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Improved determination of bovine glutaminyl cyclase activity using precolumn derivatization and reversed-phase high-performance liquid chromatography with ultraviolet detection

Toshiyuki Chikuma [a,*], Kyoji Taguchi [b], Mitsune Yamaguchi [a], Hiroshi Hojo [a], Takeshi Kato [c]

[a] *Department of Hygienic Chemistry, Showa Pharmaceutical University, 3-3165 Higashi-tamagawagakuen, Machida-shi, Tokyo 194-8543, Japan*
[b] *Department of Neuroscience, Showa Pharmaceutical University, Machida-shi, Tokyo 194-8543, Japan*
[c] *Laboratory of Molecular Recognition, Graduate School of Integrated Science, Yokohama City University, Yokohama 236-0027, Japan*

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Abstract

A sensitive, rapid and reproducible assay for the determination of glutaminyl cyclase activity is reported. This method is based on the monitoring of the absorption of L-pyroglutamic acid β-naphthylamide at 235 nm, enzymatically formed from the substrate L-glutaminyl-β-naphthylamide, after separation by high-performance liquid chromatography using a C-18 reversed-phase column by isocratic elution. The detection limit of this method is at a level as low as 0.08 nmol/ml and, the time consumed for analysis is <6.5 min per sample for separation and quantification. The optimum pH for glutaminyl cyclase activity was 8.0–8.5. The K_m and V_{max} values were 100.2 ± 2.9 μM and 332 ± 21.7 pmol/(h μg protein), respectively, with the use of enzyme extract obtained from bovine pituitary. Glutaminyl cyclase activity was strongly inhibited by zinc(II) ion and 1,10-phenanthroline. By using this assay, the stimulatory effect of bacterial lipopolysaccharide on this enzyme activity was observed in macrophage cell line RAW 264.7. Our newly developed assay would be useful for clarification of the physiological role of this enzyme.

Keywords: Glutaminyl cyclase; Enzymes

1. Introduction

Several bioactive peptides such as thyrotropin releasing hormone (TRH), luteinizing hormone releasing hormone (LH-RH), and neurotensin contain a pyroglutamyl residue at their N-terminal positions. This posttranslational modification results from cyclization of the glutaminyl residue, which is unmasked by proteolytic processing of precursor proteins by prohormone convertases [1,2]. Although the conversion from glutaminyl peptides to pyroglutamyl peptides can occur under non-enzymatic conditions, especially under the catalytic influence of phosphate ions [3], previous studies have demonstrated that the formation of pyroglutamyl residues in neuroendocrine tissues must be enzymatically catalyzed because the non-enzymatic reaction is quite slow under physiological conditions [4,5].

The enzyme responsible for the cyclization of glutaminyl peptides to pyroglutamyl peptides, glutaminyl cyclase (EC 2.3.2.5), has been identified in a number of animals [4,6] and plants [7–9]. The human cDNA for glutaminyl cyclase has been cloned and expressed in a number of bacterial expression systems [10,11].

Little is known about the biological role of glutaminyl cyclase at present. Researchers have suggested that glutaminyl cyclase is responsible for modification of storage proteins during seed germination [12], as well as in vivo modification of bioactive peptides [13–15]. These findings support the idea that the glutaminyl cyclase catalyzed reaction might be important for protection of the N-terminus of bioactive peptides against exopeptidases. Moreover, this enzymatically catalyzed N-terminal formation of the pyroglutamic acid residue could be important in developing the proper receptor binding conformation of such peptides.

* Corresponding author. Tel.: +81-42-721-1564; fax: +81-42-721-1563.
E-mail address: chikuma@ac.shoyaku.ac.jp (T. Chikuma).

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Assays currently used for detecting and estimating glutaminyl cyclase activity are based on the ability of the enzyme to convert the synthetic peptide to the corresponding pyroglutamyl peptide product. The degree of conversion is determined by radioimmunoassay using Gln-His-Pro-NH_2 as substrate [4]. The progress of the reaction also has been monitored by measurement of the product by reversed-phase high-performance liquid chromatography (HPLC) with spectrophotometric and fluorimetric detection using [Gln^1] LH-RH and Gln-Leu-Tyr-Glu-Asn-Lys-ε-(Dns)-OH, respectively [5,6]. Commonly used assays of this enzyme activity also include a spectrophotometric method to measure the ammonia released during cyclization of the dipeptide substrate Gln–Gln [16] and a continuous spectrometric assay using the chromogenic substrate Gln-*p*-nitroanilide, and fluorogenic substrates Gln-2-naphthylamide and Gln-4-methylcoumarinylamide [17].

In this paper, we describe a new and sensitive assay for glutaminyl cyclase activity, using L-glutaminyl-β-naphthylamide (Gln-βNA) as substrate, by HPLC on a reversed-phase column to achieve a rapid and selective separation of the substrate and the product formed. This system is suitable for routine analysis of glutaminyl cyclase activity. Using this assay, glutaminyl cyclase activity was for the first time discovered in the macrophage cell line RAW 264.7.

2. Experimental

2.1. Chemicals

Soybean trypsin inhibitor, bacitracin, ascorbic acid, and 1,10-phenanthroline monohydrate were purchased from Wako (Tokyo, Japan). L-Pyroglutamic acid β-naphthylamide (Pyr-βNA) and Gln-βNA were obtained from Bachem AG (Bubendorf, Switzerland). *N*-Ethylmaleimide (NEM), phenylmethylsulfonyl fluoride (PMSF), pepstatin A, diisopropylfluorophosphate (DFP), iodoacetic acid (IAA), reduced glutathione, β-naphthylamine, and *N*-2,4-dinitrophenyl-L-phenylalanine (DNP-Phe) were purchased from Sigma (St. Louis, MO, USA). Tetra-*n*-propylammonium bromide was obtained from Tokyo Kasei (Tokyo, Japan). Lipopolysaccharide (LPS) from *Escherichia coli* (serotype 055:B5) was purchased from BD Biosciences (Franklin Lakes, NJ, USA). Methanol was of chromatographic grade (Wako). Other chemicals and solvents were of analytical reagent grade.

2.2. Preparation of enzyme source

Bovine pituitaries were obtained from a local slaughterhouse. All subsequent operations were carried out at 0–4 °C unless stated otherwise. After washing the bovine pituitaries with saline, it was cut into small pieces and homogenized in nine volumes of 0.32 M sucrose with a glass–PTFE homogenizer. The homogenate was centrifuged at 100,000 × *g* for 80 min and the supernatant was used as an enzyme source.

2.3. Assay of glutaminyl cyclase activity

The assay of glutaminyl cyclase activity is based on spectrophotometric measurement at 235 nm of Pyr-βNA formed enzymatically from the substrate, Gln-βNA, after separation by HPLC. The reaction mixture contained 50 mM Tris–HCl buffer (pH 8.0), 40 μM Gln-βNA, 0.1 mM NEM, and enzyme plus water in a total reaction volume of 250 μl. Incubation was carried out at 37 °C, and the reaction was terminated by heating at 95 °C for 5 min in boiling water. After centrifugation, DNP-Phe was added to the clear supernatant as the internal standard, and an aliquot of the mixture was subjected to HPLC analysis. Net peak height of Pyr-βNA formed enzymatically from the substrate (experimental incubation) was obtained by subtracting the peak height of Pyr-βNA formed non-enzymatically (control incubation) and comparing the value with the peak height of the DNP-Phe added as an internal standard. One unit of enzyme activity was defined as the amount of enzyme necessary for conversion of 1 pmol of the substrate into the corresponding product in 1 min at 37 °C.

2.4. Chromatographic conditions

Analysis of the product was conducted using a Japan Spectroscopic HPLC system consisting of an 880-PU pump, 875-UV variable-wavelength detector, 860-CO column oven, 880-50 degasser, 880-02 gradient unit, 802-SC system controller, and 807-IT integrator. The system was operated at room temperature with a flow-rate of 0.8 ml/min employing a Finepak SIL Cl8S (particle size: 5 μm) reversed-phase column (150 mm × 4.6 mm i.d.) (Jasco) fitted with a TSK guard gel ODS-80TM (15 mm × 3.2 mm i.d.; particle size: 5 μm) (TOSOH). The mobile phase consisted of 10 mM sodium acetate buffer containing 0.1% tetra-*n*-propylammonium bromide (pH 4.0)–methanol (34:66 (v/v)). The analytes were detected at 235 nm.

2.5. Effects of various metal ions and inhibitors on enzymatic activity

Enzyme solution was preincubated in the absence or presence of various chemicals (0.1 and 1 mM) in 50 mM Tris–HCl buffer (pH 8.0) for 15 min at 25 °C. Immediately thereafter, 40 μM Gln-βNA (final concentration) was added to the reaction mixture and incubated at 37 °C for 2 h. After stopping the reaction, the samples were treated as described above.

2.6. Cell culture

The murine macrophage cell line RAW 264.7 (ATCC TIB 71; American Type Culture Collection, Rockville, MD) was cultured in Dulbecco's modified Eagle's medium (DMEM)

(Nissui, Tokyo, Japan) containing 10% heat-inactivated fetal bovine serum (FBS) (JRH Biosciences, Lenexa, KS, USA), 2 mM glutamine, 100 IU/ml penicillin, and 100 μg/ml streptomycin (Gibco BRL, Rockville, MD, USA) at 37 °C in an atmosphere of 5% CO_2 and 95% air, and then subcultured every 3 days before the experiments were performed. For experiments, cells were plated in 6 cm diameter culture dishes at a density of 5×10^5 cells/dish and then cultured for 3 days until they were nearly confluent. The medium was then replaced with fresh medium, and cells were activated with or without various concentrations of LPS followed by further culturing for the indicated periods. Thereafter, the cells were scraped from the dishes and homogenized in 20 mM calcium(II) ion, magnesium(II) ion—free phosphate-buffered saline (pH 7.2). The homogenates were used for the measurement of glutaminyl cyclase activity.

2.7. Protein determination

Protein concentration was measured by the modified Lowry method [18] using BSA as a standard protein.

2.8. Validation parameters

Validation was performed according to the current recommendations for analytical method validation, that require a standard curve with five–eight points with reproducible linear or non-linear responses and statistical fits [19]. The limit of detection (LOD), defined as the lowest concentration of the analyte which can be detected with a signal-to-noise ratio >3:1, was established by serial injection of reaction mixture spiked with different concentrations of Pyr-βNA. The limit of quantitation (LOQ) was defined as the lowest concentration of the analyte at which percent deviation from the nominal concentration (accuracy) and relative standard deviation (R.S.D.) (precision) are <15%, were determined from the linearity tests. Recovery was assessed by spiking known amounts of Pyr-βNA into the reaction mixture. The intra- and inter-assay precision and accuracy were evaluated to ensure reproducibility before actual assay. The intra-assay precision was assessed by analyzing six samples at six concentrations (0.2, 0.4, 2.0, 4.0, 20.0 and 40.0 nmol/ml) for 1 day. The inter-assay precision was assessed by using the same method on six different days. The intra- and inter-assay precision of non-enzymatically formed Pyr-βNA were also assessed by analyzing six samples for each of the periods described above.

3. Results

As pointed out in other HPLC-based enzymatic assays [20–22], the direct analysis of enzyme action, once separated from the substrate and other interfering substances, offers clear results. This HPLC–spectrophotometric detection system for the measurement of Gln-βNA and Pyr-βNA was sensitive. The calibration curve for Pyr-βNA showed good linearity within 60 nmol/ml. Slope and intercept, the standard deviations of the slope and the intercept, and the coefficients of determination were as follows: $y = (55.8987 \pm 0.2481)x + (0.9040 \pm 0.0752)$, $r^2 = 0.9991$. The slope and *y*-intercept values introduced in the equation represent the mean of five different curves in the same day.

The LOD and the LOQ were found to be 0.08 and 0.12 nmol/ml, respectively, for Pyr-βNA in our analytical system. The recovery and precision data for Pyr-βNA spiked into the reaction mixture are shown in Table 1. The recoveries (mean ± S.D., $n = 6$) at six different concentrations were close to 100%. The intra- and inter-assay precision expressed as R.S.D. values were <3.5%. The intra- and inter-assay precision of non-enzymatically formed Pyr-βNA were 2.0 and 1.6%, respectively, as R.S.D. The statistical validation indicates that the assay is satisfactory in practical use.

Fig. 1 shows the chromatograms of the reaction mixture after 2 h incubation with 9.36 μg of protein prepared from bovine pituitary supernatant. The blank incubation (Fig. 1A) contained Gln-βNA and DNP-Phe, while the standard incubation contained exogenous Pyr-βNA in addition to Gln-βNA and DNP-Phe (Fig. 1B). The retention times for Gln-βNA, Pyr-βNA, and DNP-Phe were 3.6, 4.6, and 6.0 min, respectively (Fig. 1A and B). As shown in the blank incubation (Fig. 1A), a moderate peak of Pyr-βNA, formed non-enzymatically from the substrate during incubation, was found at 4.6 min. Experimental incubation under the standard assay conditions (Fig. 1C) produced a significant amount of Pyr-βNA in 4.6 min, whereas the control

Table 1
Statistical validation for the determination of Pyr-βNA in reaction mixture

Theoretical concentration (nmol/ml)	lntraassay ($n = 6$) measured concentration mean ± S.D. (nmol/ml)	R.S.D.[a] (%)	Recovery (%)	lnterassay ($n = 6$) measured concentration mean ± S.D. (nmol/ml)	R.S.D.[a] (%)	Recovery (%)
0.2	0.200 ± 0.006	3.0	100.0	0.200 ± 0.007	3.5	100.0
0.4	0.404 ± 0.010	2.5	101.0	0.411 ± 0.009	2.2	102.8
2.0	2.082 ± 0.039	1.9	104.1	2.077 ± 0.071	3.4	103.9
4.0	4.026 ± 0.105	2.6	100.7	4.028 ± 0.066	1.6	100.7
20.0	21.931 ± 0.374	1.7	109.7	21.589 ± 0.497	2.3	107.9
40.0	43.762 ± 1.266	2.9	109.4	43.687 ± 0.521	1.2	109.2

[a] R.S.D., relative standard deviation.

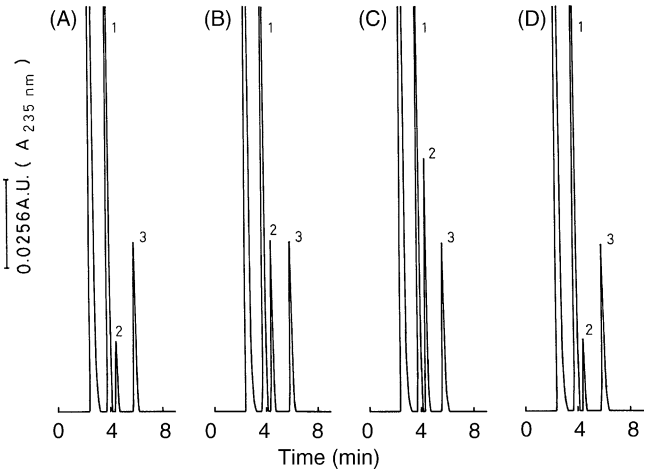

Fig. 1. HPLC elution patterns of glutaminyl cyclase activity determined using the enzyme in bovine pituitary. Conditions are described in the Section 2. Peaks: 1, Gln-βNA; 2, Pyr-βNA; 3, DNP-Phe. A 10 nmol amount of DNP-Phe (internal standard) was added to each sample after incubation. (A) Blank incubation: Gln-βNA was incubated without enzyme at 37 °C for 2 h. (B) Standard incubation: 1000 pmol of Pyr-βNA was added to the sample before incubation as a standard sample. The peak heights of Pyr-βNA and DNP-Phe correspond to 100 and 1000 pmol, respectively. (C) Experimental incubation: Gln-βNA was incubated with 9.36 μg of protein in bovine pituitary extract at 37 °C for 2 h. (D) Control incubation: a control tube without the enzyme was incubated, the same amount of active enzyme was added, and the resulting tube was kept in an ice bath before heating at 95 °C for 5 min.

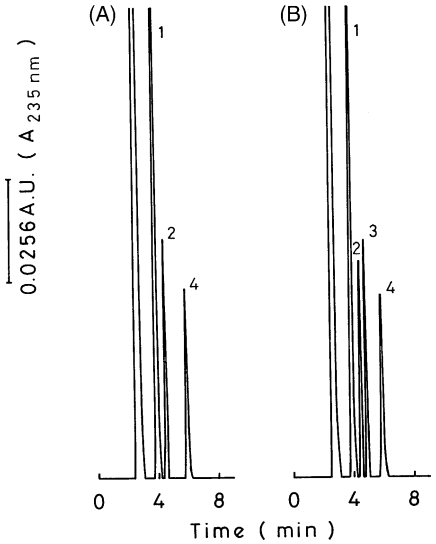

Fig. 2. HPLC elution patterns of glutaminyl cyclase activity using the enzyme in bovine pituitary. Incubation was carried out at 37 °C for 2 h with 6.69 μg of protein under the standard assay conditions (A) or without 0.1 mM NEM. Peaks: 1, Gln-βNA; 2, Pyr-βNA; 3, β-naphthylamine; 4, DNP-Phe.

incubation produced a moderate amount of Pyr-βNA (Fig. 1D). This Pyr-βNA peak also was produced non-enzymatically during incubation. In contrast, the experimental incubation under the standard assay conditions without 0.1 mM NEM produced a significant amount of β-naphthylamine in 5.0 min (Fig. 2).

The enzymatic reaction was linear for at least 4 h at 37 °C (data not shown).

The pH dependence of enzyme activity was investigated in both 50 mM Tris–HCl buffer (pH 7.5–9.0) and 50 mM EPPS–NaOH buffer (pH 7.5–8.5). Catalytic activity of the enzyme was greatest at a pH range of approximately 8.0–8.5, with little activity below pH 7.5 or above pH 9.0 (Fig. 3).

Glutaminyl cyclase activity was investigated as a function of enzyme amount. Good linearity was observed for plots of the amount of Pyr-βNA from 0.58 to 27.28 U formed enzymatically from Gln-βNA against that of the enzyme (data not shown).

Ascorbate and reduced glutathione were examined for their abilities to stimulate glutaminyl cyclase activity. The two cofactors tested had no stimulating effect on glutaminyl cyclase activity (data not shown).

A Lineweaver–Burk plot was obtained from the effect of Gln-βNA concentration on the rate of formation of Pyr-βNA by glutaminyl cyclase. The Michaelis constant (K_m) and the maximum velocity (V_{max}) toward Gln-βNA were calculated at 100.2 ± 2.9 μM and 332 ± 21.7 pmol/(h μg protein), respectively.

Ionic strength was tested as another important parameter in establishing the new glutaminyl cyclase assay in enzyme characterization. As shown in Fig. 4, activity of glutaminyl cyclase increased steadily up to 200 mM KCl and was almost constant up to 500 mM. The overall activation by increasing ionic strength was approximately 40% under the standard assay conditions without 0.1 mM NEM. In contrast, the effect of ionic strength on the conversion rate of Gln-βNA to Pyr-βNA was not found under the standard assay conditions.

We applied this standard assay to the determination of the effects of various metal ions and protease inhibitors on

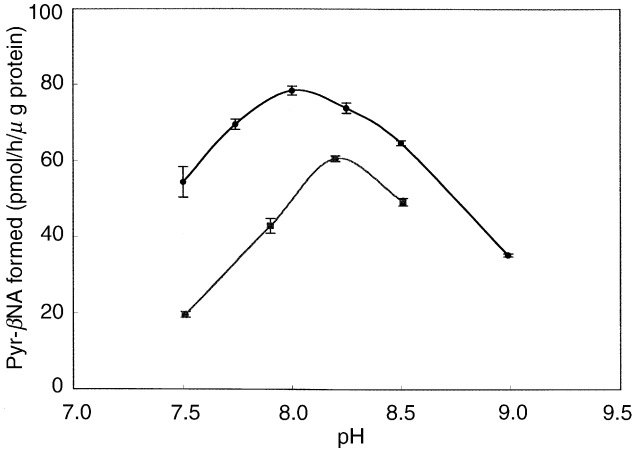

Fig. 3. Effects of pH on glutaminyl cyclase activity in bovine pituitary: (●) 50 mM Tris–HCl buffer (pH 7.5–9.0) and (■) 50 mM EPPS-NaOH buffer (pH 7.5–8.5) were used. Incubation was carried out at 37 °C for 2 h. Data are mean ± S.E.M.; (bars) values determined in three separate experiments.

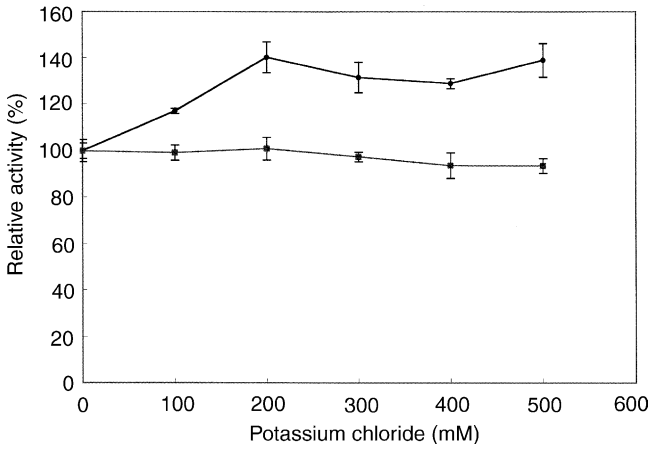

Fig. 4. Dependence of glutaminyl cyclase activity on ionic strength. Incubations were performed in 50 mM Tris–HCl buffer (pH 8.0) at different concentrations of potassium chloride between 0 and 500 mM under: (■) the standard assay conditions or (●) without 0.1 mM NEM. This corresponds to an ionic strength of 0.05–0.55 M. Data are mean ± S.E.M.; (bars) values determined in four separate experiments.

glutaminyl cyclase activity in bovine pituitary. As shown in Table 2, some metals tested inhibited the enzyme activity. Manganese(II) ion showed weak inhibition. In particular, zinc(II) ion inhibited enzyme activity completely at 0.1 mM. Furthermore, 1,10-phenanthroline (metal chelating agent) also inhibited it completely at a concentration of 1 mM. However, EDTA and dithiothreitol showed weak inhibition. Glutaminyl cyclase activity was not affected by bacitracin, soybean trypsin inhibitor, or pepstatin A. The enzyme activity was enhanced by thiol protease inhibitors such as IAA and NEM.

Finally, we investigated the effects of LSP concentration on glutaminyl cyclase activity in the macrophage cell line RAW 264.7. After 24 h incubation, the LPS produced a concentration-dependent increase of glutaminyl cyclase activity. Enzyme activity reached maximum at a LPS concentration of 0.1 μg/ml, which was about 2.4-fold over the control level, and remained constant up to 10 μg/ml of LPS (Fig. 5).

Table 2
Effects of metal ions and inhibitors on activity of glutaminyl cyclase from bovine pituitary[a]

Reagent	Final concentration (mM)	Glutaminyl cyclase activity (% of control)
None	–	100
$MgCl_2$	1	108.9 ± 0.2
$MgCl_2$	0.1	101.7 ± 0.3
$CaCl_2$	1	11 3.3 ± 0.8
$CaCl_2$	0.1	101.7 ± 0.9
$CuSO_4$	1	98.9 ± 0.2
$CuSO_4$	0.1	108.5 ± 2.9
$BaCl_2$	1	101.3 ± 0.8
$BaCl_2$	0.1	108.8 ± 0.4
$MnCl_2$	1	50.7 ± 1.6
$MnCl_2$	0.1	75.2 ± 2.5
$ZnCl_2$	1	0
$ZnCl_2$	0.1	0
$FeCl_3$	1	79.6 ± 1.9
$FeCl_3$	0.1	100.0 ± 0.6
$HgCl_2$	1	94.1 ± 0.1
$HgCl_2$	0.1	98.9 ± 1.7
EDTA	1	70.9 ± 1.4
EDTA	0.1	53.6 ± 1.6
1,10-Phenanthroline	1	0
1,10-Phenanthroline	0.1	14.8 ± 2.6
Diisopropylfluorophosphate	1	92.9 ± 2.5
Diisopropylfluorophosphate	0.1	92.1 ± 2.3
Phenylmethylsulfonyl fluoride	1	103.7 ± 0.4
Phenylmethylsulfonyl fluoride	0.1	101.5 ± 2.4
Iodoacetic acid	1	113.9 ± 1.3
Iodoacetic acid	0.1	106.5 ± 0.7
N-Ethylmaleimide	1	138.6 ± 2.5
N-Ethylmaleimide	0.1	131.8 ± 2.5
Dithiothreitol	1	29.7 ± 2.2
Dithiothreitol	0.1	59.5 ± 3.1
Bacitracin	20 μg/ml	102.3 ± 1.5
Soybean trypsin inhibitor	20 μg/ml	106.7 ± 0.1
Pepstatin A	20 μg/ml	106.0 ± 2.1

[a] *Note*: data are mean ± S.E.M. values determined in three separate experiments.

4. Discussion

The first assay used for the detection of glutaminyl cyclase activity was developed by Messer and Ottesen [8], who utilized Gln-Asn as a substrate. This assay has been modified by other workers [9,16].

We report a new assay for glutaminyl cyclase activity using an HPLC-spectrophotometric detection system with Gln-βNA as the substrate. The proposed assay offers some advantages compared to previously reported assays employing HPLC. First, all reagents, including the peptide substrate, are commercially available. Second, the substrate and product are separated clearly in <6.5 min. Third, more accurate quantitation of the product and better reproducibility were guaranteed by our method using an internal standard (DNP-Phe), compared to the results by HPLC without an internal standard [5,6]. Another HPLC–fluorimetric assay using Gln-Leu-Tyr-Glu-Asn-Lys-ε-(Dns)-OH as substrate also possesses some advantages such as high sensitivity and rapidity [5].

Moreover, we showed some physicochemical properties of glutaminyl cyclase in bovine pituitary. The optimum pH of glutaminyl cyclase from bovine pituitary is 8.0–8.5. The effects of various chemical reagents and protease inhibitors on glutaminyl cyclase activity are also investigated (Table 2).

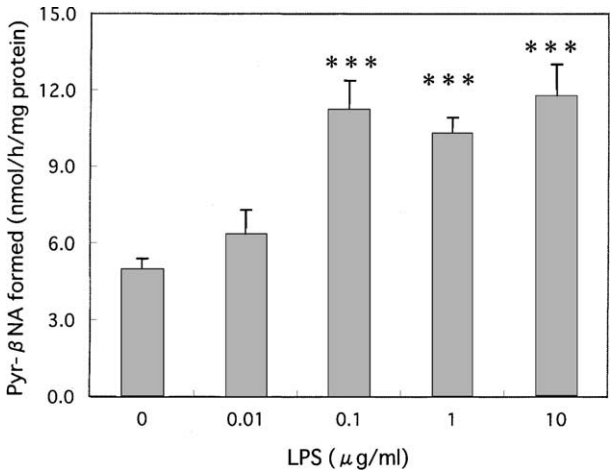

Fig. 5. Effect of LPS on glutaminyl cyclase activity in the macrophage cell line RAW 264.7. Samples of 5×10^5 cells were cultured in 6 cm plastic dishes for 3 days and then transferred to fresh medium containing LPS, and further cultured for 24 h. Data are mean ± S.E.M.; (bars) values from five to six dishes. Analysis was performed by two-tailed paired student's *t*-test: (***) $P < 0.001$ compared to the control.

The glutaminyl cyclase activity in bovine pituitary is completely inhibited by zinc(II) ion and 1,10-phenanthroline. However, there was weak inhibition by another metal chelating agent (EDTA). Busby et al. [4] reported that the activity of glutaminyl cyclase purified from porcine pituitary was stimulated by 10 mM EDTA. But EDTA was inactive against the enzyme at a final concentration of 10 mM in this inhibition study (data not shown). This difference about the inhibition pattern of EDTA may be responsible for the difference of purity of enzyme sources for inhibition studies. Optimum pH and inhibition pattern of glutaminyl cyclase activity are very similar to those of enzymes isolated from other tissues [4,8].

Macrophages, participants in both innate and specific immunity, have numerous functions such as phagocytosis, antigen processing and presentation, secretion of both pro- and anti-inflammatory cytokines, and production of reactive oxygen and nitrogen intermediates. Following stimulation with microbial products such as LPS, macrophages secrete several pro-inflammatory products, including TNFα, IL-12, IL-1, IL-6, and nitric oxide (NO), followed by secretion of the anti-inflammatory cytokines IL-10 and TGFβ [23]. Macrophage activation is controlled by a number of regulatory molecules such as prostaglandin E_2, TGFβ, vasoactive intestinal peptide, and pituitary adenylate cyclase-activating polypeptide [24–27]. In the present study, we showed that LPS produces a concentration-dependent increase of glutaminyl cyclase activity in the macrophage cell line RAW 264.7, suggesting that pyroglutamyl-peptide and/or -protein stimulated during incubation with LPS in the macrophage may participate in regulation of macrophage activation. For the confirmation of the hypothesis described above, we are currently studying the detection of pyroglutamyl-peptide and -protein stimulated with LPS in RAW 264.7.

In conclusion, we have developed a sensitive, accurate, and reliable assay for clarification of the roles of glutaminyl cyclase in vivo.

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