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Molecular properties of apelin: tissue distribution and receptor binding

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Abstract

We analyzed the tissue distribution of apelin mRNA in rats by a quantitative reverse transcription-polymerase chain reaction and that of immunoreactive apelin (ir-apelin) by an enzyme immunoassay (EIA) using a monoclonal antibody. The expression levels of apelin mRNA and ir-apelin seemed to be consistent among tissues: they were highly expressed in the lung and mammary gland. By the combination of gel filtration and EIA, we found that the molecular forms of apelin differ among respective tissues: apelin molecules with sizes close to apelin-36 (long forms) were major components in the lung, testis, and uterus, but both long and short (whose sizes were close to [$< \text{Glu}^{65}$]apelin-13) forms were detected in the mammary gland. In Scatchard analyses, the radioiodinated apelin-36 analogue bound to the receptor, APJ, with high affinity. In competitive binding assays, apelin-36 and apelin-19 far more efficiently inhibited the binding of the labeled apelin-36 analogue with APJ than [$< \text{Glu}^{65}$]apelin-13. In analyses for the dissociation of apelin from APJ, unlabeled apelin-36 replaced more rapidly the labeled apelin-36 analogue bound with APJ than [$< \text{Glu}^{65}$]apelin-13. Our results demonstrate that the long and short forms of apelin differently interact with APJ. © 2001 Elsevier Science B.V. All rights reserved.

Keywords: Apelin; APJ; Distribution; Antibody; Receptor binding

1. Introduction

Apelin is a novel bioactive peptide originally isolated as the endogenous ligand of the orphan G protein-coupled receptor (GPCR), APJ [1,2], by applying our strategy to identify ligands for orphan GPCRs [3,4]. Apelin specifically induces the promotion of extracellular acidification and inhibition of cAMP production in Chinese hamster ovary (CHO) cells transfected with APJ cDNA (CHO-A10) but not control CHO cells. Apelin is produced through processing from the C-terminal portion in the preproprotein consisting of 77 amino acid residues. Although apelin with 36 amino acid length (apelin-

Abbreviations: GPCR, G protein-coupled receptor; PCR, polymerase chain reaction; RT-PCR, reverse transcription-polymerase chain reaction; CHO, Chinese hamster ovary; CHO-A10, CHO cells expressing human APJ; HIV, human immunodeficiency virus; mAb, monoclonal antibody; HRP-apelin, HRP-conjugated [Cys^{61}]apelin-17; PBS, phosphate-buffered saline; ir-apelin, immunoreactive apelin; RACE, rapid amplification of cDNA ends; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; EIA, enzyme immunoassay; BSA, bovine serum albumin; TFA, trifluoroacetic acid; K_d , dissociation constant; B_{max} , maximal binding sites

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36) was originally isolated from bovine stomach tissue extracts, synthetic C-terminal apelin peptides with various sizes have been found to retain the agonistic activities on CHO-A10 cells. Apelin mRNA is highly expressed in the mammary gland during pregnancy and lactation and apelin is abundantly secreted in milk, especially in colostrum [5]. We have found that endogenous apelin exists in multiple molecular forms. Apelin molecules with both sizes corresponding to the long (apelin-36) and short ($[< \text{Glu}^{65}]$ apelin-13) forms have been detected in the bovine colostrum [6].

Although the main physiological functions of apelin and APJ have not been clarified yet, we have found that apelin partially suppresses cytokine production from mouse spleen cells in *in vitro* culture [5] and that apelin shows chemotactic activity on CHO-A10 cells [6]. It has been reported that the administration of apelin to rats lowers blood pressure and increases drinking behavior [7]. In addition, APJ is reportedly able to function as one of the co-receptors together with CD4 in the process of human immunodeficiency virus (HIV) infection [8–11], although CXCR4 and CCR5 are thought to function as main co-receptors [12]. APJ does not show overall high homology with chemokine receptors, such as CCR5, CCR3, and CXCR4, but it has sequence similarity to these chemokine receptors in the N-terminal region which is confirmed to be very important for the interaction with HIV [10].

In this paper, we analyzed the precise tissue distribution of apelin mRNA by reverse transcription-polymerase chain reaction (RT-PCR) and that of rat immunoreactive apelin (ir-apelin) in various tissues by an enzyme immunoassay (EIA) using a specific anti-apelin monoclonal antibody (mAb). We subsequently analyzed molecular heterogeneity of endogenous apelin by gel filtration chromatography and EIA. Finally we demonstrate here that the long (apelin-36) and short ($[< \text{Glu}^{65}]$ apelin-13) forms of apelin have quite different properties in the binding with APJ.

2. Materials and methods

2.1. Animals

Adult male or female Wistar rats (SLC Japan) and

female BALB/c mice (SLC Japan) were housed under controlled temperature and lighting conditions (lights on from 08.00 to 20.00 h) and supplied with water and food *ad libitum*. All experiments using rats and mice were conducted according to a guideline of the internal animal care and use committee.

2.2. Quantitative analyses for rat apelin mRNA by RT-PCR

Poly(A)⁺RNAs were prepared from the tissues of 8-week-old male or female Wistar rats using an mRNA Purification Kit (Amersham Pharmacia Biotech). Complement DNAs were synthesized from poly(A)⁺RNAs (160 ng) treated with deoxyribonuclease I (amplification grade, Gibco BRL) in the presence of AMV reverse transcriptase XL (Life Sciences) at 10 units in 40 μ l of the buffer provided by the manufacturer, with 2.5 μ M of random 9-mer nucleotides (Takara Shuzo) and 1 mM of dNTPs at 42°C for 30 min. According to the method detailed previously [6], we quantified rat apelin mRNA by means of a Prism 7700 Sequence Detector (Applied Biosystems) with a primer set (5'-GGCTA-GAAGAAGGCAACATGC-3', 5'-CCGCTGTCTG-CGAAATTC-3') and a hybridization probe labeled with fluorescent dyes (5'(FAM)-TGGTGAAGCC-CAGAACTTCGAGGA-(TAMRA)3'). Rat glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA expression was also measured as an internal control using Rodent GAPDH Control Reagents (Applied Biosystems) according to the manufacturer's instruction.

2.3. Synthesis of apelin peptides

Apelin-36 (i.e., LVQPRGPRSGPGPWQGGRR-KFRRQRPRLSHKGMPF), [Nle^{75} , Tyr^{77}]apelin-36 (i.e., LVQPRGPRSGPGPWQGGRRKFRRQRPRLSHKGXPY; X means norleucine), apelin-19 (i.e., RRKFRRQRPRLSHKGMPF), [Cys^{61}]apelin-17 (i.e., CFRRQRPRLSHKGMPF), apelin-15 (i.e., RRQRPRLSHKGMPF), [$< \text{Glu}^{65}$]apelin-13 (i.e., $< \text{QRPRLSHKGMPF}$), [$< \text{Glu}^{65}$, Nle^{75} , Tyr^{77}]apelin-13 (i.e., $< \text{QRPRLSHKGXPY}$; X means norleucine), [$< \text{Glu}^{65}$]apelin-13-(65–76)-peptide (i.e., $< \text{QRPRLSHKGMP}$), [$< \text{Glu}^{65}$]apelin-13-(65–75)-peptide (i.e., $< \text{QRPRLSHKGPM}$),

[<Glu⁶⁵]apelin-13-(65–74)-peptide (i.e., <QRPR-LSHKGP), [<Glu⁶⁵]apelin-13-(65–73)-peptide (i.e., <QRPRLSHKG), apelin-12 (i.e., RPRLSHKGP-MPF), and apelin-10 (i.e., RLSHKGPMPF) were synthesized using an automatic peptide synthesizer (Model 430, Applied Biosystems) as described previously [6].

2.4. Preparation of anti-apelin mAb

[<Glu⁶⁵]Apelin-13 at 2 mg was conjugated with a carrier protein, cationized bovine serum albumin (Pierce), at 2 mg in the presence of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (Pierce) and *N*-hydroxysulfosuccinimide (Pierce) in a buffer containing 50 mM 2-morpholinoethanesulfonic acid, monohydrate (Dojindo) at pH 6, and 250 mM NaCl through incubation at 4°C for 4 h. The resultant conjugates were purified with a Kwik Sep Dextran Desalting column (Pierce) according to the manufacturer's instruction. In order to raise anti-apelin antibodies, the purified conjugate at 80 µg admixed with complete or incomplete Freund's adjuvant (Difco) was subcutaneously injected into BALB/c mice (female, 8 weeks old) twice at 3 week intervals. One week after the last subcutaneous injection, 160 µg of the conjugate was intravenously injected into each mouse. Four days after the intravenous injection, spleen cells obtained from each immunized mouse were fused with mouse myeloma cells (P3-X63Ag8-U1) using polyethylene glycol 1500 (Boehringer) according to the manufacturer's instruction. A hybridoma cell line, 4G5, thus obtained was cultured in an INTEGRA CL-1000 system (Integra Biosciences). After culture for 10 days, anti-apelin mAb was purified from the resultant culture medium with a HiTrap rProtein A column (Amersham Pharmacia Biotech). The isotype of the purified antibody was determined with a Mouse mAb isotyping kit (Amersham Pharmacia Biotech).

2.5. Construction of EIA

[Cys⁶¹]Apelin-17 at 0.67 mg was mixed with 6 mg of horseradish peroxidase (HRP; Boehringer) which was previously maleimidated with *N*-(γ-maleidobutyryloxy)succinimide (Dojindo), and the mixture was incubated for 16 h at 4°C. The resultant HRP-

conjugated [Cys⁶¹]apelin-17 (HRP-apelin) was purified by gel filtration on Superose 12 HR 10/30 column (Amersham Pharmacia Biotech), and then used as a tracer. Anti-mouse IgGAM (Cappel) dissolved at 15 µg/ml in 0.1 M carbonate buffer at pH 9.5 was added to 96-well microplates (Nunc), and then incubated at 4°C overnight. After blocking the substrate with Block Ace (Dainippon Pharmaceutical), the anti-mouse IgG-coated microplates were used for following EIA. The purified anti-apelin mAb (50 µl) appropriately diluted with phosphate-buffered saline (PBS) containing 2 mM EDTA, 0.4% bovine serum albumin (BSA), 0.1 M NaCl, and 0.1% micro-O-pro-protect (Boehringer) was added to each well of the anti-mouse IgG-coated microplates. Each sample dissolved in the same buffer (50 µl) was then added to the well. After incubation at 4°C for 16 h, HRP-apelin was added to each well and incubated at 4°C for 8 h. The microplates were washed with PBS containing 0.1% Tween 20 (Sigma), and then the HRP activity retained in each well was measured with a TMB microwell peroxidase system (Kirkegaard&Perry Labs). We quantified ir-apelin on the basis of a dose-response curve using human apelin-36 as a reference.

2.6. Preparation of tissue extracts

Tissues were collected from male or female Wistar rats (8 weeks old) immediately after decapitation, and then frozen at –80°C until used. The mammary glands were collected from female Wistar rats within 1 day after parturition. Whole blood obtained from each rat was collected in a chilled tube containing 300 000 IU/ml of aprotinin and 10 mM of EDTA, and then centrifuged at 1500 × *g* for 20 min at 4°C to prepare plasma [13]. Each tissue (0.2–2 g) or plasma was boiled in water for 15 min, and then homogenized in 1 M acetic acid with a Polytron homogenizer. After centrifugation, the resultant supernatant was subjected to a Sep-Pak Vac C18 column (Waters), and then eluted with stepwise increments of 10, 30, and 50% acetonitrile containing 0.1% trifluoroacetic acid (TFA). Fractions eluted with 30% acetonitrile were diluted with 2 vols. of 20 mM CH₃COONH₄ at pH 6.4, and then applied to a HiTrap CM-Sepharose FF 5 ml column (Amersham Pharmacia Biotech). Elution from CM-Sepharose

was performed with stepwise increments of 200 and 1000 mM $\text{CH}_3\text{COONH}_4$ at pH 6.4 containing 10% acetonitrile. Fractions eluted with 1000 mM $\text{CH}_3\text{COONH}_4$ were desalted with a Sep-Pak plus C18 column, dried, and then used as samples for the EIA.

2.7. Gel filtration

The tissue extracts (i.e., fractions eluted with 1000 mM $\text{CH}_3\text{COONH}_4$ in CM-Sepharose column chromatography) prepared from the lung, testis, uterus, and mammary gland were applied to gel filtration chromatography using a Superdex Peptide PC 3.2/30 column in a SMART system (Amersham Pharmacia Biotech). Elution was performed in 40% acetonitrile containing 0.1% TFA at a flow rate of 25 $\mu\text{l}/\text{min}$.

2.8. Receptor-binding assays

Apelin analogues, [$< \text{Glu}^{65}, \text{Nle}^{75}, \text{Tyr}^{77}$]apelin-13 and [$\text{Nle}^{75}, \text{Tyr}^{77}$]apelin-36, were labeled respectively with Na^{125}I (IMS-30, Amersham Pharmacia Biotech) by the method using lactoperoxidase (Sigma) as described elsewhere [14]. After the reaction, labeled and unlabeled peptides were separated through a reverse phase-high performance liquid chromatography using a TSKgel ODS-80 TM CTR (Tosoh). Aliquots of the labeled peptide were stored at -30°C until used. The membrane fractions of CHO-A10 cells prepared by the method as described previously [6] were incubated with [^{125}I] $< \text{Glu}^{65}, \text{Nle}^{75}, \text{Tyr}^{77}$]apelin-13 or [^{125}I] $[\text{Nle}^{75}, \text{Tyr}^{77}]$ apelin-36 in 100 μl of the binding buffer containing 0.1% BSA in 96-well microplates (SEROCLUSTER, Corning Costar) at room temperature. In the binding assay using [^{125}I] $[\text{Nle}^{75}, \text{Tyr}^{77}]$ apelin-36, 1 μM of poly-L-lysine (Wako) was also added to prevent nonspecific binding. In order to determine the amounts of nonspecific binding, 1 μM of unlabeled apelin analogues was simultaneously added to the wells. After incubation, bound and free radioactivities were separated through rapid filtration using the glass fiber filter units (GF/C, Packard) of a 96-well cell harvester (Packard). The filter units were completely dried, and the Microcinti O (Packard) was added to each well. The radioactivity of each well was counted with a TopCount liquid

scintillation counter (Packard). The dissociation constant (K_d) and the number of binding sites (B_{max}) at 90 min of incubation were determined by the Scatchard method [15]. In time course analyses for the dissociation of apelin, radiolabeled apelin analogues (0.1 nM at final concentration) were incubated with the membrane fraction of CHO-A10 cells, and then radioactivities bound to the membrane were determined every 30 min for 4 h. Dissociation of the labeled apelin analogues was examined by the addition of 0.1 μM of unlabeled [$< \text{Glu}^{65}$]apelin-13 or apelin-36, respectively, at 90 min after the incubation.

2.9. Assay for cAMP production-inhibitory activity

The inhibitory activity of apelin to forskolin-induced cAMP production in CHO-A10 cells was measured as described previously [5].

3. Results

3.1. Construction of EIA to detect apelin

We established a hybridoma cell line, 4G5, secreting anti-apelin mAb, by fusing myeloma cells to spleen cells obtained from mouse immunized with apelin. Anti-apelin mAb produced by 4G5 consisted of IgG₁ heavy chains and κ light chains. By utilizing this mAb, we constructed an EIA to detect apelin. We detected apelin by the competition of samples against HRP-apelin which could be recognized by the anti-apelin mAb. In order to determine which epitope was recognized by the mAb, we examined its reactivity against various apelin peptides in the EIA. As shown in Fig. 1, apelin-36, apelin-15, and [$< \text{Glu}^{65}$]apelin-13 were detectable by this EIA, though apelin-36 was somewhat more efficiently detected than apelin-15 and [$< \text{Glu}^{65}$]apelin-13. In accordance with the deletion of the C-terminal amino acid residues in [$< \text{Glu}^{65}$]apelin-13, apelin peptides gradually lost the reactivity to the mAb, though the deletion of the C-terminal Phe⁷⁷ little affected the reactivity. The rank order of the reactivity was as follows: [$< \text{Glu}^{65}$]apelin-13 = (65–76)-peptide > (65–75)-peptide > (65–74)-peptide > (65–73)-peptide. The deletion of the N-terminal amino acid residues in [$< \text{Glu}^{65}$]apelin-13 more drastically attenuated the

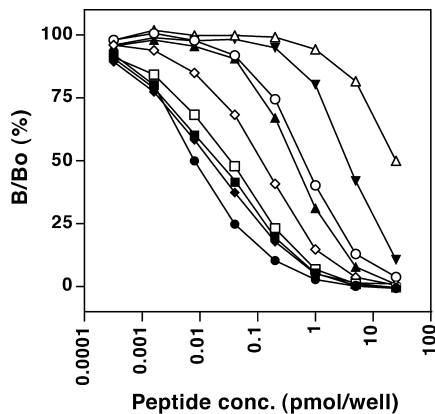


Fig. 1. Reactivity of anti-apelin mAb with apelin-related peptides. The purified anti-apelin mAb (4G5) was added to each well of the anti-mouse IgG-coated microplates. Samples (human apelin-36 (●), apelin-15 (◆), [$< \text{Glu}^{65}$]apelin-13 (■), [$< \text{Glu}^{65}$]apelin-13-(65–76)-peptide (□), [$< \text{Glu}^{65}$]apelin-13-(65–75)peptide (◇), [$< \text{Glu}^{65}$]apelin-13-(65–74)peptide (○), [$< \text{Glu}^{65}$]apelin-13-(65–73)peptide (△), apelin-12 (▲), and apelin-10 (▼)) were then added to the well, respectively. After incubation for 16 h, HRP-apelin was added to each well and incubated for 8 h. After the plates were washed, the HRP activity retained in each well was measured. Data are expressed as means in duplicate assays. B, HRP activity detected in the presence of a sample; Bo, HRP activity detected in the absence of a sample.

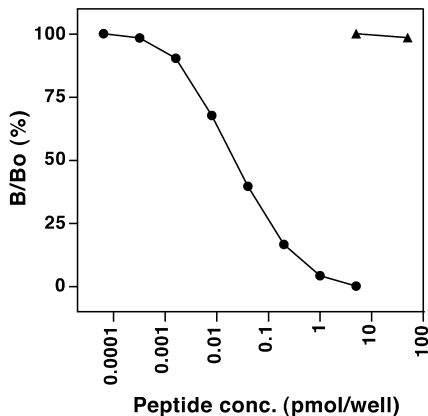


Fig. 2. Specific detection of apelin by EIA. The indicated amounts of synthetic human apelin-36 (●) were tested in the EIA under the same conditions as in Fig. 1. Other bioactive peptides (▲) including prolactin-releasing peptide-31, Arg-vasopressin, corticotropin-releasing hormone, vasoactive intestinal peptide, substance P, angiotensin II, oxytocin, pituitary adenylate cyclase-activating peptide, neurotensin, growth hormone-releasing hormone, thyrotropin-releasing hormone and gonadotropin-releasing hormone did not inhibit the binding of HRP-apelin to the mAb.

reactivity to the mAb. The rank order of the reactivity was as follows: [$< \text{Glu}^{65}$]apelin-13 > apelin-12 > apelin-10. These results suggested that the mAb essentially recognized the epitope corresponding to (65–76)-peptide in apelin.

We subsequently examined the specificity of the EIA. As shown in Fig. 2, we could detect synthetic human apelin-36 at at least 4 fmol/well by EIA. However, we could not detect the other 12 known

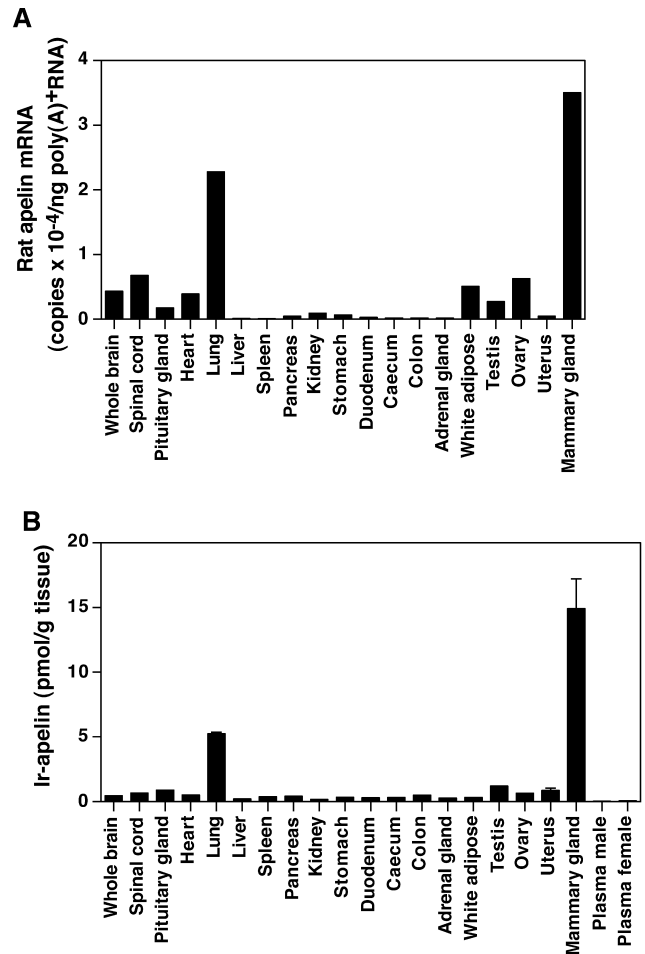


Fig. 3. Tissue distribution of apelin in rats. (A) Distribution of apelin mRNA. Poly(A)⁺RNA preparations obtained from the indicated rat tissues were subjected to quantitative RT-PCR analyses using a Prism 7700 Sequence Detector. Poly(A)⁺RNA of the mammary gland was prepared from female rats within 1 day after parturition. (B) Distribution of ir-apelin. The contents of ir-apelin in the extracts prepared from the indicated tissues were determined by EIA. A calibration curve to quantify the contents of ir-apelin was obtained using synthetic human apelin-36. Data represent means \pm S.E.M. of multiple assays. $n=4$ or 5, except for the pituitary gland and adrenal gland which were pooled from 20 and 10 rats, respectively.

peptides including angiotensin II even at 50 pmol/well.

3.2. Tissue distribution of apelin mRNA in rats

We quantitatively analyzed the precise distribution of apelin mRNA in rat tissues by RT-PCR. As shown in Fig. 3A, we detected apelin mRNA in almost all of the tissues tested, although the quantity of apelin mRNA varied considerably among them. We detected the highest level of apelin mRNA in the mammary gland of rats after parturition. A very high level of apelin mRNA was detected in the lung. In the central nervous system, apelin mRNA was detected in the multiple areas in relatively high quantity. Moderate levels of apelin mRNA were detected in the heart, adipose, testis, and ovary. We amplified the same cDNA preparations with a set of primers corresponding to GAPDH cDNA as a control. The levels of amplified GAPDH cDNA were almost consistent among the tissues within the range of 0.7×10^5 – 9.1×10^5 copies/ng poly(A)⁺RNA, except for the pituitary, heart, and mammary gland (1.1×10^6 – 2.2×10^6 copies/ng poly(A)⁺RNA) and the skeletal muscle (4.6×10^6 copies/ng poly(A)⁺RNA).

3.3. Tissue distribution of ir-apelin in rats

We measured the contents of ir-apelin in extracts prepared from rat by EIA. As shown in Fig. 3B, the highest concentration of ir-apelin (15 pmol/g tissue) was detected in the mammary gland of rats after parturition. A high concentration of ir-apelin (5.2 pmol/g tissue) was detected in the lung. In these tissues, high levels of apelin mRNA expression were also detected as described above. In the other tissues, the contents of ir-apelin were not so high, although significant levels of ir-apelin were detected in all tissues examined. In the plasma samples prepared from male and female rats, the ir-apelin concentrations were lower (i.e., 0.023 and 0.052 pmol/ml, respectively) than those in the other tissues.

3.4. Heterogeneity of endogenous apelin molecules

Molecular heterogeneity of ir-apelin among rat tissues was analyzed by gel filtration chromatography in combination with EIA. As shown in Fig. 4, in the

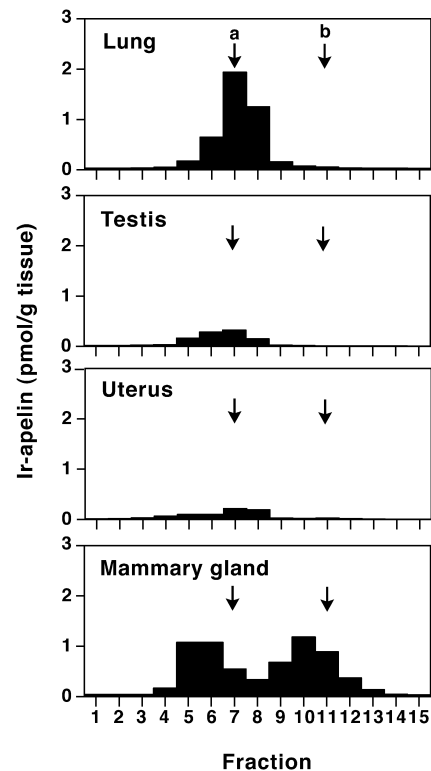


Fig. 4. Gel filtration of tissue extracts. Extracts which were prepared from the indicated tissues and partially purified were subjected to gel filtration chromatography. Ir-apelin contained in each fraction was quantified by EIA. Arrows indicate the positions of human apelin-36 (a) and [$< \text{Glu}^{65}$]apelin-13 (b) eluted in the same chromatography.

lung, testis, and uterus, ir-apelin was mainly eluted at the position corresponding to apelin-36. However, in the mammary gland, ir-apelin was eluted as two peaks at a position slightly greater than apelin-36 and at that of around [$< \text{Glu}^{65}$]apelin-13.

3.5. Properties of apelin-36 in binding to APJ

In order to analyze the receptor-binding properties of apelin-36, we prepared [^{125}I][Nle⁷⁵,Tyr⁷⁷]apelin-36. We used [Nle⁷⁵,Tyr⁷⁷]apelin-36 in the radioiodination because of the following reasons. As there was no tyrosine residue for radioiodination using lactoperoxidase in the sequence of apelin-36, we substituted Phe⁷⁷ with Tyr⁷⁷. [Nle⁷⁵,Tyr⁷⁷]Apelin-36 was almost equivalently potent to apelin-36 in the agonistic activity in the cAMP production-inhibitory assays (data not shown). In order to prevent possible oxidation at Met⁷⁵ during the labeling reactions, we

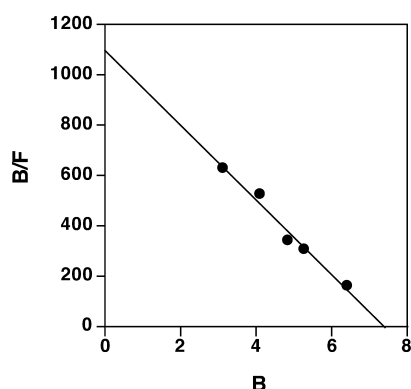


Fig. 5. Scatchard analysis for binding of radiolabeled apelin-36 with APJ. The membrane preparations of CHO-A10 cells were incubated with increasing concentrations of [125 I][Nle 75 ,Tyr 77]-apelin-36 at room temperature. The bound and free ligands were separated at the time when the binding of [125 I][Nle 75 ,Tyr 77]-apelin-36 reached equilibrium (90 min). The amounts of nonspecific binding were estimated by adding 1 μ M of unlabeled [Nle 75 ,Tyr 77]-apelin-36 to the reaction. Data are plotted as bound (B, pmol mg $^{-1}$) versus the bound/free (B/F, pmol mg $^{-1}$ nM $^{-1}$) radiolabeled ligand. Data represent means \pm S.E.M. of triplicate assays. Standard error bars are invisible because they lie inside the symbols.

substituted this residue with Nle (norleucine), because its oxidation was found to affect the agonistic activity of apelin-36 (data not shown). The substitution of Met 75 to Nle 75 did not reduce the agonistic activity. We confirmed that the agonistic activity of [Nle 75 ,Tyr 77]-apelin-36 remained after iodination (data not shown).

Scatchard plot analysis using the labeled apelin-36

Table 1

Comparison of apelin peptides in receptor-binding and cAMP production-inhibitory assays

Peptide	Receptor-binding assay ^a IC ₅₀ (nM)	cAMP assay ^b EC ₅₀ (nM)
Apelin-36	5.4 \pm 0.06	0.52 \pm 0.09
Apelin-19	8.0 \pm 0.89	0.35 \pm 0.03
[<Glu 65]-apelin-13	> 100	0.17 \pm 0.02

^aThe binding of [125 I][Nle 75 ,Tyr 77]-apelin-36 (100 pM) to the membrane preparation of CHO-A10 cells was examined in the presence of the indicated peptides. The bound and free ligands were separated after incubation for 90 min at room temperature. Data represent means \pm S.E.M. of triplicate assays.

^bThe effects of the indicated peptides on cAMP production in CHO-A10 cells were examined in the presence of 0.2 mM 3-isobutyl-1-methylxanthine and 1 μ M forskolin. Data represent means \pm S.E.M. of triplicate assays.

analogue indicated that CHO-A10 cells expressed a single class of high affinity binding sites with a K_d of 6.3 pM and a B_{max} of 7.4 pmol/mg protein (Fig. 5). In competitive binding assays, unlabeled apelin-36 and apelin-19 efficiently inhibited the binding of the labeled apelin-36 analogue with APJ (Table 1). However, [<Glu 65]-apelin-13 little inhibited this binding. In the activity to inhibit cAMP production in CHO-A10 cells, [<Glu 65]-apelin-13 was slightly more potent than apelin-36 and apelin-19 (Table 1).

In our previous study, it has been suggested that apelin-36 more hardly dissociates from APJ than [<Glu 65]-apelin-13 [6]. In order to examine directly this presumption, we examined the dissociation of radiolabeled [<Glu 65]-apelin-13 and apelin-36 ana-

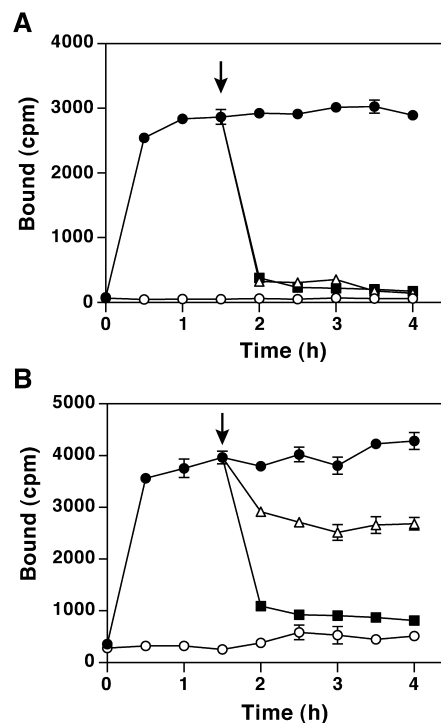


Fig. 6. The time course of association and dissociation of radiolabeled apelin analogues binding to APJ. The membrane preparations of CHO-A10 cells were incubated with 0.1 nM of [125 I][<Glu 65 ,Nle 75 ,Tyr 77]-apelin-13 (A) and [125 I][Nle 75 ,Tyr 77]-apelin-36 (B) at room temperature. Radioactivities (\bullet) bound to the membrane were measured at the indicated incubation times. Radioactivities bound to the membrane after the addition of 0.1 μ M of [<Glu 65]-apelin-13 (Δ) or apelin-36 (\blacksquare) at 90 min of incubation (arrow) were also measured. The amounts of nonspecific binding were estimated by adding 1 μ M of unlabeled apelin analogues to the reaction (\circ). Data represent means \pm S.E.M. of triplicate assays.

logues from APJ. As shown in Fig. 6A, the binding of the labeled [$< \text{Glu}^{65}$]apelin-13 analogue to the CHO-A10 cell membrane was saturated within 30 min after starting the incubation, and the labeled analogue bound to the membrane was efficiently dissociated by the addition of unlabeled [$< \text{Glu}^{65}$]apelin-13 and apelin-36 at 90 min after the incubation. There were no apparent differences in the kinetics of its dissociation induced by unlabeled [$< \text{Glu}^{65}$]apelin-13 and apelin-36. On the other hand, the binding of the labeled apelin-36 analogue to the membrane was more efficiently dissociated by the addition of unlabeled apelin-36 than [$< \text{Glu}^{65}$]apelin-13 (Fig. 6B), indicating that apelin-36 more readily dissociates from APJ than [$< \text{Glu}^{65}$]apelin-13.

4. Discussion

In the present study, we constructed a specific EIA for apelin with a minimum detectable level of 4 fmol/well. The contents of ir-apelin in rat tissues (e.g., whole brain, heart, lung and adipose) were well consistent with those of bioactive apelin measured on the basis of the inhibitory activities on cAMP production in CHO-A10 cells [5]. These results indicate that this EIA detects mainly bioactive apelin.

By the analyses for apelin mRNA and ir-apelin, we demonstrated that apelin was abundantly produced in the lung and mammary gland in rats. These results are consistent with our previous observation that stimulatory activities on CHO-A10 cells are abundantly detectable in the lung and mammary gland [5]. APJ mRNA has been found to be highly expressed in the lung in rats [6]. Taken together with the results in this study, it is suggested that apelin is produced in the lung and functions in the vicinity of the tissue. The highest levels of apelin mRNA and ir-apelin were detected in the mammary gland in rats after parturition. These results are consistent with our previous observation that apelin is abundantly secreted in bovine colostrum and milk [5]. In contrast to the case of the lung, the expression of APJ mRNA was at a low level in the mammary gland in the extent that we examined [6]. It must be elucidated in future studies whether apelin plays an important role in the mammary gland.

Of interest, apelin concentration in plasma was

considerably lower than those in other tissues. It has been recently reported that APJ is abundantly expressed in the neurons and oligodendrocytes in human, and mediates intracellular signals by apelin [16]. We therefore consider that apelin would not act as a circulating hormone and function in paracrine such as a neuromodulator/neurotransmitter. In addition, we confirmed that apelin concentration in plasma was not increased even during pregnancy and lactation in rats (data not shown), suggesting that apelin produced in the mammary gland is not delivered to other tissues in circulation. In the previous study, moderate levels of APJ mRNA were detected in the stomach and intestine in neonatal rats [6]. We therefore speculate that apelin secreted into colostrum and milk might act on neonates through oral intake. Although the contents were lower when compared with the lung and mammary gland, ir-apelin was detected in a variety of tissues. We have reported that APJ mRNA is expressed in a variety of tissues including the lung, heart, costal cartilage, placenta, spinal cord, ovary, adipose, and thyroid gland in rats when analyzed by RT-PCR [6]. In situ hybridization has shown that APJ mRNA is expressed in the central nervous system and peripheral tissues in rats [17]. These results suggest that apelin functions widely in various tissues. It has been reported that APJ mRNA is more highly expressed in neonates than in adults in rats [2,6]. We observed that apelin mRNA was also highly expressed in rat neonatal tissues including the lung (data not shown). In addition, we have found that apelin shows chemotactic activity on CHO-A10 cells [6]. We therefore speculate that apelin and APJ are related to the regulation of morphogenesis in the development of some tissues including the lung in rats.

By analyzing the molecular forms of endogenous apelin, we found that the large forms of apelin with sizes close to apelin-36 were major constituents in ordinary tissues including the lung. However, the molecular forms of apelin produced in the rat mammary gland appeared to be considerably heterogeneous; in addition to the large forms, small forms with sizes close to [$< \text{Glu}^{65}$]apelin-13 were detected. These results well agree with our previous observation that bovine colostrum and milk contain both large and short forms of apelin [6]. Although it is unclear why apelin produced in the mammary gland is so hetero-

geneous, we have found that there are differences in some biological activities between apelin-36 and [$< \text{Glu}^{65}$]apelin-13 [5,6].

In Scatchard analysis, we found that a radiolabeled apelin-36 analogue bound to APJ with a very high affinity (i.e., K_d of 6.3 pM). In our previous study, the K_d value of a radiolabeled [$< \text{Glu}^{65}$]apelin-13 analogue bound to APJ has been estimated to be 22.3 pM [6]. Our results indicate that apelin-36 binds to APJ with higher affinity than [$< \text{Glu}^{65}$]apelin-13. In the competitive binding assays, unlabeled apelin-36 and apelin-19 efficiently inhibited the binding of the radiolabeled apelin-36 analogue to APJ. However, [$< \text{Glu}^{65}$]apelin-13 did not. The basic amino acid-rich N-terminal sequence of apelin-19 (i.e., RRKFRR) thus appeared to be critical in the efficient inhibition of this binding. Besides, in the time course study, the saturated binding of the radiolabeled apelin-36 to APJ was efficiently replaced by unlabeled apelin-36 but not by [$< \text{Glu}^{65}$]apelin-13. We have reported that when CHO-A10 cells were transiently exposed to apelin and their extracellular acidification was analyzed by a Cytosensor, the stimulation of apelin-36 was long lasting whereas that of [$< \text{Glu}^{65}$]apelin-13 was temporary [6]. These results suggest that apelin-36 bound to APJ is harder to dissociate from APJ than [$< \text{Glu}^{65}$]apelin-13.

As we have reported, [$< \text{Glu}^{65}$]apelin-13 shows somewhat greater activity than apelin-36 in analyses for dose responses in the suppression of cAMP production, promotion of acidification rates, and chemotaxis for CHO-A10 cells [5,6]. We think that these differences between apelin-36 and [$< \text{Glu}^{65}$]apelin-13 might be related to the association with APJ. In this study, we could not find a significant difference in the kinetics of the association of the radiolabeled [$< \text{Glu}^{65}$]apelin-13 and apelin-36 analogues with APJ. More precise analyses will be required to clarify their differences in the association with APJ.

It has been reported that APJ could function as one of the co-receptors together with CD4 in the process of HIV infection in cells [8–11]. We and another group recently reported that apelin inhibits HIV infection in cells co-expressing CD4 and APJ, and the inhibitory activity of apelin on the infection is greater in apelin-36 than in [$< \text{Glu}^{65}$]apelin-13 [18,19]. The inhibitory activities of apelin molecules

against HIV infection seem to be correlated with the dissociation rather than the association with APJ.

Endogenous apelin has multiple molecular forms and they differently interact with the receptor. This appears to be a unique character of apelin among bioactive peptides. We believe that studies on apelin will give insight into the unknown aspects of bioactive peptides.

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