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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.

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

was measured with a CatchPoint cGMP fluorescent assay kit (Molecular Devices, Sunnyvale, CA) according to the manufacturer's instructions. Measurement of fluorescence signals was done with EnVision 2104 Multilabel Reader (Perkin-Elmer, Waltham, MA).

2.9. Data analysis and statistics

Statistical analyses of data from cGK nucleotide specificities, ligand dose-dependencies, β-galactosidase activities and cGMP ELISA were performed with GraphPad Prism 3.0 Software (GraphPad Software, San Diego, CA).

3. Results

3.1. cGMP reporter gene system construction

cGK and cAK have some comparable structural and functional features, and there might be some crosstalk between their signal transduction pathways [15,16]. Some of their signaling pathways are linked with CRE, and induction of these pathways promotes CREB binding of CRE and transactivates certain downstream target genes [16]. Consequently, we have hypothesized that expression of a large amount of cGK would result in functional replacement of cAK signaling, and attempted to develop a novel method enabling the monitoring of intracellular cGMP concentrations utilizing a cAMP/cAK/CREB/CRE signal transduction pathway. We have constructed reporter plasmids containing firefly luciferase gene controlled by a mouse mammary tumor virus (MMTV) with its 5′-LTR substituted glucocorticoid-responsive element with multiple copies of CRE [17–19], referred to as ΔMMTV/CRE reporter genes, and the expression vector encoding human cGK (Fig. 1).

3.2. Detection of intracellular cGMP with ΔMMTV/CRE reporter gene assay in a cGK dependent manner

To examine whether the ΔMMTV/CRE reporter gene in combination with cGK could be detect intracellular cGMP, we evaluated the luciferase activity of HEK293 cells harboring the ΔMMTV/CRE reporter plasmids followed by treatment of forskolin, an activator of adenylate cyclase, or the cGMP analog 8-bromo-cGMP under conditions with and without cGK overexpression. Luciferase chemiluminescence signals were detected in the living cells (Fig. 2A). The results found that the luciferase gene controlled by

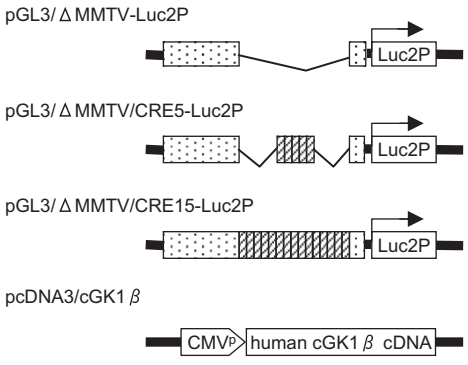

Fig. 1. Scheme of the ΔMMTV/CRE-Luc2P reporter genes and the cGK1β expression vector constructs. The glucocorticoid-responsive element of the MMTV promoter was removed (pGL3b/ΔMMTV) or substituted with multiple copies of CRE (pGL3b/ΔMMTV/CRE5-Luc2P and pGL3b/ΔMMTV/CRE15-Luc2P). MMTV promoter regions and CREs are indicated by dotted boxes and striped boxes, respectively. The human cGK1β cDNA was cloned under the control of an early cytomegalovirus (CMV) promoter (pcDNA/cGK1β).

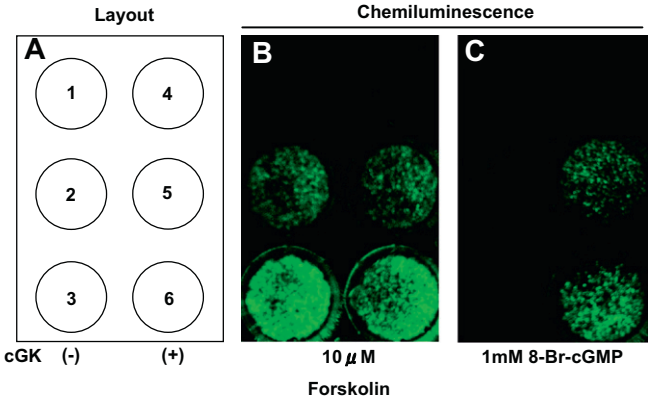

Fig. 2. Detection of 3′,5′-cyclic mononucleotide-induced chemiluminescence signals emitted by intact HEK293 cells harboring ΔMMTV/CRE reporter genes and human cGK1β expression vector. HEK293 cells were transfected with the reporter plasmids (pGL3b/ΔMMTV-Luc2P, 1 and 4; pGL3b/ΔMMTV/CRE5-Luc2P, 2 and 5; pGL3b/ΔMMTV/CRE15-Luc2P, 3 and 6) along with either an empty vector (1–3, (−)) or a cGK1β expression vector (4–6, (+)) (A). Thirty-six hours after transfection, 0.2 mM D-Luciferin plus 10 μM forskolin (B) or 1 mM 8-bromo-cGMP (C) were added. Cells were visualized by a luminescent image analyzer.

the ΔMMTV/CRE promoter was transactivated in response to elevation of intracellular cAMP by addition of forskolin independent of cGK (Fig. 2B). The intensities of luciferase signals were found to be dependent upon the number of CREs. Alternatively, in the case of addition of 8-bromo-cGMP, the luciferase activity was detected only where cGK was expressed at the same time, and was correlated with the number of CREs (Fig. 2C). No signal was detected in the control HEK293 cells (Fig. 2B and C).

3.3. Cyclic mononucleotide specificity of the ΔMMTV/CRE reporter gene assay

To analyze whether the ΔMMTV/CRE reporter gene assay with cGK was capable of detecting intracellular cGMP, HEK293 cells harboring the reporter gene with or without cGK were stimulated with 8-bromo-cAMP or 8-bromo-cGMP and subjected to an analysis of luciferase activity (Fig. 3). Transactivations of the luciferase gene from the ΔMMTV/CRE reporter plasmids were similarly observed under conditions of 8-bromo-cAMP stimulation with or without cGK (Fig. 3). These results indicate that the 8-bromo-cAMP-induced luciferase gene transactivations reflect the endogenous expression of cAK in HEK293 cells. On the other hand, the luciferase activities were detected at very low concentrations by the addition of cGMP without cGK expression, whereas they were markedly increased by cGK overexpression. These results demonstrate that the expression of luciferase activity under the condition of 8-bromo-cGMP is cGK-dependent and that endogenously expressed cAK fails to be activated by cGMP. In addition, we have confirmed that 8-bromo-cGMP-induced cGK activation was specifically inhibited by the addition of cGK antagonist Rp-8-CPT-cGMP (Supplementary Fig. S1), and that cGMP molecules synthesized with guanylate cyclases strictly activated cGK (Supplementary Fig. S2). On the basis of these results, we have designated this intracellular cGMP detection method the cGK × ΔMMTV/CRE reporter gene assay.

3.4. Analysis of dose-dependent activation of GC receptors using the cGK × ΔMMTV/CRE reporter gene assay

To assess whether the cGK × ΔMMTV/CRE reporter gene can be applied in identifying receptor-type GC agonists, we transiently transfected the reporter plasmids into HEK293 cells along with

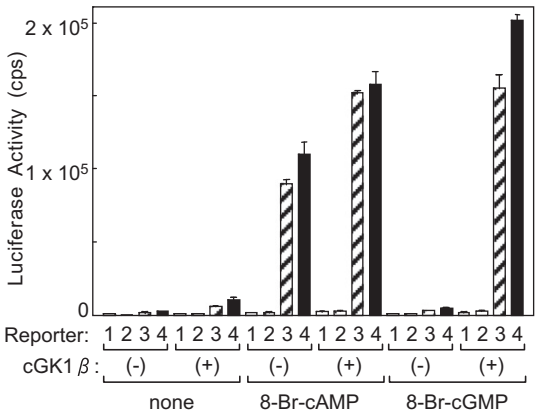

Fig. 3. cGMP reporter gene assay using HEK293 cells harboring cGK × ΔMMTV/CRE reporter genes. HEK293 cells were transiently transfected with either an empty vector (−) or pcDNA/cGK1β (+) along with the reporter gene constructs (1, pGL3b (open bars); 2, pGL3b/ΔMMTV-Luc2P (dotted bars); 3, pGL3b/ΔMMTV/CRE5-Luc2P (striped bars); 4, pGL3b/ΔMMTV/CRE15-Luc2P (closed bars)). Cells were treated with 8-bromo-cAMP or 8-bromo-cGMP at a concentration of 1 mM. The error bars indicate the mean and SEM from at least three experiments performed in triplicate.

receptor-type GC expression vectors. We found that when the cells were stimulated with various doses of each ligand, their luciferase activities increased in a dose-dependent fashion, as shown in Fig. 4A–D. In the case of the combination of ANP and GC-A, the cGMP production of GC-A was measured with an enzyme-linked immunosorbent assay (ELISA) for the purpose of verifying the result of the developed cGK × ΔMMTV/CRE reporter gene assay (Fig. 4A). The concentration values required for one-half of the maximal activation (EC_{50}) obtained from the reporter gene assay and the ELISA were found to be 1.3 and 10 nM, respectively. We were able to detect ANP at less than 100 pM with this reporter gene assay. In addition, using a reporter gene assay we obtained results in BNP, CNP and STa with EC_{50} values of 14.7, 1 and >200 nM against GC-A, GC-B and GC-C, respectively (Fig. 4B–D).

4. Discussion

We report here the construction of a simple and novel intracellular cGMP measurement system using the CRE reporter gene and overexpressed cGK. So far, it has been reported and proposed that the cAMP/cAK signal transduction pathway could interact with the cGMP/cGK signaling pathway in various tissues via activation of the CREB/CRE pathway [14,15,20]. Previous studies analyzing cGK activity using transcription of the reporter gene controlled by the CREB/CRE pathway demonstrated that high doses of cGMP concentration in comparison to the physiological condition were needed to detect a sufficient level of cGK activity. Hence, it was difficult to evaluate whether cGK may have some effects on cAMP/cAK signaling pathways *in vivo* [14]. We therefore postulated this might be due to the following two reasons—firstly, transcriptional abilities of some heterologous promoters containing CRE might be suddenly activated when they exceed a threshold, and secondly, cGK expression is at a very low level in many cell types, and it is difficult to detect cGK-specific activation without non-specific activation of cAK. Therefore, we constructed the method here presented to measure intracellular cGMP utilizing a heterologous promoter, composed of a mouse mammary tumor virus promoter and a CRE, and cGK overexpression, and we designated this system the cGK × ΔMMTV/CRE reporter gene assay (Fig. 1). As expected, reporter gene activity was associated with intracellular cGMP concentration (Figs. 2 and 3 and S1) and with cGK expression (Figs. 2 and 3 and S2). In addition, to our knowledge, this is the first report of measuring cGMP in living cells (Fig. 2). Results from the analysis of dose-dependent activation of GC receptors using the cGK × ΔMMTV/CRE reporter gene assay revealed that this system can detect very small amounts of cGMP agonistic ligands and is linear over a wide range. Comparison of this reporter assay with the

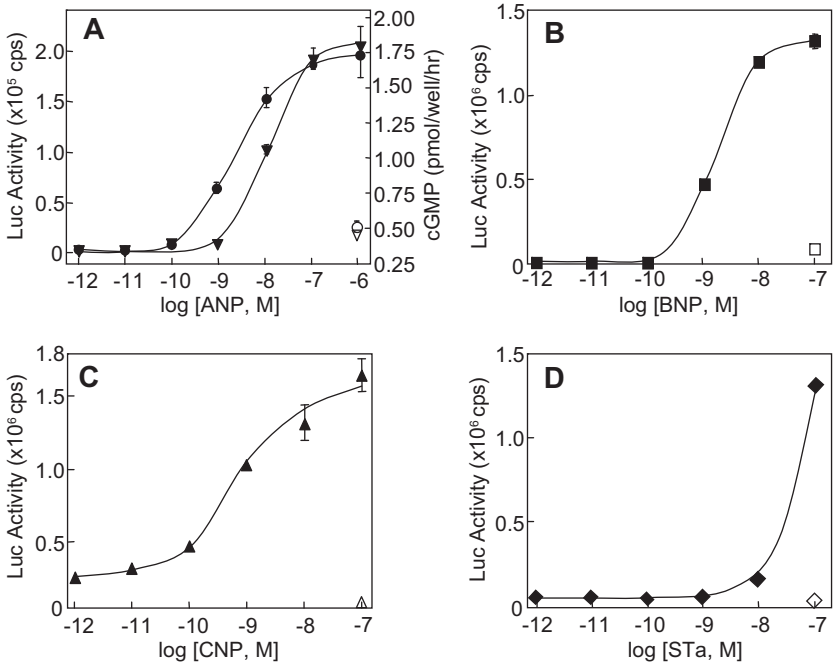

Fig. 4. Responses of HEK293 cells expressing receptor-type GCs specific to each ligand. The dose–response relationships were measured by a cGK × ΔMMTV/CRE reporter gene assay. Cells were transfected with GC-A (A and B), GC-B (C) or GC-C (D) expression vectors along with the reporter gene vectors. Dose–response curves are shown for (A) ANP (filled circles), (B) BNP (filled squares), (C) CNP (filled triangles), and (D) STa (filled diamonds), respectively. The dose–response curve for ANP obtained by ELISA is shown in (A) (filled inverted triangles). The open symbol in each panel shows the response of control HEK293 cells at indicated concentration of each ligand. Each data point shows the mean and SEM from at least three independent measurements. Data are representative of three independent experiments performed in triplicate.

ELISA method indicated that the former method was found to be considerably more sensitive than the latter (Fig. 4). Therefore, this reporter gene assay is capable of evaluating the efficacy of drugs and may be extremely useful in identifying receptor-type GC selective agonists with regard to its high sensitivity, the ability for rapid data acquisition, and simple instrumentation.

Interestingly, the EC_{50} value of STa for GC-C (>200 nM) is much higher when compared with the values of ANP and BNP for the common receptor GC-A (1.3 and 14.4 nM, respectively) and with the value of CNP for GC-B (1 nM) (Fig. 4). One possible reason for this result may be due to a paracrine mode of action of GC-C ligands. Guanylin and uroguanylin, gastrointestinal peptides and endogenous ligands for GC-C, are thought to act on neighboring cells in a paracrine fashion and might require higher doses to activate GC-C receptors than other bioactive peptides [21]. Another possibility is that GC-C may need some additional and specific cellular mechanisms, which are lacking in HEK293, to fully express its receptor activity. These mechanisms would be concerned with the determination of GC-C ligand selectivity between guanylin and uroguanylin *in vivo*. Moreover, this may be one reason why it is that the ligands of GC-D, -E, -F and -G have still not been identified. In any of these cases, this reporter gene system would be useful tool for solving these problems.

The receptor-type GC agonists screened with this reporter gene assay will have some benefit in clinical uses. Many of the physiological effects of ANP and BNP have been reported to exhibit cardioprotective activity, including a diuretic effect and vasodilation [8,9,13]. The infusion of ANP into patients with acute heart failure resulted in hemodynamic responses without tolerance or severe side effects [22]. Although ANP indeed demonstrates cardioprotective effects and its administration may be a clinically useful treatment for heart failure, it only has a half-life of 2 min in the plasma [23]. On the other hand, BNP has a stronger hypotensive effect and a longer half-life in the plasma than does ANP, but its clinical usefulness is limited by adverse effects on renal function, and thus it is mainly used as a better diagnostic marker of heart failure [24]. Hence, to develop GC-A agonists with long half-lives and little or no adverse effects would enable easy maintenance of body fluid volume and blood pressure control, especially in emergency care.

In the case of CNP, its most notable physiological effect via GC-B is to stimulate bone growth, suggesting that CNP could be used in the treatment of osteoporosis and dwarfism. The half-life of CNP in the plasma, however, is about 3 min. GC-B agonists that are stable in the plasma and have few adverse effects would be able to be maintained in long-term administration [13]. Guanylin and uroguanylin peptides have been reported to regulate water and electrolyte absorption in intestinal mucosa epithelial cells, and have an antiproliferative effect on some colon cancer cell types [25]. It may be possible to use GC-C agonists as laxatives and anticancer agents. Therefore, identification of receptor-type GC selective agonists would facilitate the development of a novel class of drugs with improved therapeutic indices and better clinical profiles.

Moreover, the cGK × ΔMMTV/CRE reporter gene assay may also be an applicable method for the following purposes: (1) screening for the specific ligands of GC-D, -E, -F and -G, (2) screening for cGMP-dependent phosphodiesterase inhibitors for the treatment of asthma, alopecia, sexual dysfunction, chronic obstructive pulmonary disease (COPD) and others, and (3) screening for soluble NO-dependent GC agonists to treat patients with hypertension or heart failure. Recently, it has been possible to screen huge amounts of synthetic compounds available from publicly- or commercially-available resources [26]. The reporter gene assay we have reported here would facilitate the high throughput screening of receptor-type GC selective agonists and the development of a novel and more effective class of drugs.

To conclude this report, we have developed a novel reporter gene assay system for measuring intracellular cGMP concentrations using the cGMP/cGK/CRE/CREB signal transduction pathways. We have shown that this system is more sensitive and has a wider range of linearity than do conventional methods. We have demonstrated the possibility of high throughput screening of agonists for receptor-type GCs.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2011.03.009.

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