Development of a Cyclic Adenosine Monophosphate Assay for G_i-Coupled G Protein-Coupled Receptors by Utilizing the Endogenous Calcitonin Activity in Chinese Hamster Ovary Cells

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ABSTRACT

Activation of G_i-coupled G protein-coupled receptor (GPCRs) by their ligands leads to inhibition of adenylyl cyclase (AC) and reduction of cyclic adenosine monophosphate (cAMP) levels in cells. The traditional cAMP assay for G_i-coupled GPCRs commonly uses forskolin, a nonspecific AC activator, to increase the basal cAMP level in cells to create an assay window for ligand detection. However, there is still a need to develop a nonforskolin-based cAMP assay because of the challenges inherent in titrating the concentration of forskolin to achieve a reliable assay window, along with issues related to the cAMP-independent effects of forskolin. Herein, we describe such an assay by utilizing the endogenous activity of the calcitonin receptor in Chinese hamster ovary (CHO) cells. The calcitonin receptor is a G_s-coupled GPCR that, when activated by calcitonin, leads to the stimulation of AC and increases cAMP in cells. Thus, we use calcitonin, instead of forskolin, to increase the basal cAMP level in CHO cells to achieve an assay window. We demonstrated that calcitonin peptides robustly increased cAMP accumulation in several CHO cell lines stably expressing well-known G_i-coupled GPCRs, such as the Dopamine D2 receptor, the Opioid μ receptor, or the Cannabinoid receptor-1. Agonists of these G_i-coupled GPCRs attenuated calcitonin-induced cAMP production in their receptor stable cell lines. On the other hand, antagonists and/or inverse agonists blocked the effects of their agonists on calcitonin-induced cAMP production. This calcitonin-based cAMP assay has been demonstrated to be sensitive and robust and exhibited acceptable assay windows (signal/noise ratio) and, thus, can be applied to screen for agonists and antagonists/inverse agonists of G_i-coupled GPCRs in high-throughput screening formats.

INTRODUCTION

G protein-coupled receptors (GPCRs) are a large group of membrane proteins that transduce various extracellular stimuli to intracellular signals and regulate many physiological functions. The binding of the extracellular ligand to the receptor changes its conformation so as to permit coupling with the heterotrimeric $G_{\alpha\beta\gamma}$ proteins, thereby catalyzing the exchange of GTP for GDP on the G_α subunits, and dissociation of G_α from $G_{\beta\gamma}$ subunits. Both the G_α subunit and $G_{\beta\gamma}$ subunits modulate the activity of downstream effectors, such as adenylyl cyclase (AC), phospholipases, small GTP-binding proteins, and ion channels.[1]

Cyclic adenosine monophosphate (cAMP) is one of the most important cellular second messengers mediating diverse physiological responses of extracellular neurotransmitters and hormones through GPCRs.[2] cAMP is formed from ATP processed by the membrane-bound enzyme, AC. The regulation of AC activity by GPCRs is mediated primarily through two distinct GTP binding proteins, G_s and G_i, for their abilities to stimulate or inhibit AC, respectively.[3,4] Ligands binding to G_s-coupled GPCRs lead to activation of AC and thereby cause an increase in intracellular cAMP. By comparison, the activation of G_i-coupled GPCRs, therefore, leads to the inhibition of AC and decreases cAMP production. This close relationship between receptor stimulation or inhibition and cellular cAMP production led to the development of methods that measure the effects of ligands on receptor activation by quantifying intracellular cAMP levels.[5,6]

Several homogenous assay platforms for cAMP measurement in whole cells or for AC activity in membrane are available.[7,8] Screening G_s-coupled receptors is generally straightforward by measuring the cAMP increase in cells, whereas screening G_i-coupled receptors in the cAMP assay can be considerably more difficult primarily due to the low basal cAMP level in cells. It is often necessary to artificially elevate the cAMP levels in cells with an AC activator, such as forskolin,[9] to maximize the inhibition signal elicited by ligands that activate G_i-coupled GPCRs. However, there are several limitations of using forskolin in the cAMP assay. It is usually a technical challenge

ABBREVIATIONS: AC, adenylyl cyclase; AMY, amylin; cAMP, cyclic adenosine monophosphate; CB1, cannabinoid receptor-1; CGRP, Calcitonin gene-related peptide; CHO, Chinese hamster ovary; CTAP, D-Phe-Cys-Tyr-D-Trp-Arg-Thr-Pen-Thr-NH2; CTR, calcitonin receptor; DAMGO, [D-Ala*,N-Me-Phe4-Gly5-ol]-enkephalin; DMSO, dimethyl sulfoxide; GPCR, G protein-coupled receptor; hCT, human calcitonin; HTRF, homogeneous time-resolved fluorescence; HTS, high throughput screening; IBMX, 3-isobutyl-1-methylxanthine; PEI, polyethyleneimine; PTX, pertusis toxin; sCT, salmon calcitonin.

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to determine the optimal concentration of forskolin in G_i-coupled GPCR cAMP assays for ligand screening. Inappropriate titration of forskolin in the cAMP assay could lead to small assay windows and variable ligand activities. Additionally, it has been demonstrated that forskolin can elicit both cAMP-dependent and cAMP-independent effects in many cells.[10] Forskolin has been shown to interact with certain proteins other than AC, including glucose transporters and ion channels.[10]

Therefore, there is a need to develop a nonforskolin cAMP assay for G_i-coupled GPCRs. In this study, we provide such an alternative method to screen ligands of G_i-coupled GPCRs in Chinese hamster ovary (CHO) cells without using forskolin. We utilized a common cell model, the CHO cells, which endogenously express the calcitonin receptor (CTR). The CTR belongs to a G_s-coupled class B GPCR.[11] It has been demonstrated that CTR, upon activation by its ligands such as calcitonin, stimulates AC and increases cAMP amounts in cells.[12–15] We used calcitonin peptide, instead of forskolin, to enhance the amount of cAMP in CHO cells to achieve assay windows for screening G_i-coupled GPCR ligands. We found that calcitonin peptides robustly increased cAMP accumulation in CHO-K1 cells, as well as several CHO stable cell lines expressing well-known G_i-coupled GPCRs such as the Dopamine D2 receptor, the Opioid μ receptor, and the Cannabinoid receptor-1. The selective agonists of the G_i-coupled GPCRs attenuated calcitonin-induced cAMP production in their respective receptor cell lines. On the other hand, their antagonists and/or inverse agonists blocked or reversed the effect of their agonists on calcitonin-induced cAMP production. This calcitonin-based cAMP method, therefore, can be applied to the screening of the agonists, antagonists, and/or inverse agonists of G_i-coupled GPCRs.

MATERIALS AND METHODS

Materials and Cell Lines

Synthetic salmon calcitonin (sCT), human calcitonin (hCT), rat amylin (rAMY), human CGRPα, and AC-66 peptides were purchased from Bachem (Torrance, CA). All other compounds used in the study, including [D-Ala*,N-Me-Phe4-GIy5-ol]-enkephalin (DAMGO), Naloxone hydrochloride, CTAP, (−)-Quinpirole hydrochloride, Nemonapride, Eticlopride hydrochloride, AM251, and AM281, were purchased from Tocris (Ellisville, MI). ^{125}I-salmon calcitonin was obtained from Perkin Elmer Life Sciences (Boston, MA). The specific activities of ^{125}I-sCT were ∼2,000 Ci/mmol. All other chemicals, including bacitracin, Bestatin, phosphoramidon, and isobutylmethylzanthine (IBMX), were purchased from Sigma Chemical (St. Louis, MO). All cell culture medium and reagents were obtained from Invitrogen (Carlsbad, CA).

Cell Lines and Cell Culture

CHO-K1 cells were purchased from ATCC (Manassas, VA) and cultured in F12 medium with 10% fetal bovine serum and 1×Penn/Strep. The Human CB1 Receptor CHO stable cell line (CHO-CB1), the human opioid μ receptor CHO stable cell line (CHO-μ), and the D2 dopamine receptor CHO stable cell line (CHO-D2) were the properties of Bristol-Myers Squibb Company (BMS) and kindly provided by the Lead Evaluation Group in BMS. All stable cell lines were cultured in F12 medium with 10% fetal bovine serum and 1×Penn/Strep, with 200 μg/mL G418.

cAMP HTRF® Assay

The cAMP assays were conducted by using a HTRF® cAMP assay kit from Cisbio (Bedford, MA). Cells were grown in medium over night in 96-well plates (∼10,000 cells/well). The medium was removed from the wells on the second day and replaced with the assay buffer (HBSS buffer, 2.5 mM HEPES, pH 7.5, 100 μM IBMX). The cells were then incubated with peptides or compounds for 30 min at 37°C. Then, the buffer in the wells was removed and replaced with 40 μL cell lysis buffer provided in the kit. After a 10 min of incubation, 10 μL of cell lysate from each well was transferred into a 384-well solid black assay plate (Perkin Elmer Life Sciences). Five microliters of diluted cAMP-d2 and 5 μL of diluted anti-cAMP cryptate conjugate were added into each well. The assay plate was incubated at room temperature for 30 min with gentle shaking. The fluorescence ratios (665 nm/620 nm) were counted in an EnVision™ 2103 Multilabel Reader (Perkin Elmer, Covina, CA). The cAMP concentrations were calculated based on a cAMP standard curve.

For automation of a cAMP assay in 384-well plate format, 100 nL of DMSO, test compounds, or salmon calcitonin stock was added to the wells of assay plate (ProxiPlate™-384 from Perkin Elmer Life Sciences) by a BioCel 1600 from Agilent Technologies, Inc. (Santa Clara, CA). Then, 5 μL of cells (2×10^6 cells/mL) suspended in PBS buffer with 100 μM IBMX was added into the wells by a Thermo Multidrop Combi (Waltham, MA). The plates were covered and incubated for 30 min at room temperature; 5 μL/well of D2-conjugate and 5 μL/well of anti-cAMP Cryptate in HTRF lysis buffer provided by a HTRF cAMP dynamic 2 Jumbo kit from Cisbio were added sequentially by Thermo Multidrop Combi. After incubation for 1 h at room temperature, the fluorescence ratios (665 nm/620 nm) from the assay plates were measured on an EnVision 2103 Multilabel Reader (Perkin Elmer).

Receptor Binding Assay

For cell membranes preparation, cells were collected and lysed in ice-cold buffer containing 20 mM Tris-HCL (pH 7.5), 1 mM EDTA and 1× protease inhibitor cocktails (1 tablet/50 mL) (Roche Applied Science, Indianapolis, IN) and homogenized using a tissue homogenizer. After a low-speed centrifugation ($300\times g$ for 5 min), the supernatant was collected and centrifuged again at $38{,}000\times g$ for 30 min. The pellet was subsequently resuspended by passing through a 26G needle in buffer containing 50 mM Tris-HCL (pH 7.5), 2.5 mM EDTA, and 5 mM MgCl2, and stored at −80°C in aliquots after protein measurement using the Coomassie-Plus Protein Assay Reagent (Pierce, Rockford, IL)

The receptor radioligand binding assays were conducted in 100 μL binding buffer (25 mM HEPES, pH 7.4, 5 mM EDTA, 1 mM MgCL2, 0.1% BSA, and 1 μg/mL leupeptin in a 96-well plate). CHO-K1 cell membrane fractions were resuspended in the binding buffer to a final concentration of 25 μg protein/well and mixed with a range of

concentrations of ^{125}I-sCT from 0 to 250 pM. For nonspecific binding detection, a final concentration of 100 nM cold sCT was included in wells. After 2 h of incubation at 37°C, the binding reactions were stopped by filtration through GF/B plates that were presoaked with 0.3% PEI in a Unifilter®-96 Cell Harvester (PerkinElmer, Shelton, CT). After three washes with cold PBS, the plates were dried in air for 2 h at room temperature. After addition of 30 μL MicroScint 20 scintillation fluid to all wells, the plates were sealed and the radioactivity was counted by a TopCount® NXT high throughput screening (HTS) (PerkinElmer) for 1 min.

Data Analysis and Calculation of Z′ Values

Data from *in vitro* receptor binding and cAMP assays were analyzed by nonlinear regression using the PRISM® software (version 5; Graphpad, San Diego, CA).

For calculating the Z′ value, which is used as a measure of the assay quality and its suitability for HTS,[42] the assay was performed as described above, using assay buffer without salmon calcitonin (sCT) as negative control, in multiple 384-well plates. The Z′ factor was calculated according to the following equation:

$$Z' = 1 - \frac{(3\sigma_{c+} + 3\sigma_{c-})}{|\mu_{c+} - \mu_{c-}|}$$

where σ_{c+} and σ_{c-} are standard deviations of positive and negative controls, and μ_{c+} and μ_{c-} are means of positive and negative controls, respectively.

RESULTS

Demonstration of Endogenous CTR in CHO Cells by cAMP Stimulation and Receptor Binding Activities

CHO cells are one of the most widely applied mammalian cells in the study of the GPCR functions and drug discovery due to their easy transfection and culture, high expression of proteins with accurate post-translational modifications, and validated G protein signal pathways.[16,17] The endogenous expression of CTR in CHO cells has been observed from both receptor binding analysis and cAMP responses to CTR ligand peptides.[12–15]

To further demonstrate the endogenous activity of functional CTR in CHO cells, we tested the effects of several CTR agonist peptides on cAMP production in CHO-K1 cells. Cells were incubated individually with salmon calcitonin (sCT), human calcitonin (hCT), rat amylin (rAMY), or human CGRP (hCGRP) for 30 min in the presence of 100 μM IBMX, a phosphodiesterase inhibitor. The whole cell cAMP accumulation was then measured by a commercial cAMP assay kit. As shown in *Figure 1A*, all four peptides dose-dependently stimulated cAMP production in CHO-K1 cells. Salmon calcitonin exhibited an EC_{50} of 0.114 nM, which was ~100-fold more potent than its human counterpart (EC_{50}: 1.79 nM) and ~3,000-fold more potent than the human amylin (EC_{50}: 372.7 nM) and hCGRP (EC_{50}: 780.3 nM). The rank order of potencies of these peptides suggests that the cAMP response in CHO-K1 cell were primarily mediated by the CTR, which was defined by the high affinity for calcitonin peptides

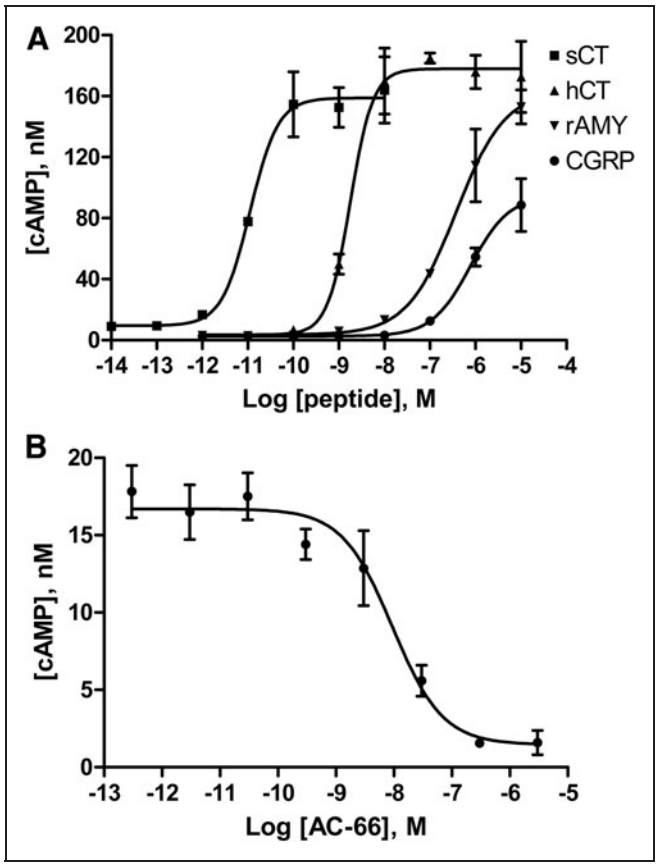


Fig. 1. Cyclic adenosine monophosphate (cAMP) production in Chinese hamster ovary (CHO)-K1 cells. **(A)** Dose–response of four calcitonin receptor agonist peptides. cAMP concentrations were measured after cells were treated with indicated doses of salmon calcitonin (sCT), human calcitonin (hCT), rat amylin (rAMY), or human CGRP (hCGRP) for 30 min in the presence of 100 μM IBMX. The EC_{50} values are calculated to be approximately 0.114 nM for sCT, 1.79 nM for hCT, 372 nM for rAMY, and 780 nM for hCGRP. **(B)** Inhibition of sCT-induced cAMP production by a calcitonin receptor antagonist AC66. Cells were preincubated with indicated doses of AC66 for 30 min before addition of 0.5 nM of sCT to the cell medium. The whole cell cAMP accumulation was measured after 30-min incubation at 37°C. The IC_{50} value of AC66 was approximately 9.3 nM. Data shown represent the mean ± SEM of triplicate wells ($n = 3$) from at least two individual experiments.

and relatively low affinity for amylin and CGRP.[11,14,15,18] Consistent with this observation, AC66, a specific antagonist of the CTR, was able to block sCT-induced cAMP accumulation (*Fig. 1B*). Further, a specific binding activity of ^{125}I-sCT was detected with an observed Kd value of 0.179 nM for the CHO-K1 cell membranes in the receptor binding studies (*Fig. 2*). The CTR density on the CHO-K1 cell membrane was estimated to be 27.6 fmol/mg. Additionally, the binding activity of ^{125}I-sCT was blocked by the CTR antagonist AC66 (data not shown). These results further demonstrate the presence of endogenous functional CTR in CHO-K1 cells.

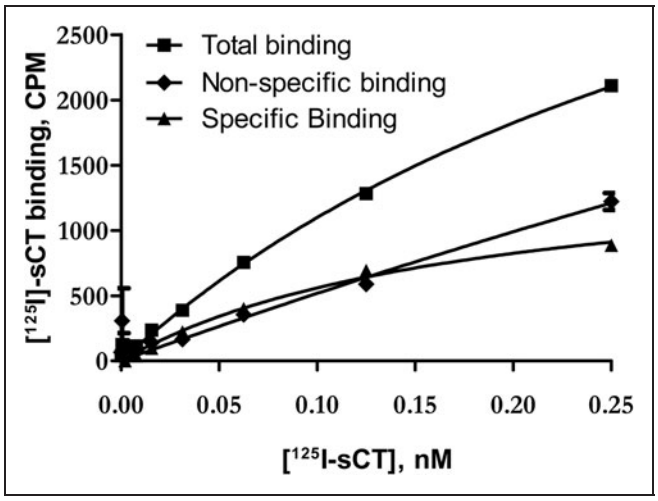


Fig. 2. Specific binding of ^{125}I-salmon calcitonin (sCT) on the CHO-K1 cell membrane. CHO-K1 cell membranes at 25 μg protein/well were incubated with a range of concentrations of ^{125}I-sCT for 2 h at 37°C in the absence of cold sCT (Total binding), and in the presence of 100 nM cold sCT (nonspecific binding). The specific binding of ^{125}I-sCT (▲) was calculated by subtracting the nonspecific 125I-sCT binding (◆) from the total binding (■). Data were analyzed by nonlinear regression using the PRISM® software (version 5; Graphpad, San Diego, CA). In all experiments, each data point shown represents the mean ± SEM of triplicate wells ($n = 3$) from at least two individual experiments.

Effects of Agonists and Antagonists of the Opioid μ Receptor on sCT-Induced cAMP Production in CHO Cells

To explore a cAMP assay for G_i-coupled GPCRs using calcitonin instead of forskolin, we assessed the effect of sCT on cAMP accumulation in a CHO cell line stably expressing the opioid μ receptor (CHO-μ cells). The opioid μ receptor has been well demonstrated to couple to the G_i protein family and inhibit AC upon activation by its ligands.[19–22] As shown in *Figure 3A*, sCT dose-dependently increased cAMP production in the CHO-μ cells, with an EC_{50} value of 29.6 pM. The sCT-induced cAMP responses were robust and the maximal ratio of signal/noise was over 15-fold. We further examined if the sCT-induced cAMP production in this cell line could be inhibited by the μ-receptor selective agonist DAMGO. The CHO-μ cells were treated with a range of DAMGO doses for 10 min, and then sCT at a final concentration of 500 pM was added to the cells and cAMP amounts in

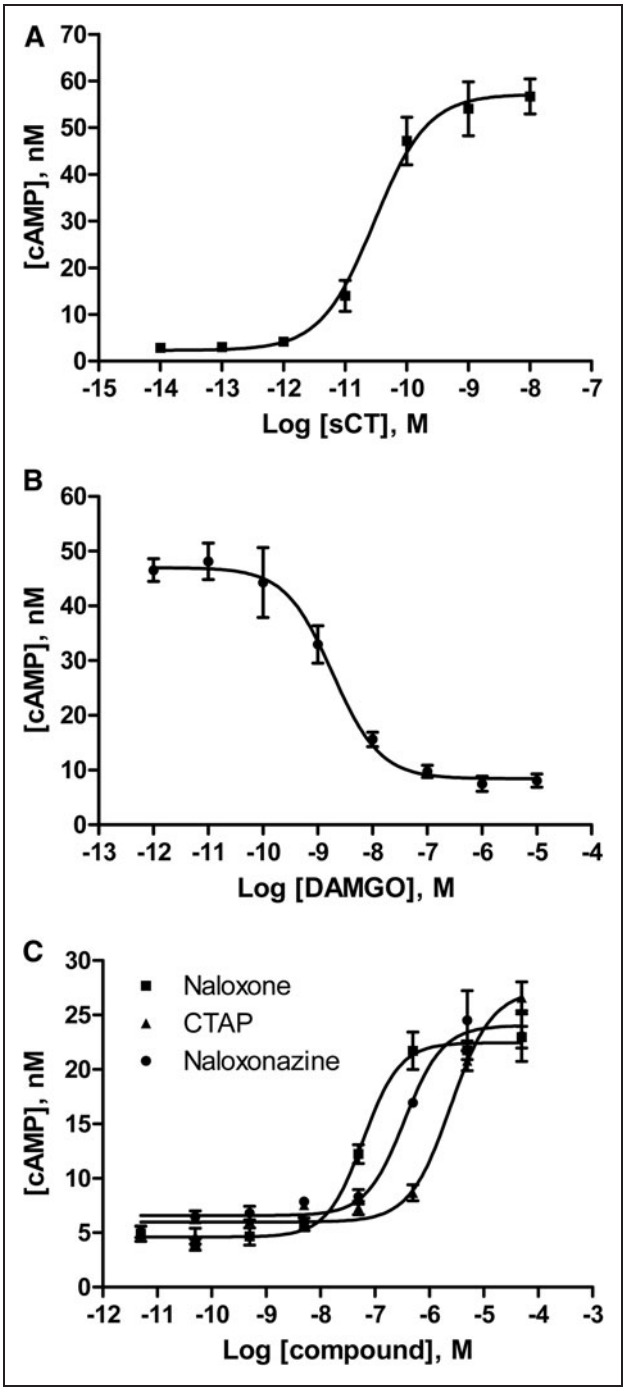
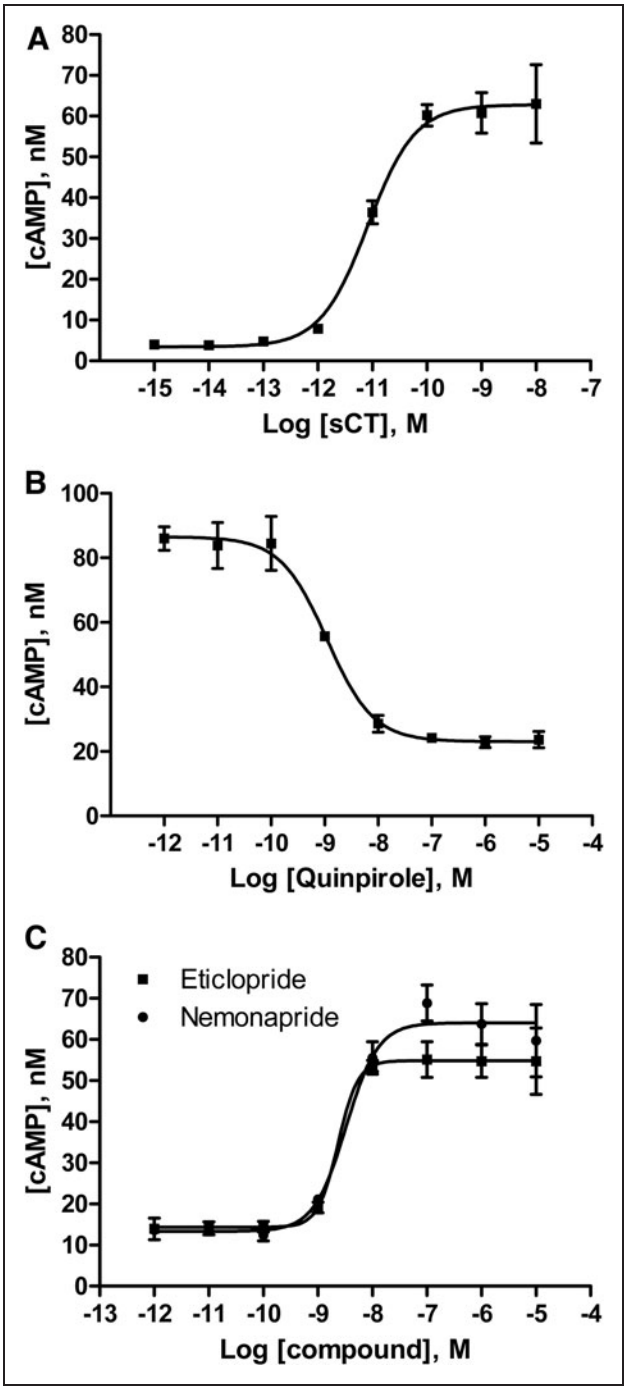


Fig. 3. cAMP production in CHO-μ cells. **(A)** Dose–response of salmon calcitonin (sCT). cAMP concentrations were measured after the cells were incubated with the indicated doses of sCT for 30 min in the presence of 100 μM IBMX. The EC_{50} of sCT was calculated to be approximately 29.6 pM. **(B)** Dose-dependent inhibition of sCT-induced cAMP production by [D-Ala*,N-Me-Phe4-Gly5-ol]-enkephalin (DAMGO). The CHO-μ cells were first treated with the indicated doses of DAMGO for 10 min, and then sCT at 500 pM was added to the cell medium. cAMP amounts in cells were measured after a 30-min incubation at 37°C. The EC_{50} value of DAMGO was approximately 1.86 nM. **(C)** Dose-dependent inhibition of DAMGO activity by the μ receptor antagonists Naloxone, CTAP, and Naloxonzine. CHO-μ cells were first incubated with 100 nM of DAMGO and an indicated antagonist for 30 min at 37°C. The cAMP amounts in cells were measured after an additional 30-min treatment with 500 pM of sCT. The IC_{50} values of Naloxone, CTAP, and Naloxonzine were approximately 61.1 nM, 2.47 μM, and 349 nM, respectively. In all experiments, each data point shown represents the mean ± SEM of triplicate wells ($n = 3$) from at least two individual experiments.

cells were measured after a 30 min incubation at 37°C. The results indicated that DAMGO dose-dependently inhibited sCT-induced cAMP production in the CHO-μ cells, with an EC_{50} value around 1.86 nM (*Fig. 3B*), which were in line with published data.[23–26]

To further explore the utility of calcitonin-based cAMP to screen antagonists of G_i-coupled GPCRs, we assessed the activities of several well-known μ receptor antagonists such as Naloxone, Naloxonzine, and CTAP in CHO-μ cells.[27] CHO-μ cells were coincubated with DAMGO and an antagonist for 30 min at 37°C. The cAMP amounts in cells were measured after an additional 30 min incubation with 500 pM of sCT. As shown in *Figure 3C*, Naloxone, Naloxonzine, and CTAP dose-dependently reduced the DAMGO inhibition on sCT-induced cAMP production. The IC_{50} values of Naloxone, Naloxonzine, and CTAP in this experiment were 61 nM, 349 nM, and 2.47 μM, respectively. These results suggested that the calcitonin-based cAMP assay could be applied to screen both agonists and antagonists of opioid μ receptor in CHO cells.



Effects of Agonists and Antagonists of the Dopamine D2 Receptor on sCT-Induced cAMP Production in CHO Cells

We further applied the sCT-based cAMP assay in another well-known G_i-coupled GPCR, the dopamine D2 receptor.[28,29] We first determined the effect of sCT in the cAMP assay in a CHO cell line stably expressing human dopamine D2 receptor (CHO-D2 cells). Results in *Figure 4A* showed that sCT elicited robust cAMP production in a dose-dependent manner in CHO-D2 cells, with an EC_{50} value of 8.2 pM. To further determine the inhibitory effect of the D2 receptor agonist quinpirole in sCT-induced cAMP production, the CHO-D2 cells were pretreated with multiple doses of quinpirole for 30 min, and then sCT at 500 pM was added to cells for an additional 30 min before whole cell cAMP amounts were measured. The results shown in *Figure 4B* indicated that quinpirole inhibited sCT-induced cAMP production with an EC_{50} value around 1.1 nM, which were in line with published values.[30,31] To detect the effects of D2 antagonists, we treated CHO-D2 cells with the D2 receptor antagonists (eticlopride and nemonapride) individually[32] in the presence of quinpirole (10 nM) for 30 min, and then cAMP production was measured followed by another 30-min incubation in the presence of sCT. As expected, both eticlopride and nemonapride dose-dependently decreased quinpirole's inhibition on sCT-induced cAMP production in the CHO-D2 cell line with IC_{50}s of 2.3 nM and 3.2 nM, respectively (*Fig. 4C*). These results provide another example of the calcitonin-

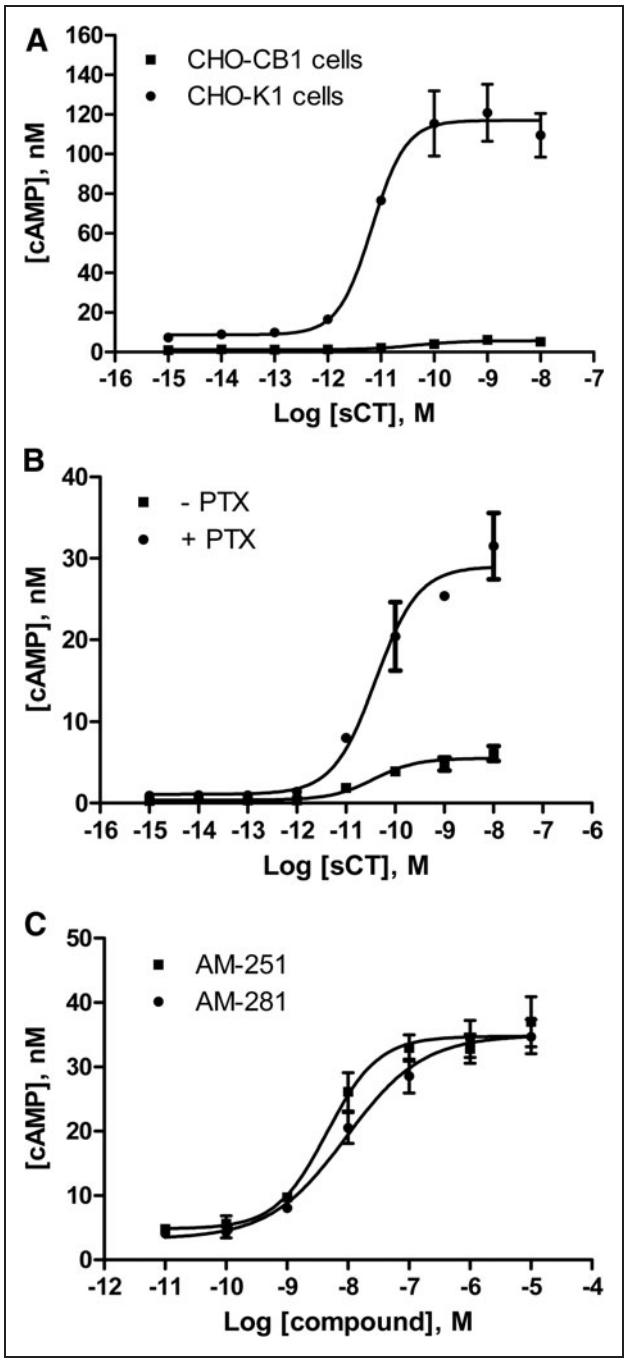
Fig. 4. cAMP production in CHO-D2 cells. **(A)** Dose–response of salmon calcitonin (sCT). cAMP concentrations were measured after cells were incubated with the indicated doses of sCT for 30 min in the presence of 100 μM IBMX. The EC_{50} of sCT was approximately 8.2 pM. **(B)** Dose-dependent inhibition of sCT-induced cAMP production by a D2 receptor agonist quinpirole. The CHO-D2 cells were first treated with the indicated doses of quinpirole for 10 min, and then sCT at 500 pM final concentration was added to the cell medium and cAMP amounts in cells was measured after a 30-min incubation at 37°C. The EC_{50} of quinpirole was approximately 1.1 nM. **(C)** Dose-dependent inhibition of quinpirole activities by D2 receptor antagonists eticlopride and nemonapride. CHO-D2 cells were incubated with 10 nM of quinpirole and indicated doses of an antagonist for 30 min at 37°C. The cAMP amounts in cells were measured after an additional 30-min incubation with 500 pM of sCT. The IC_{50} values of eticlopride and nemonapride were approximately 2.3 nM and 3.2 nM, respectively. In all experiments, each data point shown represents the mean ± SEM of triplicate wells ($n = 3$) from at least two individual experiments.

based cAMP assay to detect agonists and antagonists of a G_i-coupled GPCR.

Effects of Inverse Agonists of the CB1 Receptor on sCT-Induced cAMP Production in CHO Cells

Cannabinoid receptor 1 (CB1) has been shown to couple to G_i proteins upon the activation by its agonists.[33] It has been reported that overexpression of the human CB1 receptor in some cell types, including CHO, resulted in constitutive activation of the receptor.[34–36] Consistent with these observations, we found that the maximal sCT-induced cAMP production was much lower in the CHO-CB1 stable cell line than the CHO-K1 parental cells (5.7 nM vs 117 nM) (*Fig. 5A*). Overnight treatment with pertussis toxin (PTX), which specifically uncouples the interaction of the $G_{\alpha i}$ protein with its receptor,[37] resulted in ~5-fold increase of sCT-induced cAMP accumulation (from 5.5 nM to 29 nM) in the CHO-CB1 cells (*Fig. 5B*). These results suggested that CB1 receptors in our CHO-CB1 stable cell line were constitutively active to suppress cAMP production.

Since the CB1 receptors were constitutively active in the CHO-CB1 cell line we used, we investigated the effects of CB1 inverse agonists such as AM251 and AM281 in the sCT-induced cAMP assay.[38,39] As expected, treatment of CHO-CB1 cells with either AM-251 or AM-281 lead to dose-dependent increases in cAMP production in the presence of sCT (*Fig. 5C*). The EC_{50} values of AM-251 and AM-281 were determined as 4.5 nM and 9.3 nM, respectively, which were close to the values obtained in the forskolin-based cAMP assay (data not shown) and were also in line with the published values.[40,41] These results demonstrated that the calcitonin-based cAMP assay could be useful to screen inverse agonists of constitutive G_i-coupled GPCRs in CHO cells. However, this calcitonin-based cAMP assay may not be appropriate to detect CB1 agonists due to the receptor constitutive activity in the recombinant CHO cell line.



Assay Automation in a 384-Well Plate Format for High-Throughput Screening

We further adapted this assay in an automated HTS format using 384-well plates in CHO-μ cells as an example. For this purpose, suspension cells were used for the assay after cell density optimization. An independent sCT titration experiment suggested ~78 pM of EC_{50} for the peptide in this assay format (*Fig. 6A*). The uniformity and signal-to-noise ratio of the sCT-based cAMP assay was further de-

Fig. 5. cAMP production in CHO-CB1 cells. **(A)** Dose–response of sCT in CHO-CB1 cells and CHO-K1 parental cells. cAMP accumulation was measured after cells were incubated with the indicated doses of salmon calcitonin (sCT) for 30 min in the presence of 100 μM IBMX. sCT stimulated a robust cAMP production in CHO-K1 cells with an EC_{50} of approximately 6.7 pM, whereas sCT had only minor effect on cAMP production in CHO-CB1 cells. **(B)** Effect of PTX on sCT-induced cAMP accumulation in the CHO-CB1 cells. The cells were preincubated with 50 μg/mL of PTX overnight (~16 hrs). cAMP amounts were measured after a treatment of sCT for 30 min. PTX treatment increased cAMP production by ~6-fold in the presence of sCT. **(C)** Activities of CB1 receptor inverse agonists AM-251 and AM-281. CHO-CB1 cells were incubated with the indicated compounds for 30 min at 37°C. The cAMP amounts in cells were measured after an additional 30-min incubation with 500 pM of sCT. The IC_{50} values of AM-251 and AM-281 were approximately 4.5 nM and 9.3 nM, respectively. In all experiments, each data point shown represents the mean ± SEM of triplicate wells ($n = 3$) from at least two individual experiments.

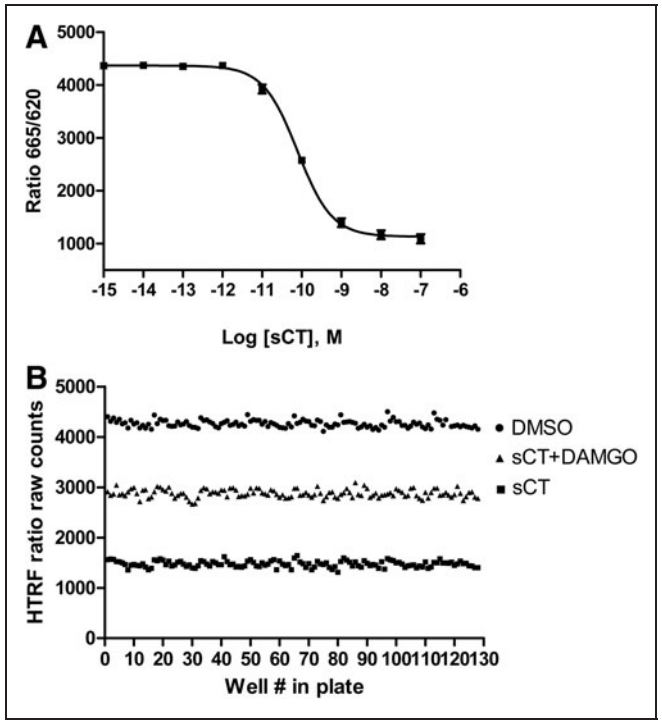


Fig. 6. Automation of sCT-based cAMP assay in CHO-μ cells in 384-well plate formats. **(A)** Dose–response of sCT-induced cAMP production represented as the HTRF ratio of 625 nm/660 nm. Each data point shown represents the mean ± SEM of triplicate wells ($n = 3$) from at least two individual experiments. The EC_{50} of sCT was approximately 78 pM. **(B)** Determination of the Z′ factor. Cells were treated with either DMSO (●) as negative control or background ($n = 128$), sCT at 100 nM, (■) as positive control or max signal ($n = 128$), or sCT (100 nM) plus DAMGO (10 nM) (▲) ($n = 128$). The plates were incubated for 30 min before the cAMP was measured by a HTRF cAMP assay. The Z′ factor was calculated to be 0.85 based on the formula given in the Materials and Methods section.

termined over multiple experiments in a 384-well format in CHO-μ cells. The Z′ factor,[42] which is a measure of the reproducibility and quality of the assay, was found to be 0.84 (*Fig. 6B*), indicating that the assay is ideal for application to HTS format and screening of compound libraries (*Table 1*).

Comparison of the sCT-Based and Forskolin-Based cAMP Assays in Automated Format

Since the forskolin-based cAMP assay has been well adapted in the HTS field to screen G_i-coupled GPCRs, we compared the sCT-based assay to the forskolin-based method in cAMP production in CHO-μ cells. As shown in the *Figure 7A*, sCT stimulated the dose-dependent accumulation of cAMP in CHO-μ cells, with an observed EC_{50} value of 0.55 nM. In contrast, forskolin strongly activated cAMP production in a dose-dependent mode as well. However, there was no obvious saturation of the cAMP response curve up to a 100 μM concentration of forskolin. Therefore, the EC_{50} values of forskolin could not be calculated from the experiment. We further compared

Table 1. Example High-Throughput Screening Assay Protocol Table

Step	Parameter	Value	Description
1	Compounds or DMSO	0.1 μL	Compounds in DMSO
2	sCT peptide	0.1 μL	100 nM stock in DMSO
3	Suspended cells	5 μL	2×10^6 cells/mL in PBS
4	Incubation time	0.5 h	Room temperature
5	D2 conjugate	5 μL	In lysis buffer
6	Anti-cAMP Cryptate	5 μL	In lysis buffer
7	Incubation time	1 h	Room temperature
8	Assay readout	665 and 620 nm	Fluorescence reader

Step Notes
1. Proxiplate-384 assay plate from Perkin Elmer.
2. Compounds and sCT peptide stock solution should be made in DMSO.
3. Cells are suspended in PBS buffer with 0.1 mM IBMX.
4. Cells are added by Thermo Multidrop Combi at high speed.
5. D2 conjugate and anti-cAMP Cryptate are dissolved in the lysis buffer from the HTRF cAMP dynamic 2 Jumbot kit from Cis-Bio.
6. The plates should be covered or lidded during the incubation.
7. The fluorescence ration (665 nm/620 nM) are counted in an EnVision 2103 Multilabel Reader.

cAMP, cyclic adenosine monophosphate.

the activities of the μ-receptor agonist DAMGO in the sCT-based assay (*Fig. 7B*) and forskolin-based assay (*Fig. 7C*). In this study, we used the 2 nM (~EC_{50}) or 10 nM (~EC_{90}) of sCT, and 1 μM or 10 μM of forskolin (doses commonly used in HTS). The results in the *Figure 7B* showed that DAMGO dose-dependently inhibited sCT-induced cAMP production in the CHO-μ cells. The EC_{50} values of DAMGO in the assay using cells stimulated with 2 nM or 10 nM of sCT were calculated to be 0.354 nM and 0.399 nM, respectively (*Fig. 7B*). In contrast, the EC_{50} values of DAMGO in the forskolin assay were 0.311 nM at 1 μM forskolin and 1.33 nM at 10 μM forskolin, a 4.2-fold difference (*Fig. 7C*).

DISCUSSION

The calcitonin-based cAMP assay described here represents an alternative nonforskolin-based cAMP assay for G_i-coupled GPCRs. This assay utilizes the endogenous G_s protein-coupled CTR in CHO cells to generate a cAMP assay window to detect the cAMP-lowering effects of G_i-coupled GPCR ligands. This assay has been examined in several well-known G_i-coupled GPCRs and demonstrated a robust and reproducible signal to screen agonists, antagonists, and/or inverse agonists of G_i-coupled GPCRs.

There are several unique properties of the sCT-based cAMP assay in comparison with the commonly used forskolin-based cAMP

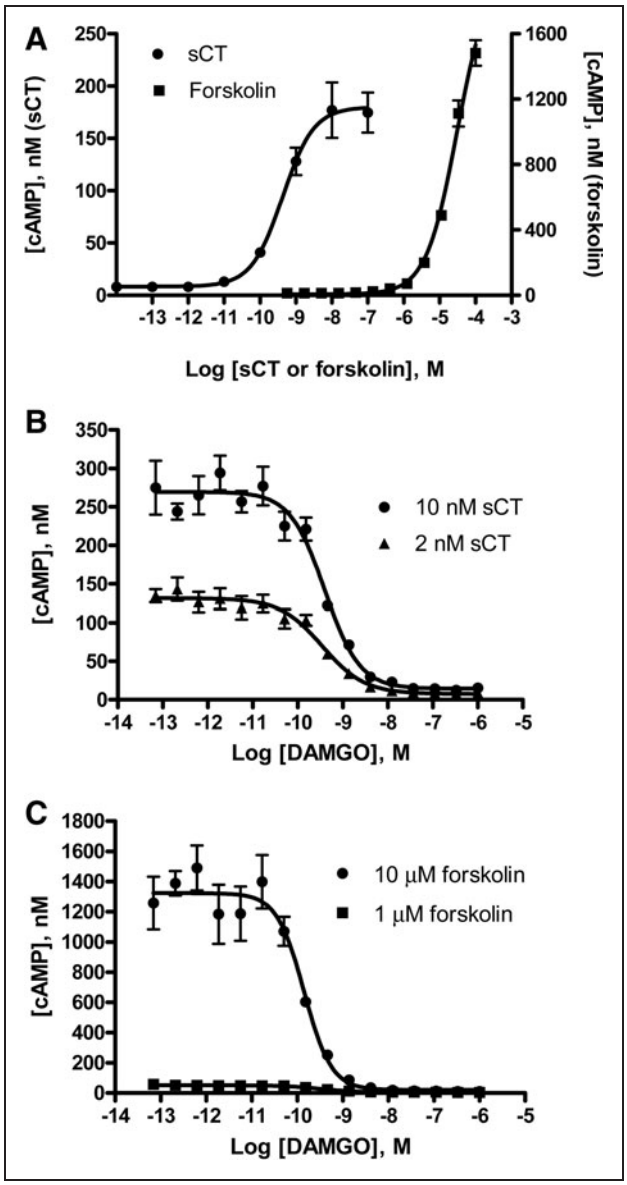


Fig. 7. Comparison of the sCT-based cAMP assay and the forskolin-based cAMP assay in CHO-μ cells in 384-well plate formats. **(A)** Dose–responses of sCT-induced and forskolin-induced cAMP production. The EC_{50} of sCT was approximately 0.55 nM. The EC_{50} of forskolin could not be calculated since no saturation of the response was achieved. **(B)** Dose-dependent inhibition of sCT-induced cAMP production by DAMGO. The CHO-μ cells were treated with the indicated doses of DAMGO with 2 nM or 10 nM of sCT for 30 min in room temperature (~25°C). cAMP amounts in cells were measured by a HTRF cAMP assay. The EC_{50} value of DAMGO was approximately 0.39 nM and 0.35 nM, respectively. **(C)** Dose-dependent inhibition of forskolin-induced cAMP production by DAMGO. The CHO-μ cells were treated with the indicated doses of DAMGO with 1 μM or 10 μM of forskolin for 30 min in room temperature (~25°C). cAMP amounts in cells were measured by a HTRF cAMP assay. The EC_{50} value of DAMGO was approximately 0.31 nM and 1.33 nM, respectively. Each data point shown represents the mean ± SEM of triplicate wells ($n = 3$) from at least two individual experiments.

method. We observed that the calcitonin-based cAMP assay generated more consistent, less variable EC_{50} values for ligands than the forskolin-based method. sCT, which activates G_s-coupled CTR, stimulated a moderate and dose-dependent cAMP production, which reaches a plateau at a sub-nM concentration of the peptide in CHO cells. Therefore, it is easy to titrate an optimal dose of sCT such as EC_{90} value for compound screening. We found that the sCT assay tolerates the use of either an EC_{50} or an EC_{90} dose of sCT for ligand screening. For example, DAMGO produced very similar EC_{50}s in activating the opioid μ-receptor by using either the EC_{50} or EC_{90} dose of sCT (*Fig. 7B*). However, different doses of sCT may generate different signal/noise ratios, which in turn could affect the Z′ factor in HTS. To achieve the best assay window and assay sensitivity, we recommend to use the EC_{90} dose of sCT for ligand screening. By contrast, forskolin, which is a strong AC activator, stimulates a much higher cAMP production than that of sCT in CHO cells. There was no obvious saturation of the cAMP response curve of forskolin up to 100 μM. Therefore, it is difficult to determine the optimal dose of forskolin for ligand screening. The different doses of forskolin used in the assay could generate significantly different EC_{50} values for Gi-GPCR ligands. For example, we found that DAMGO produced a ~4-fold difference in EC_{50} values in activating the opioid μ-receptor using 1 or 10 μM of forskolin (*Fig. 7C*). Therefore, it is more important to titrate an optimal dose for forskolin than sCT for ligand compound screening. Practically, sCT can be dissolved in either aqueous buffer solution or DMSO and thus is easy to operate in cAMP assays. By comparison, forskolin has very poor aqueous solubility and needs to be dissolved in organic solvents such as DMSO before applying to cells.

CTR is a member of the Class B GPCRs that use polypeptides as their endogenous ligands.[43] Previous HTS efforts to discover small-molecule ligands acting at family B GPCRs have met with frustration. There are a few reports so far describing the discovery of nonpeptide small molecules for the CTR.[44,45] Therefore, there could be a low incidence of false-positive small molecule compounds that directly modulate CTR activity in the sCT-based cAMP assay in HTS. Nevertheless, these potential false-positive compounds can be removed from the HTS hit list by counter screening with the CHO-k1 parental cells.

In conclusion, the calcitonin-based cAMP assay described here represents an alternative cAMP assay for G_i-coupled GPCRs. Our results suggest that the sCT-based assay seems to be a more robust and reproducible assay than the forskolin-based method in screening agonists, antagonists, and/or inverse agonists of Gi-GPCRs.

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AUTHOR DISCLOSURE STATEMENT

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