Results

Cloning of human APJ and its expression in CHO cells

By using the degenerate primers, which were designed on the basis of consensus sequence corresponding to the first and sixth transmembrane domains among known 7TMRs, I isolated a cDNA fragment possibly encoding 7TMR-like protein. I subsequently isolated a cDNA clone with a full coding region from a human amygdala cDNA library by utilizing the cDNA fragment as a probe. The amino acid sequence from the isolated cDNA (Fig. 1) was fully corresponded to that of APJ reported by O'Dowd *et al.* (O'Dowd *et al.*, 1993).

I subsequently constructed an expression plasmid for APJ by inserting the cDNA into the downstream of SR α promoter in the expression vector, and introduced it into CHO *dhfr*$^-$ cells. The transformed CHO cells thus obtained (CHO-A10 cells) were used to detect APJ ligand in tissue samples through microphysiometric assays (Tatemoto *et al.*, 1998).

Cloning of rat *apj* cDNA and its tissue distribution

I isolated a rat *apj* cDNA by RACE method from rat brain poly(A)$^+$RNA. The isolated cDNA encoded an open reading frame with 377 amino acid length. The amino acid sequences of rat and human APJ are aligned in Fig. 1. Amino acid identity in the sequences of rat and human APJ was 87.2%. The N-terminal region which was supposed to have an important role in HIV infection in human (Farzan *et al.*, 1998) was also highly conserved between the two species. I confirmed that CHO cells expressing rat APJ as well as CHO-A10 cells specifically responded to [pGlu]apelin-13

in a dose-dependent manner in the assay using a Cytosensor (data not shown).

Quantitative analyses for rat *apj* mRNA by RT-PCR

I analyzed the precise distribution of *apj* mRNA in rat tissues by reverse transcription-polymerase chain reaction (RT-PCR). As shown in Fig. 2, I detected *apj* mRNA in almost all tissues tested, although their quantity considerably varied among the tissues. The highest expression was detected in the lung in infants, and a comparable level of expression was also detected in the adult lung. To be interested, *apj* mRNA expression tended to be higher in the tissues (e.g., the kidney, stomach, and intestine) in infants than those in adults. In the peripheral tissues of adults, moderate levels of expression were widely detected in the heart, thymus, kidney, adrenal gland, adipose, ovary, uterus, femur, costal cartilage, and placenta. Similar levels of expression were also detected in the central nervous system in adults, such as the hypothalamus, medulla oblongata and spinal cord. In these experiments, the levels of *g3pdh* mRNA expression were almost consistent among the tissues within the range of 0.7×10^5 to 9.1×10^5 copies/ng of poly(A)$^+$RNA except for pituitary, heart, and mammary gland (1.1×10^6 to 2.2×10^6 copies/ng of poly(A)$^+$RNA) and skeletal muscle (4.6×10^6 copies/ng of poly(A)$^+$RNA).

Analyses for reaction patterns in extracellular acidification induced by apelin

I exposed CHO-A10 cells with [pGlu]apelin-13 and apelin-36 for relatively long time (7 min 2 s). This treatment enabled us to discern differences in the reaction patterns of extracellular acidification induced by

the two peptides (Fig. 3). The promotion of acidification rate induced by [pGlu]apelin-13 reached a maximum at 7 cycles under the experimental conditions used here. The maximal acidification rate induced by [pGlu]apelin-13 at 0.1 nM was 140%, and it reached a plateau (i. e., approximately 180%) at 1 to 10 nM. By the removal of [pGlu]apelin-13, the elevated acidification rates gradually declined and returned to the basal level by 20 cycles at all doses examined. The promotion of acidification rates induced by apelin-36 reached a maximum at the same cycles as [pGlu]apelin-13. Although the maximum acidification rates induced by apelin-36 at 1 nM was lower (i. e., 120%) than that by [pGlu]apelin-13, it reached the same level of a plateau as [pGlu]apelin-13 at 10 to 100 nM. However, the patterns of the acidification rates after removal of the samples were quite distinctive between [pGlu]apelin-13 and apelin-36: the elevated acidification rates induced by apelin-36 at 10 to 100 nM were kept even after 20 cycles. At 20 cycles, re-stimulation with [pGlu]apelin-13 could induce a significant raise of the acidification rates comparable to that in the first exposure. In contrast, the same treatment with apelin-36 did not induce further elevation of the acidification rates at 10 to 100 nM doses (data not shown).

In order to explore a mechanism that apelin caused extracellular acidification in CHO-A10 cells, I examined the effects of enzyme inhibitors. Since apelin could effectively inhibit the forskolin-stimulated cAMP production in CHO-A10 cells (Tatemoto *et al.*, 1998; Habata *et al.*, 1999), it has been expected that APJ couples to the inhibitory G protein, Gi. I thus first tested the effects of PTX treatment. As shown in Fig. 4, both [pGlu]apelin-13 and apelin-36 actions were obviously suppressed by the PTX treatment, suggesting that the signal transduction pathway stimulated by both peptides was transduced by Gi. On the other hand, when I treated CHO-A10 cells with

MIA, the specific inhibitor for Na^+/H^+ exchanger (NHE) (Neve *et al.*, 1995), the acidification rates promoted by both peptides were also evidently suppressed (Fig. 5), suggesting that they induced the promotion of the extracellular acidification through NHE. These results using the two inhibitors indicate that the promotion of acidification induced by both peptides are caused through essentially the same signal transduction pathways.

Binding properties of apelin to APJ

In our previous study, [pGlu]apelin-13 has been found to be the most potent agonist in the promotion of the extracellular acidification rates when determined in the microphysiometric assay (Tatemoto *et al.*, 1998). I radioiodinated an analogue of apelin-13 (i.e., [pGlu65,Nle75,Tyr77]apelin-13) to use for receptor binding experiments. I used this analogue because of following reasons. As there was no tyrosine residue for radioiodination using lactoperoxidase in the sequence of [pGlu]apelin-13, I substituted Phe77 with Tyr77. [pGlu65, Tyr77]apelin-13 was equivalently potent to the [pGlu]apelin-13 in the microphysiometric assay (data not shown). In addition, [pGlu65, Nle75, Tyr77]apelin-13 was designed to prevent possible oxidization at Met65 during the labeling reactions. The activity of [pGlu]apelin-13 was significantly decreased by oxidation (data not shown). The substitution of Met75 to norleucine (Nle75) did not reduce the agonistic activity. By both the microphysiometric and cAMP-inhibitory assays, I confirmed that the agonistic activity of [pGlu65, Nle75, Tyr77]apelin-13 remained after the iodination (data not shown).

[^{125}I][pGlu65, Nle75, Tyr77]apelin-13 specifically bound to intact CHO-A10 cells and their membrane preparations. Scatchard's plot analysis of

[^{125}I][pGlu65, Nle75, Tyr77]apelin-13 binding to the membranes of CHO-A10 cells represented a single class of high affinity binding sites with the *Kd* of 22.3 ± 2.7 pM and the Bmax of 3.01 ± 0.07 pmol/mg of protein (Fig. 6). In the competitive binding experiments, [pGlu65]apelin-13 and human apelin-36 effectively inhibited the binding of [^{125}I][pGlu65, Nle75, Tyr77]apelin-13 to the CHO-A10 cell membranes, and their inhibitory dose of 50% were 1.4 ± 0.1 nM and 4.8 ± 0.24 nM, respectively (Fig. 7).

In order to assess the dissociation of [pGlu]apelin-13 and apelin-36 to APJ, I compared the binding of [^{125}I][pGlu65, Nle75, Tyr77]apelin-13 to CHO-A10 cells treated with [pGlu]apelin-13 or apelin-36. At first, I examined the time-course of the association of [^{125}I][pGlu65, Nle75, Tyr77]apelin-13 on intact CHO-A10 cells and found that the labeled ligand rapidly bound to the cells by 30 min (Fig. 8A). I thus exposed CHO-A10 cells to the excess amount of each peptide at the concentration of 1 μ M for 90 min, washed out unbound peptides, and then determined the amount of [^{125}I][pGlu65, Nle75, Tyr77]apelin-13 binding to the CHO-A10 cells. The radiolabeled ligand effectively bound to the cells even after the pretreatment with the high concentration of [pGlu]apelin-13 (Fig. 8B). The time-course kinetics of [^{125}I][pGlu65, Nle75, Tyr77]apelin-13 binding to the cells pretreated with [pGlu]apelin-13 was close to that of untreated cells. In contrast, the binding of radiolabeled ligand to CHO-A10 cells treated with apelin-36 was kept a very low level, suggesting that the dissociation of apelin-36 from APJ is considerably lower than that of [pGlu]apelin-13.

Chemotactic action of apelin

As functional characterization of APJ, I examined the chemotactic action of apelin on CHO-A10 cells. As shown in Fig. 9, [pGlu]apelin-13

showed a potent chemotaxis-inducing activity. Apelin-36 also induced the chemotactic movement of the cells, however, its potency was weaker than [pGlu]apelin-13. The dose-response curves for [pGlu]aplein-13 and apelin-36 were typically bell-shaped. With checkerboard analysis, addition of apelin in the upper chamber did not induce the migration and inhibited the migration toward the ligand in the lower chamber (data not shown), indicating that the migration in response to apelin was chemotactic but not chemokinetic.

Gel filtration analysis for molecular forms of apelin in bovine colostrum

A peptide-enriched fraction was prepared from bovine colostrum by a combination of C_{18} reversed phase and CM-sepharose ion exchange chromatographies. In order to analyze the molecular forms of endogenous apelin, I subjected this fraction to gel filtration, and biologically active apelin in each fraction was detected by the cAMP production-inhibitory assay utilizing CHO-A10 cells. As shown in Fig. 10, two peaks of the activity were detected at positions corresponding to those of synthetic apelin-36 and [pGlu]apelin-13 eluted. However, these fractions did not show such activities on mock-transfected CHO cells (data not shown). These results indicate that both long and short forms of apelin corresponding to apelin-36 and [pGlu]apelin-13 respectively are produced at least in bovine colostrum.