



Allosteric modulation of adenosine A1 and cannabinoid 1 receptor signaling by G-peptides

Anja M. Touma | Rabia U. Malik | Tejas Gupte | Sivaraj Sivaramakrishnan

Department of Genetics, Cell Biology, and Development, University of Minnesota, Minneapolis, MN, USA

Correspondence

Sivaraj Sivaramakrishnan, Department of Genetics, Cell Biology, and Development, 6-140 MCB, 420 Washington Avenue SE, Minneapolis, MN 55455, USA.
Email: sivaraj@umn.edu

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Abstract

While allosteric modulation of GPCR signaling has gained prominence to address the need for receptor specificity, efforts have mainly focused on allosteric sites adjacent to the orthosteric ligand-binding pocket and lipophilic molecules that target transmembrane helices. In this study, we examined the allosteric influence of native peptides derived from the C-terminus of the G α subunit (G-peptides) on signaling from two Gi-coupled receptors, adenosine A₁ receptor (A₁R) and cannabinoid receptor 1 (CB₁). We expressed A₁R and CB₁ fusions with G-peptides derived from G α s, G α i, and G α q in HEK 293 cells using systematic protein affinity strength modulation (SPASM) and monitored the impact on downstream signaling in the cell compared to a construct lacking G-peptides. We used agonists N⁶-Cyclopentyladenosine (CPA) and 5'-N-Ethylcarboxamidoadenosine (NECA) for A₁R and 2-Arachidonoylglycerol (2-AG) and WIN 55,212-2 mesylate (WN) for CB₁. G-peptides derived from G α i and G α q enhance agonist-dependent cAMP inhibition, demonstrating their effect as positive allosteric modulators of Gi-coupled signaling. In contrast, both G-peptides suppress agonist-dependent IP₁ levels suggesting that they differentially function as negative allosteric modulators of Gq-coupled signaling. Taken together with our previous studies on Gs-coupled receptors, this study provides an extended model for the allosteric effects of G-peptides on GPCR signaling, and highlights their potential as probe molecules to enhance receptor specificity.

KEYWORDS

adenosine-5'-(N-ethylcarboxamide), GTP-binding proteins, N(6)-cyclopentyladenosine, receptor, adenosine A₁, receptor, cannabinoid, CB₁, SCH 442 416, signal transduction

Abbreviations: 2-AG, 2-Arachidonoylglycerol; A₁R, Adenosine type 1 receptor; A_{2A}R, Adenosine type 2A receptor; A_{2B}R, Adenosine type 2B receptor; β 2-AR, β 2-adrenergic receptor; CB₁, cannabinoid type 1 receptor; CPA, N⁶-Cyclopentyladenosine; D₁R, Dopamine receptor; G protein, GTP-binding protein; GPCR, G protein-coupled receptor; IP, Inositol Phosphate; NECA, 5'-N-Ethylcarboxamidoadenosine; PTX, pertussis toxin; SPASM, systematic protein affinity strength modulation; V_{1A}-R, Vasopressin 1A receptor; WN, WIN 55,212-2 mesylate. Anja M. Touma and Rabia U. Malik are contributed equally to this work.

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1 | INTRODUCTION

G protein-coupled receptors (GPCRs) have been the most successful class of drug targets in clinical medicine, due in part to their widespread distribution and important roles in physiology.¹ The pharmacological success of GPCRs derives from their selective coupling to specific heterotrimeric G proteins, triggering the corresponding physiological response. Recent drug discovery efforts have focused on the development of allosteric modulators for GPCRs.² Allosteric modulators have the potential to increase receptor specificity by targeting sequence motifs unique to receptor family subtypes and isoforms. Furthermore, allosteric modulators require the presence of an orthosteric ligand, providing physiological context-dependent control of GPCR signaling.³ Therefore, compared to orthosteric ligands, large doses of allosteric modulators can be administered with a lower risk of target-based toxicity.² An emerging target site for allosteric modulators is the GPCR-G protein-binding interface. The GPCR-G protein-binding interface contains sequence divergent structural elements including three intracellular loops and the GPCR C-tail.⁴ However, the intrinsically disordered nature of the loop and C-tail, combined with the potential for binders in these regions to disrupt GPCR-G protein coupling has limited efforts to rationally design allosteric modulators that target the GPCR-G protein interface.⁵

In this study, we examine the potential for the G protein α subunit C-terminus (G-peptide) to serve as an allosteric modulator of GPCR signaling. The G-peptide is a well-established determinant of GPCR-G protein coupling selectivity.^{6,7} The G-peptide interacts at the cytosolic GPCR-G protein interface, which is distinct from the orthosteric ligand-binding pocket. The GPCR interaction with a cognate G-peptide triggers nucleotide exchange in the $G\alpha$ subunit (GDP to GTP) resulting in G protein activation and downstream signaling. While interactions with noncognate G-peptides do not precipitate G protein activation, we have recently shown that noncognate interactions alter receptor conformation resulting in enhanced ligand efficacy.^{8,9} Previous studies show that while the noncognate G-peptide interactions are transient, the GPCR conformational state persists following dissociation resulting in the allokaïric modulation (AKM) of downstream signaling.^{8,9} Allokaïric modulators bind asynchronously with the ligand and rely on the temporal persistence of GPCR conformation to exert their influence on orthosteric ligand efficacy.⁹ Our previous studies focused on the Gs-coupled β 2-adrenergic (β 2-AR) and dopamine (D1R) receptors, which show enhanced cyclic AMP generation in the presence of a noncognate Gq protein. Likewise, the Gq-coupled V1 vasopressin receptor (V1R) shows enhanced IP₁ levels in the presence of the noncognate Gs protein.⁸ In this study, we examine the potential for allokaïric modulation of two canonical Gi-coupled receptors, adenosine type 1 (A1R) and cannabinoid type 1 (CB1) using G-peptides derived from Gs, Gi, and Gq subtypes.

While β 2-AR and D₁R principally signal through Gs, and A₁R and CB₁ primarily signal through Gi. However, A₁R and CB₁ display signaling through multiple G proteins with A₁R signaling through Gi and Gq, and CB₁ signaling through Gi, Gq, and Gs.¹⁰⁻¹³ CB₁, the most widely expressed GPCR in the central nervous system, primarily

signals through Gi producing euphoria and analgesia upon binding tetrahydrocannabinol (THC) in the brain.^{14,15} CB₁ has also been shown to signal through Gq in human embryonic kidney (HEK) 293 cells after treatment with WIN55,212-2 (WN)¹² and through Gs in rat globus pallidus, HEK 293, COS-7, CHO, and 3T3 cells after treatment with WN.^{10,13,16} However, the physiological effects of CB₁ signaling through Gs, Gq, and non-G protein-mediated pathways is less clear since there have not been biased ligands identified that specifically target these pathways. A₁R is another example of a promiscuous receptor that can activate different signal transduction pathways in an agonist-dependent manner. A₁R is ubiquitously expressed and most well known for being antagonized by caffeine, producing stimulant effects.¹⁷ While A₁R canonically signals through Gi, there is evidence that A₁R has a diverse G protein-activating profile where A₁R can adopt agonist-specific conformations, arising from small changes in ligand structure, which lead to the differential activation of G proteins including Gi and Gq.¹¹ This promiscuity of coupling in these canonical Gi receptors allows us to examine the allosteric effects of the G-peptide on multiple G protein signaling pathways.

The goal of this focused study is to examine the allosteric effects of G-peptides derived from three distinct $G\alpha$ C-termini peptides ($G\alpha_s$, $G\alpha_i$, and $G\alpha_q$) on signaling from two promiscuous Gi-coupled receptors (A₁R and CB₁). The C-termini of three G proteins, $G\alpha_s$, $G\alpha_i$, and $G\alpha_q$, will be referred to as s-pep, i-pep, and q-pep (or collectively as G-peptides) throughout this manuscript. We expressed A₁R and CB₁ fusions with the s-, i-, or q-pep in HEK 293 cells using systematic protein affinity strength modulation (SPASM) and monitored the impact on downstream signaling in the cell compared to a construct lacking this G-peptide, referred to henceforth as no-pep. We have extensively reported on this SPASM technique, which allows systematic control of the intramolecular interaction between a GPCR and a G-peptide.^{6,8,18,19} This technology allows us to directly compare the influence of different G-peptides on the cognate G protein signaling pathways in cells. While this is a tethered system, we have shown that these engineered GPCR constructs yield similar results to reconstituted systems of GPCR membranes and recombinant G proteins with regards to allokaïric modulation of G protein activation.^{8,19} Hence, despite the synthetic nature of our approach, it provides insight into the impact of receptor interactions with G-peptides on downstream signaling.

To investigate the allosteric effects of G-peptides on Gi-coupled receptors, we used N⁶-Cyclopentyladenosine (CPA) and 5'-N-Ethylcarboxamidoadenosine (NECA) for A₁R and 2-Arachidonoylglycerol (2-AG) and WIN 55,212-2 mesylate (WN) for CB₁. Our current study confirms what we previously found in Gs-coupled receptors β 2-AR and D₁-R, where s-pep and q-pep positively modulate canonical Gs signaling.⁸ cAMP response at high concentrations of 2-AG and WN is enhanced by q-pep (~30% and 95% increase in cAMP, respectively). Likewise, cAMP stimulation by WN at CB₁ is enhanced by s-pep (~40% increase). In contrast, i-pep diminishes cAMP response from CB₁ for both 2-AG and WN (30 and 50% decreases, respectively). At low concentrations of 2-AG, WN, and CPA (nmol/L) we observed inhibition of cAMP,

associated with signaling through Gi. We found that the presence of q-pep or i-pep enhanced canonical Gi signaling in A₁R after activation by CPA (~35% increase), and in CB₁ after activation by WN (~700% increase) and 2-AG (~125% increase), respectively. These findings extend our previously reported allosteric effects of G-peptides to Gi-coupled signaling.^{8,9} At high concentrations of 2-AG, WN, CPA, or NECA (μmol/L), stimulation of inositol phosphate (IP₁) is observed, associated with signaling through Gq. We found that the presence of different G-peptides universally inhibits IP₁ signaling through Gq (decreases ranging from 30% to 65%), with the exception of s-pep (~50% increase) on CB₁ following activation by WN. Taken together, our data provide an extended model for the allosteric effects of distinct G-peptides on signaling through Gs, Gi, and Gq pathways and highlight the ability of G-peptides to differentially impact signaling in a receptor and ligand-dependent manner.

2 | MATERIALS AND METHODS

2.1 | Reagents and buffers

5'-N-Ethylcarboxamidoadenosine (NECA), pertussis toxin (PTX), and forskolin were purchased from Sigma-Aldrich. 2-Arachidonoylglycerol (2-AG), N⁶-Cyclopentyladenosine (CPA), WIN 55,212-2 mesylate (WN), [SCH 442416 \(SCH\)](#), and [PSB 1115 \(PSB\)](#) were purchased from Tocris. cDNA encoding Gα_{i2} isoform 1, Gα_q, and the long splice variant of Gα_s were acquired from GE (Open Biosystems). Human A₁R was acquired from DNASU Plasmid Repository. *Mus musculus* CB₁ was acquired from transOMIC technologies. DNA transfection reagents X-tremeGENE HP and Mirus-LT DNA were purchased from Roche and Mirus, respectively. Buffer A is phosphate-buffered saline (PBS pH 7.4; Gibco™), 800 μmol/L ascorbic acid, and 0.2% dextrose (w/v). Buffer B (Stimulation Buffer 2; Cisbio) is 10 mmol/L HEPES, 1 mmol/L CaCl₂, 0.5 mmol/L MgCl₂, 4.2 mmol/L KCl, 146 mmol/L NaCl, 5.5 mmol/L glucose, 50 mmol/L LiCl₂, pH 7.4.

2.2 | Molecular cloning

For mammalian HEK 293 expression, all GPCR and Gα constructs were cloned into a pCDNA5/FRT vector (ThermoFisher). GPCR sensors were cloned with a modular scheme. Each GPCR sensor contained (from N- to C-terminus): a full length GPCR (A₁R or CB₁), mCitrine, 10 nm ER/K linker, mCerulean, and a Gα subunit C-terminal peptide corresponding to Gα_s, Gα_i, Gα_q, (s-pep, i-pep, or q-pep, respectively) or a control peptide (no-pep), consisting of repeating (Gly-Ser-Gly)₄ residues. A (Gly-Ser-Gly)₄ linker was inserted between all protein domains as part of the primer sequence to allow for free rotation between domains. All sensors also contained either an N-terminal HA-tag or a His-tag. All constructs were confirmed by sequencing.

2.3 | Mammalian cell preparation and sensor expression

HEK293T-Flp-In (HEK293T, ThermoFisher) cells were cultured in DMEM media (ThermoFisher) supplemented with 10% FBS (v/v) (Millipore Sigma), 4.5/gL D-glucose, 1% Glutamax (ThermoFisher), 20 mmol/L HEPES, pH 7.5 at 37°C in a humidified atmosphere at 5% CO₂. HEK293T cells were plated onto six-well tissue culture treated plates at ~30% confluence. Cells were transfected 16-20 hours later with X-tremeGENE HP DNA transfection reagent. Transfection conditions including the amount of DNA (1.4-4 μg DNA + 4.2-6 μL reagent) and the length of transfection (control no-pep sensors: 18-24 hours; sensors containing s-, i-, or q-pep: 22-32 hours) were optimized to consistently yield equivalent levels of sensor expression across different conditions. Where indicated, 12 hours after transfection, cells were incubated with 100 ng/mL PTX for 16 hours. Experiments were conducted at 60%-80% transfection efficiency (evaluated on a Nikon tissue-culture microscope enabled with fluorescence detection using 20x and 40x magnification). At the time of the experiment, 60%-90% of transfected cells expressed predominantly plasma membrane localized sensor with minimal localization to the intracellular compartments. Sensor integrity, localization, and sensor expression were tracked for all experiments to ensure consistency. Each experiment was performed at equivalent sensor expression and matched OD of the cell suspension using the following steps. Cells were first resuspended by gentle pipetting into their original media, spun down (350 g, 3 minutes), and washed once with Buffer A or B for cAMP or IP₁ assays, respectively. Subsequently, cells were resuspended in an appropriate volume of the same buffer to reach a 0.3 OD measured at A_{600 nm}. Sensor expression was measured by mCitrine fluorescence. mCitrine fluorescence was held within 1.6-2.4 × 10⁶ counts-per-second (cps) for a cell OD of 0.3. Sensor integrity was confirmed by measuring the mCitrine (Horiba Fluoromax-4; excitation 490 bandpass 8 nm; emission range 500-600 bandpass 4 nm; emission maximum 525 nm) to mCerulean fluorescence ratio (excitation 430 bandpass 8 nm; emission range 450-600 bandpass 4 nm; emission maximum 475 nm). Experiments were conducted at mCitrine to mCerulean fluorescence ratio of 1.7-2.1.

2.4 | cAMP assays

HEK293T cells expressing indicated sensor were harvested 28-32 h posttransfection (X-tremeGENE HP) to assess cAMP levels using the bioluminescent cAMP Glo assay (Promega). Cells were gently suspended in their original media, counted using a hemocytometer, and spun down (350 g, 3 minutes). Cells were resuspended in an appropriate volume of Buffer A to reach 4 × 10⁶ cells/mL density. Cell suspensions were aliquoted into 384-well opaque plates (5 μL per well). Where indicated, cells were preincubated with 100 nmol/L of the [adenosine type 2A receptor \(A2AR\)](#) selective antagonist, [SCH 442416 \(SCH\)](#), and 1 μmol/L of the [adenosine type 2B receptor \(A2BR\)](#)

selective antagonist, PSB 1115 (PSB) in 10 $\mu\text{mol/L}$ forskolin for 15 minutes at 37°C. Cells were incubated with CPA or NECA (for A_1R) or 2-AG or WN (for CB_1) for 15 minutes with 10 $\mu\text{mol/L}$ forskolin at 37°C. Subsequently, cells were lysed and the protocol was followed according to the manufacturer's recommendation (Promega). Luminescence was measured using a microplate reader (SpectraMax M5e, Molecular Devices). cAMP levels were evaluated by subtracting relative luminescence units (RLUs) in the absence and presence of agonists. Each experiment was performed in quadruplicate and independently repeated at least three times ($N > 3$). For experiments involving comparisons between multiple sensors, equivalent sensor expression was first verified using fluorescence measurements (see previous section) and data for all four sensors were collected together (Figure S3).

2.5 | IP_1 assays

HEK293T cells expressing the indicated sensor were harvested 28–32 h posttransfection (X-tremeGENE HP) to assess IP_1 levels using the IP-One HTRF assay kit (Cisbio). Cells were gently suspended in their original media, counted using a hemocytometer, and spun down (350 g, 3 minutes). An appropriate volume of Buffer B (StimB buffer) was added to reach 3×10^6 cells/mL density. Where indicated, cells were preincubated with 1 $\mu\text{mol/L}$ of the $A_{2B}R$ selective antagonist, PSB 1115 (PSB) for 30 minutes at 37°C. Cells were incubated with 100 $\mu\text{mol/L}$ of CPA or NECA (for A_1R) or 100 $\mu\text{mol/L}$ of 2-AG or WN (for CB_1) at 37°C for a total incubation time of 30 or 120 minutes. The manufacturer's protocol was modified to achieve a high signal to noise ratio as follows: 70 μL of suspension was incubated for 1 hour with 2 μL IP_1 conjugated to d2 dye diluted in 13 μL of lysis buffer (Cisbio) and 2 μL terbium cryptate-labeled anti- IP_1 monoclonal antibody also diluted in 13 μL of lysis buffer. 80 μL of each reaction suspension was then transferred and split between 4 wells (20 μL /well) on a 384-well opaque plate. IP_1 spectra were collected by exciting samples at 340 nm (bandpass 15 nm). Emission counts were recorded from 600 to 700 nm using a long pass 475 nm filter (FSQ GG475, Newport). Raw IP_1 signal was calculated as the ratio of fluorescence emissions at 665nm and 620nm. Data were corrected by subtracting the untransfected IP_1 ratio from cells expressing transfected sensor. Data are presented as a change in IP_1 ratio following drug treatment. Each experiment included four repeats per condition and was independently repeated at least three times ($N > 3$).

2.6 | Statistical analysis

Data are represented as mean values \pm SEM. All experiments were repeated for at least three independent trials, with three to six technical repeats per condition ($N > 3$). Statistical analysis was performed using GraphPad Prism 7.0c (Graphpad Software, Inc). To assess significance across experimental repeats, pooled or un-pooled data underwent subsequent pairwise ANOVA analysis. Tukey's post hoc test was performed to assess significance when evaluating

comparisons between multiple conditions with P -values $*P \leq .05$; $**P \leq .01$; $***P \leq .001$; $****P \leq .0001$; $*****P \leq .00001$.

2.7 | Nomenclature of targets and ligands

Key protein targets and ligands in this article are hyperlinked to corresponding entries in <http://www.guidetopharmacology.org>, the common portal for data from the IUPHAR/BPS Guide to PHARMACOLOGY,²⁰ and are permanently archived in the Concise Guide to PHARMACOLOGY 2019/20: G protein-coupled receptors.²¹

3 | RESULTS

3.1 | SPASM sensor design

SPASM sensors were developed for two cognate Gi-coupled receptors, adenosine A_1 receptor (A_1R) and cannabinoid type 1 (CB_1) (Figure 1A). From N- to C-terminus, each SPASM sensor contains a GPCR, mCitrine (to monitor sensor integrity), 10 nm ER/K linker, mCerulean (for matching receptor expression), and a 27-amino acid peptide derived from the $\alpha 5$ -helix at the C-terminus of the $G\alpha$ subunit (s-pep, i-pep, q-pep, or no-pep). We chose the 10 nm linker based on previous work, where we found that a shorter linker corresponded to a higher effective concentration of the protein interaction (Figure S1, left).²² We had previously shown that a peptide derived from $G\alpha s$ (s-pep) could enhance G_s signaling through $\beta 2$ -AR, and we confirmed this in Figure S1 with $\beta 2$ -AR producing a significant increase in cAMP when tethered to the s-pep (Sp) by either a 10 or 20 nm linker.⁸ However, we observed no significant increase in cAMP production by $\beta 2$ -AR when tethered to s-pep by a 30 nm linker (Figure S1). We therefore used a 10 nm linker to tether peptides to GPCRs for subsequent experiments, since it appeared that the effective concentrations enforced by either a 10 or 20 nm linker were required to modulate signaling. The $G\alpha$ C-terminal peptides have been shown to be essential for activation by the GPCR but do not themselves trigger downstream effectors.^{6,8,23–27} In previous studies we have shown the ability of SPASM sensors to be expressed and localized primarily to the plasma membrane in HEK 293 cells.²⁸ Our SPASM sensors are therefore designed to modulate the interaction between the attached receptor (A_1R or CB_1) and endogenous G proteins in cells, allowing one to study the impact of the tethered $G\alpha$ peptides on canonical GPCR signaling.¹⁹ SPASM A_1R and CB_1 constructs lacking a C-terminal peptide (no-pep) were used to measure background cAMP and IP_1 levels and for characterization of ligand dose-response.

3.2 | Impact of $G\alpha$ C-terminal peptides on cAMP response in the Cannabinoid (CB_1) Receptor

Cells expressing the CB_1 sensor display potentiation of forskolin-stimulated cAMP accumulation with signaling dominated by G_s in response to 30 $\mu\text{mol/L}$ of the CB_1 agonists 2-Arachidonoylglycerol

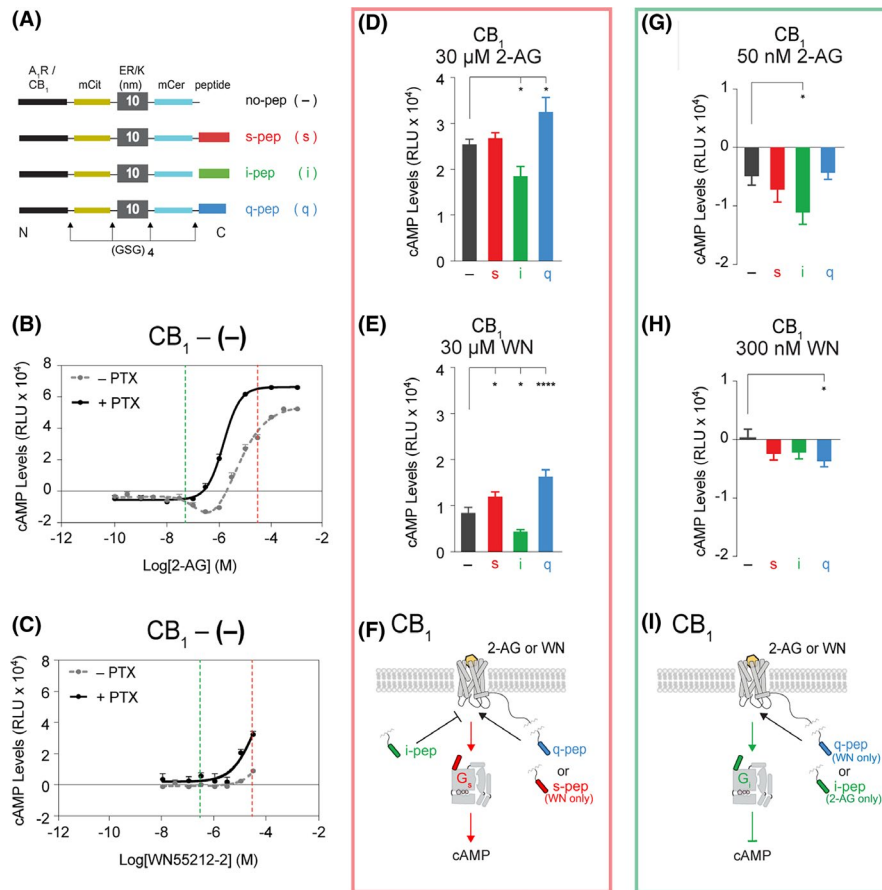


FIGURE 1 $G\alpha$ peptides differentially impact G_s and G_i signaling in Cannabinoid (CB_1) receptors. A, SPASM sensors for characterization of second messenger response. Schematics of the A_1R and CB_1 GPCR peptide sensors containing C-terminal $G\alpha$ peptides corresponding to s-, i-, or q- 5 α helices separated with Gly-Ser-Gly (GSG)₄ linkers to ensure rotational freedom. The no-pep (-) construct lacks the $G\alpha$ C-terminal peptide. Forskolin-stimulated cAMP dose-response curves of B, CB_1 agonist, 2-Arachidonoylglycerol (2-AG), and C, WIN 55,212-2 mesylate (WN55212-2) in a CB_1 no-pep (-) sensor (representative curves from N = 2 independent biological replicates composed of ≥ 3 technical repeats each). cAMP levels shown in the absence (gray line) and presence (black line) of pertussis toxin (PTX) treatment. Ligands potentiate forskolin-stimulated cAMP accumulation at 30 $\mu\text{mol/L}$, suggesting G_s bias (B and C, red dashed lines). 2-AG and WIN 55,212-2 mesylate (WN) inhibit forskolin-stimulated cAMP at 50 nmol/L and 300 nmol/L, respectively, suggesting G_i bias (B and C, green dashed lines). cAMP levels of tethered CB_1 sensors after stimulation by forskolin and 30 $\mu\text{mol/L}$ 2-AG (D) or WN (E) (N = 5 independent biological replicates). F, summary of $G\alpha$ peptide influence on G_s signaling and cAMP production in CB_1 . Inhibition of forskolin-stimulated cAMP by tethered CB_1 sensors after stimulation by 50 nmol/L 2-AG (G) (N = 8 independent biological replicates) or 300 nmol/L WN (H) (N = 6 independent biological replicates). I, summary of $G\alpha$ peptide influence on G_i signaling and cAMP inhibition in CB_1 . GPCR- $G\alpha$ C-terminal peptide sensors are compared with the no-pep (-) control. Results are expressed as mean \pm SE. **** $P < .0001$; * $P < .05$

(2-AG) (Figure 1B, red dashed line) or WIN 55,212-2 mesylate (WN) (Figure 1C, red dashed line).^{10,13} Representative dose-response curves with untransfected HEK 293 cells are shown in Figure S2 with stimulation by 2-AG (Figure S2, green) or WN (purple). We observed no potentiation of forskolin-stimulated cAMP accumulation in untransfected HEK 293 cells in response to a range of 2-AG and WN concentrations (Figure S2), suggesting any potentiation of forskolin-stimulated cAMP accumulation can be attributed to transfected CB_1 receptors rather than endogenous receptors in the HEK 293 cells. CB_1 appeared to inhibit forskolin-stimulated cAMP accumulation with signaling dominated by G_i in response to 50 nmol/L 2-AG (Figure 1B, green dashed line) or 300 nmol/L WN (Figure 1C, green dashed line). To characterize G_i signaling in CB_1 , dose-response curves were performed for both 2-AG and WN (Figure 1B and C,

respectively) in the presence (black lines) or absence (gray lines) of pertussis toxin (PTX). cAMP levels increased in response to PTX treatment in 2-AG-stimulated CB_1 (Figure 1B, black line), indicating that cAMP inhibition in the absence of PTX is likely due to signaling through G_i . 2-AG or WN can be used at high concentrations (30 $\mu\text{mol/L}$, Figure 1B and C, red dashed lines) to characterize the impact of peptides on cAMP stimulation and G_s signaling (Figure 1D and E) and at low concentration (50 or 300 nmol/L, Figure 1B and C, green dashed lines) to characterize cAMP inhibition and signaling through G_i in CB_1 (Figure 1G and H).

We examined the allosteric modulation of $G\alpha_s$, $G\alpha_i$, and $G\alpha_q$ peptides on forskolin-stimulated cAMP accumulation in the promiscuous G_i -coupled receptor, cannabinoid type 1 (CB_1). SPASM sensors with s-, i-, or q-pep fusions, in addition to a no-pep control (-), were

expressed in HEK 293 cells as shown previously.²⁸ Cells expressing the CB₁ sensors were treated with high concentrations (30 μmol/L) of 2-AG (Figure 1D) or WN (Figure 1E) to stimulate cAMP production through the G_s pathway (Figure 1B and C). The q-pep sensor was found to increase signaling through G_s in CB₁, as evidenced by a significant increase in cAMP levels (Figure 1D and E, blue bars). This finding in a Gi-coupled receptor extends our previous results where q-pep exhibited enhanced signaling in the G_s pathway in G_s-coupled receptors.⁸ S-pep sensors also increased signaling through G_s in CB₁ after stimulation by WN (Figure 1E, red bar). In contrast, the presence of i-pep inhibited G_s signaling in CB₁ after stimulation by 2-AG or WN, decreasing cAMP levels (Figure 1D and E, green bars). These findings are also summarized in the schematic (Figure 1F) with q-pep (blue) and s-pep (red) stimulating G_s signaling and i-pep (green) inhibiting signaling through G_s.

Gα peptides affected signaling through Gi-mediated inhibition of forskolin-stimulated cAMP accumulation in CB₁. To target Gi signaling, HEK 293 cells expressing CB₁ SPASM sensors were treated with low concentrations of 2-AG (50 nmol/L) or WN (300 nmol/L), conditions resulting in cAMP inhibition (Figure 1B and C). The i-pep increased the inhibition of cAMP production after stimulation by 2-AG (Figure 1G, green bar) compared to the no-pep (-) sensor. Treatment with WN leads to an increase in Gi signaling with q-pep but not with i-pep (Figure 1H, blue bar). The agonist-dependent enhancement of Gi signaling by both i-pep and q-pep is summarized in the schematic (Figure 1I).

3.3 | Impact of Gα C-terminal peptides on cAMP inhibition in the Adenosine (A₁R) Receptor

Cells expressing the A₁R no-pep (-) sensor display Gi-mediated inhibition of forskolin-stimulated cAMP accumulation after stimulation by 50 nmol/L of the A₁R agonist, N⁶-Cyclopentyladenosine (CPA) (Figure 2A, green dashed line). Pertussis toxin (PTX) treatment inhibits Gi signaling, allowing for differentiation between the G_s- and Gi-mediated effects on cAMP.²⁹ cAMP levels increased in response

to PTX treatment in CPA-stimulated A₁R (Figure 2A, black line), indicating that cAMP inhibition in the absence of PTX is likely due to signaling through Gi. To characterize the impact of different Gα peptides on Gi inhibition of forskolin-stimulated cAMP accumulation in a promiscuous Gi-coupled receptor, cells expressing the different A₁R peptide sensors at equivalent levels were treated with 50 nmol/L of CPA resulting in cAMP inhibition, dominated by Gi (Figure 2B). The i-pep and q-pep both increased signaling through Gi in A₁R after stimulation by CPA, as evidenced by a significant increase in cAMP inhibition (Figure 2B, *green and blue bars*, respectively). To address potential variability in individual sensor response, for each experiment equivalent sensor expression was verified using fluorescence measurements (see methods) and data for all four peptide sensors were collected together (supplemental Figure S3). This phenomenon is summarized in a schematic (Figure 2C) showing the presence of i-pep (green) and q-pep (blue) increasing signaling through Gi and inhibiting cAMP.

Despite the potentiation of forskolin-stimulated cAMP accumulation at high concentrations of CPA (Figure 2A), the cAMP accumulation appears to be the result of stimulation of endogenous HEK 293 cell receptors rather than G_s signaling through A₁R receptors. Untransfected HEK 293 cells treated with 30 μmol/L CPA showed higher potentiation of forskolin-stimulated cAMP accumulation than was seen with HEK 293 cells transfected with A₁R (Figure S4). Under the same conditions, pretreatment with 100 nmol/L of the A_{2A}R selective antagonist, SCH 442 416 (SCH), and 1 μmol/L of the A_{2B}R selective antagonist, PSB 1115 (PSB), resulted in complete inhibition of cAMP production. The slight decrease in forskolin-stimulated cAMP accumulation in cells transfected with A₁R without antagonist pretreatment can likely be attributed to increased Gi signaling by transfected A₁R receptors. We performed the same control experiments with untransfected HEK 293 cells treated with 30 μM NECA and found equivalent potentiation of forskolin-stimulated cAMP accumulation as compared to A₁R-transfected cells (Figure S4). Pretreatment with A_{2A}R and A_{2B}R selective antagonists, SCH and PSB, did not change cAMP accumulation in untransfected cells. However, pretreatment with SCH and PSB in A₁R-transfected cells

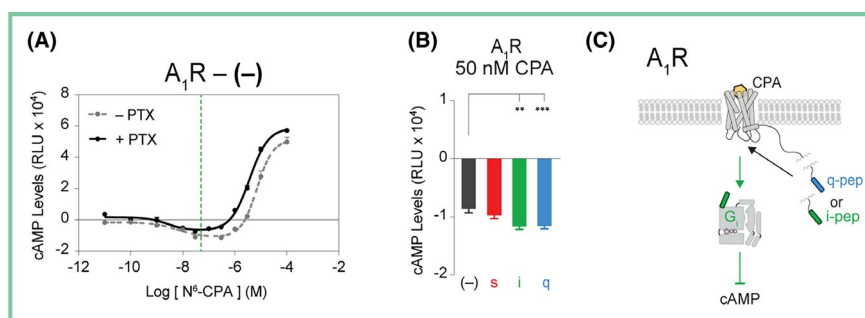


FIGURE 2 Characterization of cAMP modulation in adenosine receptor (A₁R) by SPASM sensors. Forskolin-stimulated cAMP dose-response curves of (A), A₁R agonist, N⁶-Cyclopentyladenosine (CPA). cAMP levels shown in the absence (*gray line*) and presence (*black line*) of pertussis toxin (PTX) treatment. 50 nmol/L CPA inhibits forskolin-stimulated cAMP, suggesting Gi bias (A, *green dashed line*). B, Inhibition of forskolin-stimulated cAMP by tethered A₁R peptide sensors after stimulation by 50 nmol/L CPA. C, Summary of Gα peptide influence on Gi signaling and cAMP inhibition. GPCR-Gα C-terminal peptide sensors are compared with the no-pep (-) control. Results are expressed as mean ± SE. ***P < .001; **P < .01. N = 8 independent biological replicates

reduced cAMP accumulation by 50%. In both cases, treatment with either 30 $\mu\text{mol/L}$ CPA or NECA appears to increase forskolin-stimulated cAMP accumulation due to endogenous receptors in the HEK 293 cells. A representative dose-response curve shows potentiation of forskolin-stimulated cAMP accumulation in untransfected HEK 293 cells in response to a range of CPA and NECA concentrations (Figure S2). We therefore could not characterize the impact of G α peptides on Gs signaling in A₁R.

3.4 | C-terminal G α Peptides Inhibit Gq Signaling from Promiscuous Receptors

Previous work from our lab suggests that the effect of noncanonical G proteins on IP₁ signaling are more receptor specific.⁸ We found that Gs enhances IP₁ production and signaling through Gq in the vasopressin receptor (V_{1A}-R) but not the $\alpha 1$ adrenergic receptor ($\alpha 1$ -AR).⁸ In the current study we examined the impact of G α peptides on Gq signaling and IP₁ production in A₁R and CB₁ receptors. A dose-response study of NECA (Figure 3A, *black line*) and CPA (*gray lines*) with A₁R no-pep (-) sensors revealed maximum IP₁ signal at 100 $\mu\text{mol/L}$ ligand (*blue dotted line*). To rule out G $\beta\gamma$ -dependent PLC- β activation, we performed IP₁ dose-response assays in the absence (Figure 3A, *dark gray line*) and presence (*light gray line*) of pertussis toxin (PTX)

treatment. Regardless of CPA concentration, no reduction in IP₁ production was observed in PTX-treated cells compared to untreated cells, suggesting the observed IP₁ production is due to A₁R signaling through the PTX-insensitive Gq pathway. Additionally, to rule out Gq signaling through endogenous HEK 293 A_{2B}R receptors, IP₁ levels were assessed in untransfected HEK 293 cells after stimulation by 100 $\mu\text{mol/L}$ CPA or NECA (Figure S5). Regardless of pretreatment with 1 $\mu\text{mol/L}$ of the A_{2B}R selective antagonist PSB 1115 (PSB), significant IP₁ production occurred in A₁R transfected cells but not in untransfected HEK 293 cells, suggesting IP₁ production resulted from Gq signaling through A₁R and not endogenous A_{2B}R (Figure S5). A₁R SPASM sensors with tethered s-, i-, or q-pep, in addition to a no-pep (-) sensor lacking a peptide, were expressed in HEK 293 cells to equivalent levels. IP₁ assays were performed with each of the A₁R sensor constructs after stimulation by 100 $\mu\text{mol/L}$ CPA (Figure 3B, left) or NECA (right). Constructs containing the s-pep, i-pep, or q-pep inhibited IP₁ production regardless of ligand, as summarized in the schematic (Figure 3C).

To examine the impact of the G α peptides on Gq signaling in a second promiscuous receptor, CB₁, we first performed assays to identify the optimal concentration of ligand to use for characterization. A dose-response study of WN (Figure 3D, *black line*) and 2-AG (*gray lines*) on CB₁ no-pep (-) sensors revealed maximum IP₁ signal at 100 $\mu\text{mol/L}$ ligand (*blue dotted line*). To rule out G $\beta\gamma$ -dependent

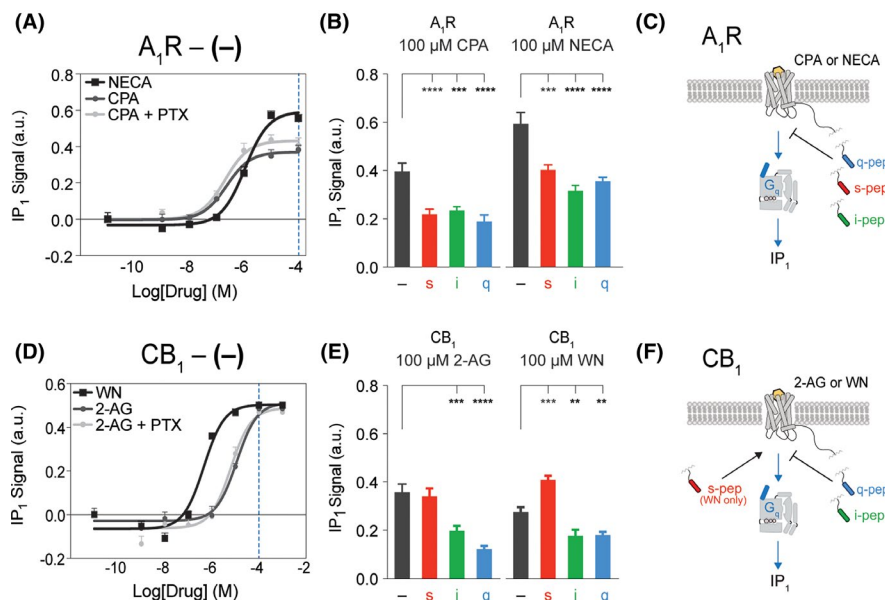


FIGURE 3 G α q and G α i peptides inhibit signaling through Gq. A, IP₁ dose-response curve of A₁R agonists, CPA (*gray lines*) (representative curves from N = 2 independent biological replicates composed of ≥ 3 technical repeats each) and NECA (*black line*) (N = 3 technical repeats), with A₁R-no-pep (-) sensor. IP₁ levels shown in the absence (*dark gray line*) and presence (*light gray line*) of pertussis toxin (PTX) treatment. 100 $\mu\text{mol/L}$ CPA or NECA stimulate IP₁ (A, *blue dashed line*). B, IP₁ signal from A₁R after stimulation by 100 $\mu\text{mol/L}$ CPA (left) (N = 3 independent biological replicates) or NECA (right) (N = 4 independent biological replicates) in the presence of different G α C-terminal peptides compared to no-pep (-) control. C, summary of G α peptide influence on Gq signaling and IP₁ production in A₁R. D, IP₁ dose-response curve of CB₁ agonists, 2-AG (*gray lines*) (N = 5 independent biological replicates) and WN (*black line*) (N = 4 independent biological replicates), with CB₁-no-pep (-) sensor. IP₁ levels shown in the absence (*dark gray line*) and presence (*light gray line*) of pertussis toxin (PTX) treatment. 100 $\mu\text{mol/L}$ 2-AG or WN stimulate IP₁ (D, *blue dashed line*). E, IP₁ signal from CB₁ G α C-terminal peptide sensors after stimulation by 100 $\mu\text{mol/L}$ 2-AG (left) or WN (right) compared to no-pep (-) control (N = 3 technical repeats). F, summary of G α peptide influence on Gq signaling and IP₁ production in CB₁. Results are expressed as mean \pm SE. ****P < .0001; ***P < .001; **P < .01

PLC- β activation, we performed IP₁ dose-response assays in the absence (Figure 3D, dark gray line) and presence (light gray line) of pertussis toxin (PTX) treatment. Regardless of 2-AG concentration, no reduction in IP₁ production was observed in PTX-treated cells compared to untreated cells, suggesting the observed IP₁ production is due to CB₁ signaling through the PTX-insensitive Gq pathway. An IP₁ assay was performed on HEK 293 cells expressing SPASM sensors with s-, i-, or q-pep fusions, in addition to a sensor lacking a peptide no-pep (-), after stimulation by 100 μ mol/L 2-AG (Figure 3E, left) or WN (right). Consistent with A₁R, the i-pep and q-pep inhibited signaling through Gq in CB₁, as evidenced by reduction of IP₁ signal (Figure 3E, green and blue bars, respectively). The s-pep significantly enhanced signaling through Gq after stimulation by 100 μ mol/L WN (Figure 3E, right, red bar). The influence of i-pep, q-pep, and s-pep on Gq signaling and subsequent IP₁ production in CB₁ is summarized in the schematic (Figure 3F). We have summarized these findings in supplemental model Figure S6, highlighting how G α C-terminal peptides differentially influence signaling in each of these promiscuous receptors.

4 | DISCUSSION

In this study we demonstrate the allosteric modulation of two Gi-coupled receptors, A₁R and CB₁, using peptides derived from the C-terminus of the G α subunit (G-peptides). G-peptides derived from G α i and G α q (i-pep and q-pep) enhance agonist-dependent cAMP inhibition, demonstrating their function as positive allosteric modulators of Gi-coupled signaling. In contrast, i-pep and q-pep suppress agonist-dependent IP₁ levels suggesting that they function as negative allosteric modulators of Gq-coupled signaling. Taken together with our previous studies focused on Gs-coupled receptors, our findings reinforce the potential of G-peptides to allosterically modulate signaling from class A GPCRs.^{8,9} While allosteric modulation of GPCR signaling has gained prominence to address the need for receptor specificity, efforts have mainly focused on allosteric sites adjacent to the orthosteric ligand-binding pocket and lipophilic molecules that target transmembrane helices.² In contrast, here we use as G-peptides as probe molecules to demonstrate allosteric modulation through the GPCR-G protein binding interface.

The two Gi-coupled receptors (CB₁ and A₁R) examined in this study have also been reported to signal to varying degrees through other G proteins.^{10-13,16} While traditionally described as a Gi-coupled receptor, it has been demonstrated that A₁R can couple to Gs and Gq in response to CPA or NECA, suggesting A₁R can adopt agonist-specific conformations arising from small differences in ligand structure leading to differential G protein activation.¹¹ However, previous studies emphasize A₁R signaling through Gi and contradict signaling through Gs.^{30,31} Our data suggest any apparent Gs signaling by A₁R, measured by potentiation of forskolin-induced cAMP production, cannot be distinguished from activation of endogenous A_{2A}R or A_{2B}R receptors by A₁R agonists. We saw significantly higher

potentiation of forskolin-induced cAMP production in untransfected HEK 293 cells compared to A₁R-transfected HEK 293 cells in response to CPA, suggesting CPA is likely stimulating endogenous Gs-coupled receptors (Figure S4). Further investigation revealed A_{2A}R and A_{2B}R specific antagonists could inhibit this potentiation of cAMP in untransfected cells, suggesting any potentiation of forskolin-induced cAMP production likely resulted from stimulation of endogenous A_{2A}R or A_{2B}R receptors. Therefore, we could not independently examine A₁R signaling through the Gs pathway. The A_{2B}R receptor is also known for signaling through Gq, however, control experiments confirmed Gq signaling likely occurred through A₁R and not A_{2B}R since no significant IP₁ production was seen in untransfected HEK 293 cells (Figure S5). In accordance with a previous studies, we confirmed CB₁ did indeed signal through Gs, as no significant potentiation of forskolin-induced cAMP production was observed in untransfected HEK 293 cells stimulated by the CB₁ agonists 2-AG or WN (Figure S2).^{10,13,16} We therefore used CB₁ to examine the impact of G-peptides on Gs signaling, with findings consistent with our previous report for the Gs selective β 2-AR receptor (Figure 1D-F).⁸

Our data contrast with previous studies that report inhibition of GPCR signaling by native cognate G-peptides.³²⁻³⁴ In these studies, minigene vectors were used to overexpress cognate G-peptides in cells at arbitrarily high concentrations, in order to identify and selectively inhibit cognate G protein engagement with the receptor. Accordingly, we have previously shown that high concentrations of cognate G-peptides (100 μ mol/L s-pep) can competitively inhibit signaling from Gs-coupled receptors.^{8,9} In contrast, we find that noncognate G-peptides can bind weakly to the receptor and serve as positive allosteric modulators.^{8,9} While no significant positive allosteric effects were noted in studies with minigene vectors encoding noncognate G-peptides, these could be attributed to the variation and/or lack of control in expression since saturating levels would result in inhibition.³²⁻³⁴ To alleviate the confounding effects of G-peptide concentration, we used the SPASM constructs to provide equivalent effective concentrations of distinct G-peptides across different receptor-ligand-pathway combinations. Furthermore, the ER/K linker in the SPASM sensors provides an effective concentration of approximately 10 μ mol/L,¹⁸ which is significantly lower than our previously reported threshold for competitive inhibition by cognate G-peptides. Using this technology, we observe differential effects of G-peptides on distinct pathways emerging from the same receptor. Specifically, while both i-pep and q-pep augment Gi-mediated cAMP inhibition, they suppress IP₁ accumulation downstream of Gq activation. Given that sensor expression levels were matched between cAMP and IP₁ assays and the ER/K linked G-peptides (i-pep and q-pep) are presented at equal effective concentrations, it is unlikely that inhibition of Gq signaling stems from a simple competitive inhibition mechanism. Instead, the differential effects of G-peptides likely stem from the dynamic conformational landscape of GPCRs.^{35,36}

We propose a model wherein transient interactions with G-peptides alter receptor conformation. The receptor does not

form a stable ternary complex with the G-peptide and therefore at low concentrations (10 $\mu\text{mol/L}$) does not interfere with the kinetics of the receptor-cognate G protein interaction.⁹ However, the altered receptor conformation triggered by G-peptide binding impacts ligand efficacy for cognate G protein activation, resulting in positive or negative allosteric modulation of downstream responses. The inability of the G-peptides, especially those derived from noncognate G proteins, to form stable interactions with the receptor has been previously observed in A₁R-Gi fusions.³⁷ The lack of stable ternary complex formation with noncognate G proteins has been suggested as a kinetic proofreading mechanism to prevent noncognate GPCR-G protein coupling.³⁷ Nonetheless, we have previously shown that both cognate and noncognate G-peptide interactions influence receptor conformation.⁹ Transient interactions of the G-peptide at the cognate G protein binding site on the receptor stabilize a distinct receptor conformational state. This conformational state persists following G-peptide dissociation enabling increased efficacy of subsequent cognate G protein coupling and enhanced downstream signaling.⁹ Given that the G-peptide and cognate G protein share the same binding site, albeit staggered in time, we propose that the G-peptides function as allosteric modulators (AKMs) of cognate GPCR signaling. Allokairy is an established concept in enzymatic reactions, wherein increased substrate concentrations can increase maximal reaction rates, especially if the substrate stabilizes a distinct active enzyme conformation.³⁸ AKMs can bind asynchronously with the orthosteric ligand and rely on temporally persistent conformational states of the enzyme to exert their effects.⁹ G-peptides as AKMs provide access to the entire GPCR-G protein interaction interface for allosteric modulation, without necessarily competing with cognate G protein coupling. Targeting the GPCR-G protein interface offers the potential to enhance receptor specificity, especially given the three intrinsically disordered loop regions with considerable isoform specific sequence homogeneity.

ETHICS STATEMENT

No animals, human tissue, human volunteers, or patients were used in this study.

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AUTHORSHIP CONTRIBUTIONS

Touma, Malik, and Sivaramakrishnan participated in research design. Touma, Malik, and Gupte conducted experiments.

Touma, Malik, Gupte, and Sivaramakrishnan performed data analysis.

Touma and Sivaramakrishnan wrote manuscript.

DATA AVAILABILITY STATEMENT

Additional information and requests for data and/or reagents should be directed to the corresponding author, Dr Sivaramakrishnan. Please contact sivaraj@umn.edu.

ORCID

Anja M. Touma  <https://orcid.org/0000-0001-9953-9695>

Sivaramakrishnan  <https://orcid.org/0000-0002-9541-6994>

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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