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Enzyme Immunoassay of Calcitonin Gene-Related Peptide-like Immunoreactive Substance in Human Plasma and Saliva

TOSHIAKI NAGANO*, KAZURO IKAWA and MASAHARU TAKEYAMA

Department of Clinical Pharmacy, Oita Medical University†

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A sensitive and specific double-antibody enzyme immunoassay (EIA) for a calcitonin gene-related peptide-like immunoreactive substance (CGRP-IS) was developed. In competitive reactions, the CGRP-antibody was incubated with CGRP standard (or sample) and β-D-galactosidase-labeled synthetic human αCGRP fragment [residue (8-37)](delayed addition). Free and antibody-bound enzyme haptens were separated by using an anti-rabbit IgG coated immunoplate. Activity of the enzyme on the plate was fluorometrically determined. The present immunoassay allows the detection of 2.1 to 19.5 fmol/mL of CGRP. Using the proposed EIA, CGRP-ISs in human saliva and plasma were determined.

The levels of CGRP-IS in human saliva were about 3.2 fmol/mL, which were much higher than that in human plasma.

Key words —— calcitonin gene-related peptide, enzyme immunoassay, calcitonin gene-related peptide(8-37)-β-D-galactosidase, fluorometric detection, human plasma, human saliva

Introduction

Calcitonin gene-related peptide (CGRP) is 37 amino acids peptide formed by modification of the primary RNA transcript of the rat calcitonin gene.[1] The transcription product is processed in distinct mRNAs encoding either calcitonin or CGRP, which significantly differ in their amino acid sequences. CGRP-like peptides, the distribution of which is extended in the central nervous system and peripheral nerves[2]. In rat[3] and man[4-7] two calcitonin genes have been isolated, and they encoded two different forms of CGRP, α and βCGRP. Such intraspecies variants have not yet been isolated from porcine, bovine, rabbit and chicken tissues. A second gene encoding a closely related peptide, βCGRP only differing in 3 amino acids has been described, and receptor for both α and β CGRP has been isolated from human placenta. The peptides has been detected immunochemically in human thyroid, pituitary and brain. CGRP-IS that has been isolated and characterized from human medullary thyroid carcinoma tissue possesses several potent biological activities, including inhibition of gastric acid secretion and food intake[8]. The peptides effect on both the endocrine and the exocrine pancreas and the vasodilation, being the most powerful vasoactive substance described

† 大分県大分郡挾間町医大ケ丘1-1 ; 1-1,Idaigaoka, Hasama-machi, Oita, 879-5593 Japan

to date.

An RIA of CGRP has been developed by several groups[9,10] using ^{125}I-human CGRP. However, in terms of safety, sensitivity and ease of handling, RIA methods are still less than satisfactory. In this report, we detailed a sensitive and specific enzyme immunoassay (EIA) for detecting CGRP using αCGRP (8-37)-linked β-D-galactosidase [CGRP(8-37)-β-gal] as a marker antigen, a secondary-antibody-coated immunoplate and 4-methylumbelliferyl β-D-galactopyranoside (MUG) as a fluorogenic substrate.

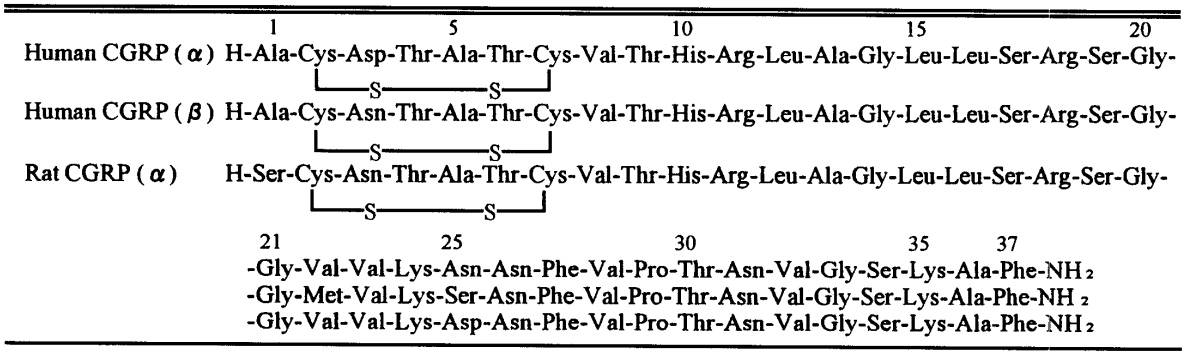

Fig. 1. Structures of Calcitonin Gene-related Peptides

Materials and Methods

Materials

Synthetic human αCGRP and its fragment (position 8-37), rat CGRP. sub stance P (SP), luteinizing hormone releasing hormone (LHRH), human calcitonin (hCT), gastrin 17 (Gas), cholecystokinin (CCK), β-endorphin (End) and somatostatin (SS) were purchased from Peptide Institute Inc. (Osaka, Japan). Other synthetic peptides [secretin (Sec), gastrin releasing peptide (GRP), vasoactive intestinal polypeptide (VIP) and peptide histidine isoleucine (PHI)]were supplied from Prof. H. Yajima (Kyoto University, Kyoto, Japan). Human βCGRP, bovine serum albumin (BSA), polyoxyethylene sorbitan monolaurate (Tween 20), *N*-(ε-maleimidocaproyloxy)succinimide(EMC-succinimide) and 4-methylumbelliferyl β-D-galactopyranoside (MUG) were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). β-D-Galactosidase (β-gal from *Escherichia coli*) and goat anti-rabbit lgG (0612-0081) were purchased from Boehringer Mannheim Corp. (Mannheim, Germany) and Cappel Laboratories (Malvern, PA, U.S.A.), respectively.

An antiserum against human αCGRP (CA 1132) was purchased from Affiniti Research Products Ltd. (Nottingham, UK) and was reconstituted to 150 mL with an assay buffer (0.05 mol/L phosphate buffer, pH 7.0, containing 0.5 g/dL BSA, 1 mmol/L $MgCl_2$ and 250 kallikrein inhibitor units /mL aprotinin). All other chemicals were of analytical reagent grades.

Preparation of Plasma and Saliva Extracts

Human plasma and saliva samples were obtained from five healthy male volunteers. Blood and saliva samples were collected in a chilled tube containing 500 kallikrein inhibitor units/mL aprotinin and 1.2 mg/mL EDTA. Afier centrifugation (1670 g, 4℃, 20 min), plasma (1 mL) and saliva (1 mL) samples were diluted fivefold by 4 vol% acetic acid (AcOH), and loaded on reversed-

phase C_{18} cartridges (Sep-Pak C_{18}, Millipore Corp., Milford, MA, U.S.A.). After washing with 4 vol % AcOH (10 mL), the CGRPs were eluted with 70 vol% acetonitrile (MeCN) in 0.5 vol% AcOH, pH 4.0 (2 mL). Eluates were concentrated by spin-vacuum evaporation, lyophilized, reconstituted to 100 μL with the assay buffer and subjected to EIA. The recovery of αCGRP from plasma and saliva with this extracting procedure was 91±2 and 94±5% (n=6).

Preparation of Enzyme-labeled Antigen

αCGRP fragment(8-37) was conjugated with β-gal by EMC-succinimide according to the method of Kitagawa *et al.*[11] CGRP(8-37) (0.40 mg) dissolved in 0.05 mol/L phosphate buffer pH 7.0 (0.80 mL) was mixed with EMC-succinimide (0.20 mg) in tetrahydrofuran (70 μL) at 20℃ for 60 min. The EMC-CGRP(8-37) thus obtained was purified *via* separation through a Sephadex G-25 column (1.5×50 cm) pre-equilibrated with 0.05 mol/L phosphate buffer, pH 7.0, to elute the column. Individual fractions (1.8 mL each) that showed absorbance at 260 nm were collected. The purified EMC-CGRP(8-37) fractions were combined with β-gal (7.6 mg) by mixing them at 20℃ for 60 min. The β-gal conjugate was then applied to a Sephacryl S-300 column (1.5×52 cm) and eluted with 0.05 M phosphate buffer pH 7.0, containing 1 mmol/L $MgCl_2$. Individual fractions (1.8 mL each) that showed absorbance at 260 nm were collected. The fractions containing β-gal activity were collected and stored at 4℃ after addition of 0.2 g/dL BSA and 0.1 g/dL NaN_3.

Assay Procedure for CGRP

The assay buffer mentioned above was used for plasma and saliva samples assay. Secondary-antibody-coated immunoplates were prepared as previously reported using Microwell MaxiSorp F 8 plates (Nunc, Roskilde, Denmark) and anti-rabbit IgG[12]. A test tube containing 100 μL of CGRP-antiserum, CA1132 and 100 μL of sample (or standard) were mixed and incubated at 4℃ for 24 h. And then, diluted enzyme-labeled antigen [CGRP(8-37)-β-gal](50 μL) was added, and the test tube was incubated at 4℃ for additional 24 h. One hundred microliters of the antigen-antibody solution for each sample was added to the secondary antibody-coated immunoplate. The plate was incubated at 4℃ for overnight, washed 4 times with a buffer (0.01 mol/L phosphate buffer, pH 7.0, containing 0.15 mol/L NaCl and 0.05 vol% Tween 20), and then 200 μL of 0.1 mmol/L MUG in a substrate buffer (0.05 mol/L phosphate buffer, pH 7.0, containing 1 mmol/L $MgCl_2$) was added to each well. The plate was again incubated at 37℃ for 3 h. The fluorescence intensity (λ_{EX}360 nm, λ_{EX} 450 nm) of each well was measured with an MTP-100 F microplate reader (Corona Electric, Ibaraki, Japan).

HPLC of Plasma and Saliva Extracts

HPLC was performed using a reversed-phase C18 column (Cosmosil 5 C18, Nacalai Tesque, Kyoto, Japan). The HPLC consisted of a model 610 dual pump system (Millipore Corp., Milford, MA, U.S.A.). The plasma and saliva samples (1 mL), purified by the Sep-Pak C_{18} cartridge described above, were reconstituted to 100 μL with 0.1 vol% trifluoroacetic acid (TFA) and passed through the column. CGRP-ISs were eluted with a linear gradient of MeCN (from 5 vol% to 50 vol% over 45 min) in 0.1 vol% TFA. The flow rate was 1 mL/min and the fraction size was 1 mL. Eluted fractions were then concentrated by spin-vacuum evaporation, lyophilized, and reconstituted to 100 μL with an assay buffer prior to undergoing EIA.

Results

Standard Curve

Typical calibration curves for the CGRP-EIA are shown (**Fig. 2**). When plotted as a semilogarithmic function, a linear displacement of enzyme-linked αCGRP(8-37) by synthetic αCGRP was noted, between 2.1 and 176 fmol/mL. The minimum amount of αCGRP detectable by this EIA system was 2.1 fmol/mL (0.084 fmol/well), approximately the same sensitivity as obtained using the RIA methods. The affinity of human βCGRP about CA 1132 was higher than that of human α CGRP between 20 and 105 fmol/mL. But more than 105 fmol/mL, the affinity of human αCGRP was much higher than that of human βCGRP. The affinity of CA 1132 to rat CGRP was as well as that of human αCGRP from 2.1 fmol/mL to 20 fmol/mL. More than 20 fmol/mL, the affinity of the antiserum to rat CGRP was lower than that of human αCGRP.

Specificity of the Antiserum, CA 1132

Immunospecificity of the antiserum (CA 1132) was examined by EIA using CGRP-β-gal. The displacement curves of CGRP and other endogenous peptides are shown (Fig.2). hCT, SP, SS, hGRP, Sec, PHI, LHRH, End, Gas, VIP and CCK minimally inhibited the binding of CGRP(8-37)-β-gal with the CGRP-antibody. Thus, the CGRP-antiserum, CA 1132, recognizes CGRP and can distinguish CGRP from other endogenous peptides.

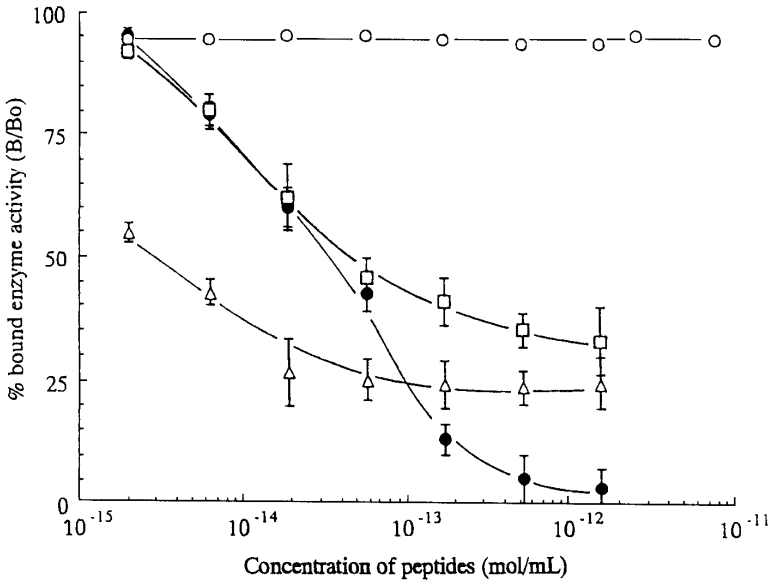

Fig. 2. Competitive Inhibition of CGRP and Various Peptides in the EIA
Each vertical bar represents the standard deviation ($n=6$).
● : human α CGRP, △ : human β CGRP, □ : rat CGRP, ○ : hCT
Inhibition patterns of SP, SS, hGRP, Sec, PHI, LHRH, End, Gas, VIP, hCT and CCK are essentially the same as that of hCT.

Measurements of CGRP-Immuoreactive Substances (CGRP-ISs) in Human Plasma and Saliva by EIA

The proposed EIA was applied to the determination of CGRP-ISs in human plasma and saliva samples from five male volunteers. Concentrations of CGRP-ISs in human plasma and saliva were 0.9±0.7 fmol/mL and 3.2±1.9 fmol/mL, respectively (**Table 1**) .

HPLC of Human Plasma and Saliva Extracts

Human plasma and saliva extracts were subjected to reversed phase HPLC in order to study the

Table 1. The Levels of CGRP-ISs in Human Plasma and Saliva

Subject	Sex	Age	Plasma (fmol/mL)	Saliva (fmol/mL)
1	M	26	1.2	1.2
2	M	48	0.3	4.2
3	M	29	0.4	1.2
4	M	40	1.9	4.2
5	M	35	0.6	5.4
Mean±S.D.			0.9±0.7	3.2±1.9

M ; Male

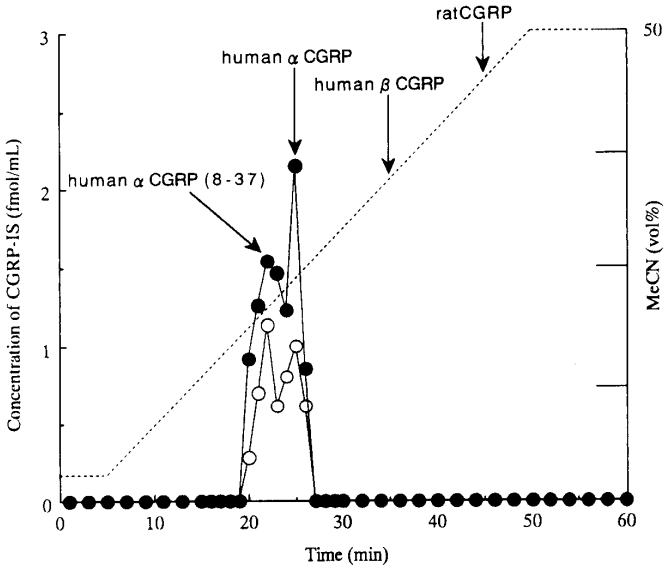

Fig. 3. Elution Profiles of Plasma and Saliva Extracts by HPLC on a C 18 Column

CGRP-ISs extracted from plasma (1 mL) and saliva (1 mL) samples by Sep-Pak C_{18} were dissolved in 10vol% MeCN in 0.1vol% TFA and injected onto HPLC. The MeCN gradient is indicated by the dotted line. Fractions were lyophilized and their content of CGRP-IS was measured. Synthetic human α CGRP, CGRP(8–37), human β CGRP and rat CGRP were run in a separate chromatography under the same condition and the fractions containing this compound is indicated by the arrow. Column, Cosmosil 5 C 18 AR (4.6 ×150 mm) ; flow rate, 1 mL/min ; fraction volume, 1 mL.

● : Saliva, ○ : Plasma

molecular variants of CGRP-ISs present in human plasma and saliva. Extracts from human plasma and saliva by a Sep-Pak C_{18} cartridge were applied to reversed phase HPLC on a C18 column, and the elution profiles revealed the presence of two immunoreactive peaks (**Fig. 3**). One was eluted at the position corresponding to that of standard synthetic αCGRP and the other eluted earlier at a position corresponding to that of standard αCGRP (8-37). The immunoreactive peaks of human β CGRP (35 min) and rat αCGRP (45 min) were not detected in plasma and saliva.

Discussion

Using β-gal-labeled α-CGRP(8-37) as a marker antigen, an anti-rabbit IgG coated immunoplate as a bound/free (B/F) separator and MUG as a fluorogenic substrate, we developed a highly sensitive and specific EIA for the quantitation of CGRP. Since 1985, RIA methods developed for CGRP have been used widely, however these methods have several disadvantages due to the use of radioisotopes. The EIA detailed in this report retains the advantages of the RIA systems while minimizing the disadvantages. This EIA is highly sensitive (20 fmol/mL) and specific for αCGRP. The calibration curve was linear in the range of 2.1 to 176 fmol/mL. The antibody of CGRP, CA 1132 was found to have some crossreactivity to human βCGRP, but βCGRP was not detected in human plasma and saliva confirmed by HPLC methods. Thus it is possible for detecting αCGRP in human plasma and saliva.

The levels of αCGRP-IS in plasma from five healthy volunteers was 0.9±0.7 fmol/mL. The levels of αCGRP-IS in saliva was 3.2±1.9 fmol/mL, which was more than threetimes higher than those in plasma. The CGRP-neuron is present in sialaden.[13] Thus topically released αCGRP was diluted by saliva and the level of αCGRP-IS in saliva was higher than that in plasma.

The molecular heterogeneity in human plasma and saliva was also examined by HPLC. The main αCGRP-IS in plasma and saliva were eluted at the same elution volumes of synthetic α CGRP with a minor peak at the same elution volumes of αCGRP(8-37). No other αCGRP-IS in plasma and saliva were detected in HPLC elution fractions by present EIA.

This simple and sensitive EIA will be useful for measurement of human αCGRP in biological fluids and tissues.

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