previously reported for numerous agonist-receptor systems, Ang IV is internalized as a functional complex with its AT_4 receptor.

In conclusion, we have shown that AT_4 receptor undergoes an agonist-dependent internalization and recycling process in BAEC. Internalization of Ang IV was drastically reduced by hyperosmolar sucrose, and to a lesser extent, by monensin and NH_4Cl. The major proportion of internalized and externalized Ang IV was recovered intact. The function of receptor-mediated endocytosis may be to regulate, either positively or negatively, ligand-initiated signaling events. Further work is necessary to better define the different molecular mechanisms responsible for ligand-mediated endocytosis of AT_4 receptor and the functional significance of this process.

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REFERENCES

Baker KM, Aceto JF. 1990. Angiotensin II stimulation of protein synthesis and cell growth in chick heart cells. Am J Physiol 259:H610–H618.

Basu SK, Goldstein JL, Anderson RGW, Brown MS. 1981. Monensin interrupts the recycling of low density lipoprotein receptors in human fibroblasts. Cell 24:493–502.

Beisiegel U, Schneider WJ, Goldstein JL, Anderson RGW, Brown MS. 1981. Monoclonal antibodies to the low density lipoprotein receptor as probes for study of receptor-mediated endocytosis and the genetics of familial hypercholesterolemia. J Biol Chem 256:11923–11931.

Benya RV, Kusui T, Pradhan TK, Battey JF, Jensen RT. 1995. Expression and characterization of cloned human bombesin receptors. Mol Pharmacol 47:10–20.

Berg T, Blomhoff R, Naess L, Tolleshaug H, Drevon CA. 1983. Monensin inhibits receptor-mediated endocytosis of asialoglycoproteins in rat hepatocytes. Exp Cell Res 148:319–330.

Bernier SG, Fournier A, Guillemette G. 1994. A specific binding site recognizing a fragment of angiotensin II in bovine adrenal cortex membranes. Eur J Pharmacol 271: 55–63.

Bernier SG, Servant G, Boudreau M, Fournier A, Guillemette G. 1995. Characterization of a binding site for angiotensin IV on bovine aortic endothelial cells. Eur J Pharmacol 291:191–200.

Bernier SG, Bellemare JML, Escher E, Guillemette G. 1998. Characterization of AT_4 receptor from bovine aortic endothelium with photosensitive analogues of angiotensin IV. Biochemistry 37:4280–4287.

Brazsko JJ, Kupryszewski G, Witczuk B, Wisniewski K. 1988. Angiotensin II-(3-8)-hexapeptide affects motor activity, performance of passive avoidance and a conditioned avoidance response in rats. Neuroscience 27:777–783.

Briand SI, Bellemare JML, Bernier SG, Guillemette G. 1998. Study on the functionality and molecular properties of AT_4 receptor. Endocrine Res 24:315–323.

Brown MS, Anderson RGW, Basu SK, Goldstein JL. 1982. Recycling of cell-surface receptors: observations from the LDL receptor system. Cold Spring Harb Symp Quant Biol 46:713–721.

Brown MS, Anderson RGW, Goldstein JL. 1983. Recycling receptors: the round-trip itinerary of migrant membrane proteins. Cell 32:663–667.

Chabry J, Gaudriault G, Vincent JP, Mazella J. 1993. Implication of various forms of neurotensin receptors in the mechanism of internalization of neurotensin in cerebral neurons. J Biol Chem 268:17138–17144.

Chabry J, Botto JM, Nouel D, Beaudet A, Vincent JP, Mazella J. 1995. Thr-422 and Tyr-424 residues in the carboxyl terminus are critical for the internalization of the rat neurotensin receptor. J Biol Chem 270:2439–2442.

Champion HC, Garrison KA, Estrada LS, Potter JM, Kadowitz PJ. 1996. Analysis of responses to angiotensin I and angiotensin I-(3-10) in the mesenteric vascular bed of the cat. Eur J Pharmacol 309:251–259.

Cheng DY, DeWitt BJ, Dent EL, Nossaman BD, Kadowitz PJ. 1994. Analysis of responses to angiotensin IV in the pulmonary vascular bed of the cat. Eur J Pharmacol 261:223–227.

Conchon S, Monnot C, Teutsch B, Corvol P, Clauser E. 1994. Internalization of the rat AT1a and AT1b receptors: pharmacological and functional requirements. FEBS Lett 349: 365–370.

Delorenzi A, Locatelli F, Romano A, Nahmod V, Maldonado H. 1997. Angiotensin II (3-8) induces long-term memory improvement in the crab *Chasmagnathus.* Neurosci Lett 226:143–146.

Ebner R, Derynck R. 1991. Epidermal growth factor and transforming growth factor-alpha: differential intracellular routing and processing of ligand-receptor complexes. Cell Regul 2:599–612.

Felder S, Miller K, Moehren G, Ullrich A, Schlessinger J, Hopkins CR. 1990. Kinase activity controls the sorting of the epidermal growth factor receptor within the multivesicular body. Cell 61:623–634.

Fine RE, Ockleford CD. 1984. Supramolecular cytology of coated vesicles. Int Rev Cytol 91:1–43.

Fraker PJ, Speck JC. 1978. Protein and cell membrane iodinations with a sparingly soluble chloramide 1,3,4,6-tetrachloro-3a,6a-diphrenylglycoluril. Biochem Biophys Res Commun 80:849–857.

Gardiner SM, Kemp PA, March JE, Bennett T. 1993. Regional heamodynamic effects of angiotensin II (3-8) in conscious rats. Br J Pharmacol 110:159–162.

Garland AM, Grady EF, Payan DG, Vigna SR, Bunnett NW. 1994. Agonist-induced internalization of the substance P (NK1) receptor expressed in epithelial cells. Biochem J 303:177–186.

Garrison EA, Kadowitz PJ. 1996. Analysis of the responses to angiotensin I-(3-10) in the hindlimb vascular bed of the cat. Am J Physiol 270:H1172–H1177.

Gaudriault G, Nouel D, Dal Farra C, Beaudet A, Vincent JP. 1997. Receptor-induced internalization of selective peptidic μ and δ opioids ligands. J Biol Chem 272:2880–2888.

Gershengorn MC, Osman R. 1996. Molecular and cellular biology of thyrotropin-releasing hormone receptors. Physiol Rev 76:175–191.

Gladhaug IP, Christoffersen T. 1988. Rapid constitutive internalization and externalization of epidermal growth factor receptors in isolated rat hepatocytes. J Biol Chem 263:12199–12203.

Haberl RL, Decker PJ, Einhaupl KM. 1991. Angiotensin degradation products mediate endothelium-dependent dilation of rabbit brain arterioles. Circ Res 68:1621–1627.

Hall KL, Venkateswaran S, Hanesworth JM, Schelling ME, Harding JW. 1995. Characterization of a functional angiotensin IV receptor on coronary microvascular endothelial cells. Regul Pept 58:107–115.

Handa RK, Krebs LT, Harding JW, Handa SE. 1998. Angiotensin IV AT_4-receptor system in the rat kidney. Am J Physiol 274:F290–F299.

Hanesworth JM, Sardinia MF, Krebs LT, Hall KL, Harding JW. 1993. Elucidation of a specific binding site for angiotensin II (3-8), angiotensin IV, in mammalia heart membranes. J Pharmacol Exp Ther 266:1036–1042.

Hansen SH, Sandvig K, Van Deurs B. 1993. Clathrin and HA2 adaptors: effects of potassium depletion, hypertonic medium, and cytosol acidification. J Cell Biol 121:61–72.

Harding JW, Cook VI, Miller-Wing AV, Hanesworth JM, Sardinia MF, Hall KL, Stobb JW, Swanson GN, Coleman JKM, Wright JW, Harding EC. 1992. Identification of an AII(3-8) [AIV] binding site in guinea pig hippocampus. Brain Res 583:340–343.

Helenius A, Mellman I, Wall D, Hubbard A. 1983. Endosomes. Trends Biochem Sci 8:245–250.

Heuser JE, Anderson RGW. 1989. Hypertonic media inhibit receptor-mediated endocytosis by blocking clathrin-coated pit formation. J Cell Biol 108:389–400.

Jans DA, Peters R, Zsigo J, Fahrenholz F. 1989. The adenylate cyclase-coupled vasopressin V2-receptor is highly laterally mobile in membranes of LLC-PK1 renal epithelial cells at physiological temperature. EMBO J 8:2481–2488.

Jans DA, Peters R, Fahrenholz F. 1990. Lateral mobility of the phospholipase C-activating vasopressin V1-type receptor in A7r5 smooth muscle cells: a comparison with the adenylate cyclase-coupled V2-receptor. EMBO J 9:2693–2699.

Kerins DM, Hao Q, Vaughan DE. 1995. Angiotensin induction of PAI-1 expression in endothelial cells is mediated by the hexapeptide angiotensin IV. J Clin Invest 96:2515–2520.

Krebs LT, Kramar EA, Hanesworth JM, Sardinia MF, Ball AE, Wright JW, Harding JW. 1996. Characterization of the binding properties and physiological action of divalinal-angiotensin IV, a putative AT_4 receptor antagonist. Regul Pept 67:123–130.

Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. 1951. Protein measurement with the Folin phenol reagent. J Biol Chem 193:265–275.

Lutz W, Sanders M, Salisbury J, Kuman R. 1990. Internalization of vasopressin analogs in kidney and smooth muscle cells: Evidence for receptor-mediated endocytosis in cells with V2 or V1 receptors. Proc Natl Acad Sci USA 87:6507–6511.

Mellman I, Fuchs R, Helenius A. 1986. Acidification of the endocytic and exocytic pathways. Ann Rev Biochem 55: 663–700.

Mollenhauer HH, Morré DJ, Rowe LD. 1990. Alteration of intracellular traffic by monensin; mechanism, specificity and relationship to toxicity. Biochim Biophys Acta 1031: 225–246.

Riva L, Galzin AM. 1996. Pharmacological characterization of a specific binding site for angiotensin IV in cultured porcine aortic endothelial cells. Eur J Pharmacol 305:193–199.

Robinson MS. 1994. The role of clathrin, adaptors and dynamin in endocytosis. Curr Opin Cell Biol 6:538–544.

Robinson MS, Watts C, Zerial M. 1996. Membrane dynamics in endocytosis. Cell 84:13–21.

Roettger BF, Rentsch RU, Pinon D, Holicky E, Hadac E, Larkin JM, Miller LJ. 1995. Dual pathways of internalization of the cholecystokinin receptor. J Cell Biol 128:1029–1041.

Sandvig K, Sundan A, Olsnes S. 1985. Effect of potassium depletion of cells on their sensitivity to diphtheria toxin and pseudomonas toxin. J Cell Physiol 124:54–60.

Sandvig K, Olsnes S, Petersen OW, van Deurs B. 1987. Acidification of the cytosol inhibits endocytosis from coated pits. J Cell Biol 105:679–689.

Sorkin A, Carpenter G. 1993. Interaction of activated EGF receptors with coated pit adaptins. Science 261:612–615.

Sorkin A, Waters CM. 1993. Endocytosis of growth factor receptors. BioEssays 15:375–382.

Stein BS, Sussman HH. 1986. Demonstration of two distinct transferrin receptor recycling pathways and transferrin-independent receptor internalization in K562 cells. J Biol Chem 261:10319–10331.

Swanson GN, Hanesworth JM, Sardinia MF, Coleman JKM, Wright JW, Hall KL, Miller-Wing AV, Stobb JW, Cook VI, Harding EC, Harding JW. 1992. Discovery of a distinct binding site for angiotensin II (3-8), a putative angiotensin IV receptor. Regul Pept 40:409–419.

Tartakoff AM. 1983. Perturbation of vesicular traffic with the carboxylic ionophore monensin. Cell 32:1026–1028.

Thomas WG, Thekkumkara TJ, Motel TJ, Baker KM. 1995. Stable expression of a truncated AT1A receptor in CHO-K1 cells. The carboxyl-terminal region directs agonist-induced internalization but not receptor signaling or desensitization. J Biol Chem 270:207–213.

Timmermans PBMWM, Wong PC, Chiu AT, Herblin WF, Benfield P, Carini DJ, Lee RJ, Wexler RR, Saye JAM, Smith RD. 1993. Angiotensin II receptors and angiotensin II receptor antagonists. Pharmacol Rev 45:205–251.

van Dyke RW, Hornick CA, Belcher J, Scharschmidt BF, Havel RJ. 1985. Identification and characterization of ATP-dependent proton transport by rat liver multivesicular bodies. J Biol Chem 260:11021–11026.

van Renswoude J, Bridges KR, Harford JB, Klausner RD. 1982. Receptor-mediated endocytosis of transferrin and the uptake of fe in K562 cells: Identification of a nonlysosomal acidic compartment. Proc Natl Acad Sci USA 79: 6189–6190.

Wang L, Eberhard M, Erne P. 1995. Stimulation of DNA and RNA synthesis in cultured rabbit cardiac fibroblasts by angiotensin IV. Clin Sci 88:557–562.

Wang S, Clemmons A, Strader C, Bayne M. 1998. Evidence for hydrophobic interaction between galanin and the GalR1 galanin receptor and GalR1-mediated ligand internalization: Fluorescent probing with a fluorescein-galanin. Biochemistry 37:9528–9535.

Watts C. 1985. Rapid endocytosis of the transferrin receptor in the absence of bound transferrin. J Cell Biol 100: 633–637.

Whittaker J, Hammond VA, Taylor R, Alberti KG. 1986. Effects of monensin on insulin interactions with isolated hepatocytes. Evidence for inhibition of receptor recycling and insulin degradation. Biochem J 234:463–468.

Wright JW, Krebs LT, Stobb JW, Harding JW. 1995. The angiotensin IV system: functional implications. Front Neuroendocrin 16:23–52.

Yamashiro DJ, Maxfield ER. 1987. Acidification of morphologically distinct endosomes in mutant and wild-type Chinese hamster ovary cells. J Cell Biol 105:2723–2733.

Zapf A, Hsu D, Olefsky JM. 1994. Comparison of the intracellular itineraries of insulin-like growth factor-1 and insulin and their receptors in rat-1 fibroblasts. Endocrinology 134:2445–2452.

Zapf-Colby A, Olefsky JM. 1998. Nerve growth factor processing and trafficking events following TrkA-mediated endocytosis. Endocrinology 139:3232–3240.