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A guanosine 3′,5′-cyclic monophosphate (cGMP) reporter system based on the G-kinase/CREB/CRE signal transduction pathway

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ABSTRACT

Guanylate cyclases constitute a gene family of enzymes that synthesize the second messenger guanosine 3′,5′-cyclic monophosphate (cGMP) and play important roles in diverse physiological functions. Here we report a novel, simple and highly sensitive method for measurement intracellular cGMP concentrations using a cAMP-responsive element (CRE) and cGMP-dependent protein kinase (cGK). Transient transfection of the CRE reporter plasmid, encoding a luciferase reporter gene under the control of a modified promoter containing a CRE, and a cGK expression vector into HEK293 cells followed by treatment with 8-bromo-cGMP showed a dose dependent increase in luciferase activity. Moreover, HEK293 cells expressing GC-A or GC-B natriuretic peptide receptors and harboring this reporter system responded to specific ligands in a dose dependent manner. Our results indicate that this reporter gene method enables high throughput screening of receptor-type GC selective agonists in the treatment of cardiovascular diseases and homeostatic dysfunctions.

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1. Introduction

The guanylate cyclases (GCs) constitute a family of isoenzymes that play important physiological and pathophysiological roles via the synthesis of guanosine 3′,5′-cyclic monophosphate (cGMP), an intracellular signal transducer, from guanosine triphosphate (GTP), in response to some hormone stimulation. In mammals, seven receptor-type GC (membrane-bound GC) genes and four soluble GC genes have been identified [1,2]. They all share a highly conserved catalytic domain of about 250 amino acids. Three of seven receptor-type GCs are known as bioactive peptide binding proteins and the all soluble GCs are thought to bind nitric oxide and carbon oxide in mammals [1,2].

The known receptor-type GC genes in mammals are referred to as guanylate cyclase-A (GC-A) to -G (GC-G), and their products are all single-chain polypeptides and are composed of four characteristic subdomains: an N-terminal extracellular domain responsible for ligand binding, a short hydrophobic transmembrane domain, an intracellular kinase homology domain and a C-terminal GC catalytic domain [1]. Atrial natriuretic peptide (ANP) and brain natriuretic peptide (BNP) are both considered to bind to GC-A (also called NPR-A and NPR-1), and C-type natriuretic peptide (CNP) is known to preferentially bind to GC-B (also called NPR-B and NPR-2) rather than to GC-A [3,4]. Guanylin and uroguanylin both are gastrointestinal peptide hormones, and they have been reported to bind to GC-C, an *Escherichia coli* heat-stable enterotoxin receptor [5,6].

Physiological and pathophysiological effects of bioactive peptides that bind to receptor-type GCs have been widely analyzed *in vivo*. ANP is mainly secreted from the cardiac atria, and plays a role in vasodilation, lowering blood pressure and promoting water and sodium excretion [7,8]. BNP has similar biological properties to ANP and appears to be antagonistic to the renin/angiotensin II/aldosterone system [9]. The most obvious physiological effect of CNP, as has been reported, is to stimulate bone growth [10,11]. Guanylin and uroguanylin are thought to regulate water and electrolyte absorption in the gastrointestinal tract [5,6,12]. Therefore, receptor-type GCs are thought to be targets for a range of diseases such as acute and chronic heart failure, hypertension, dwarfism, constipation and arterial sclerosis, and this underscores the necessity of developing a receptor-type GC subtype selective agonist.

Although the physiological importance of bioactive peptides has been widely recognized, there remain many unanswered questions related to the cellular response mediated through the intracellular cGMP signal transduction machinery. This is due to the limited utilization of the cGMP signal transduction system in organs and the instability of cGMP against hydrolysis by cGMP-specific phosphodiesterase, which does not allow the accurate determination of cGMP concentrations. cGMP has been reported to be capable of activating three classes of proteins: ion channels,

Abbreviations: GC, guanylate cyclase; cGK, cGMP-dependent protein kinase; CRE, cAMP-responsive element; CREB, CRE binding protein; cAK, cAMP-dependent protein kinase; MMTV, mouse mammary tumor virus.

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phosphodiesterases and protein kinases. The cell-specific responses evoked by receptor-type GCs are mediated by the cooperative interactions of these proteins [13]. cGMP-dependent protein kinase (cGK) has many structural and functional features that are highly similar to those found in cAMP-dependent protein kinase (cAK), and has similar substrate specificities [14]. Therefore, phosphorylation of cellular proteins by cGK leads to transactivation of multiple target genes, which creates crosstalk between cAMP and cGMP signaling pathway [15,16].

We report here the development of a novel intracellular cGMP detection method utilizing a signaling pathway activating cAMP-response element (CRE)-mediated transcription with phosphorylated CRE binding protein (CREB) and overexpressed cGK in HEK293 cells. Moreover, using this detection method, we have constructed a simple and highly sensitive reporter gene assay system for use in the high throughput screening of receptor-type GC selective agonists.

2. Materials and methods

2.1. Reporter genes construction

MMTV8–29 (VG028), mouse mammary tumor virus (MMTV) genome, was obtained from the Human Healthcare Science Research Resources Bank (Osaka, Japan). To construct the modified promoter ΔMMTV containing a CRE, the glucocorticoid-responsive element (GRE) located in the MMTV promoter was replaced with a CRE derived from the corticotropin-releasing hormone gene, as previously reported [17–19]. In brief, MMTV 5′-LTR regions located at both GRE sites were amplified in separate PCRs. PCR fragments encoding the upstream (position −1200 to −190) and the downstream (position −88 to +1) segments from the GRE were generated using specific sets of primers. The primer sets used for the PCRs are given in Supplementary Table S1. The resulting PCR fragments were subcloned into pGL3-basic-Luc2P, and the luciferase gene in pGL3-basic (Promega, Medison, WI) was substituted with a synthetic destabilized luciferase gene in pGL4.11 (Promega), and was referred to as pGL3b/ΔMMTV-Luc2P. Oligonucleotide cassettes with five tandemly-repeated CRE motifs were synthesized as complementary pairs of primers with HindIII sites at the 5′-end (see Supplementary Table S2 for detailed sequence), and they were subcloned into the pGL3b/ΔMMTV-Luc2P. The plasmid with one unit inserted was designated as pGL3b/ΔMMTV/CRE5-Luc2P and that inserted into three was designated pGL3b/ΔMMTV/CRE15-Luc2P. All constructs were sequenced to be able to confirm the orientation and integrity of the oligonucleotide.

2.2. Cloning of human GC-A, GC-B, GC-C and G-kinase 1β

Total RNA was prepared from HEK293 cells (for isolation of human GC-A), T84 human colonic adenocarcinoma cells (for isolation of human GC-C and G-kinase 1β) and SK-N-MC human neuroblastoma cells (for isolation of human GC-B). The first strand of cDNA was made using Superscript II reverse transcriptase (Invitrogen, Carlsbad, CA) with an oligo(dT) primer. The full-length coding regions of human GC-A, GC-B, GC-C and G-kinase 1β cDNAs were amplified by PCR using specific primers. The primer sets used for the PCRs are given in Supplementary Table S3. The PCR products were subcloned into mammalian expression vector pcDNA3.1(+) (pcDNA) (Invitrogen). To verify the cDNAs encoding a full-length region, constructed plasmids were subjected by DNA sequencing.

2.3. Cell culture and transfection

HEK293 (RIKEN BRC, Tsukuba, Japan) cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum. Cells were seeded at a density of 1×10^4 cells/cm^2 on type I collagen-coated 12-well or 96-well plates (IWAKI, Japan). Transient transfection was performed using the FuGene6 (Roche, Mannheim, Germany), as described in the manufacturer's instructions.

2.4. cGMP reporter signal detection in living cells

Cells seeded on 12-well plates were transfected with 500 ng of pGL3b/ΔMMTV-Luc2P, pGL3b/ΔMMTV/CRE5-Luc2P or pGL3b/ΔMMTV/CRE15-Luc2P along with either 500 ng of pcDNA or pcDNA/G-kinase 1β. After 36 h transfection, forskolin (Nacalai Tesque, Kyoto, Japan) or 8-bromo-cGMP (Sigma, St. Louis, MO) was added to the culture media containing 0.2 mM D-Luciferin (Nacalai Tesque) in each well at a final concentration of 10 μM and 1 mM, respectively. Four hours later, cells were analyzed with a LAS 4000miniEPUV Luminescent Image Analyzer (Fuji Film, Tokyo, Japan).

2.5. cGMP reporter gene assay in multi well plate

The transfection of cells plated on 96-well plates was done as described above except for using 100 ng of plasmids per well. In addition, as an internal control for transfection efficiency, cells were transfected with 10 ng of the pcDNA/β-galactosidase per well. Thirty-six hours after transfection, the cells were treated with 8-bromo-cAMP (Sigma) or 8-bromo-cGMP, respectively, at a concentration of 1 mM for each. Four hours later, the cells in each well were lysed with 100 μl of Passive Lysis Buffer (Promega). For each well, 50 μl of cell lysate was transferred to a 96-well white plate and 50 μl of Luciferase Assay Substrates (Promega) was added. Luciferase activity (in counts per second, cps) was measured using the Luminous CT-9000D Luminometer (Dia-Iatron, Tokyo, Japan).

2.6. β-Galactosidase activity measurement

Each cell lysate in the cGMP reporter gene assay prepared was subjected to an assay for β-galactosidase activity using the Galacto-Star System (Applied Biosystems, Bedford, MA), as recommended by the supplier. Luciferase reporter gene activities were normalized with the β-galactosidase activities of untreated cells.

2.7. Dose–response analysis of GC receptors employing cGMP reporter gene assay

The human ANP, BNP and CNP were purchased from Peptide Institute Inc. (Osaka, Japan). *E. coli* heat-stable enterotoxin STa was obtained from Sigma. HEK293 cells plated on 96-well plates were transfected with 30 ng of pcDNA/GC-A, pcDNA/GC-B or pcDNA/GC-C along with 30 ng of pcDNA/G-kinase 1β, 30 ng of pGL3b/ΔMMTV/CRE5-Luc2P and 10 ng of pcDNA/β-galactosidase. Thirty-six hours after transfection, the cells were stimulated with appropriate ligands for 4 h at concentrations between 1 pM and 100 nM, except for ANP (1 pM to 1 μM). Cells were subjected to a luciferase assay as described above.

2.8. cGMP enzyme-linked immunosorbent assay (ELISA)

Cells on 96-well plates were transfected with 100 ng of pcDNA encoding human GC-A, GC-B or GC-C. After 36 h transfection, culture media was replaced with assay buffer composed of 1 mM isobutylmethylxanthine (IBMX), 0.1% bovine serum albumin, 137.93 mM NaCl, 5.33 mM KCl, 0.441 mM KH_2PO_4, 4.17 mM $NaHCO_3$, and 0.338 mM Na_2HPO_4 with 20 mM Hepes–NaOH (pH 7.5). The human ANP in the assay buffer was added at various concentrations to each well and incubated for 1 h. The intracellular cGMP

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