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Biochimica et Biophysica Acta 1452 (1999) 25–35

BIOCHIMICA ET BIOPHYSICA ACTA

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Apelin, the natural ligand of the orphan receptor APJ, is abundantly secreted in the colostrum

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Received 30 June 1999; received in revised form 9 August 1999; accepted 12 August 1999

Abstract

By using a strategy that we have developed to search for the ligands of orphan seven-transmembrane-domain receptors [S. Hinuma et al., Nature 393 (1998) 272–276], we have recently identified a natural ligand, apelin, for the orphan 7TMR, APJ [K. Tatemoto et al., Biochem. Biophys. Res. Commun. 251 (1998) 471–476]. In this paper, we isolated rat and mouse apelin cDNAs, and analyzed the tissue distribution of apelin mRNA in rats. Although apelin mRNA was widely detected in a variety of tissues, the highest expression of apelin mRNA was detected in the mammary gland of pregnant rats. In the mammary gland, biologically active apelin and its mRNA considerably increased during pregnancy and lactation, and reached a maximal level around parturition. Moreover, a large amount of apelin (14–93 pmol/ml) was found to be secreted in the bovine colostrum, and it was still detectable even in commercial bovine milk. Since apelin partially suppressed cytokine production by mouse spleen cells in response to T cell receptor/CD3 cross-linking, the oral intake of apelin in the colostrum and milk might modulate immune responses in neonates.

Keywords: Apelin; APJ; Mammary gland; Milk; Colostrum

1. Introduction

Various sorts of biologically active molecules, including hormones, neurotransmitters, and chemokines, transmit signals into cells by binding to so-called seven-transmembrane-domain receptors (7TMRs). Since these receptors are coupled to guanine nucleotide-binding protein (G protein) to transduce intracellular signals, they are also called G protein-coupled receptors [3,4]. Recent progress in both cDNA and genome DNA analyses has led to the

Abbreviations: 7TMR, seven-transmembrane-domain receptor; G protein, guanine nucleotide-binding protein; CHO, Chinese hamster ovary; HIV, human immunodeficiency virus; RT, reverse transcription; PCR, polymerase chain reaction; RACE, rapid amplification of cDNA ends; G3PDH, glyceraldehyde 3-phosphate dehydrogenase; EIA, enzyme immunoassay; TFA, trifluoroacetic acid; IBMX, 3-isobutyl-1-methylxanthine; EST, expressed sequence tag; mAbs, monoclonal antibodies; IL, interleukin; IFN-γ, interferon-γ; BB, borate buffer

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0167-4889/99/$ – see front matter
PII: S0167-4889(99)00114-7

discovery of numerous 7TMR-like genes. Since most of their endogenous ligands are unidentified, they are referred to as 'orphan' 7TMRs [5]. We have recently developed a strategy to identify the ligands of orphan 7TMRs by monitoring the specific signal transductions induced in Chinese hamster ovary (CHO) cells expressing orphan 7TMRs [1,2]. Employing this method, we first succeeded in identifying the natural ligand of the orphan 7TMR, hGR3. The peptidic ligand identified for hGR3 was found to show a specific prolactin-release-promoting activity, and so was named prolactin-releasing peptide [1].

We subsequently searched for the natural ligand of another orphan 7TMR, APJ, which was originally isolated from human genomic DNA [6]. APJ encodes a protein slightly resembling the angiotensin II receptor, having about 30% amino acid sequence identity overall. However, angiotensin II did not interact with APJ when expressed in fibroblasts [6] and CHO cells [2]. In order to detect the specific signal transduction in CHO cells expressing APJ, we utilized a Cytosensor which could measure extracellular acidification induced by the interaction of ligands and receptors [7]. We found that peptide-enriched fractions prepared from bovine stomach tissue extracts showed a specific activity to promote the acidification rate in CHO cells expressing APJ [2]. By monitoring this activity, we purified a ligand peptide for APJ from this extract. We subsequently isolated human and bovine cDNAs based on the sequence of the purified peptide. These cDNAs encoded preproproteins consisting of 77 amino acid residues. Since the purified peptide sequence was situated in their C-terminal portions, we predicted that a peptide of 36 amino acid in length, including almost all of the C-terminal portion, would comprise a mature form. When synthesized, this peptide showed a specific acidification-rate-promoting activity to CHO cells expressing APJ. We named it apelin after APJ endogenous ligand [2].

Although CXCR4 and CCR5 are thought to function as main coreceptors with CD4 in the process of human immunodeficiency virus (HIV) type 1 infection [8], APJ has recently been reported to be one of several alternative coreceptor candidates [9–12]. Although APJ does not show high overall homology with chemokine receptors, including CCR5, CCR3, and CXCR4, APJ shows sequence similarity to these chemokine receptors in the N-terminal region, which is confirmed to be very important for the interaction with HIV [9]. It is thus a very interesting question whether or not apelin might influence HIV infection via APJ.

In this paper, we isolated rat and mouse apelin cDNAs, and then analyzed the precise tissue distribution of rat apelin mRNA by quantitative reverse transcription-polymerase chain reaction (RT-PCR). Apelin mRNA was widely distributed in rat tissues, and most highly expressed in the mammary gland during pregnancy and lactation. We will subsequently demonstrate here the secretion of large amounts of apelin peptides into the colostrum and milk, and discuss the physiological significance of these findings.

2. Materials and methods

2.1. Cloning of mouse and rat apelin cDNAs

We isolated mouse and rat apelin cDNAs from the poly(A)$^+$ RNA fractions prepared from mouse and rat brain, respectively, by the 3′ and 5′ rapid amplification of cDNA ends (RACE) method using a Marathon cDNA amplification kit (Clontech, Palo Alto, CA, USA) in a manner similar to that used previously for the cloning of human and bovine apelin cDNAs [2]. We designed primers, 5′-CTGGCAGGGAGGCAGGAGGAA-3′ and 5′-GCAGGAGGAAATTTCGCAGACAGC-3′ for 3′ RACE, and 5′-GAAGAGAATTCATCTGTGGAGTA-3′ and 5′-ACCGGCACCGGGAGGGCACTT-3′ for 5′ RACE. PCR was carried out in combination with the adapter primers provided with the kit. The first set of PCR reactions for RACE were carried out under the following conditions: 94°C for 2 min; 5 cycles of 98°C for 10 s and 72°C for 2 min; 5 cycles of 98°C for 10 s and 70°C for 2 min; 25 cycles of 98°C for 10 s and 68°C for 2 min. The resultant PCR products were then used as the templates for the second set of nested PCR under the conditions: 94°C for 2 min; 5 cycles of 98°C for 10 s and 72°C for 2 min; 5 cycles of 98°C for 10 s and 70°C for 2 min; 30 cycles of 98°C for 10 s and 68°C for

2 min. DNA sequences thereby obtained were analyzed with a DNA sequencer (model 377, ABI, Foster City, CA, USA), and entire mouse and rat apelin cDNA sequences stretching 234 bp in total were determined respectively on the basis of these sequences.

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 (Japan SLC, Shizuoka, Japan). The mammary glands obtained from three female rats in various reproductive stages were pooled and then their poly(A)$^+$ RNA fractions were prepared using Isogen (Nippon Gene, Toyama, Japan) followed by an mRNA Purification Kit (Pharmacia, Uppsala, Sweden). Complement DNAs were synthesized from poly(A)$^+$ RNAs (160 ng) treated with deoxyribonuclease I (amplification grade, Gibco BRL, Grand Island, NY) in the presence of 2.5 μM of random 9-mer nucleotides (Takara Shuzo, Kyoto, Japan), and 10 units of AMV reverse transcriptase XL (Life Sciences, Petersburg, FL, USA) at 42°C for 30 min. We quantified rat apelin mRNA by means of a Prism 7700 Sequence Detector (ABI) [13,14] with a primer set (5′-GGCTAGAAGAAGGCAACATGC-3′, 5′-CCGCTGTCTGCGAAATTTC-3′) and a hybridization probe labeled with fluorescent dyes (5′(FAM)-TGGTGAAGCCCAGAACTTCGAGGA-(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, 1 μM of each primer, and 200 nM probe. PCR was conducted under the following conditions: 50°C for 2 min for the reaction of uracil *N*-glycosylase to prevent the reamplification of PCR products carried over; 95°C for 10 min for the activation of AmplyTaq Gold DNA polymerase; and 45 cycles of 95°C for 15 s and 57°C for 80 s for amplification. In order to obtain a calibration curve, we amplified a known amount of a plasmid encompassing rat apelin cDNA in the same manner as the samples. A good linear relationship was obtained between the amount of rat apelin cDNA input and the release of the reporter dye within the range of 10–10^6 copies. Rat glyceraldehyde 3-phosphate dehydrogenase (G3PDH) mRNA was also measured as an internal control using Rodent G3PDH Control Reagents (ABI) according to the manufacturer's instruction, in the same manner as used for the quantification of rat apelin cDNA. The contents of G3PDH mRNA in the poly(A)$^+$ RNA fractions obtained from the rats tended to decrease along with the progress of pregnancy and lactation (data not shown).

2.3. Synthesis of apelin peptides

[pGlu]Apelin-13 (pERPRLSHKGPMPF), apelin-13 (QRPRLSHKGPMPF), apelin-17 (KFRRQRPRLSHKGPMPF), and apelin-36 (LVQPRGPRSGPGPWQGGRRKFRRQRPRLSHKGPMPF) were synthesized using an automatic peptide synthesizer (Model 430, ABI) as described previously [2].

2.4. Assay for inhibition of cAMP production by apelin

CHO cells with introduced human APJ cDNA were established as described previously [2]. The inhibitory activity of apelin to cAMP production by these cells was measured in the presence of 0.2 mM 3-isobutyl-1-methylxanthine (IBMX, Sigma, St. Louis, MO, USA) and 1 μM forskolin (Wako, Osaka, Japan) in an in vitro culture, and the amount of cAMP was determined with a cAMP enzyme immunoassay (EIA) system (Amersham, Buckinghamshire, UK) as described elsewhere [15]. As a control, we used CHO dhfr$^-$ cells, a parent cell line, prior to transfecting the expression vector plasmid containing human APJ cDNA, in order to assess non-specific activities in the samples.

2.5. Quantification of apelin based on cAMP-production-inhibitory activity

In order to determine the content of apelin, we obtained tissues from female Wistar rats (8 weeks old) immediately after decapitation. They were pooled (about 1.5–5 g), if necessary, and then kept on ice until used for the quantification. Each tissue was boiled in 20 ml of water for 15 min, supplemented with up to 1 M acetic acid, and homogenized

using a Polytron homogenizer. The supernatant prepared by centrifugation was supplemented with up to 0.1% trifluoroacetic acid (TFA), and then applied to a Sep-Pak Vac C18 20 cc column (5 g, Waters, Milford, MA, USA). Elution was performed in stepwise increments of 10%, 30%, and 50% CH_3CN in 0.1% TFA in water. The 30% CH_3CN fraction was further fractionated by HiTrap CM-Sepharose FF 5 ml (Pharmacia) in stepwise increments of 0.1, 0.2, and 1 M CH_3COONH_4 (pH 6.4) containing 10% CH_3CN. The 1 M CH_3COONH_4 fraction desalted using a Sep-Pak plus C18 cartridge (Waters) was lyophilized, and then applied to the cAMP-production-inhibitory assay using CHO cells expressing human APJ.

Colostrum and milk from Holstein cows (3–5 years old) were kindly supplied by Hinode Rakunou (Tsukuba, Japan). Pasteurized bovine milk was purchased at markets in Tsukuba. Rat milk was collected and pooled from five Wistar rats after parturition. In order to determine the content of apelin, milk was boiled for 15 min, an equal volume of 1 M acetic acid added, and homogenized by a Polytron homogenizer. After centrifugation, the resultant supernatant was fractionated in the same manner as the rat tissues, and then subjected to the cAMP-production-inhibitory assay. The amounts of apelin in the tissue and milk extracts were quantified on the basis of a calibration curve obtained using synthetic [pGlu]apelin-13.

2.6. *Analyses for effects of apelin on cytokine production*

Spleens were aseptically removed from female BALB/c mice (8 weeks old, Charles River Japan, Atsugi, Japan), and single cell suspensions were prepared by gently squeezing the spleen between two glass slides in Hanks' balanced salt solution supplemented with HEPES, 5 U/ml of heparin, and 5% NuSerum (Becton Dickinson Labware, Bedford, MA, USA). The cells were suspended in RPMI 1640 medium supplemented with HEPES, L-glutamine, penicillin G, streptomycin, 2-mercaptoethanol, and 10% NuSerum, and cultured at 2.5×10^6 cells/ml in 96-well flat-bottomed microplates (Nippon InterMed, Tokyo, Japan) pre-coated with 2 μg/ml of anti-CD3 monoclonal antibodies (mAbs) (145-2C11, Cedarlane Laboratories, Ontario, Canada) in the presence or absence of apelin peptides. After culturing for 24 h, the supernatants were harvested to measure the cytokines produced.

The concentrations of interleukin-2 (IL-2), interleukin-4 (IL-4), and interferon-γ (IFN-γ) were determined by sandwich EIA. The capture antibodies used were rat anti-mouse IL-2 mAbs (Genzyme, Cambridge, MA, USA), rat anti-murine IL-4 mAbs (clone BVD4-1D11, Endogen, Cambridge, MA, USA), and hamster anti-murine IFN-γ mAbs (Genzyme). Detection antibodies used were rabbit anti-mouse IL-2 polyclonal antibodies (Becton Dickinson), rat anti-murine IL-4 mAbs (clone BVD6-24G2, Endogen), and rat anti-mouse IFN-γ mAbs (clone XMG1, 2, Upstate Biotechnology, Lake Placid, NY, USA). The 96-well microplates were coated with 100 μl of the capture antibodies (2 μg/ml) diluted with borate buffer (BB) at pH 8.5. After overnight incubation at room temperature, the wells were blocked with 1% BSA in BB for 30 min at room temperature. The wells were washed three times with BB containing 0.05% Tween 20, and then the culture supernatants were incubated for 90 min at room temperature. After the wells were washed with BB containing 0.05% Tween 20, the detection antibodies corresponding to the capture antibodies were appropriately diluted and added to the wells. After incubation for 90 min, the wells were washed with BB containing 0.05% Tween 20, and peroxidase-conjugated monoclonal mouse anti-rat IgG1 (Experimental Immunology, Belgium) or peroxidase-conjugated monoclonal rat anti-rabbit IgG (Zymed Laboratories, San Francisco, CA, USA) antibodies were added to the wells, respectively. The wells were then incubated for 90 min, washed with BB containing 0.05% Tween 20, and supplemented with *o*-phenylenediamine (1 mg/ml) in phosphate buffer at pH 7 containing 0.2 μl/ml of H_2O_2. After a final incubation for 40 min, the colorimetric reaction was stopped with 1.5 N H_2SO_4. Absorbance at 492 nm minus that at 630 nm was measured by a MTP-32 microplate reader (Corona Electric, Ibaraki, Japan). The concentrations of cytokines in the culture supernatants were determined on the basis of calibration curves obtained using standard cytokines. As standard cytokines, we used recombinant mouse IL-2, IL-4, and IFN-γ (Genzyme).

3. Results

3.1. Cloning of cDNAs encoding mouse and rat apelin preproproteins

Based on the sequence of a mouse expressed sequence tag (EST) in the GenBank/EMBL database (accession number W33327), which possessed significant homology to the bovine apelin sequence [2], we isolated the mouse and rat cDNAs from the poly-$(A)^+$ RNA fractions prepared from mouse and rat brain, respectively, by means of RACE. The amino acid sequences encoded by bovine, human, rat and mouse cDNAs are aligned in Fig. 1. These cDNAs encoded preproproteins of 77 amino acid residues in length. Their N-terminal portions, comprising 22 amino acid residues, were rich in hydrophobic amino acid residues, and showed a typical profile of secretory signal sequences. Since it has been proven that endogenous mature apelin peptide purified from bovine stomach tissue extracts starts at Leu-42 and includes almost all of the C-terminal portion [2], similar endogenous apelin peptides were expected to exist in mice and rats. In comparing these sequences, the C-terminal portion from positions Trp-55 to Phe-77 was absolutely conserved among the four species tested.

3.2. Tissue distribution of rat apelin mRNA

We quantitatively analyzed the distribution of apelin mRNA in rat tissues by RT-PCR, and detected apelin mRNA in various tissues tested. In the peripheral tissues, we detected a very high level of apelin mRNA in the lung; moderate levels of apelin mRNA in the heart, adipose tissue, testis, and ovary (data not shown). Interestingly, we detected the highest level of apelin mRNA in the mammary gland of the lactating rats. We therefore determined the change of apelin mRNA expression levels in the mammary gland during pregnancy and lactation in rats. We repeated several similar experiments, and show a representative result in Fig. 2. Although the expression of apelin mRNA was not particularly high in control virgin rats, it gradually increased in accordance with the development of the mammary gland during pregnancy, and reached a maximal level around parturition. High levels of apelin mRNA were consistently detected during lactation.

3.3. Inhibition of cAMP production in CHO cells expressing human APJ by apelin

Since the C-terminal portion of preproapelin contains a number of basic amino acid residues which

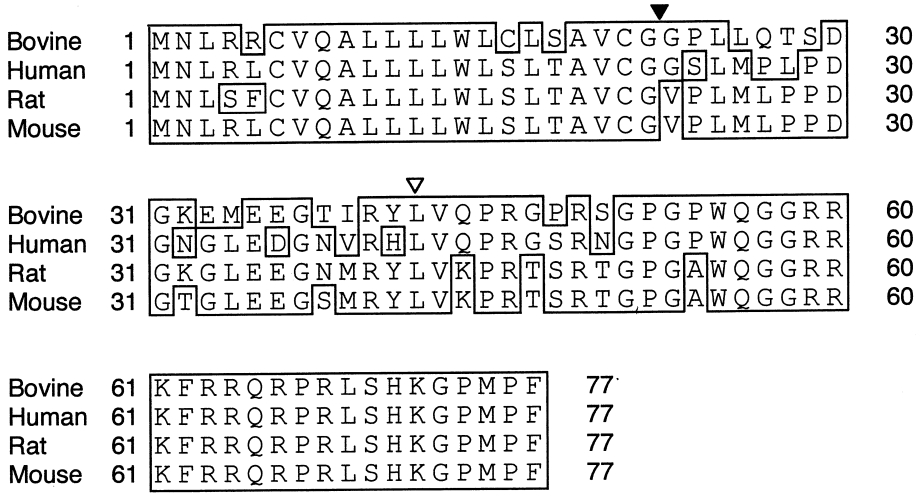

Fig. 1. Amino acid sequences of bovine, human, rat, and mouse apelin preproproteins. Amino acid residues identical in at least two species are boxed. The filled arrowhead indicates the predicted cleavage site of secretory signal peptides. The open arrowhead indicates the N-terminus of apelin-36, which was originally identified as an endogenous apelin in the bovine stomach tissue extracts. The nucleotide sequence data of apelin cDNAs will appear in the DDBJ/EMBL/GenBank databases with the accession numbers AB023492, AB023493, AB023494, and AB023495, respectively.

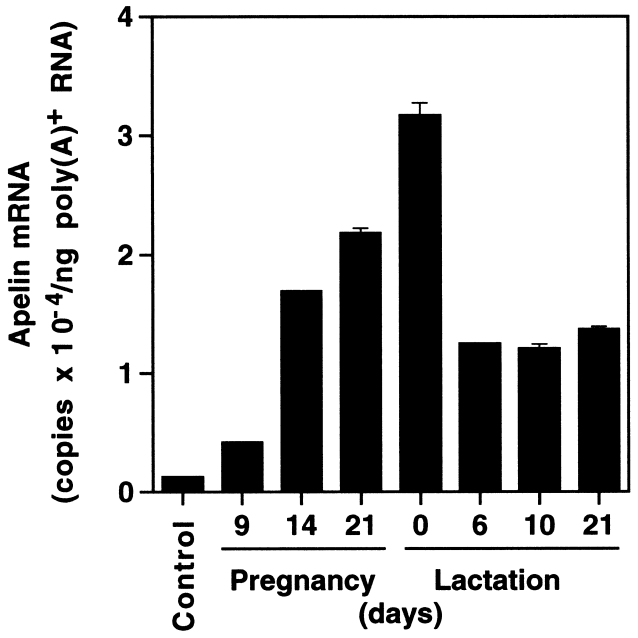

Fig. 2. Increased expression of rat apelin mRNA in the mammary gland during pregnancy and lactation. Mammary glands obtained from three female rats during pregnancy (indicated days after copulation) and lactation (indicated days after parturition) and control virgin rats were pooled respectively, and then their poly(A)$^+$ RNA preparations were subjected to quantitative RT-PCR analyses using a Prism 7700 Sequence Detector. Parturition was observed between day 21 of pregnancy and day 0 of lactation. The amounts of apelin mRNA were determined in quadruplicate assays per sample. Values represents mean ± S.E.M.

are potential cleavage sites by peptidases, it is expected that various sizes of apelin would exist as endogenous ligands. We hence synthesized various length of apelin peptides including apelin-17, apelin-13, and [pGlu]apelin-13. [pGlu]Apelin-13 was synthesized as a pyroglutaminated form of apelin-13, because Glu at the N-terminus of bioactive peptides is frequently pyroglutaminated in vivo through processing [16–20]. As shown in Fig. 3, forskolin-stimulated cAMP production in CHO cells expressing human APJ was inhibited by the addition of [pGlu]apelin-13, apelin-13, apelin-17, and apelin-36 in a dose-dependent fashion, with IC_{50} values of 0.17, 0.16, 0.28, and 0.52 nM, respectively. By the higher concentrations of these apelin peptides, the forskolin-stimulated cAMP production was suppressed to nearly the basal level. However, they showed no significant inhibition of the forskolin-stimulated cAMP production in CHO dhfr$^-$ cells, a parent cell line of CHO cells which does not express human APJ, even at 10^{-7} M, indicating that the inhibition of cAMP production was induced in CHO cells by the specific interaction between APJ and apelin.

3.4. The distribution of biologically active apelin in rat tissues

We quantified the contents of bioactive apelin in rat tissues based on the specific inhibitory activity to the forskolin-stimulated cAMP production in CHO cells expressing human APJ. We repeated several similar experiments, and show a representative result in Fig. 4A. Among the tissues examined, a high content of apelin was detected only in the lung, while moderate levels of apelin were detected in the heart, ovary, and brain. A small amount of apelin was also detected in the adipose tissue.

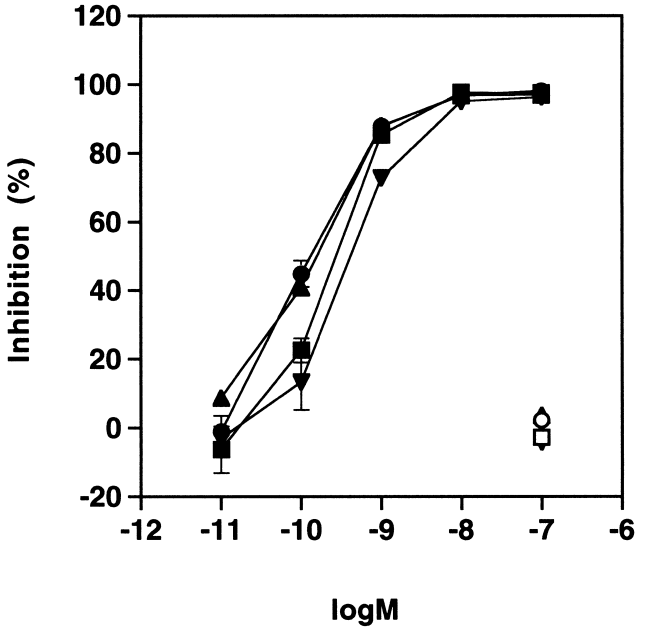

Fig. 3. Inhibition of forskolin-stimulated cAMP production in CHO-A10 cells by apelin. The effects of [pGlu]apelin-13 (●,○), apelin-13 (▲,△), apelin-17 (■,□), and apelin-36 (▼,▽) at the indicated concentrations added to CHO cells expressing human APJ or CHO dhfr$^-$ cells respectively, and cAMP production were examined in the presence of 0.2 mM IBMX and 1 μM forskolin. The inhibition of the cAMP production is indicated as percentage of inhibition against maximum cAMP production by CHO cells without apelin. Values represent mean ± S.E.M. in triplicate assays.

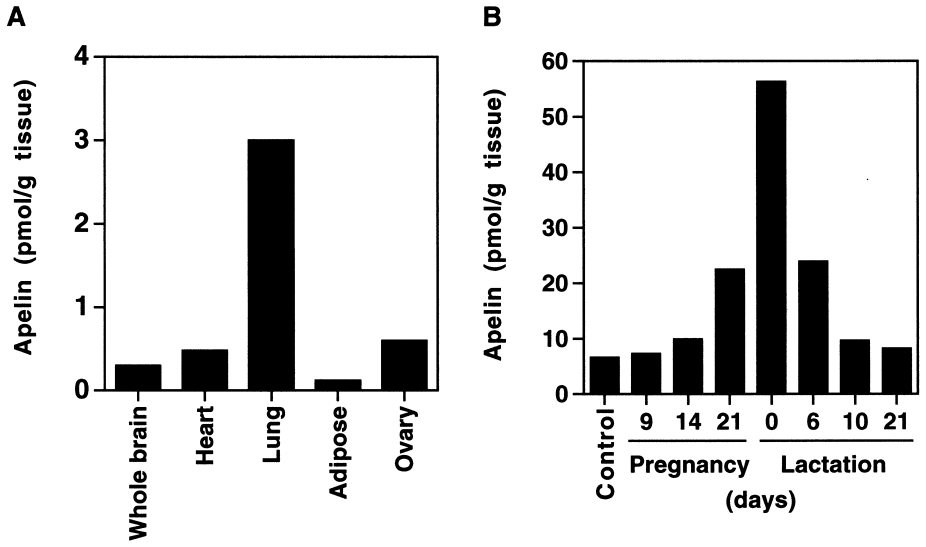

Fig. 4. Distribution of apelin in rat tissues. The contents of apelin in the indicated tissues were determined on the inhibitory activities of their extracts to forskolin-stimulated cAMP production in CHO cells expressing human APJ. A calibration curve to quantify the contents of apelin was obtained using synthetic [pGlu]apelin-13. (A) The contents of apelin in the rat tissues. (B) The contents of apelin in the mammary gland during pregnancy and lactation. Mammary glands obtained from female rats during pregnancy (indicated days after copulation) and lactation (indicated days after parturition) and control virgin rats were pooled respectively, and then subjected to the assay. The tissues were prepared at the same time as the experiments of Fig. 2.

In the mammary gland, we detected larger amounts of apelin than in the other tissues. In accordance with the increase of apelin mRNA (Fig. 2), the content of bioactive apelin also increased as pregnancy progressed (Fig. 4B). The content of apelin gradually increased during pregnancy and reached a peak around parturition. Then it gradually decreased to the basal level during the lactating period. The kinetics of the apelin increment appeared to parallel those of apelin mRNA during pregnancy. However, although the expression of apelin mRNA was sustained at the higher level during the lactating period, apelin decreased to the basal level at 21 days after parturition.

3.5. Secretion of apelin in colostrum and milk

Based on the results that apelin and its mRNA were highly expressed in the mammary gland during pregnancy and lactation, we expected that apelin would be secreted in milk from the mammary gland. Accordingly, we performed the cAMP-production-inhibitory assay and detected a high concentration of apelin (45.8 pmol/ml) in rat milk collected from

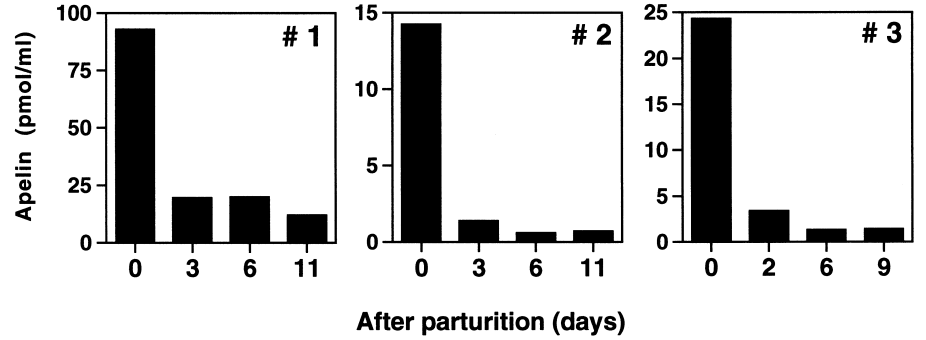

Fig. 5. Contents of apelin in bovine colostrum and milk. The contents of apelin were quantified on the basis of the cAMP-production-inhibitory activities in a manner similar to that described in Fig. 4. Bovine colostrum and milk were obtained from three Holstein cows (No. 1–3) on the indicated days after parturition.

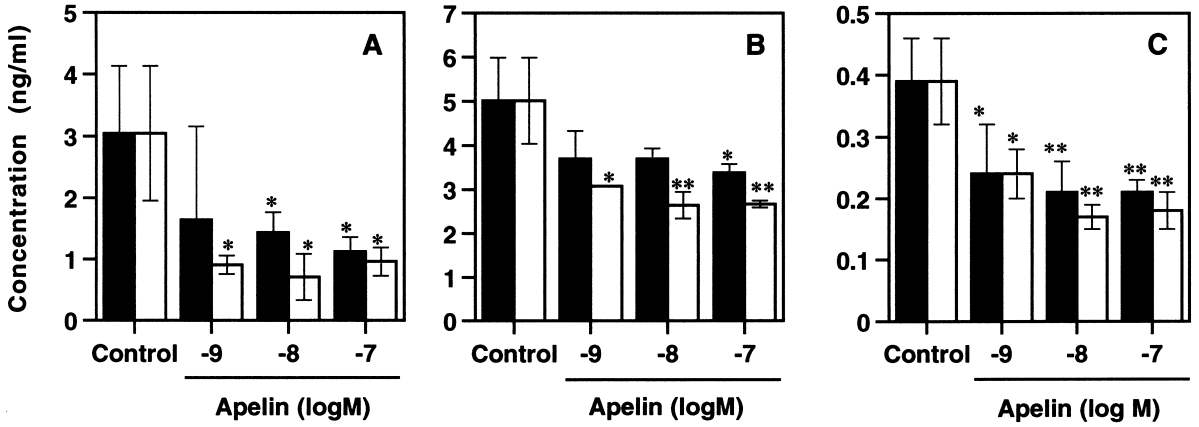

Fig. 6. Suppression of cytokine production in mouse spleen cells by apelin. Spleen cells obtained from BALB/c were cultured in microplates coated with anti-CD3 mAbs in the presence or absence (control) of apelin-36 (closed columns) and [pGlu]apelin-13 (open columns) at the indicated concentrations. The amounts of IFN-γ (panel A), IL-2 (panel B), and IL-4 (panel C) in culture supernatants were determined by EIA. Values represent mean ± S.D. in triplicate assays. $^{**}P < 0.01$; $^{*}P < 0.05$, as compared with a control in Student's *t*-test.

day 1 to 14 after parturition (data not shown). In addition, we detected apelin in commercially available bovine milk, at a concentration ranging from 0.6 to 1.2 pmol/ml in several different lots (data not shown).

We subsequently examined the change of apelin content in bovine milk during the lactating period (Fig. 5). For this time course study, we obtained colostrum and milk samples from three Holstein cows 0–11 days after parturition. Although the content of apelin in the colostrum and milk varied considerably among the cows examined, we detected higher levels of apelin in the colostrum than in the milk in all cows, and the apelin content drastically decreased within several days after parturition.

3.6. Modulation of cytokine productions by apelin

Since many components capable of modulating immune responses are secreted in milk [21], we examined whether or not apelin has such activities. In Fig. 6, as a representative result of several experiments, both apelin-36 and [pGlu]apelin-13 partially suppressed IFN-γ, IL-2, and IL-4 productions from BALB/c mouse spleen cells stimulated with immobilized anti-CD3 mAbs in an in vitro culture. However, [pGlu]apelin-13 was more potent in this activity than apelin-36, which seemed to parallel the results of the cAMP-production-inhibitory assay. We observed that the proliferation of mouse spleen cells in response to CD3 cross-linking was also partially suppressed by these peptides (data not shown). These results suggest that apelin plays a role in regulating immune responses.

4. Discussion

Based on the information of mouse EST in the database, we isolated apelin cDNA from rat and mouse brains. Bovine, human, rat, and mouse apelin cDNAs encoded preproproteins of the same length of 77 amino acids. When the amino acid sequences of apelin preproproteins were compared among these species, their amino acid identity ranged from 76% to 95%. The amino acid sequences of a mature apelin peptide (e.g., apelin-36) appeared to be more conserved among the species (i.e., 86–100% amino acid identity). In particular, their C-terminal regions from Trp-55 to Phe-77, a segment of 23 amino acid residues in length, were completely conserved among the species, suggesting that the C-terminal region is functionally important in apelin. Indeed, since apelin-13, [pGlu]apelin-13, and apelin-17 showed a stronger cAMP-production-inhibitory activity to CHO cells expressing human APJ than apelin-36, it is unequivocal that a core structure essential for the interaction between apelin and APJ is localized at the C-terminal region of apelin.

We synthesized [pGlu]apelin-13 as a pyroglutamin-

ated form of apelin-13, and the two peptides were equipotent in both the acidification-rate-promoting and cAMP-production-inhibitory assays. Since [pGlu]apelin-13 is structurally more stable than apelin-13, we mainly used [pGlu]apelin-13 in this study. In these two assays, differences among the potency of the apelin peptides appeared to be greater in the acidification-rate-promoting assay than in the cAMP-production-inhibitory assay [2]. The former assay principally detects proton excretion as a total output of cellular events. On the other hand, the latter detects the change of the intracellular second messenger, cAMP. As the extracellular proton excretion is an event downstream in a cascade of the intracellular changes, the amplified differences among the apelin peptides might be more readily detected in the acidification-rate-promoting assay. With respect to the signal transduction pathway of APJ, we could not detect Ca^{2+} mobilization or the release of arachidonic acid metabolites in CHO cells expressing human APJ after the addition of [pGlu]apelin-13 and apelin-36 (data not shown). These results suggest that APJ is coupled to Gi but not to Gs or Gq.

Among the rat tissues tested, the mammary gland of lactating rats showed the highest expression of apelin and its mRNA. Prior to pregnancy, the expression levels of apelin and its mRNA in female rats were not particularly high, but they gradually increased in parallel with the development of the mammary gland during pregnancy, and reached a maximum level around late pregnancy and parturition. Even after parturition, high levels of apelin and its mRNA were still detected in the mammary gland. We therefore expected that apelin would be secreted in milk. Indeed, apelin was detected in not only bovine and rat milk, but also human milk. In our preliminary experiments, about 0.2 pmol/ml of apelin was detected in human milk (data not shown). In the time course study using bovine milk, the highest level of apelin content was detected in the colostrum, and it rapidly decreased within several days after parturition. A certain level of apelin is likely to exist consistently in bovine milk during lactation, because we could detect apelin in commercially available bovine milk. Our results demonstrated that apelin produced in the mammary gland was actually secreted in the colostrum and milk.

Based on the following results of our previous study [2], we expected that endogenous apelin would have multiple isoforms: (1) the chromatographic behavior of apelin extracted from the bovine stomach was heterogeneous; (2) in the bovine apelin preproprotein sequence, downstream of the secretory signal sequence with 22 amino acid residues, there were many arginine and lysine residues (i.e., Lys-32, 61, and 72, and Arg-40, 46, 49, 59, 60, 63, 64, 66, and 68) which are potential proteolytic cleavage sites; (3) synthetic apelin peptides shorter than apelin-36 (e.g., apelin-17, apelin-13, and [pGlu]apelin-13) showed evident activities to interact with APJ. Since apelin was found to be abundantly secreted in milk, we analyzed the molecular heterogeneity of endogenous apelin in milk. By the purification of apelin from bovine milk, we found that at least three different apelin isoforms, starting at the N-terminal amino acid residues, Leu-42, Gly-47, and Ser-50, respectively, existed in the milk (data not shown). In the Western blot analyses using rabbit polyclonal antibody obtained by immunizing apelin-17 conjugated with thyroglobulin, we found that there were at least five apelin isoforms having different molecular weights in the bovine colostrum (data not shown). Although we have not been able to directly demonstrate the existence of apelin-13 as an endogenous ligand, we observed that apelin-13 was actually produced when apelin cDNA was expressed in CHO cells (data not shown). These observations appear to support the idea that endogenous apelin is very heterogeneous.

Milk is not only a rich source of nutrients, but it is also known to contain a variety of biologically active substances, including peptides (e.g., peptide fragments derived from the digestion of casein or albumin, gastrointestinal regulatory peptides, somatostatin, calcitonin, gonadotropin-releasing hormone, thyrotropin-releasing hormone), growth factors, enzymes, steroids, prostaglandins, and immunoglobulins [21–25]. A number of studies indicate that some of these substances are biologically active in neonates. We demonstrated here that apelin was quite abundantly secreted in colostrum and milk. When we examined the effects of apelin-36 and [pGlu]apelin-13 on cytokine production from mouse spleen cells in response to CD3 cross-linking in an in vitro culture, the productions of IFN-γ, IL-2, and IL-4 were partially suppressed by these peptides, sug-

gesting that apelin plays a role in regulating immune responses. The question of whether or not mononuclear cells express APJ remains controversial: one report has indicated that APJ mRNA could be induced in human peripheral blood mononuclear cells by stimulation with IL-2 and phytohemagglutinin [9]; another has reported that APJ mRNA was undetectable in these cells even after similar stimulation [12], although APJ transcripts were detected in a particular T cell line. We think that apelin would act on mouse spleen cells via APJ expressed in a certain subpopulation of T cells, although further studies will be needed to confirm this hypothesis. Transforming growth factor-β ordinarily suppresses immune responses including cytokine production, but it might act to enhance innate immunity if administered orally [26]. We therefore think that apelin secreted in milk might work to the advantage of neonates by helping them survive through the modulation of immune responses.

Apelin has unique characteristics, such as exogenous secretion and a diverse molecular heterogeneity. We believe that further studies on apelin will provide new insights into unknown physiological regulatory mechanisms and the vital function of the colostrum and milk.

Acknowledgements

We thank Drs. Yasuhiro Sumino, Osamu Nishimura, Yasuo Sugiyama, Kozo Shimakawa, Yoshihiro Ishibashi, and Hirokazu Matsumoto for their helpful advice and collaboration. We also thank Mr. Toshihiko Akabane and Drs. Ichiro Tatsuno and Tadao Saito for their kind supply of the bovine and human milk and instruction on the use of milk, respectively.

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