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Pharmacological characterization of receptor guanylyl cyclase reporter cell lines

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ABSTRACT

Receptor guanylyl cyclases are implicated in a growing number of pathophysiologies and, therefore, represent an important target class for drug development. We report here the generation and pharmacological characterization of three particulate guanylyl cyclase (pGC) reporter cell lines. Plasmid constructs encoding the natriuretic peptide receptors GC-A and GC-B, and the heat-stable enterotoxin receptor GC-C, were stably transfected in a parental reporter cell line expressing a cyclic nucleotide-gated (CNG) cation channel, acting as the biosensor for intracellular cGMP. In our reporter cell lines pGC activity can be monitored in living cells in real-time . By using different natural as well as synthetic receptor ligands of the natriuretic and guanylin peptide families, we show that our reporter assay monitors pGC activity with very high sensitivity. In contrast to previous findings, we could detect significant stimulation of GC-A and GC-B by each of the natriuretic peptides ANP, BNP and CNP. In addition, the clearance receptor ligand Cys-ANF(4–18) and the ANP receptor antagonist Arg-ANF(6–18) were characterized as partial GC-A agonists. The results imply that our novel pGC reporter cell lines are well suited for the characterization of receptor pharmacology and may be used for natural ligand characterization of guanylyl cyclase orphan receptors.

1. Introduction

Guanylyl cyclases (GCs) are a family of enzymes that catalyze the conversion of guanosine triphosphate (GTP) to the intracellular second messenger 3′,5′-cyclic guanosine monophosphate (cGMP). The enzyme family comprises both soluble and particulate isoforms (sGC and pGC). In contrast to sGC, which is activated by the gaseous messenger nitric oxide (NO), membrane-bound receptor guanylyl cyclases are regulated by diverse extracellular agonists, including peptide hormones and bacterial toxins (Lucas et al., 2000; Tamura et al., 2001; Potter, 2005; Garbers et al., 2006).

Seven different mammalian receptor guanylyl cyclases, named GC-A through GC-G, have been identified up to now. GC-A, also known as natriuretic peptide receptor-A or -1 (NPR-A or NPR-1), is the receptor for atrial and brain natriuretic peptides (ANP and BNP). GC-B or natriuretic peptide receptor-B or -2 (NPR-B or NPR-2) is mainly activated by C-type natriuretic peptide (CNP). GC-C was first characterized as a receptor for heat-stable bacterial enterotoxins (STa). Later on guanylin and uroguanylin, small peptides with homology to STa, were suggested as the natural endogenous ligands for GC-C (Vandraager, 2002; Forte, 2004; Potter et al., 2006; 2009). Recently, different ligands have been proposed as GC-D agonists, namely guanylin, uroguanylin and the gaseous messenger CO_2 (Hu et al., 2007; Leinders-Zufall et al., 2007; Duda and Sharma, 2008; Cockerham et al., 2009; Sun et al., 2009). Up to now, no ligands have been identified for the orphan receptors GC-E, GC-F and GC-G (Tamura et al., 2001; Garbers et al., 2006).

Receptor guanylyl cyclases play essential roles in cardiovascular and gastrointestinal (patho-)physiology, reproduction biology, cell proliferation, bone growth and sensory signal transduction and, therefore, are important pharmacological targets. Accordingly, the receptor guanylyl cyclase agonists ANP and BNP (carperitide and neseritide) have been approved for the treatment of heart failure. Additional natural peptides (e.g., CNP, DNP and urodilatin) as well as designer peptides (e.g., CD-NP) are currently evaluated as potential therapeutics. As a drawback, all these approaches require parenteral application of the respective peptides. Therefore, small molecule pGC modulators with oral bioavailability would be very desirable as both pharmacological tools and novel therapeutics (Forte, 2004; Walther and Stepan, 2004; Dickey et al., 2008; Woodard and Rosado, 2008; Boerrigter et al., 2009; Kuhn, 2009; Potter et al., 2006; 2009). In addition, guanylin and its derivatives (e.g., linaclotide and guanilib) are explored for the treatment of GI disorders and colon cancer (Forte, 2004; Harris and Crowell, 2008; www.callistopharma.com).

We report here the generation and pharmacological characterization of three stably transfected cell lines, recombinantly expressing

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the receptor guanylyl cyclases GC-A, GC-B or GC-C, respectively. In our reporter cell lines, activation of the membrane-bound guanylyl cyclases can be monitored with unique sensitivity in living cells in real-time .

2. Materials and methods

2.1. Generation of stable receptor guanylyl cyclase reporter cell lines

A recombinant CHO cell line expressing cytosolic apoaequorin was stably cotransfected with a plasmid construct encoding the bovine CNGA2 channel (GB ID: X55010) and a plasmid providing zeocin resistance, as has been described previously (referred to here as the cGMP reporter cell line; Wunder et al., 2005a). For the generation of the receptor guanylyl cyclase reporter cell lines, the parental cGMP reporter cell line was transfected with plasmid constructs encoding rat GC-A (GB ID: NM_012613), rat GC-B (GB ID: NM_53838), or human GC-C (GB ID: NM_004963), respectively. Stably transfected clones were obtained by G-418 (Geneticin) selection and were characterized by ANP, CNP or uroguanylin stimulation (data not shown). Positive clones were purified once by the limited dilution technique and one clonal cell line was selected for further experiments.

2.2. Quantitative real-time RT-PCR analysis

Quantitative TaqMan analysis was performed using the Applied Biosystems PRISM 7900 sequence detection system. Human tissue mRNA probes were obtained from Ambion, Inc. (Austin, TX, USA), Cell Applications, Inc. (San Diego, CA, USA), Clontech Laboratories (Mountain View, CA, USA) and Stratagene (La Jolla, CA, USA). RNA probes from the reporter cell lines and various rat tissues (Sprague Dawley, Charles River) were isolated by using TRIZOL Reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturers protocol. Probes were treated with DNase and reverse transcribed using random hexamers. Equal probe efficiencies were assured by titration of corresponding plasmid constructs. Normalization was performed using the housekeeping gene L32 as control, and relative expression was calculated using the formula: relative expression $= 2^{(20-(C_t(\text{probe})-C_t(\text{L32})))}$. The parameter C_t is defined as the threshold cycle number at which the amplification plot passed a fixed threshold above baseline. The resulting expression is given in arbitrary units.

The following primers and fluorescent probes were used: GC-A: forward primer: 5′-GACCTACTGCCCTGCTGTTC-3′; probe: 5′-(FAM)CCTCCCTGTGCCAGAGGTGACAGAG(TAMRA)-3′; reverse primer: 5′-GAGAGGTGGAAGCTGGACAC-3′; GC-B: forward primer: 5′-TGGCTCTTAGGAGAGCGAAA-3′; probe: 5′-(FAM)TCCTGGACTCCTGTAAATCCCAAGCA(TAMRA)-3′; reverse primer: 5′-GCCCATCCAAGTACCAACAG-3′; GC-C: forward primer: 5′-CTGGGATGAAGGACCAGAAA-3′; probe: 5′-(FAM)ACCTGCCAACCCCTCCTACTGTGG(TAMRA)-3′; reverse primer: 5′-TTGCAAACGCTGTTGATTCT-3′.

2.3. Cell culture conditions and aequorin luminescence measurements

Cells were cultured as described previously (Wunder et al., 2009). Cells were passaged using enzyme-free/Hanks'-based cell dissociation buffer. All cell culture reagents were obtained from Invitrogen (Carlsbad, CA). Luminescence measurements were performed on opaque 384-well microtiter plates. Either 2500 cells/well or 1500 cells/well were plated and were cultured for 24 h or 48 h, respectively. After removal of the cell culture medium, cells were loaded for 3 h with 0.6–5 μg/ml coelenterazine in Ca^{2+}-free Tyrode (130 mM NaCl, 5 mM KCl, 20 mM HEPES, 1 mM $MgCl_2$, 4.8 mM $NaHCO_3$ at pH 7.4) at 37 °C and 5% CO_2. Receptor ligands were added for 6 min in Ca^{2+}-free Tyrode containing 0.1% BSA. IBMX (0.2 mM) was used to prevent cGMP degradation by endogenous phosphodiesterases. Immediately before adding calcium ions (final concentration 3 mM), measurement of the aequorin luminescence was started by using a charge-coupled device (CCD) camera (Hamamatsu Corporation, Shizuoka, Japan) in a light tight box. Alternatively, a conventional luminometer may also be used (Wunder et al., 2005a). Luminescence was monitored continuously for 60 s. For the real-time detection of cGMP generation, coelenterazine loading and agonist stimulation were performed in Tyrode containing 2 mM calcium ions (Wunder et al., 2005a).

2.4. cGMP enzyme immunoassay

For the measurement of intracellular cGMP, 5000 cells/well were seeded on 384-well MTPs and cultured for 2 days. The medium was removed, the cells were washed once, and test compounds were added in tyrode supplemented with 0.2 mM IBMX for 15 min at 37 °C. Intracellular cGMP was measured by using a commercially available cGMP immunoassay kit (HTRF, CisBio, Gif-sur-Yvette, France).

2.5. Compounds and receptor ligands

Mini-ANP, (Des-Gln^{18}, des-Ser ^{19}, des-Gly ^{20,22}, des-Leu ^{21})-Atrial Natriuretic Peptide(4-23) amide (referred to here as Cys-ANF(4-18)), Arg^{6}, β-cyclohexyl-Ala^{8}, D-Tic ^{16}, Arg^{17}, Cys^{18})-Atrial Natriuretic Factor(6-18) amide (referred to here as Arg-ANF (6-18)) and all other natural receptor ligands were purchased from Bachem (Bubendorf, Switzerland). IBMX (3-isobutyl-1-methylxanthine) was obtained from Sigma-Aldrich (Taufkirchen, Germany).

2.6. Statistics

The data are presented as mean values with standard deviation errors. The GraphPad Prism software (Version 4.02, GraphPad Software Inc., San Diego, CA) was used for curve-fitting and calculation of the half-maximal effective and inhibitory concentrations (EC_{50} and IC_{50}, respectively). For the determination of pEC_{50} and pIC_{50} values, three to six independent experiments were performed in quadruplicate. pEC_{50} and pIC_{50} values are given as means ± S.E.M.

3. Results

3.1. Generation of the recombinant receptor guanylyl cyclase reporter cell lines

A cGMP reporter cell line, recombinantly expressing apoaequorin and the CNG channel CNGA2, was generated as has been described previously (Wunder et al., 2005a). This cell line was stably transfected with plasmid constructs encoding the receptor guanylyl cyclases GC-A, GC-B, or GC-C, respectively. Active clones were characterized by stimulation using ANP, CNP or uroguanylin (data not shown). One clonal receptor cell line was used for further characterization, referred to here as the GC-A, GC-B and GC-C reporter cell lines. In these cell lines, receptor guanylyl cyclase activity can be monitored via aequorin luminescence stimulated by calcium influx through the CNG channel.

3.2. Analysis of pGC expression by quantitative real-time RT-PCR

To verify the recombinant pGC expression in our reporter cell lines, we analyzed the expression levels by quantitative RT-PCR and compared them to the expression in different native rat (GC-A and GC-B) and human (GC-C) tissues (Fig. 1). As expected, we were able to detect very high expression levels of GC-A, GC-B and GC-C in the respective reporter cell lines. Comparably high tissue pGC expression levels were only found for GC-A in lung. In addition, expression of GC-A and GC-B could be detected in heart, kidney, skeletal muscle, small intestine and aortic smooth muscle cells, albeit at clearly lower levels. Significant tissue GC-C expression was only detected in small intestine.

3.3. Characterization of the GC-A reporter cell line

The receptor guanylyl cyclase GC-A, also referred to as ANP or BNP receptor, has been characterized as a receptor for the members of the natriuretic peptide family. Therefore, we initially characterized our newly established GC-A cell line by stimulation using various natriuretic peptides. As shown in Fig. 2A, ANP and BNP stimulated concentration-dependent luminescence signals on the GC-A cell line with very high potency and respective pEC_{50} values of 10.70 ± 0.09 and 10.39 ± 0.10. Unexpectedly, CNP was also able to fully stimulate luminescence responses, however, with significantly lower potency ($pEC_{50} = 7.91 \pm 0.13$). We further characterized our novel reporter cell line by testing the effects of additional, naturally occuring natriuretic peptides. As shown in Fig. 2B, stimulation of the GC-A cell line with the ANP derivatives urodilatin and atriopeptins I-III resulted in concentration-dependent luminescence signals with pEC_{50} values of 10.12 ± 0.07, 9.59 ± 0.05, 10.55 ± 0.08 and 10.64 ± 0.07, respectively.

To correlate aequorin luminescence signals with intracellular cGMP content, the GC-A reporter cell line was stimulated with ANP, BNP or CNP, and cGMP concentrations were measured using a commercially available cGMP immunoassay. As shown in Fig. 3, ANP, BNP and CNP stimulated cGMP synthesis with pEC_{50} values of 9.57 ± 0.07, 9.34 ± 0.05 and 6.29 ± 0.06, respectively. Similar maximal cGMP levels were reached after stimulation with ANP, BNP and CNP.

Next, we characterized the activity of the commercially available synthetic peptides mini-ANP, Cys-ANF(4-18) and Arg-ANF(6-18) on the GC-A reporter cell line. Mini-ANP is an ANP derivative with reduced size, which has been characterized as a full ANP receptor agonist (Li et al., 1995). Cys-ANF(4-18) is a clearance receptor ligand (Maack et al., 1987) and Arg-ANF(6-18) has been described as an ANP receptor antagonist (von Geldern et al., 1990). As expected, mini-ANP fully stimulated the GC-A receptor cell line with a pEC_{50} value of 7.77 ± 0.07 (Fig. 4A). In contrast, Cys-ANF(4-18) and Arg-ANF(6-18) were identified as partial agonists on the GC-A receptor, which stimulated luminescence signals with pEC_{50} values of 6.49 ± 0.07 and 6.21 ± 0.03, respectively. Both peptides consistently

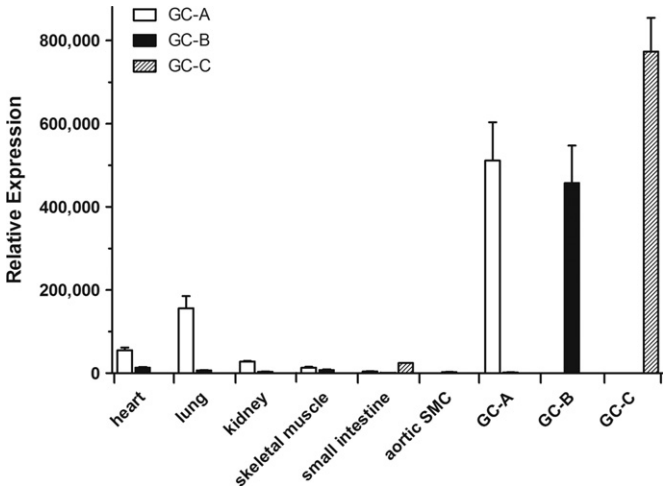

Fig. 1. Characterization of pGC expression levels. Quantitative real-time RT-PCR analysis was performed on different native rat (GC-A and GC-B) and human (GC-C) tissues, aortic smooth muscle cells (SMC) and the three pGC reporter cell lines using specific oligonucleotide probes detecting rat GC-A and GC-B, as well as human GC-C. Expression levels were normalized to the housekeeping gene L32. Relative expression is given in arbitrary units. Data are presented as means with S.E.M. ($n=3$).

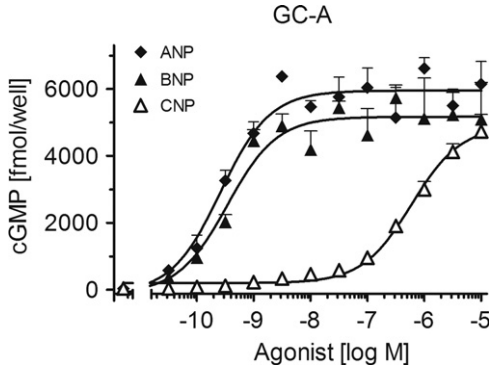

Fig. 3. Intracellular cGMP formation in GC-A reporter cells measured by a cGMP enzyme immunoassay. Cells were incubated for 15 min with ANP (♦), BNP (▲) or CNP (△). Data are presented as means with S.E.M. ($n=4$).

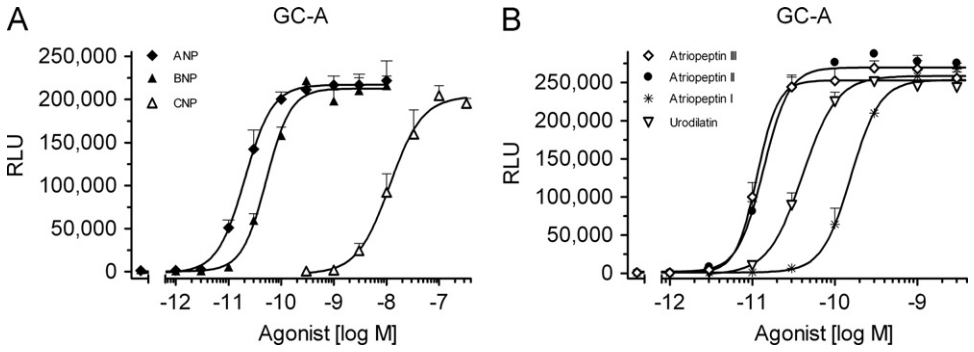

Fig. 2. Stimulation of the GC-A reporter cell line by members of the natriuretic peptide family. (A) Luminescence signals mediated by stimulation with ANP (♦), BNP (▲) and CNP (△). (B) Luminescence signals mediated by stimulation with atriopeptin I (*), atriopeptin II (●), atriopeptin III (◇) and urodilatin (▽). Receptor ligands were added for 6 min in Ca^{2+}-free Tyrode and luminescence measurements were started immediately before the addition of Ca^{2+} (final concentration 3 mM). Results are expressed as relative light units (RLU), and data are presented as means with SD.

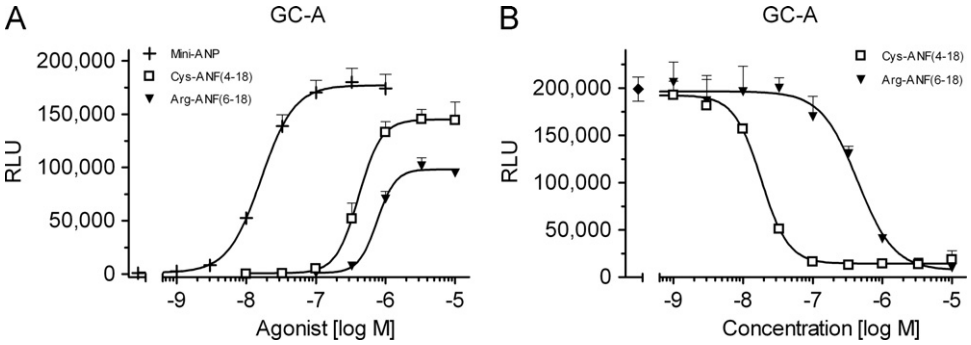

Fig. 4. Characterization of synthetic ligands on the GC-A reporter cell line. (A) Luminescence signals generated by stimulation with mini-ANP (+), Cys-ANF(4-18) (□) and Arg-ANF(6-18) (▼). (B) Inhibition of 0.3 nM ANP-mediated luminescence signals by Cys-ANF(4-18) (□) and Arg-ANF(6-18) (▼). Receptor ligands were added for 6 min in Ca^{2+}-free Tyrode and luminescence measurements in (A) were started immediately before the addition of Ca^{2+} (final concentration 3 mM). (B) 10 min later, 0.3 nM ANP was added and luminescence signals were continuously monitored for 90 s. Data are presented as means with SD.

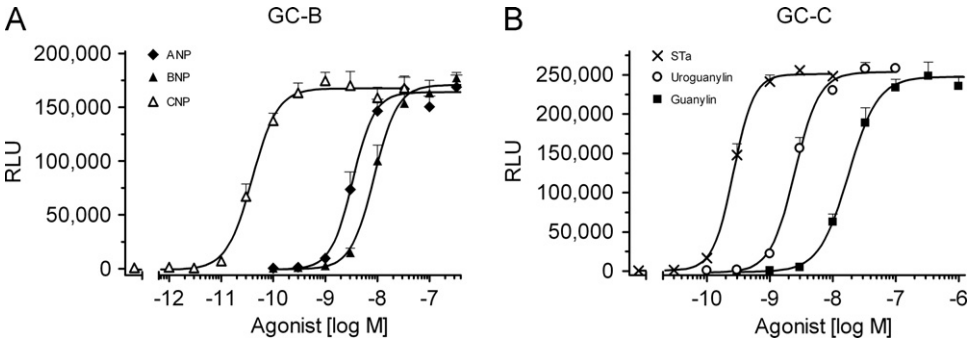

Fig. 5. Characterization of GC-B and GC-C reporter cell lines. (A) Stimulation of the GC-B reporter cell line with ANP (◆), BNP (▲) and CNP (△). (B) Stimulation of the GC-C reporter cell line with *E. coli* heat-stable enterotoxin STa (x), uroguanylin (○) and guanylin (■). Receptor ligands were added for 6 min in Ca^{2+}-free Tyrode and luminescence measurements were started immediately before the addition of Ca^{2+} (final concentration 3 mM). Data are presented as means with SD.

stimulated luminescence responses with efficacies in the range of ~80% and ~50%, respectively (Fig. 4A). In addition, Cys-ANF(4-18) and Arg-ANF(6-18) antagonized 0.3 nM ANP-mediated luminescence signals with pIC_{50} values of 7.79 ± 0.03 and 6.43 ± 0.04, respectively (Fig. 4B).

We also tested if guanylin, uroguanylin and *E. coli* heat-stable enterotoxin (STa) were able to stimulate the receptor guanylyl cyclase GC-A . However, members of the guanylin peptide family were not able to stimulate significant luminescence signals on the GC-A reporter cell line when tested up to 10^{-6} M (data not shown). In addition, we characterized the activity of the various members of the natriuretic and guanylin peptide families on the parental cGMP reporter cell line. However, none of the receptor agonists stimulated significant luminescence signals (data not shown).

3.4. Characterization of the GC-B reporter cell line

The receptor guanylyl cyclase GC-B is also known as the CNP receptor, since it is most potently stimulated by this natriuretic peptide. Activation of GC-B by ANP and BNP has not been unequivocally demonstrated (Koller et al., 1991; Ohyama et al., 1992; Suga et al., 1992). Therefore, our newly established GC-B reporter cell line was characterized by stimulation using the natriuretic peptides ANP, BNP and CNP. As shown in Fig. 5A, CNP stimulated luminescence signals with very high potency ($pEC_{50} = 10.43 \pm 0.04$). ANP and BNP also fully stimulated the reporter cell line, however, with clearly lower potencies ($pEC_{50} = 8.51 \pm 0.07$ and 8.07 ± 0.08). Guanylin, uroguanylin and STa were also tested on the GC-B receptor cell line. However, these peptides did not stimulate any significant luminescence signals when tested up to 10^{-6} M (data not shown).

3.5. Characterization of the GC-C reporter cell line

The receptor guanylyl cyclase GC-C has been characterized as a receptor for the various members of the guanylin peptide family (Vandraager, 2002). Therefore, we characterized our newly established GC-C reporter cell line by stimulation using guanylin, uroguanylin and *E. coli* heat-stable enterotoxin (STa). As shown in Fig. 5B, the three peptides fully stimulated the reporter cell line, however, with different potencies. The heat-stable enterotoxin STa stimulated luminescence reponses with the highest potency ($pEC_{50} = 9.59 \pm 0.04$), followed by uroguanylin ($pEC_{50} = 8.74 \pm 0.07$) and guanylin ($pEC_{50} = 7.87 \pm 0.05$). We also tested the ligand selectivity of the GC-C receptor by application of ANP, BNP and CNP. However, none of the three natriuretic peptides was able to stimulate significant luminescence reponses when tested up to 10^{-6} M (data not shown).

3.6. Characterization of peptides derived from the atrial natriuretic peptide prohormone.

Besides ANP, three additional distinct peptides are derived from the ANP prohormone (proANP): long-acting natriuretic peptide (LANP), vessel dilator (VDL) and kaliuretic peptide (KP). These peptides were previously shown to possess potent cardiovascular activities (Vesely, 2007). In addition, all three peptides are able to stimulate intracellular cGMP synthesis by particulate guanylyl cyclase activation (Vesely et al., 1987a, 1987b).

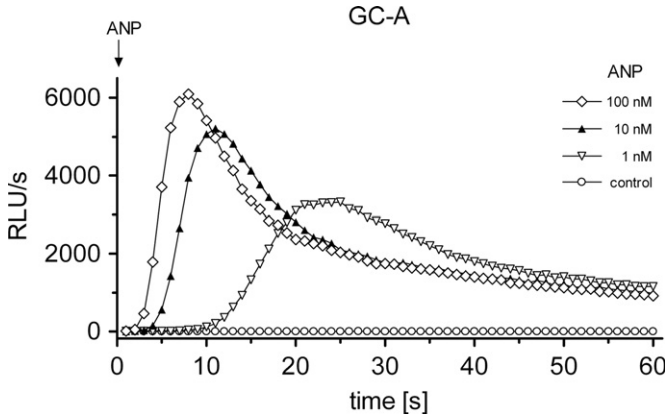

Fig. 6. Real-time detection of intracellular cGMP accumulation. Kinetics of the luminescence signals generated by ANP on the GC-A reporter cell line in the presence of 2 mM calcium ions. Cells were stimulated with 0 nM (o), 1 nM (▽), 10 nM (▲), and 100 nM (◇) ANP.

Therefore, we characterized the activity of LANP, VDL and KP on our GC-A, GC-B and GC-C reporter cell lines. However, none of these peptides was able to significantly stimulate luminescence signals when tested up to 10^{-6} M (data not shown).

3.7. Real-time detection of cGMP synthesis

To monitor intracellular cGMP accumulation in real-time, increasing concentrations of ANP (1–100 nM) were added to the GC-A reporter cell line in the presence of 2 mM calcium ions ("kinetic mode"; Wunder et al., 2005a). Luminescence measurements were started immediately before agonist addition. As shown in Fig. 6, ANP stimulated concentration-dependent luminescence signals on the GC-A receptor cell line with transient signal kinetics. Lag times in the range of 9 s (at 0.01 nM) to 2 s (at 100 nM) were observed. At the highest ANP concentration of 100 nM, maximum luminescence signals were reached after 8 s.

4. Discussion

We have shown previously that reporter cell lines expressing the homomeric, cGMP-sensitive CNGA2 channel and the calcium-sensitive photoprotein aequorin are well suited for the identification and characterization of modulators of the NO/cGMP signaling pathway. Using this cGMP reporter assay platform, we have identified and characterized sGC activators and stimulators, PDE inhibitors and modulators of NO synthesis (Wunder et al., 2005a, 2005b, 2007, 2009; Mittendorf et al., 2009). In this report, we describe an additional application of the cGMP reporter assay: the functional expression and pharmacological characterization of membrane-bound guanylyl cyclases.

In recent years, receptor guanylyl cyclases have been implicated in the pathophysiology of a growing number of indications and, therefore, have become important targets for new drug development. As a consequence, functional assays to monitor pGC activity which can be used for drug discovery are highly appreciated. However, conventional cGMP RIA and ELISA assays do not provide the necessary throughput. Only few assay formats suitable for HTS applications are currently available, namely ALPHAscreen (Perkin Elmer, Waltham, MA), the HitHunter cGMP assay (DiscoveRx, Freemont, CA), and a cGMP HTRF assay (Cisbio, Gif-sur-Yvette, France). However, these assays include cell lysis and do not allow real-time measurements of receptor activation in living cells. Further drawbacks are low signal-to-background windows, requirement for special plate readers and high costs. Our novel pGC reporter system provides an alternative to these assay formats. Receptor guanylyl cyclase activity is detected with very high sensitivity in our stable reporter cell lines. In addition, in contrast to other assay formats, high signal-to-background ratios (>100) are consistently observed and receptor activation can be monitored in real-time . As a drawback, agents that are capable of unspecifically inducing increases in intracellular calcium concentration will cause an increase in aequorin luminescence. However, these agents are likely to show similar effects on the parental cGMP reporter cell line and, thereby, can be identified.

In the literature, EC_{50} values in the range of 0.4–100 nM for the activation of GC-A by ANP or of GC-B by CNP, and of 150–300 nM for STa-mediated GC-C activation have been reported. These results were obtained by classical cGMP RIA measurements (Schulz et al., 1990; de Sauvage et al., 1991; Lowe and Fendly, 1992; Ohyama et al., 1992; Suga et al., 1992; Li et al., 1995; Schoenfeld et al., 1995; Dickey et al., 2008). Using our novel pGC reporter cell lines, corresponding EC_{50} values for GC-A and GC-B activation of ~0.02–0.04 nM and for GC-C activation of ~0.2 nM were determined. Therefore, the sensitivity of our reporter assay is at least 10 to 100-fold higher compared to cGMP RIA measurements with respect to the detection of pGC activity. The unexpectedly high sensitivity of this novel assay format is likely due to the direct plasma membrane colocalization of the membrane-bound guanylyl cyclases with the CNG channel used as the cGMP biosensor. The high assay sensitivity might also be due to signal amplification caused by calcium influx through the CNG channel and to cGMP compartmentation. As has been shown in cardiomyocytes, cGMP synthesized by the ANP receptor is located in subsarcolemmal microdomains and shows only limited diffusion into the cytosol (Castro et al., 2006; Fischmeister et al., 2006). In addition, due to the necessary cell lysis step, conventional cGMP assays only allow the determination of total intracellular cGMP, which impaires the detection of pGC activity.

Using our novel GC-A and GC-B reporter cell lines, we could show that ANP, BNP and CNP significantly stimulate GC-A as well as GC-B receptors. This is an important finding, since the significance of activation of GC-A by CNP and of GC-B by ANP and BNP is under controversial discussion in the literature (Koller et al., 1991; Ohyama et al., 1992; Suga et al., 1992; Schoenfeld et al., 1995; Dickey et al., 2008; Woodard and Rosado, 2008). However, since in these studies receptor stimulation has been determined by RIA measurements, the observed discrepancies are likely due to the much lower sensitivity of the RIA assay for the detection of pGC activity. In addition, differences in receptor activation may also be observed when using receptors from different species (Dickey et al., 2008).

Stimulation of GC-A by CNP was further corroborated by using a cGMP enzyme immunoassay. We could show that CNP significantly stimulated cGMP synthesis in the GC-A reporter cell line. CNP stimulation resulted in similar maximal cGMP levels compared to stimulation by ANP and BNP. Therefore, by luminescence measurements and cGMP determination we could clearly show that CNP is a full agonist of rat GC-A . A similar finding has recently been published (Dickey et al., 2008).

We also characterized the activity of the commercially available synthetic receptor ligands mini-ANP, Cys-ANF(4-18) and Arg-ANF(6-18), which have been described as full ANP receptor agonist, clearance receptor ligand and ANP receptor antagonist, respectively (Maack et al., 1987; von Geldern et al., 1990; Li et al., 1995). As expected, mini-ANP was characterized as a potent GC-A agonist. Unexpectedly, Cys-ANF(4-18) and Arg-ANF(6-18) could be characterized as partial ANP receptor agonists. Whereas Cys-ANF(4-18) nearly fully stimulated the GC-A reporter cell line, Arg-ANF(6-18) showed rather low efficacy. Therefore, the observed

biological effects of Cys-ANF(4-18) might be due to a combined effect related to clearance receptor binding and direct ANP receptor stimulation.

Recently, different ligands have been proposed as GC-D agonists, namely guanylin and uroguanylin (Leinders-Zufall et al., 2007), uroguanylin but not guanylin (Duda and Sharma, 2008) and bicarbonate ions (Hu et al., 2007; Sun et al., 2009). Up to now, the proposed GC-D ligands are controversely discussed and should be confirmed independently. In this respect, our novel pGC reporter assay is an ideal tool to verify the putative GC-D ligands. However, we have not been able to show significant stimulation of GC-D by guanylin, uroguanylin or bicarbonate ions (F. Wunder, unpublished data).

In summary, the results imply that our pGC reporter cell lines are well suited for the characterization of receptor pharmacology. Like our previously described cell-based sGC, nitric oxide and PDE reporter assays, the receptor guanylyl cyclase assays described in this report were also successfully transferred to the 1536-well MTP format (data not shown). This opens up the possibility to identify novel pGC ligands and modulators by uHTS (Wunder et al., 2005a, 2005b, 2007, 2009). This novel reporter system may, therefore, be used for drug discovery and for natural ligand characterization of guanylyl cyclase orphan receptors like GC-D and GC-G .

Disclosure statement

A patent application for the cGMP reporter assay has been filed. Cell lines are available to the academic community upon request.

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