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Pharmacological and kinetic characterization of ADM1 and CGRP1 receptor reporter cell lines

Frank Wunder, Annegret Rebmann, Andreas Geerts, and Bernd Kalthof

Lead Discovery Wuppertal, Bayer HealthCare AG, Pharma Research Center, Aprather Weg 18a, D-42096 Wuppertal, Germany

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Running title: Characterization of ADM1 and CGRP1 receptor cell lines

Corresponding author:

Dr. Frank Wunder
Bayer HealthCare AG
Lead Discovery Wuppertal
Pharma Research Center
Aprather Weg 18a
D-42096 Wuppertal, Germany
PHONE: ++ 49-202-364107
FAX: ++ 49-202-364470
E-MAIL: frank.wunder@bayerhealthcare.com

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Abbreviations: ADM, adrenomedullin; cAMP, 3',5'-cyclic adenosine monophosphate; CNG, cyclic nucleotide-gated; CGRP, calcitonin gene-related peptide; CRLR, calcitonin receptor-like receptor; GPCR, G protein-coupled receptor; IBMX, 3-isobutyl-1-methylxanthine; IMD, intermedin; MTP, microtiter plate; RAMP, receptor-activity-modifying protein; RLU, relative light unit; uHTS, ultra-high-throughput screening

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Abstract

Adrenomedullin (ADM) and calcitonin gene-related peptide (CGRP) receptors and their respective ligands play important roles in cardiovascular (patho-)physiology. Functional expression of ADM and CGRP receptors requires the presence of the calcitonin receptor-like receptor (CRLR) together with receptor-activity-modifying proteins (RAMPs). We have characterized the expression patterns of CRLR and RAMP1-3 in human cardiovascular-related tissues by quantitative PCR. We could identify high expression levels of CRLR, RAMP1 and RAMP2 in human heart and various blood vessels. RAMP3 expression in these tissues was detectable, however, at significantly lower levels. In addition, we describe here a novel, aequorin luminescence-based GPCR reporter assay that enables the real-time detection of receptor activation in living cells. In the assay system, intracellular cAMP levels are monitored with high sensitivity by utilizing a modified, heteromultimeric cyclic nucleotide-gated (CNG) channel mediating calcium influx. Gq-coupled receptor activation is detected via aequorin luminescence stimulated by calcium release from intracellular stores. Using this novel reporter assay, we established and characterized stable ADM1 and CGRP1 receptor cell lines. The peptide ligands ADM, CGRP1 and CGRP2 were characterized as potent agonists at their respective receptors. In contrast, intermedin acted as a weak agonist on both receptors and showed only partial agonism on the ADM1 receptor. Agonist activities were effectively antagonized by the receptor antagonists ADM(22-52) and CGRP(8-37). Various vasoactive ADM fragments were also characterized but showed no activity on the ADM1 receptor cell line. In addition, luminescence signal kinetics after activation of Gs- and Gq-coupled receptors were found to be markedly different.

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Introduction

Adrenomedullin (ADM) and the calcitonin gene-related peptides (CGRP1 and CGRP2) are members of the calcitonin family of peptides (Muff *et al*., 1995; Wimalawansa, 1997). These peptides play a pivotal role in cardiovascular physiology and pathophysiology, and are involved in the regulation of the vascular tone, cardiac output, smooth muscle cell proliferation, and fluid as well as electrolyte homeostasis (Kurihara *et al*., 2003; Brain and Grant, 2004; Muff *et al*., 2004; Ishimitsu *et al*., 2006). Functional ADM and CGRP receptors are heterodimeric complexes and require co-expression of the calcitonin-receptor-like receptor (CRLR) together with associated receptor-activity-modifying proteins (RAMPs), which regulate CRLR transport to the plasma membrane and determine ligand specificity. Activation of these receptors leads to the stimulation of intracellular cAMP synthesis (McLatchie *et al*., 1998; Poyner *et al*., 2002; Conner *et al*., 2004). In recent years, ADM and CGRP, as well as their respective receptors have gained considerable attention and have become targets for new drug development (Doggrell, 2001; Ishimitsu *et al*., 2006). Recently, intermedin (IMD) was identified as a novel member of the calcitonin peptide family. IMD also shows cardiovascular activity, which might be related to activation of ADM and CGRP receptors (Roh *et al*., 2004).

Various functional assays to monitor GPCR activation and signaling have been developed and are used for the characterization of GPCR pharmacology and drug discovery (Williams, 2004; Jacoby *et al*., 2006; Kostenis, 2006). Recently, novel fluorescence-based assays using cyclic nucleotide-gated (CNG) channels as biosensors to detect intracellular cAMP levels and GPCR activity have been introduced (Rich *et al*., 2001; Fagan *et al*., 2001; Reinscheid *et al*., 2003). In these

reports, homomeric CNG channels were described as biosensors to monitor intracellular cAMP levels. However, native CNG channels usually form heterotetrameric complexes of two or three different types of subunits, and the ligand sensitivity and selectivity are determined by their particular subunit composition (Kaupp & Seifert, 2002). Native olfactory channels are comprised of three different subunits, CNGA2, CNGA4 and CNGB1b. Upon heterologous co-expression of these subunits, functional CNG channels with increased cAMP sensitivity are observed (Bradley *et al*., 1994; Liman and Buck, 1994; Sautter *et al*. 1998; Bönigk *et al.*, 1999). In addition, the ligand specificity and sensitivity can also be shifted by mutations in the cyclic nucleotide-binding domain. Thereby, a CNGA2 mutant, CNGA2(T537A), with increased cAMP and decreased cGMP sensitivity could be identified (Altenhofen *et al*., 1991).

We have shown previously that the homomeric, olfactory CNGA2 channel is well suited for the detection of intracellular 3',5'-cyclic guanosine monophosphate (cGMP) generation in an uHTS assay format (Wunder *et al*., 2005a). We describe in this report the development of a novel, highly sensitive, luminescence-based cAMP and GPCR reporter assay. To achieve optimal cAMP sensitivity, three different subunits, CNGA2(T537A), CNGA4 and CNGB1b, were co-expressed in an aequorin reporter cell line. In addition, we describe here the pharmacological and kinetic characterization of newly established ADM1 and CGRP1 receptor cell lines, using this novel reporter technology.

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Materials and Methods

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), Analytical Biological Services, Inc. (Wilmington, DE, USA), Clontech Laboratories (Mountain View, CA, USA) and Stratagene (La Jolla, CA, USA) and were reverse transcribed using random hexamers. Probes were carefully designed to cross exon boundaries and comparable probe efficencies were assured by titration of corresponding plasmid constructs. Normalization was performed using β-actin as control, and relative expression was calculated using the formula: relative expression = $2^{(15-(Ct(probe)-Ct(actin)))}$. The parameter Ct 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: CRLR: forward primer: 5'-CATGGACAAATTATACCCAGTGT-3'; probe: 5'-(FAM)CACCCACGAGAAAGTGAA-GACTGCA(TAMRA)-3'; reverse primer: 5'-TCCAATTATGGTCAGGTAAAACAA-3'; RAMP1: forward primer: 5'-GGAGACGCTGTGGTGTGAC-3'; probe: 5'-(FAM)ACCATCAGGAGCTACAGGGAGCTGG(TAMRA)-3'; reverse primer: 5'-CATGTGCCAGGTGCAGTC-3'; RAMP2: forward primer: 5'-CTCAGCCTCTTCCC-ACCAC-3'; probe: 5'-(FAM)CACACCAGGGTCAGAAGGGGG(TAMRA)-3'; reverse primer: 5'-TTCCAGCAAAATTGGACAGC-3'; RAMP3: forward primer: 5'-AAGGTGGACGTCTGGAAGTG-3'; probe: 5'-(FAM)CCTGTCCGAGTTCATCGTG-TACTATGAGA(TAMRA)-3'; reverse primer: 5'-ATCTCGGTGCAGTTGGTGA-3'; β-

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actin: forward primer: 5'-CGTCTTCCCCTCCATCGT-3'; probe: 5'-(FAM)AGGCACCAGGGCGTGATGGT(TAMRA)-3'; reverse primer: 5'-GG-AATCCTTCTGACCCATGC-3'

Generation of the parental GPCR reporter cell line

A recombinant CHO cell line expressing cytosolic apoaequorin was cotransfected with a pcDNAI plasmid construct encoding the bovine CNGA2 channel (acc.no. X55010) with one amino acid substitution (CNGA2(T537A); Altenhofen *et al*., 1991), a pcDNAI construct containing the rat CNGA4 cDNA (acc.no. U12623; Bradley *et al*., 1994) and pZeoSV (zeocin resistance). Positive clones were identified by 8-Br-cAMP and 8-Br-cGMP stimulation (data not shown) and were purified by the limited dilution technique. One purified clone was then cotransfected with a pcDNA1.1/Amp plasmid construct encoding the CNGB1b channel (acc.no. AJ000515; Sautter *et al*., 1998) and a plasmid providing hygromycin resistance. Stable transfected clones were characterized by 8-Br-cAMP and forskolin stimulation (data not shown), and were again purified by the limited dilution technique. One clonal cell line, referred to here as the GPCR reporter cell line, was selected for further experiments. All plasmid vectors were purchased from Invitrogen (Carlsbad, CA, USA).

Generation of ADM1 and CGRP1 receptor cell lines

The parental GPCR reporter cell line was cotransfected with a pcDNA3 plasmid construct encoding the human CRLR receptor (acc.no. U17473) and pcDNA1.1/Amp constructs containing either the human RAMP1 cDNA (acc.no. NM_005855) or the

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human RAMP2 cDNA (acc.no. NM_005854), according to McLatchie *et al*. (1998). Stably transfected cell clones were obtained by geneticin selection and were characterized by ADM and CGRP1 stimulation (data not shown). Positive clones were purified by the limited dilution technique and two clonal cell lines were selected for further characterization, referred to here as the ADM1 receptor (CRLR/RAMP2) and CGRP1 receptor (CRLR/RAMP1) cell lines.

Cell culture conditions and aequorin luminescence measurements

Cells were cultured at 37°C and 5% CO_2 in Dulbecco's modified Eagle's medium/NUT mix F12 with L-glutamine, supplemented with 10% (v/v) inactivated fetal calf serum, 1 mM sodium pyruvate, 0.9 mM sodium bicarbonate, 50 U/ml penicillin, 50 µg/ml streptomycin, 2.5 µg/ml amphotericin B, 0.6 mg/ml hygromycin B and 0.25 mg/ml zeocin. In addition, 1 mg/ml geneticin was added to the cell culture medium used for the ADM1 and CGRP1 receptor cell lines. Cells were passaged using enzyme-free/Hank's-based cell dissociation buffer. All cell culture reagents were obtained from Invitrogen (Carlsbad, CA, USA).

Luminescence measurements were performed on opaque 384-well microtiter plates (MTPs). 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 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. Agonists and antagonists were added for 6 min in Ca^{2+}-free tyrode containing 0.1% BSA. IBMX (0.2 mM) was used to prevent cAMP degradation by endogenous phosphodiesterases. Immediately before adding Ca^{2+} ions (final concentration 3

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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 cAMP generation and calcium release from intracellular stores, coelenterazine loading and agonist stimulation were performed in tyrode containing 2 mM calcium ions (Wunder *et al*., 2005a).

Transient transfections

Transient transfections were performed using lipofectamine^{TM} 2000 from Invitrogen (Carlsbad, CA, USA) according to the manufacturer's standard protocol. Luminescence measurements were performed on 384-well MTPs 24 h or 48 h after transfection of plasmid constructs encoding β2 adrenergic (acc.no. NM_000024), bradykinin B2 (acc.no. NM_000623), vasopressin V1A (acc.no. NM_000706) or endothelin A receptors (acc.no. NM_001957).

Detection of cAMP by radioimmunoassay (RIA)

For the measurement of intracellular cAMP, the ADM1 receptor cell line (20,000 cells/well) was seeded on 24-well MTPs and cells were cultured for 2 days. The medium was removed, the cells were washed once, and agonist stimulation was performed in tyrode supplemented with 0.2 mM IBMX for 15 min at 37°C. After removal of the supernatant, cAMP was extracted overnight with 70% EtOH at -20°C.

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Intracellular cAMP was measured by using a commercially available RIA kit (IBL, Hamburg, Germany). Measurements were performed in triplicate.

Compounds

ADM(1-21) and ADM(16-21) were purchased from Phoenix Pharmaceuticals (Burlingame, CA, USA). All other human peptide agonists and antagonists were obtained from Bachem (Bubendorf, Switzerland). Forskolin, 3-isobutyl-1-methylxanthine (IBMX), ATP, 8-bromoguanosine-3',5'-cyclic monophosphate (8-Br-cGMP) and 8-bromoadenosine-3',5'-cyclic monophosphate (8-Br-cAMP) were obtained from Sigma-Aldrich (Taufkirchen, Germany).

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 ± SEM.

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Results

Analysis of CRLR and RAMP expression in human cardiovascular-related tissues

Since we were interested in the cardiovascular pharmacology of ADM and CGRP receptors, we analysed the expression patterns of CRLR and RAMP1-3 by quantitative RT PCR in human heart, various blood vessels and kidney. As shown in Fig. 1, we were able to detect very high expression levels of CRLR, RAMP1 and RAMP2 in heart ventricle and atrium. In addition, we could verify medium to high expression of these transcripts in all blood vessels studied. RAMP3 expression was also detected in heart and blood vessels, however, at significantly lower levels. In primary endothelial cells from coronary arteries, high levels of CRLR and RAMP2 mRNA were found, whereas RAMP1 and RAMP3 expression could not be detected. Similar results were obtained with primary endothelial cells from aorta and pulmonary arteries, as well as with HUVEC cells (data not shown). Low expression levels of CRLR, RAMP1 and RAMP2 could also be detected in primary smooth muscle cells from aorta, pulmonary and coronary artery. In contrast, RAMP3 expression could not be detected in these cells (data not shown). In human kidney, high expression of CRLR and RAMP2, and medium levels of RAMP1 and RAMP3 transcripts were detected.

Generation of the recombinant GPCR reporter cell line

A CHO cell line expressing cytosolic apoaequorin was cotransfected with a plasmid construct encoding a mutated CNGA2 channel with one amino acid substitution

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(CNGA2(T537A); Altenhofen *et al*., 1991) and a second construct encoding the CNGA4 channel (Bradley *et al*., 1994). Positive clones were identified by 8-Br-cAMP and 8-Br-cGMP stimulation (data not shown) and were purified by the limited dilution technique. One purified clone was subsequently transfected with a plasmid construct encoding the CNGB1b channel (Sautter *et al*., 1998). Stable transfected clones were characterized by 8-Br-cAMP and forskolin stimulation (data not shown) and were again purified by the limited dilution technique. In our reporter system, ligand-mediated activation of Gs-coupled GPCRs can be monitored in real-time via soluble adenylyl cyclase activation and calcium influx through a cAMP-gated cation channel, acting as the intracellular cAMP sensor. In addition, activation of Gq-coupled receptor and stimulation of the PLC/IP_3 pathway is detected via aequorin luminescence stimulated by calcium release from intracellular stores (Fig. 2).

As shown in Fig. 3, we compared different stable CNG channel expressing cell lines for their ability to detect intracellular cAMP synthesis. Therefore, we tested forskolin-induced luminescence signals using our cGMP reporter cell line expressing the CNGA2 subunit only (Wunder *et al*., 2005a), in comparison to our newly established cell lines expressing a combination of CNGA2(T537A) and CNGA4, or a combination of CNGA2(T537A), CNGA4 and CNGB1b subunits. The cGMP reporter cell line showed only minor stimulation by forskolin, significant stimulation could only be observed at very high concentrations starting from 10 μM (Fig. 3A). In contrast, the CNGA2(T537A)/CNGA4 cell line already showed an increased forskolin response (pEC_{50} = 5.73 ± 0.01; Fig. 3B). As shown in Fig. 3C, high cAMP-sensitivity was achieved by heterologous expression of a combination of CNGA2(T537A), CNGA4 and CNGB1b subunits. Using this heteromultimeric CNG channel, forskolin stimulated luminescence signals with a pEC_{50} value of 6.62 ± 0.04. Accordingly, one

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clonal cell line expressing this CNG channel subunit combination (CNGA2(T537A)/CNGA4/CNGB1b) was used for further experiments (referred to here as the GPCR reporter cell line).

Generation and characterization of ADM1 and CGRP1 receptor cell lines

According to the results of our TaqMan expression analysis, the parental GPCR reporter cell line was cotransfected with CRLR and RAMP2 plasmid constructs encoding the ADM1 receptor. For the generation of the CGRP1 receptor cell line, cotransfection with CRLR and RAMP1 encoding plasmids was performed. Active clones were identified by ADM and CGRP1 stimulation and were purified once by the limited dilution technique. One clonal cell line of both transfection series was selected and used for further characterization (referred to here as the ADM1 and CGRP1 receptor cell lines).

We characterized these newly established cell lines by testing the effects of different members of the human calcitonin family of peptides. Stimulation of the ADM1 receptor cell line with ADM, CGRP2 and CGRP1 resulted in concentration-dependent luminescence signals with pEC_{50} values of 9.45 ± 0.08, 7.95 ± 0.03 and 6.87 ± 0.01, respectively (Fig. 4A). As expected, stimulation of the CGRP1 reporter cell line showed that CGRP1 and CGRP2 are potent agonists with pEC_{50} values of 9.50 ± 0.12 and 9.53 ± 0.10, respectively (Fig. 4B). ADM stimulated the CGRP1 receptor cell line with lower potency (pEC_{50} = 8.44 ± 0.03). In contrast, calcitonin and amylin did not stimulate any luminescence signals on both receptor cell lines at concentrations up to 1 μM. We also tested the agonists ADM, CGRP1 and CGRP2 on the parental GPCR reporter cell line. However, the agonists did not induce

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significant luminescence signals (data not shown). Next, we correlated agonist-stimulated luminescence signals with intracellular cAMP accumulation measured by a cAMP radioimmunoassay. As shown in Fig. 4C, stimulation of the ADM1 receptor cell line by ADM resulted in intracellular cAMP accumulation (pEC_{50} = 9.48 ± 0.06). The ADM-mediated cAMP increase was inhibited (pEC_{50} = 7.84 ± 0.04) by the addition of the ADM receptor antagonist ADM(22-52).

We also tested various shorter C-terminally amidated ADM peptides, which were previously shown to possess cardiovascular activities (Watanabe *et al*., 1996; Champion *et al*., 1999), for their ability to stimulate the ADM1 receptor cell line. ADM(1-21), ADM(16-21), ADM(16-31) and ADM(22-52) did not stimulate any signals when tested up to 1 μM. In contrast, ADM(13-52) stimulated the ADM1 receptor cell line with the same potency as the full length agonist ADM (data not shown). The ADM1 receptor cell line was also simultaneously stimulated with a combination (up to 1 μM each) of ADM(1-21) and ADM(22-52), or a combination of ADM(16-21) and ADM(22-52). However, no luminescence stimulation was observed. We also tested the activity of pro-ADM(1-20) on the ADM1 receptor cell line. However, pro-ADM(1-20) was not able to mediate receptor stimulation (data not shown).

Modulation by receptor antagonists

We next sought to determine whether the agonist-stimulated luminescence signals could be inhibited by the addition of the receptor antagonists ADM(22-52) and CGRP(8-37). As shown in Fig. 5A, ADM(22-52) and CGRP(8-37) concentration-dependently inhibited 3 nM ADM-stimulated signals on the ADM1 receptor cell line with pIC_{50} values of 7.57 ± 0.05 and 5.67 ± 0.11, respectively. In contrast, on the

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CGRP1 receptor cell line CGRP(8-37) inhibited 3 nM CGRP1-stimulated luminescence signals more potently (pIC_{50} = 7.31 ± 0.12), whereas ADM(22-52) showed only minor effects (pIC_{50} < 5.52; Fig. 5B). As shown in Fig. 5C, the antagonist ADM(22-52), when applied to the ADM1 receptor cell line at concentrations of 0.1 μM and 1 μM, shifted the pEC_{50} values of ADM-mediated luminescence signals from 9.16 ± 0.02 to 8.34 ± 0.01 and 7.76 ± 0.01, respectively. Using the CGRP1 receptor cell line, the antagonist CGRP(8-37), at concentrations of 0.1 μM and 1 μM, shifted the pEC_{50} values for CGRP1-mediated luminescence signals from 9.23 ± 0.01 to 8.20 ± 0.04 and 7.51 ± 0.04, respectively (Fig. 5D). In addition, we tested the truncated ADM fragments ADM(1-21), ADM(16-21), ADM(16-31) for their antagonistic activity on the ADM1 receptor cell line. However, none of the shorter peptides was able to antagonize 3 nM ADM stimulated luminescence signals (data not shown).

Characterization of intermedin

Next, we characterized the activity of human IMD, a newly discovered member of the calcitonin family of peptides (Roh *et al*., 2004). As shown in Fig. 6A, IMD showed only partial agonism on the ADM1 receptor cell line and stimulated luminescence signals with a pEC_{50} value of 8.27 ± 0.06. The activity of IMD was potently inhibited (pEC_{50} < 6) in the presence of the antagonist ADM(22-52). On the CGRP1 receptor cell line, IMD acted as a full agonist and luminescence signals were stimulated with a pEC_{50} value of 7.74 ± 0.09. In the presence of the antagonist CGRP(8-37), IMD activity on the CGRP1 receptor was potently antagonized (pEC_{50} < 6; Fig. 6B).

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Real-time detection of cAMP synthesis

To monitor Gs-coupled receptor activation and cAMP synthesis in real-time, increasing concentrations (1-100 nM) of the agonists ADM and CGRP1 were added to the reporter cell lines 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. 7A, ADM concentration-dependently stimulated luminescence signals on the ADM1 receptor cell line with slow signal kinetics. Lag times in the range of 22 s (at 0.01 nM) to 8 s (at 10 and 100 nM) were observed. CGRP1 induced luminescence signals with similar kinetics on the CGRP1 receptor cell line, with lag times ranging from 18 s (0.01 nM) to 7 s (100 nM), respectively (Fig. 7B). At the highest agonist concentration of 100 nM, maximal signals stimulated by ADM and CGRP1 were reached after 31 s and 34 s, respectively.

Next, we measured the luminescence signal kinetics mediated by endogenous, Gq-coupled P2Y receptors after stimulation with the P2Y agonist UTP (Burnstock, 2004). Compared to ADM- and CGRP1-mediated signals, UTP (1-1000 μM) stimulated luminescence signals with much faster activation kinetics (Fig. 7C). At the lowest UTP concentration used, a lag time of 4 s was observed. At higher UTP concentrations, the signals started to rise with a lag time of 1 s, and maximal signals were reached after 4-5 s. Similar results were obtained after stimulation of purinergic receptors with UDP and ATP (data not shown). Simultaneous application of UTP (100 μM) and ADM (100 nM) to the ADM1 receptor cell line resulted in luminescence signals with two maxima, which were reached after 4 s and 36 s, respectively (Fig. 7D).

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To further corroborate these findings, we studied the luminescence signals generated by activation of transiently transfected human bradykinin B2 (BK2) receptors. Stimulation of the Gq-coupled BK2 receptors (Zhang *et al*., 2001) with bradykinin (BK) resulted in luminescence signals with fast kinetics (Fig. 7E). Similar results were obtained after (Arg^8)-vasopressin (AVP) stimulation of transiently transfected, Gq-coupled human arginine vasopressin 1A (V1A) receptors (Liu and Wess, 1996; data not shown).

Stimulation of transiently transfected endothelin A (ET_A) receptors, previously shown to couple to both the IP_3 and the cAMP pathway (Aramori and Nakanishi, 1992), with endothelin-1 (ET-1) resulted in luminescence signals which peaked after 3 s and 37 s, respectively (Fig. 7F). The agonists isoproterenol, BK, AVP and ET-1 did not stimulate significant luminescence signals on the GPCR reporter cell line in the absence of transiently transfected receptors (data not shown).

In additional experiments we characterized the luminescence signal decrease after ADM and CGRP1 stimulation. By sequential addition of ADM or tyrode control, followed by UTP stimulation we could show that the observed signal decrease is not due to aequorin consumption (supplementary Fig. S1A). However, by sequential application of ADM or tyrode control, followed by isoproterenol stimulation of transiently transfected, Gs-coupled β2 adrenoceptors we could show that the signal decrease is likely caused by CNG channel inactivation (supplementary Fig. S1B).

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Discussion

CNG channels have properties that stimulated us to characterize them as tools for the sensitive detection of the intracellular second messenger molecules cGMP and cAMP. We have previously shown 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 cGMP/NO signaling pathway. Using this reporter assay platform, we have identified and characterized activators of soluble guanylyl cyclase, phosphodiesterase inhibitors, as well as modulators of nitric oxide synthesis (Wunder *et al*., 2005a; 2005b; 2007).

In this report we have described a novel approach using a recombinant aequorin reporter cell line expressing a modified, heteromultimeric CNG channel. The reporter cell line enables the monitoring of Gs-coupled receptor activation and stimulation of cAMP production, which is linked to Ca^{2+}-influx through the cAMP-gated cation channel. In addition, activation of Gq-coupled receptors can be detected via aequorin luminescence stimulated by calcium release from intracellular stores.

Recently, similar fluorescence-based assays using homomeric CNG channels to monitor GPCR activity have been introduced. However, the observed forskolin responses, reflecting the assay sensitivity and suitability of these CNG channels as cAMP biosensors, as well as the reported dynamic ranges are usually low, despite the fact that modified versions of the CNGA2 channel were used (Rich *et al*., 2001; Fagan *et al*., 2001; Reinscheid *et al*., 2003).

Native CNG channels of olfactory sensory neurons display high cAMP sensitivity and are composed of three different subunits (Bönigk *et al*., 1999). Accordingly, we tested heteromultimeric CNG channels, comprised of two or three olfactory channel

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subunits, for their suitability as cAMP biosensors. To achieve maximal cAMP sensitivity, we used a mutated CNGA2 subunit with increased cAMP and decreased cGMP sensitivity (Altenhofen *et al*., 1991), instead of the wild-type CNGA2. We found that the combination of CNGA2(T537A), CNGA4 and CNGB1b shows superior forskolin sensitivity and, therefore, is best suited for the detection of Gs-coupled GPCR activation. In addition, very high signal-to-background ratios (S/B > 100) were observed. The cAMP sensitivity of the GPCR reporter assay was tested by a comparison of agonist-stimulated luminescence signals and intracellular cAMP formation measured by RIA. The results show that the cAMP sensitivity of the luminescence assay is comparable to the sensitivity of the RIA measurements. However, a high cAMP background signal was measured by RIA which was not seen in the luminescence measurements. This might be related to intracellular cAMP compartimentalization and to the fact that CNG channels only detect local changes in cAMP levels near the cell membrane (Rich *et al*., 2000).

ADM, CGRP1 and CGRP2, three members of the calcitonin family of peptides, play pivotal roles in cardiovascular physiology, possess potent vasodilatory activites and have been implicated in the pathophysiology of hypertension, heart and renal failure, circulatory shock and migraine (Doggrell, 2001; Kurihara *et al*., 2003; Brain and Grant, 2004; Muff *et al*., 2004; Ishimitsu *et al*., 2006). The pioneering work of McLatchie *et al*. (1998), who could show that the so-called RAMP proteins regulate CRLR receptor membrane transport and determine receptor pharmacology, has enabled functional ADM and CGRP receptor expression and characterization. It has now been widely accepted that functional ADM receptors with similar pharmacology (ADM1 and ADM2 receptors) are generated by coexpression of CRLR with RAMP2

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or RAMP3, respectively. In addition, the CGRP1 receptor is generated by coexpression of CRLR with RAMP1 (Poyner *et al*., 2002, Conner *et al*., 2004).

Since we were interested in the establishment of reporter cell lines expressing cardiovascular ADM and CGRP receptors, we studied the expression patterns of CRLR and RAMP1-3 in human cardiovascular-related tissues by quantitative PCR. We were able to detect high expression levels of CRLR, RAMP1 and RAMP2 in human heart and all blood vessels studied. And although we were able to detect RAMP3 in these tissues, the expression levels were found to be significantly lower. Interestingly, in cultured vascular endothelial cells, high levels of CRLR and RAMP2 transcripts were found, whereas RAMP1 and RAMP3 expression could not be detected. Therefore, it seems likely that ADM and CGRP peptides exert their cardiovascular activities primarily by stimulation of ADM1 and CGRP1 receptors.

According to the results of our expression analysis, we used our novel GPCR reporter cell line to establish the ADM1 (CRLR/RAMP2) and CGRP1 (CRLR/RAMP1) receptor cell lines. We characterized both cell lines using the various members of the calcitonin family of peptides. The results show that ADM acted as a potent agonist on the ADM1 receptor cell line, whereas CGRP2 and CGRP1 were about 30-fold and 350-fold less potent, respectively. In contrast, CGRP1 and CGRP2 were nearly equipotent agonists on the CGRP1 receptor cell line, with ADM being about 10-fold less potent. IMD, a novel member of this peptide family (Roh *et al*., 2004), activated both receptors with similar potencies. However, IMD was characterized as a partial agonist on the ADM1 receptor and as a full agonist on the CGRP1 receptor. In addition, IMD potency on both receptor cell lines was about 15- to 60-fold lower compared to ADM and CGRP potencies on their respective receptors. Given that IMD and ADM posses comparable vasodilatory potencies *in vivo* (Roh *et al*., 2004),

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we speculate that the observed IMD *in vivo* activity cannot be attributed solely to activation of ADM1 and CGRP1 receptors. Therefore, we speculate that additional, specific IMD receptors might exist. We also characterized the activity of amylin and calcitonin. However, these peptides did not stimulate any luminescence signals.

In addition, we characterized the competitive antagonists ADM(22-52) and CGRP(8-37) on both receptor cell lines. As anticipated, ADM(22-52) was an effective antagonist of the ADM1 receptor, but had very weak activity on the CGRP1 receptor. In contrast, CGRP(8-37) was effective at antagonizing agonist action on both receptors, with about 40-fold higher potency at the CGRP1 receptor. Both antagonists showed competitive behaviour and induced right-shifts of the ADM and CGRP1 concentration-response curves at their respective receptors. These results correspond well to previously reported data (McLatchie *et al*., 1998; Aiyar *et al*., 2001; Poyner *et al*., 2002; Bailey and Hay, 2006).

We also studied the activity of shorter ADM peptides, which have been shown to possess vasodilator or vasopressor activities (Watanabe *et al*., 1996; Champion *et al*., 1999). However, none of the shorter ADM peptides, with the exception of ADM(13-52) stimulated the recombinant ADM1 receptor. In addition, none of these peptides, except ADM(22-52), antagonized ADM responses on the ADM1 receptor. We therefore speculate that the reported cardiovascular activities are due to stimulation of receptors different from the ADM1 receptor. In addition, we could show that the simultaneous addition of ADM(1-21) or ADM(16-21) with ADM(22-52) did not stimulate any luminescence signals. The six-membered ring structure of ADM(16-21) has been implicated in receptor activation, whereas the C-terminal tail was shown to be necessary for ADM binding to its receptor (Eguchi *et al*., 1994; Champion *et al*.,

1999). Therefore, the N-terminal ADM ring structure must be physically coupled to the C-terminal tail of the peptide for being able to activate the receptor.

Taken together, the results of the pharmacological characterization of the ADM1 and CGRP1 receptor cell lines are in good agreement with literature data and imply that the heteromultimeric CNG channel used in our reporter cell lines represents an optimized cAMP biosensor suitable to monitor GPCR activity.

As an additional interesting application, the CNG channel assay technology allows the real-time detection of cAMP synthesis within living cells. Accordingly, we characterized the luminescence signal kinetics of our receptor cell lines in the presence of calcium ions. Under these conditions, luminescence signals stimulated by activation of Gs-coupled ADM1 and CGRP1 receptors started to rise after prolonged lag times, whereas UTP-mediated activation of endogenous, Gq-coupled purinergic P2Y receptors stimulated signals with fast kinetics. To further corroborate this finding, we studied the luminescence signal kinetics after stimulation of transiently transfected BK2 and V1A receptors, which were previously characterized as Gq-coupled receptors (Liu and Wess, 1996; Zhang *et al*., 2001). As expected, activation of both receptors stimulated luminescence signals with fast kinetics and a single peak. In addition, we also studied luminescence signal kinetics mediated by ET_A receptor activation, previously shown to positively couple to both the IP_3 and the cAMP pathway (Aramori and Nakanishi, 1992). In accordance with this finding, fast and slow luminescence peaks were observed after ET_A receptor stimulation. Therefore, activation of Gs- and Gq-coupled receptors is characterized by different signal kinetics, which can be used to identify intracellular receptor coupling and signaling pathways. We speculate that the difference in signal kinetics is due to compartimentalized cAMP synthesis and restricted diffusion, as described for

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cardiomyocytes (Fischmeister *et al*., 2006). This hypothesis is further supported by the observation of slow signal kinetics measured in CHO cells by FRET-based, cytosolic cAMP sensors after Gs-coupled receptor activation (Nikolaev *et al*., 2004).

The luminescence signals induced by ADM1 and CGRP1 receptor activation were found to be transient, which might be due to aequorin consumption or CNG channel inactivation. By sequential application of different agonists we could show that the signal decrease is likely caused by Ca^{2+}/calmodulin-dependent CNG channel inactivation. A Ca^{2+}/calmodulin-binding site has been identified in the N-terminal region of the CNGA2 channel (Liu *et al*., 1994).

The ADM1 and CGRP1 receptor reporter assays have now been successfully transferred to the 1536-well MTP format with minor variations (unpublished data). Therefore, these reporter cell lines provide further examples for the implementation of the CNG channel assay technology in an ultra-high-throughput screening (uHTS) format (Wunder *et al*., 2005a; 2005b; 2007). In summary, the results presented in this report show that our novel reporter assay is well-suited for the characterization of GPCR pharmacology, but can also be used for uHTS purposes.

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Figure legends

Figure 1: Characterization of CRLR and RAMP1-3 expression in human cardio-vascular tissues. Quantitative real-time RT-PCR analysis was performed on a panel of human cardiovascular-related tissue cDNAs using specific oligonucleotide probes. Expression levels were normalized to β-actin. Abbreviations: EC, endothelial cells; scler., sclerotic

Figure 2: Schematic presentation of the GPCR reporter cell line. Ligand-mediated activation of Gs-coupled receptors stimulates cAMP synthesis by adenylyl cyclase (AC) and opening of the cAMP-gated, heteromultimeric CNG channel, comprised of CNGA2(T537A), CNGA4 and CNGB1b subunits. Calcium ions from the extracellular solution enter the cell through the CNG channel and are detected by aequorin luminescence measurements. Gq-coupled receptor activation and stimulation of the phospholipase C (PLC)/inositol 1,4,5-trisphosphate (IP_3) pathway is detected via aequorin luminescence stimulated by calcium release from the endoplasmic reticulum (ER).

Figure 3: Characterization of stable cell lines expressing different CNG channel subunit combinations. Concentration-dependent luminescence signals generated by forskolin stimulation for 6 min in Ca^{2+}-free tyrode using cell lines expressing (A) CNGA2, (B) CNGA2(T537A) and CNGA4, or (C) CNGA2(T537A), CNGA4 and CNGB1b. 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 ± SD.

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Figure 4: Characterization of the ADM1 and CGRP1 receptor reporter cell lines. Concentration-dependent luminescence signals generated after stimulation of (A) the ADM1 receptor cell line or (B) the CGRP1 receptor cell line with ADM (●), CGRP1 (◊), CGRP2 (Δ), calcitonin (▼) or amylin (○). Agonists were added for 6 min in Ca^{2+}-free tyrode and measurements were started immediately before the addition of Ca^{2+} (final concentration 3 mM). (C) Intracellular cAMP accumulation measured by RIA. The ADM1 cell line was stimulated for 15 min with ADM in the absence (●) or presence (◊) of 1 μM antagonist ADM(22-52). Data are presented as means ± SD.

Figure 5: Characterization of receptor antagonists. Stimulation of (A) the ADM1 receptor cell line with 3 nM ADM or (B) the CGRP1 receptor cell line with 3 nM CGRP1 in the presence of increasing concentrations of the antagonists (○) ADM(22-52) and (▲) CGRP(8-37). (C) Concentration-dependent luminescence signals generated by ADM stimulation of the ADM1 receptor cell line in the absence (●) or presence of 0.1 μM (+) and 1 μM (○) ADM(22-52) in Ca^{2+}-free tyrode. (D) Concentration-dependent luminescence signals generated by CGRP1 stimulation of the CGRP1 receptor cell line in the absence (◊) or presence of 0.1 μM (x) and 1 μM (▲) CGRP(8-37) in Ca^{2+}-free tyrode. Agonists and antagonists were added for 6 min. Measurements were started immediately before the addition of Ca^{2+} (final concentration 3 mM). Data are presented as means ± SD.

Figure 6: Characterization of IMD activity on ADM1 and CGRP1 receptors. (A) Concentration-dependent luminescence signals generated after stimulation of the ADM1 receptor cell line with ADM (●) or IMD in the absence (∗) or presence (○) of 1

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μM antagonist ADM(22-52). (B) Stimulation of the CGRP1 receptor cell line with CGRP1 (◊) or IMD in the absence (*) or presence (▲) of 1 μM antagonist CGRP(8-37). Agonists and antagonists were added for 6 min. Measurements were started immediately before the addition of Ca^{2+} (final concentration 3 mM). Data are presented as means ± SD.

Figure 7: Real-time detection of cAMP generation and calcium release from intracellular stores. Kinetics of the luminescence signals generated by (A) ADM or (B) CGRP1 in the presence of 2 mM Ca^{2+} ions: control (○), 0.1 nM (▲), 1 nM (◊), 10 nM (●), and 100 nM (Δ) agonist. (C) Kinetics of the luminescence signals generated by UTP in the presence of 2 mM Ca^{2+} ions: 1 μM (▲), 10 μM (◊), 100 μM (●), and 1000 μM (Δ) UTP. (D) Luminescence signals generated by the simultaneous addition of 100 μM UTP and 100 nM ADM. (E, F) Luminescence signals generated by 100 nM BK stimulation of transiently transfected BK2 receptors (E) or 10 nM ET-1 stimulation of transiently transfected ET_A receptors (F). Experiments were performed using the ADM1 receptor cell line (A, C, D), the CGRP1 receptor cell line (B), or the parental GPCR reporter cell line (E, F).

Figure 1

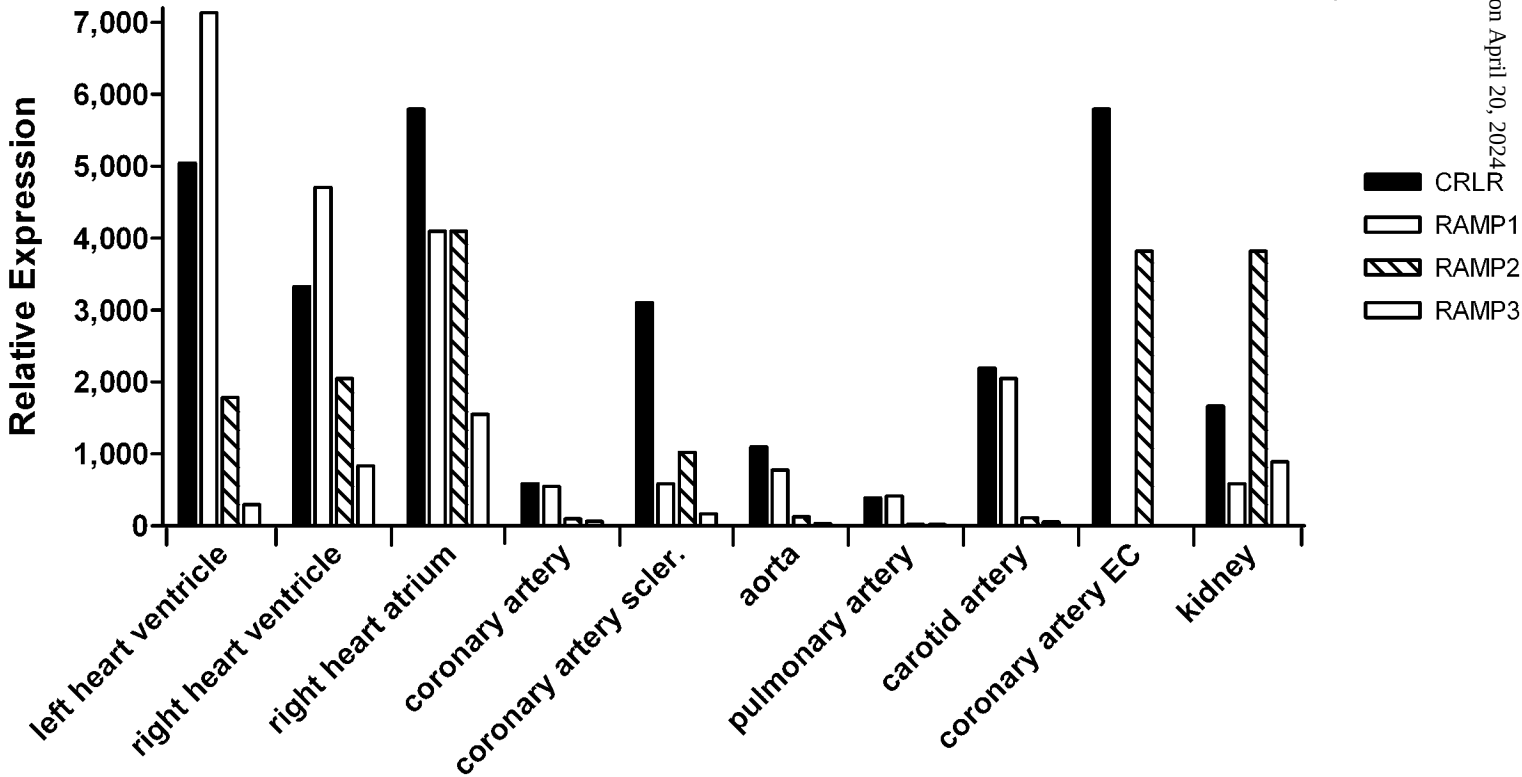
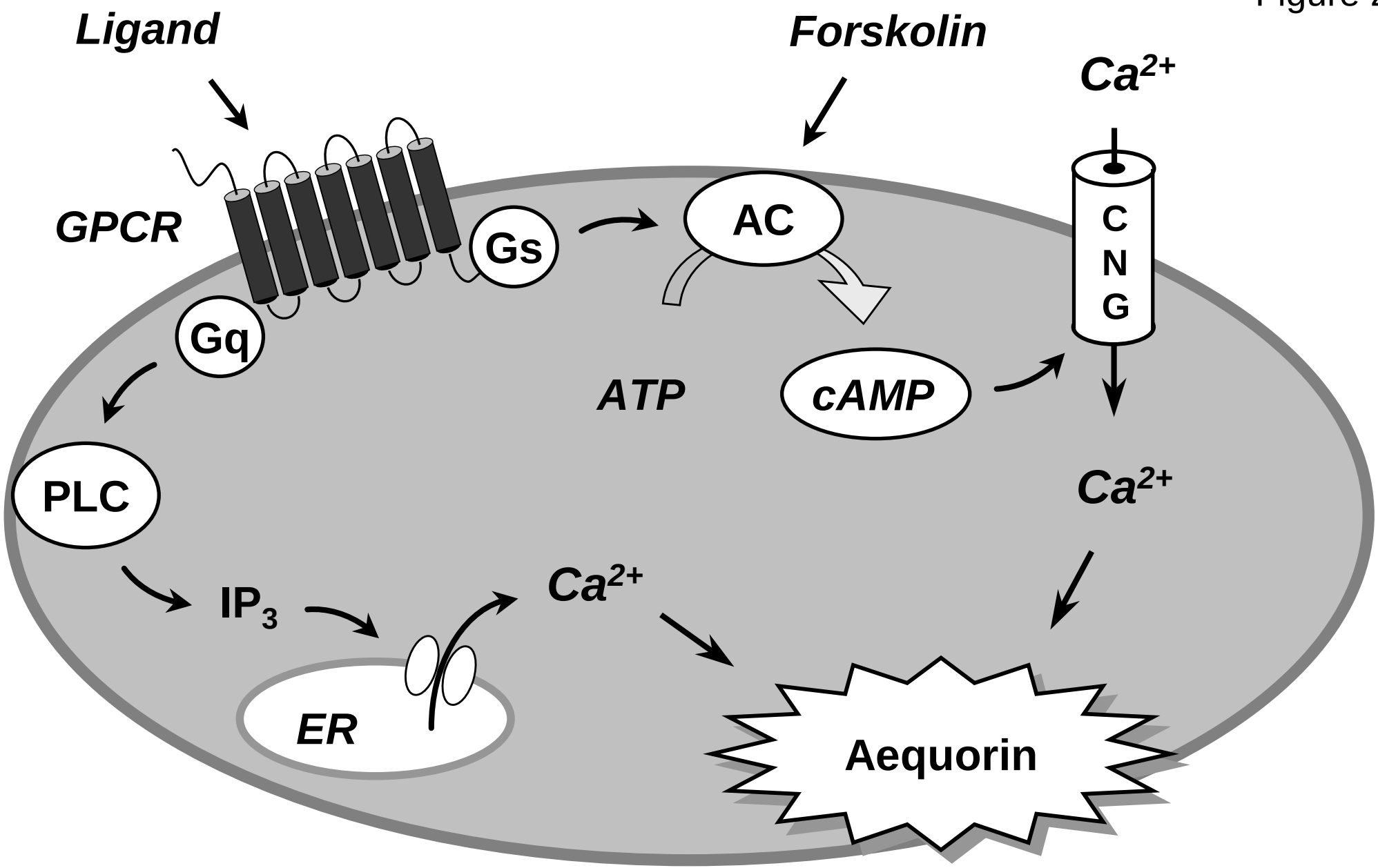


Figure 2



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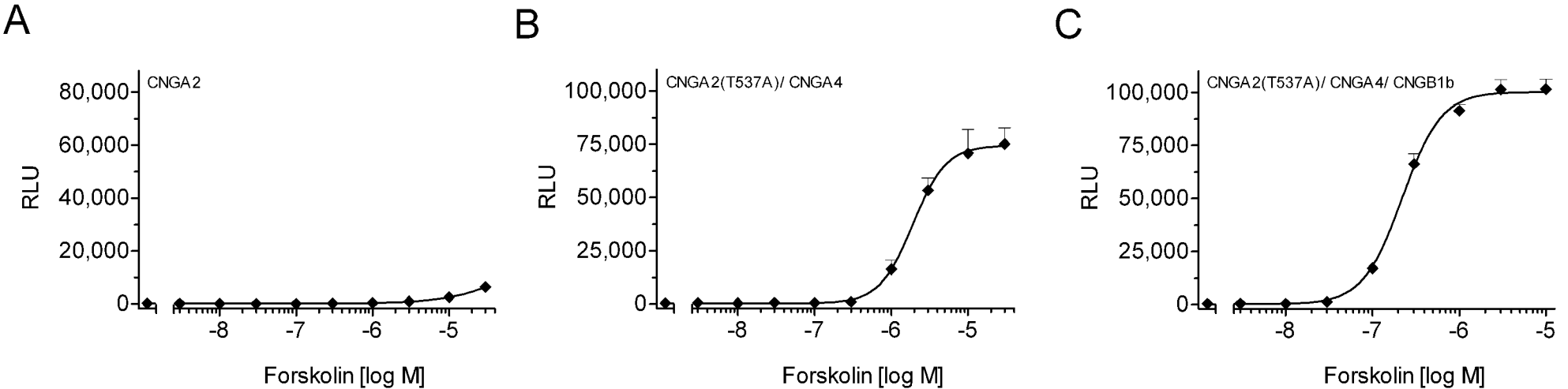

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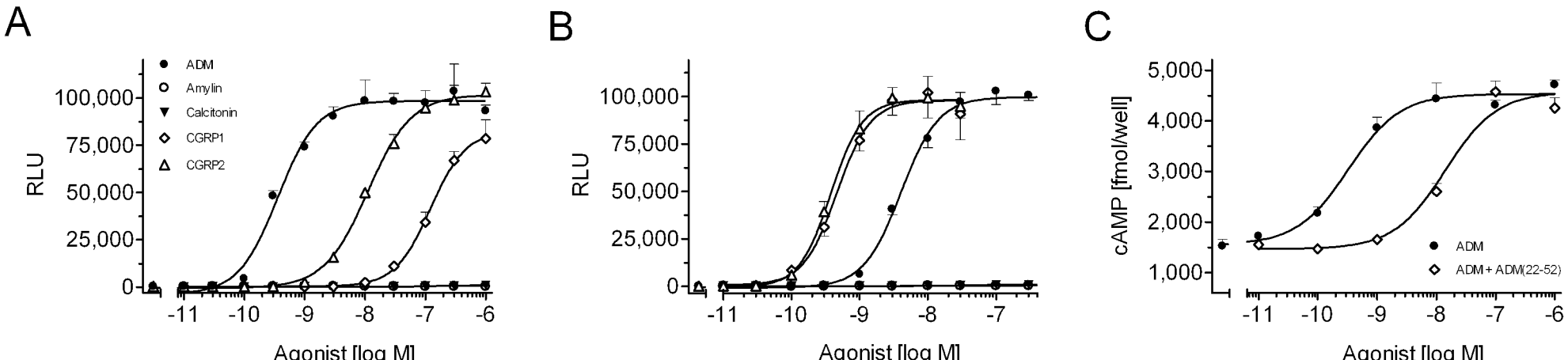
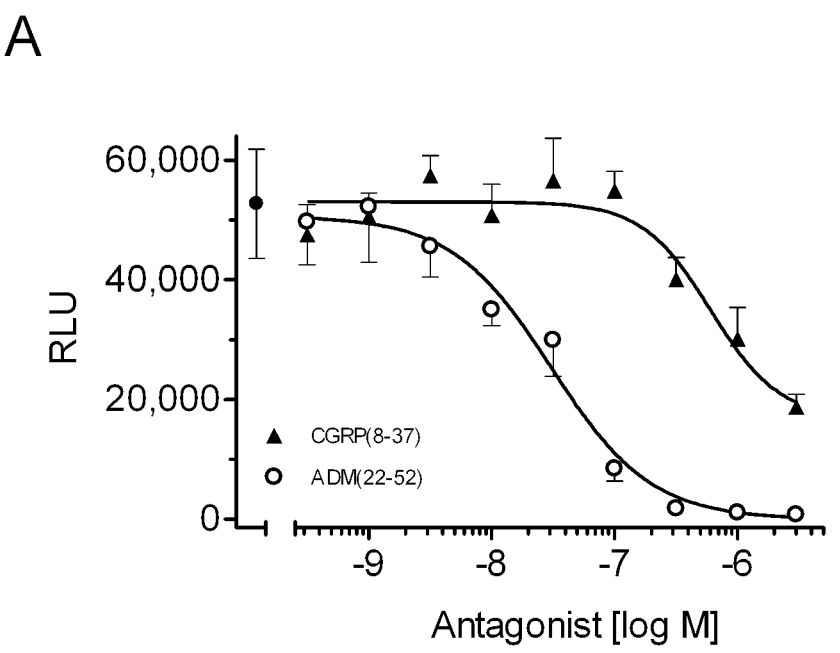
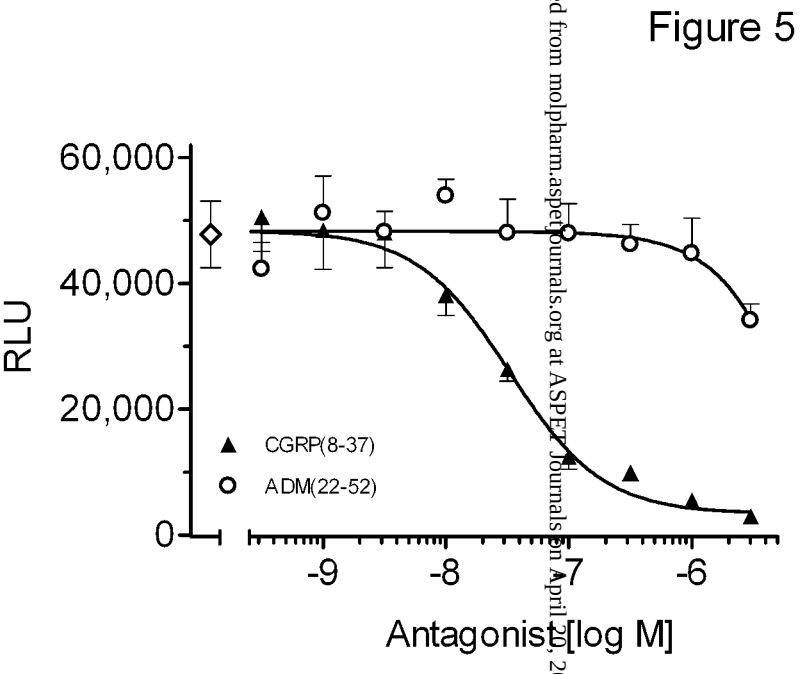
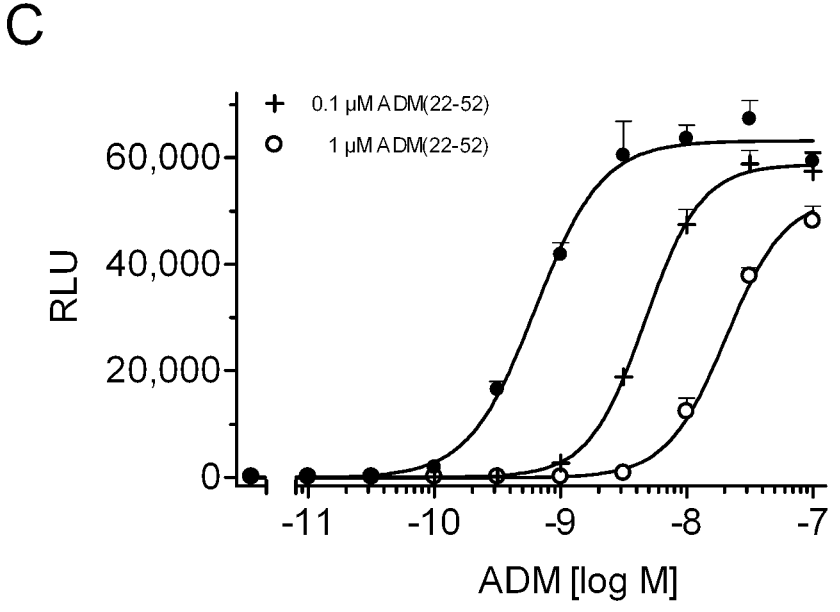

A
ADM
Amylin
Calcitonin
CGRP1
CGRP2
RLU
100,000
75,000
50,000
25,000
0
-11
-10
-9
-8
-7
-6
Agonist [log M]
B
RLU
100,000
75,000
50,000
25,000
0
-11
-10
-9
-8
-7
Agonist [log M]
C
cAMP [fmol/well]
5,000
4,000
3,000
2,000
1,000
ADM
ADM + ADM(22-52)
-11
-10
-9
-8
-7
-6
Agonist [log M]

Figure 5







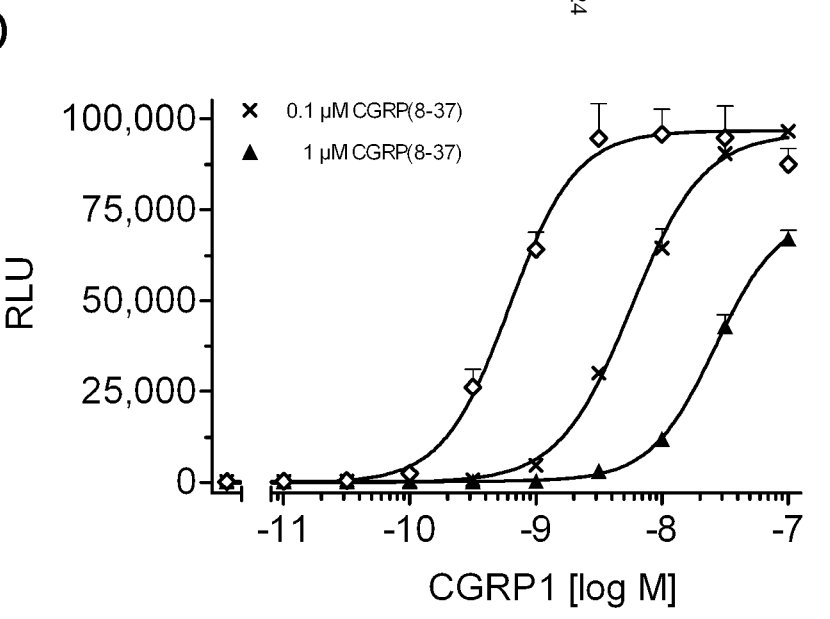


Figure 6

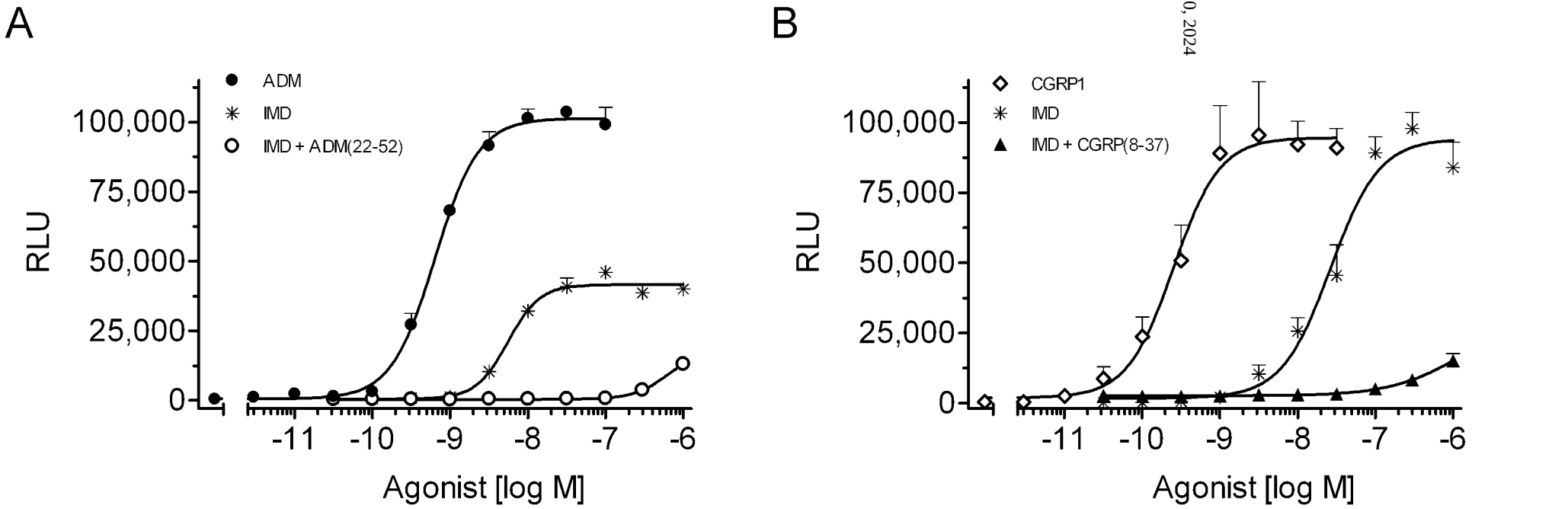


Figure 7