Materials and Methods

Isolation of human *apj* cDNA

Using the degenerate primers, 5'-CGTGG(G/C)C(A/C)T(G/C)(G/C) TGGGCAAC(A/G/C/T)(C/T)CCTG-3' and 5'-GT(A/G/C/T)G(A/T)(A/G) (A/G)GGCA(A/G/C/T)CCAGCAGA(G/T)GGCAAA-3', which were designed on the basis of consensus sequence corresponding to the first and sixth transmembrane domains among known 7TMRs, an *apj* cDNA fragment was isolated from a human amygdala cDNA by a PCR. PCR was performed in a reaction mixture (100 μ l in total) containing the primers mentioned above (1 μ M), 1 ng human amygdala cDNA (Clontech, CA, USA), 0.25 mM dNTPs, 5 units of Taq DNA polymerase (Takara Shuzo, Kyoto, Japan), and the reaction buffer provided by the manufacturer of the polymerase at 96 °C for 5 min followed by at 65 °C for 5 min and then 30 cycles at 95 °C for 60 s, at 45 °C for 60 s, and at 60 °C for 3 min. The PCR products obtained were subcloned into a cloning vector, pCR II, with a TA cloning kit (Invitrogen, CA, USA). I analyzed the DNA sequences of inserts in the plasmids using a DNA sequencer (Applied Biosystems Inc., CA, USA). An *apj* cDNA with a full coding region was then isolated from a human amygdala cDNA library (Clontech) by a plaque hybridization technique with a ^{32}P-labeled *apj* cDNA fragment according to the method described previously (Hinuma *et al.*, 1994).

Preparation of CHO cells expressing APJ

The *apj* cDNA with the entire coding region was inserted into the

downstream of SR α promoter in the expression vector, pAKKO-111H (Hinuma *et al.*, 1994). The plasmid DNA of the resultant expression vector was transfected into CHO *dhfr*$^-$ cells with a Genetransfer (Wako Pure Chemicals Inc., Osaka). Transformed CHO cells were selected and maintained in Minimum Essential Medium alpha medium without deoxyribonucleosides and ribonucleosides (GIBCO BRL, NY, USA), supplemented with 10% dialyzed fetal bovine serum (GIBCO BRL) and antibiotics.

Cloning of rat *apj* cDNA

Rat *apj* cDNA was isolated from poly(A)$^+$RNA of rat brain by 5' and 3' rapid amplification of cDNA ends (RACE) method using a Marathon cDNA amplification kit (Clontech, CA, USA). Primers used were, 5'-GAC AAAGATGAGGTAGCTGCTGAG-3' (F1) and 5'-GTCGAGCGTTAGCC ACTGGCC-3' (F2) for 5' RACE, and 5'-TGTTACTTCTTCATTGCCC AAACCAT-3' (R1), 5'-TGGGGTGTCCTCCACTGCTGT-3' (R2) and 5'-ACTCAGAGTGGGCCTGGGAGG-3' (R3) for 3' RACE, respectively. The first PCR was carried out using F2 for 5'RACE or R3 for 3'RACE in combination with the adapter primer 1 provided with the kit in a 25-μl reaction mixture prepared with appropriately diluted cDNAs, 0.2 μM of primers, 1.25 units of ExTaq DNA polymerase (Takara) treated with TaqStart antibody (Clontech), 0.1 mM of dNTPs, and the reaction buffer supplemented with the polymerase. Amplification in the first PCR was conducted under the following conditions: 94 ℃ for 2 min for the denaturation of the template and the activation of ExTaq DNA polymerase; 5 cycles of 98 ℃ for 10 s and

72 ℃ for 2 min; 5 cycles of 98 ℃ for 10 s and 70 ℃ for 2 min; 25 cycles of 98 ℃ for 10 s and 68 ℃ for 2 min. The second PCR was carried out using F1 and F2 for 5'RACE, or R1, R2 and R3 for 3'RACE with 1.0 μl of reaction mixture of the first PCR in combination with adapter primers 1 and 2 provided with the kit, and final amplification step was elongated to 33 cycles. The DNA sequence of the cDNA fragments amplified were determined with a DNA sequencer (model 377, Perkin-Elmer Applied Biosystems Inc., CA, USA) and analyzed with the computer software, DNASIS (Hitachi Software Engineering, Yokohama, Japan). A cDNA fragment with the entire open reading frame was amplified from rat heart cDNA with a primer set (5'-AAGCACCCTCAGACC ACTTACTC-3' and 5'-TTTGCAAGGCTC CTTCCCTTTCC-3'). PCR was carried out in a 25-μl reaction mixture prepared with appropriately diluted cDNAs, 0.2 μM of primers, 1.25 units of KlenTaq DNA polymerase (Clontech) treated with TaqStart antibody (Clontech), 0.1 mM of dNTP, and the reaction buffer supplemented with the polymerase. Amplification was conducted under the following conditions: 94 ℃ for 2 min for the denaturation of the template and the activation of KlenTaq DNA polymerase; 30 cycles of 98 ℃ for 10 s and 68 ℃ for 30 s for amplification; 72 ℃ for 1 min for extension.

Quantitative analyses for rat *apj* mRNA

Poly(A)$^+$ RNAs were prepared from the tissues of adult (8 to 12-week-old) and neonate Wistar rats, and cDNAs were synthesized from these poly(A)$^+$ RNAs according to the method as described elsewhere (Habata *et al.*, 1999). I quantified rat *apj* mRNA by means of a Prism 7700 Sequence Detector (ABI) with a primer set (5'-CCACCTGGTGAAGACTCTCTACA-

3' and 5'-TGACGTAACTGATGCAGGTGC-3') and a hybridization probe labeled with fluorescent dyes (5'(FAM)-TGACAGCTTCCTCATGAATG TCTTT CCC-(TAMRA)3'). PCR was carried out in a 25-μl reaction mixture prepared with a TaqMan PCR Core Reagent Kit (ABI) containing an appropriately diluted cDNA solution, 200 nM of each primer, and 100 nM of probe. PCR was performed under the following conditions: 50 ℃ for 10 min for the reaction of uracil-N-glycosylase to prevent the re-amplification of PCR products carried over; 95 ℃ for 2 min for the activation of AmpliTaq Gold DNA polymerase; and 43 cycles of 95 ℃ for 15 s and 55 ℃ for 90 s for the amplification. The quantification of *apj* mRNA in neonate tissues was carried out principally under the conditions described above, but the amplification of its cDNA was performed at 45 cycles of 95 ℃ for 15 s and 57 ℃ for 90 s. In order to obtain a calibration curve, the known amount of a rat *apj* cDNA fragment was amplified in the same manner as the samples. A good linear relationship was obtained between the amount of rat *apj* cDNA input and the release of the reporter dye within the range of 10 to 10^6 copies. Rat *glyceraldehyde-3-phosphate dehydrogenase* (*g3pdh*) mRNA was also measured as an internal standard using Rodent G3PDH Control Reagents (ABI) according to the manufacturer's instruction, in the same manner as used for the quantification of rat *apj* cDNA.

Synthesis of apelin and its analogue

Human apelin-36, [pGlu]apelin-13 and [pGlu65,Nle75,Y77]apelin-13 were synthesized using an automatic peptide synthesizer (Model 430, ABI) and purified by reversed phase-high performance liquid chromatography (RP-HPLC) as described previously (Tatemoto *et al.*, 1998).

Microphysiometric assays

Extracellular acidification rates were measured with a Cytosensor (Molecular Devices Corp., CA, USA) as described previously (Tatemoto *et al.*, 1998). CHO-A10 cells were dispersed by the treatment with trypsin, and then suspended at 3×10^5 cells/ml in the culture medium. The cell suspensions were dispensed into cell capsules (Molecular Device) at 0.9 ml/capsule and cultured overnight. Then each cell capsule was immobilized in a sensor chamber equipped with a pH-sensitive silicon sensor and set to the instrument. The cells were continuously loaded with a low-buffered RPMI 1640 medium (Molecular Device) until the rate of acidification became constant. In order to determine the effects of apelin, acidification rates were measured every 120 s (flow on at 100 μ l/min for 80 s; flow off for 8 s; measuring acidification rates for 30 s). In the assay to examine the reaction patterns of [pGlu]apelin-13 with apelin-36, the peptides were incubated with CHO-A10 cells for 7 min 2 s.

For treatment with purtussis toxin (PTX), CHO-A10 cells were seeded at 9×10^4 cells/capsule, cultured overnight, and then 100 ng/ml of PTX (P-9452, Sigma Chemical Co., MO, USA) was added to culture medium 24 h before setting the capsules to workstation. Methyl-isobutyl amirolide (MIA, Research Biochemicals Inc., MA, USA) was dissolved in the low-buffered RPMI 1640 medium at 10 μ M and exposed to the cells by switching the fluid path.

Radioiodination of apelin analogue

An analogue of apelin, [pGlu65, Nle75, Tyr77]apelin-13 (TABLE) was

radioiodinated with $Na^{125}I$ (IMS-30, Amersham, UK) by the method using lactoperoxidase (Sigma) as descried elsewhere (Ohtaki *et al.*, 1990). After the reaction, the labeled and unlabeled peptides were separated by RP-HPLC. Aliquots of the labeled peptide were stored at –30℃ until used.

Receptor binding assays using membrane preparations

CHO-A10 cells were grown to semi-confluency in 150-cm^2 tissue culture flasks, and then harvested by treatment with 5 mM ethylenediaminetetraacetic acid (EDTA) in phosphate-buffered saline (PBS). The cells were pelleted by centrifugation, and then suspended in an ice cold binding buffer: 50 mM Tris-HCl at pH7.5 containing 5 mM EDTA, 0.5 mM phenylmethylsulfonyl fluoride (Wako), 0.1 μ g/ml pepstatin A (Peptide Institute, Osaka, Japan), 20 μ g/ml Leupeptin (Peptide Inst.), and 4 μ g/ml E-64 (Peptide Inst.). The cells were homogenized with a Polytron homogenizer (Kinematica GmbH, Switzerland), and the homogenate was centrifuged at 1,000$\times g$ for 5 min to remove debris. The resultant supernatants were centrifuged at 100,000$\times g$ for 1 hr at 4 ℃. The pellets obtained were suspended in the small volume of the binding buffer. The protein concentration was quantified with a Coomassie Protein Assay Reagent (PIERCE, IL, USA). Aliquots of the membrane preparations thus obtained were stored at –80 ℃ until used.

The membrane preparations of CHO-A10 cells were incubated with [^{125}I][pGlu65, Nle75, Tyr77]apelin-13 in 100 μ l of the binding buffer containing 0.1% bovine serum albumin (BSA) in 96-well microplates at room temperature for 90 min. In order to determine the amounts of nonspecific

binding, unlabeled [pGlu65, Nle75, Tyr77]apelin-13 was simultaneously added to the wells. After incubation, bound and free radioactivities were separated through rapid filtration using glass-fiber filter units (GF/C, Packard Instrument Company, CT, USA) of a 96-well cell harvester (Packard). The filter units were entirely dried up, 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}) were determined by Scatchard's method (Scatchard, 1949).

Receptor binding assays using intact cells

CHO-A10 cells were seeded at 1×10^5 cells/well in 24-well tissue culture plates and grown for 2 days. Prior to the binding experiments, the cells were washed 3 times with Hanks' Balanced Salt Solution (HBSS) containing 0.05% BSA. In order to determine the amount of nonspecific binding, 1 μM of unlabeled [pGlu65, Nle75, Tyr77]apelin-13 was added to the wells. The cells were incubated with 200 pM of [^{125}I][pGlu65, Nle75, Tyr77]apelin-13 for time desired at room temperature. After the incubation, the cells were washed 4 times with HBSS containing 0.05% BSA, and then lysed with 0.2 N NaOH containing 1% sodium dodecyl sulfate (SDS). Radioactivity of the cell lysate was measured with a γ-counter (Beckman, CA, USA). The binding of radiolabeled apelin to the cells after exposure to unlabeled apelin was determined as follows. CHO-A10 cells prepared as described above were pre-incubated with 1 μM of [pGlu]apelin-13 or apelin-36 for 90 min, then washed 4 times with HBSS containing 0.05% BSA to remove unbound peptides. Then the cells were incubated with the

radiolabeled apelin and the amount of the labeled apelin bound was determined as described above.

Chemotactic assays

Chemotactic assay was performed with a 96-well microchemotaxis chamber (Neuro Probe, MD, USA). [pGlu]apelin-13 and apelin-36 were diluted with Dulbecco's modified minimum essential medium (DMEM) supplemented with 0.5% BSA (DMEM/BSA), and 37 μ l of each diluted solution was added to the lower chamber, respectively. A polyvinylpyrolidone-free polycarbonate framed filter with 5-μ m pores (Neuro Probe), after pre-coated with 10 μ g/ml bovine fibronectin (Yagai Research Center, Yamagata, Japan), was used to separate the upper and lower chambers. CHO-A10 cells and mock transfected CHO cells were harvested and suspended in DMEM/BSA. Cell suspensions at 1×10^5 cells/200 μ l/well were added to the upper chamber. The chemotaxis chamber was incubated at 37 ℃ for 4 hr in a CO_2 incubator with 95% air and 5% CO_2. After the cells not migrating on the upper surface of the filter were scraped off, cells migrating to the bottom of the filter were fixed and stained with Diff-Quick (International Reagent Corporation, Hyogo, Japan), and the absorbance at 595 nm was measured with a Benchmark microplate reader (Bio-Rad Laboratories, CA, USA).

Gel filtration analysis of bovine colostrum

Colostrum obtained from Holstein cows was boiled for 15 min, supplemented with up to 1 M acetic acid, and homogenized using a Polytron

homogenizer. The clear supernatant prepared by centrifugation was fractionated as described previously (Tatemoto *et al.*, 1998). In brief, the fraction eluted with 30% acetonitrile in C_{18} open column (Prep C18, Waters) chromatography was applied to HiPrep CM-Sepharose FF column (Pharmacia). The eluate with 0.5 M ammonium acetate was treated with acetone, and desalted with Sep-Pak C_{18} column (Waters). After lyophilization, this fraction was separated by Superdex Peptide gel filtration column (Pharmacia) chromatography. Synthetic apelin-36 and [pGlu]apelin-13 were applied on the same chromatography column and separated in order to determine fraction in which they were eluted. Apelin present in each fraction was detected on the basis of the cAMP production-inhibitory activity on CHO-A10 cells stimulated with forskolin as described previously (Habata *et al.*, 1999).