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Dynamic mass redistribution reveals diverging importance of PDZ-ligands for G protein-coupled receptor pharmacodynamics

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Abstract

G protein-coupled receptors (GPCRs) are essential membrane proteins that facilitate cell-to-cell communication and co-ordinate physiological processes. At least 30 human GPCRs contain a Type I PSD-95/DLG/Zo-1 (PDZ) ligand in their distal C-terminal domain; this four amino acid motif of X-[S/T]-X-[φ] sequence facilitates interactions with PDZ domain-containing proteins. Because PDZ protein interactions have profound effects on GPCR ligand pharmacology, cellular localization, signal-transduction effector coupling and duration of activity, we analyzed the importance of Type I PDZ ligands for the function of 23 full-length and PDZ-ligand truncated (PDZ) human GPCRs in cultured human cells. SNAP-epitope tag polyacrylamide gel electrophoresis revealed most Type I PDZ GPCRs exist as both monomers and multimers; removal of the PDZ ligand played minimal role in multimer formation. Additionally, SNAP-cell surface staining indicated removal of the PDZ ligand had minimal effects on plasma membrane localization for most GPCRs examined. Label-free dynamic mass redistribution functional responses, however, revealed diverging effects of the PDZ ligand. While no clear trend was observed across all GPCRs tested or even within receptor families, a subset of GPCRs displayed diminished agonist efficacy in the absence of a PDZ ligand (i.e. HT2RB, ADRB1), whereas others demonstrated enhanced agonist efficacies (i.e. LPAR2, SSTR5). These results demonstrate the utility of label-free functional assays to tease apart the contributions of conserved protein interaction domains for GPCR signal-transduction coupling in cultured cells.

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Competing Financial Interests Statement

The authors have no competing financial interests.

Author Contributions

N.D.C., K.-S.L., J.L.W.-M., N.S., A.W.-Y. and C.H. designed experiments.

N.D.C., K.-S.L., A.C., J.L.W.-M., T.K., J.-M.P., D.-A.H., M.E., A.S., A.E.C., A.W.-Y. and C.H. performed experiments.

N.D.C., N.S., A.W.-Y. and C.H. wrote the manuscript.

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Graphical Abstract

Keywords

G protein-coupled receptor; Label-free signaling; PDZ domain; Pharmacology

1. Introduction

G-protein coupled receptors (GPCRs) are essential for cell-to-cell communication and regulation of physiological events. To perform their designated functions, GPCRs must specifically interact with key proteins, the most thoroughly characterized being the heterotrimeric G-proteins ($G\alpha$, β and γ), which transmit the energy of agonist-GPCR binding to cellular response [1]. Interestingly, proteomic (i.e. affinity purification/mass spectrometry) and yeast-based (i.e. 2-hybrid) screening approaches developed over the last decade permitted high-throughput, unbiased identification of numerous novel GPCR-interacting proteins [2,3]. Indeed, GPCRs are expressed as intricate macromolecular complexes in cell membranes, with the GPCR acting as the central hub of signaling networks; a dynamic scaffold that temporally and spatially directs cellular traffic.

With this next era of GPCR molecular pharmacology comes the promise of innovative approaches to drug discovery. Targeting interaction interfaces between GPCRs and associated proteins may permit molecular tweaking of distinct GPCR signaling events, simultaneously inhibiting signaling events that are toxic whilst enhancing those that are beneficial. This endeavor is in its infancy, requiring thorough identification of GPCR interacting proteins with cell-type accuracy, and identifying divergent downstream signaling cascades linked to individual GPCR interaction modules.

Thus far, assessing how interacting proteins contribute to GPCR function has been limited to reductionist outputs: second messenger formation (i.e. cAMP/cGMP, Ca^{2+} , ERK1/2), enzyme activity (i.e. phospholipase C, protein kinase A/C), bioluminescence/fluorescence energy transfer (BRET/FRET), cellular localization with high resolution microscopy and arrestin-association. Although informative, these assays are narrow in scope, each unable to identify unknown components of GPCR signaling networks. Label-free dynamic mass redistribution (DMR) technology represents an innovative approach to analyze complex

GPCR signaling networks [4–6]. This assay involves passing polarized light through the glass bottom of a biosensor microtiter plate seeded with cells, then measuring shifts in the wavelength of reflected light over time. The shifts in wavelength are due to changes in intracellular mass near the membrane in response to exogenous stimulation, such as an agonist. As small as 1 picometer (pm) changes in wavelength can be reliably detected, and the direction of the overall change in cellular mass is indicated by whether the response is positive or negative. A positive response indicates an overall increase of cellular mass towards the cell membrane, whereas a negative response is indicative of cellular mass moving away from the membrane [7]. Similar to classic organ-tissue bath assays, which in effect are a summation of all the signaling events linking GPCR-stimulation to a contraction/relaxation event, DMR responses represent holistic changes in cellular mass and permit divergent GPCR signaling cascades to be analyzed without the need of a cell reporter. This is particularly useful for directly comparing GPCRs that couple to varying G proteins such as G α s (i.e. β -adrenergic receptors), G α i (i.e. β_2 -adrenergic receptors) or G α q (i.e. β_1 -adrenergic receptors) [6].

Remarkably, at least 30 human GPCRs contain putative Type I PSD-95/DLG/Zo-1 (PDZ) ligands on their distal C-terminus with amino acid sequence X-[S/T]-X-[φ] [8]. This small protein-protein interaction domain permits GPCRs to associate with one or more of the ~180 PDZ domain-containing proteins encoded in the human genome. Once bound, PDZ-proteins may modulate GPCR pharmacodynamic properties via scaffolding effector proteins in close proximity, organizing GPCR complexes as discrete microdomains in cells, or linking GPCRs to non-canonical signaling events [2,3]. We previously demonstrated the Type I PDZ α_{1D} -adrenergic receptor (AR) forms a macromolecular complex with PDZ-proteins scribble (SCRIB) and multiple isoforms of syntrophin (SNTA, SNTB1, and SNTB2), which impart functionality and distinct cellular localization to the receptor [9–13]. The specific contributions of each PDZ protein for ADRA1D function and agonist efficacy in human cells was determined by DMR technology. SCRIB and syntrophins bind C-terminal ADRA1D PDZ-ligands on discrete protomers within ADRA1D homodimers/oligomers, and in doing so, differentially regulate agonist efficacy [13].

Given the importance of PDZ-protein interactions for GPCR function, we subjected 23 human Type I PDZ GPCRs to label-free DMR assays in human cells with and without PDZ ligand truncation. Although no definitive role of the PDZ ligand was identified across all GPCRs tested or even within GPCR families, we identified 9 GPCRs for which the PDZ ligand differentially regulated receptor activation.

2. Materials and Methods

2.1. Plasmids, chemicals and antibodies

Human GPCR cDNAs were purchased from the Missouri S&T cDNA resource center (cdna.org) or cloned from human brain cDNA library (kindly provided by Prof. Ning Zheng, HHMI, University of Washington Department of Pharmacology). cDNAs were subcloned into pSNAPf/pCLIPf (New England Biolabs) using In-Fusion HD cloning technology (Clontech).

GPCR agonists (–)-isoproterenol hydrochloride (I6504), 5-hydroxytryptamine hydrochloride (H9523), clonidine hydrochloride (C7897), somatostatin (S9129), interleukin-8 (CXCL8, SRP3098), histamine dihydrochloride (H7250), UTP (U6875) and benzeneacetamide (C4494) were purchased from Sigma; [Ala¹⁷]-melanin concentrating hormone (3434), sphingosine-1-phosphate (1370) and galanin 1–30 (1179) from Tocris Bioscience.

SNAP-surface 782 substrate from New England Biolabs (S9142S). Topro-3 iodide (T3605) is from Life Technologies. Anti-HA mouse mAb (6E2, #2367) from Cell Signaling. IRdye 680 goat antimouse IgG and IRdye 800cw goat antirabbit IgG from Li-Cor.

2.2. Cell Culture and Reagents

Human embryonic kidney (HEK) 293T cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum and 2 mM L-glutamine. Cells were transfected with 1mg/ml polyethylenimine (PEI) and used 24–48 hr post transfection.

2.3. SNAP Cell Surface Assays

HEK293T cells were seeded in 6 well plates at 8×10^5 cells/well. Cells were transfected with SNAP-tagged cDNA constructs/PEI and replated in 96 well black optical bottom cell culture plates. Cell density was ~ 90% confluency prior to assay commencement. SNAP-Surface 782 substrate was diluted in DMEM to designated concentrations and incubated at 37°C/5% CO₂ for 30 min. Cells were washed, fixed with 4% paraformaldehyde, then incubated with 1:10000 nuclear stain TOPRO-3 to normalize for cell number. Plates were analyzed with the LI-COR Odyssey Scanner (Li-Cor Biotechnology) and signal intensity quantified.

2.4. SNAP-PAGE

HEK293T cells were transfected with SNAP-tagged proteins. 48 hours after transfection, cells were lysed with 50 mM Tris-HCl, 150 mM NaCl, 1% NP40, and 0.1% Tween 20 buffer. Final concentration of 0.5 μM BG-782 substrate and 1 mM DTT were added to lysates for substrate binding reaction and samples were incubated for 30 minutes @37°C in the dark. Samples were then run on SDS-PAGE without boiling and gels were imaged using LI-COR Odyssey Scanner.

2.5. Label-free Dynamic Mass Redistribution (DMR) Assays

HEK293T cells were seeded at ~500k/well in Corning Epic sensor microplates and cultured for 24 hr. Cells were washed 3x with HBSS buffer and transferred to Corning Epic BT reader @37°C. Baseline DMR measurements were recorded for 1 hr. Compounds were added with the Sorenson Biosciences 96-well Benchtop Pipettor and agonist DMR responses were recorded for 1 hr. Data were exported to Microsoft Excel using Epic Analyzer Software. DMR background responses (buffer triggered responses) were subtracted from all datasets, as they are thought to occur as a result of (1) buffer bulk refractive index difference between the assay buffer and compound solution, (2) temperature mismatch or (3) a mechanical issue resulting from movement of the Epic 384 well plate between the Epic BT apparatus and the Sorenson Pipettor, as previously described in [7]. Error bars are displayed in agonist-concentration response curves, and not Epic DMR traces, to improve figure

clarity. For Schild Plot analysis, data were analyzed using linear regression analysis using the method first described in [14].

2.6. Data Analysis

Data were analyzed with GraphPad Prism 6 software and expressed mean $\pm$ SEM. Differences in agonist-stimulated DMR responses were tested for significance using student's t-test ($p < 0.05$).

3. Results

3.1. Importance of Type I PDZ ligands for GPCR protein expression

Before examining the potential effects of PDZ-ligands for GPCR DMR responses, we ensured that any observed effects were not a result of significant changes in GPCR expression. Thus, we first examined the impact of PDZ ligand truncation (PDZ) on GPCR protein levels. To do so, full length and PDZ GPCRs were subcloned into the pSNAP vector to add N-terminal human O⁶-alkylguanine-DNA alkyltransferase epitope tags. SNAP technology eliminates the need for traditional antibody-based Western blotting [15], which is highly problematic for transmembrane spanning GPCRs; commercially available GPCR antibodies are of questionable value, often detecting numerous non-specific artifacts [16].

SNAP-tagged GPCRs were transfected into HEK293T cells and imaged on a polyacrylamide gel in the 700–800 nm wavelength range by LI-COR (Fig. 1). We consistently observed a GPCR band at the predicted monomer size, but also as higher order bands, which may represent homo/heterodimers, and/or higher order oligomers. In many cases GPCRs displayed multiple bands of similar size to the full length receptor (i.e. CXCR1–3, HTR2A, SSTR1, SSTR2), possibly representing post-translation modifications (i.e. glycosylation). Smaller bands in the 20–36 kDa range were observed for CXCR2, CXCR3, and P2RY12.

While most WT and PDZ GPCRs examined were expressed at similar densities, several exhibited differential expression. For example, PDZ ADRA2B expression was diminished compared to WT ADRA2B at all observed fragments. Interestingly, increased levels of lower molecular weight fragments in the PDZ versions of SSTR3 and GALR1 were observed, whereas decreased levels of a lower molecular weight fragment were observed for PDZ CXCR3 as compared to the WT receptor. These observations suggest that in several instances, the PDZ ligand regulates expression levels and cleavage events involved in receptor processing.

3.2. Role of Type I PDZ ligands for GPCR membrane localization

We next employed SNAP cell surface expression assays to determine if PDZ-ligand truncation significantly impacted the ability of GPCRs to be trafficked to the plasma membrane. WT and PDZ SNAP-GPCRs were transfected into HEK293T cells, seeded in 96-well glass bottom plates, fixed and incubated with increasing concentrations of the cell-impermeable SNAP-substrate BG782 (Fig. S1). As shown, no detectable SNAP-staining in untransfected (UT) or empty vector (SNAP) transfected HEK293T cells is present.

HTR2A demonstrated the greatest cell surface expression of all GPCRs tested and was used to normalize expression of all other GPCRs (Fig. S1, data summarized in Table 1). Only 4 receptors achieved > 50% HTR2A cell surface expression levels: P2RY1 (86.61 ± 3.83), LPAR2 (58.82 ± 4.74), SSTR3 (58.46 ± 6.51) and CXCR1 (56.27 ± 2.89). As previously reported by us and others [17–21], minimal ADRA1D cell surface expression was achieved in HEK293T cells, as it requires co-transfection of syntrophins and/or SCRIB, or truncation of the N-terminal domain. Other receptors that achieved minimal cell surface expression included MCHR2 (3.38 ± 7.79), C3AR1 (5.65 ± 0.96) and ADRA2B (7.26 ± 0.91).

Overall, deletion of the PDZ ligand produced subtle effects on cell surface expression. In certain cases, PDZ ligand truncation caused a >10% reduction in cell surface expression (HTR2A, SSTR3, P2YR1, MCHR2, C3AR1), or a >10% increase (LPAR2, SSTR5, HRH3). However, in no examples did removal of the PDZ ligand completely abolish GPCR cell surface expression, suggesting that this domain is not absolutely essential for plasma membrane localization for the GPCRs as examined by SNAP cell-surface assays.

3.3. DMR measures direct activation of GPCRs

Although others have demonstrated that observed DMR signals generated by the addition of GPCR agonists are induced by heterotrimeric G-protein signal transduction pathways [6], we wished to confirm that our DMR responses are also due to direct GPCR signaling. Thus, concentration-response curves (CRCs) were generated for the β -agonist isoproterenol in HEK293T cells expressing ADRB1, in the absence and presence of increasing amounts of the competitive β -adrenergic receptor antagonist propranolol (Fig. 2). Propranolol progressively inhibited 3 μ M isoproterenol-stimulated DMR curves (Fig. 2a), and rightward-shifted isoproterenol DMR CRCs (Fig. 2b, Fig. S2). Schild plot analysis calculated the propranolol functional affinity constant for inhibiting isoproterenol DMR responses to be 4.57 nM ($pA_2 = -8.34 \pm 0.49$ M) (Fig. 2c), which is in the range of previously reported propranolol functional affinities [21,22]. The slope of the Schild plot was 1.04 ± 0.08 , indicative of one-site competition mode of antagonist binding. Taken together, these observations validate that DMR responses are a direct result of GPCR stimulation.

3.4. DMR signatures of Type I PDZ GPCRs

Confident that PDZ ligand truncated GPCRs can still achieve significant levels of protein and plasma membrane expression and that DMR responses are the result of direct activation of GPCRs, we next subjected all WT and PDZ GPCRs to label-free DMR. GPCR cDNAs were expressed in HEK293T cells and DMR signatures were recorded for 30–60 minutes following agonist stimulation. The time point at which the greatest DMR signal was achieved was determined and termed time-to-peak (TTP) response. TTP was used to create CRCs, from which agonist potencies (EC_{50}) and maximal DMR responses (Max) were calculated (Table 2). A plethora of GPCR signatures were observed (Figs. S2–S6). With the exception of S1PR2, all maximum DMR values were positive, suggesting that activation of these receptors leads to an overall increase in cellular mass towards the membrane. The majority of WT GPCRs produced biphasic curves, with a rapid TTP occurring in the initial 5–10 minutes. After reaching TTP, certain GPCRs reached a steady state DMR which remained stable for the remainder of the experiment (HTR2A, HTR2C, MCHR2, ADRA2B,

ADRB1). Other GPCRs displayed either a rapid, (CXCR1, CXCR2, P2YR1, P2YR2, HRH3) or prolonged (SSTR1, SSTR4, GALR1) return to baseline DMR. The greatest DMR responses were stimulated by the ADRB1 (255.12 ± 3.74) and ADRB2 (251.48 ± 15.45) subtypes; other GPCRs that produced robust DMR responses included HTR2A (118.1 ± 10.5), P2YR12 (170.04 ± 2.93) and P2YR1 (116.19 ± 2.22). In certain cases GPCRs produced very weak, if any, DMR responses (CXCR3, CXCR5, C3AR1). Agonist potencies ranged from the subnanomolar (Interleukin-8 for CXCR1 and CXCR2; Galanin 1–30 for GALR1) to micromolar concentrations (UTP for P2Y receptors).

3.4.1. GPCR DMR responses affected by PDZ ligand truncations—We next focused on DMR responses in the absence of PDZ ligands. As expected multiple GPCRs required an intact PDZ ligand for DMR response. For example, the PDZ ligand is required for isoproterenol activation of ADRB1, which gradually reaches a steady state and remains relatively stable. Similarly, the PDZ ligand is also required for interleukin-8 (IL-8) activation of CXCR1. However, unlike the DMR response of ADRB1 activation, activation of CXCR1 is rapid and transient (Fig. 3). In addition to ADRB1 and CXCR1, deletion of the PDZ ligand in MCHR2 and S1PR2 also lead to decreased DMR response to agonist, suggesting the PDZ domain is required for DMR responses by these receptors (Fig. 4).

In addition to the above examples, where the PDZ ligand was required for DMR responses, we also observed multiple receptors in which deletion of the PDZ ligand resulted in an enhanced DMR response. For example, agonist stimulation of WT HTR2B produced a modest DMR response (Fig. 5a). However, the maximal response more than doubled when the PDZ ligand was deleted (Fig. 5b). Similarly, the maximal response more than tripled when the PDZ ligand was deleted from SSTR5 (Fig. 5c, 5d). In addition to HTR2B and SSTR5, deletion of the PDZ ligand enhanced the maximal DMR response in SSTR2, LPAR2, and GALR1, suggesting the PDZ ligand inhibits signaling by these receptors (Fig. 6).

Interestingly, no definitive trends were observed across the entire set of GPCRs tested or within GPCR families when the PDZ ligand was deleted. For example, within the 5-HT receptor family, deletion of the PDZ ligand in HTR2B resulted in an increased DMR response upon the addition of 5-HT, whereas deletion of the PDZ ligand in HTR2A or HTR2C had no effect compared to the WT receptors (Fig. S3). This was also apparent for the somatostatin family of receptors, although the shape of the DMR signature was vastly different. PDZ ligand removal did not affect SSTR1, SSTR3, or SSTR4 DMR responses. In striking contrast, PDZ ligand removal enhanced the efficacy of both SSTR2 and SSTR5 somatostatin-stimulated DMR responses (Fig. S4). Combined, our DMR results highlight the diversity and importance of PDZ ligands for GPCR functional responses, and represents an alternative method to functionally classify GPCRs.

Discussion

With the recent advances in methodologies to provide high resolution GPCR crystal structures, and biophysical approaches to study GPCR signaling at the level of protein-protein interaction with BRET-FRET, it is increasingly clear that GPCR cell signaling is an

intricately complex, tightly orchestrated physiological event. Given GPCRs can selectively associate with numerous accessory proteins, which in turn, impart specific functional properties to a GPCR, novel functional assays are required that provide a readout that encompasses all downstream signaling events with temporal accuracy [24,25].

Our study demonstrates the utility in using label-free DMR assays to analyze complex GPCR signaling events in real time, and to assess the contribution of conserved protein interaction domains for GPCR signaling. In this case, we examined the Type I PDZ ligand, which is found on no less than 30 human GPCRs of broad physiological significance, each of which is coupled to myriad molecular signaling events and regulates a wide variety of physiological processes. This subfamily of GPCRs activates divergent cellular responses, thus making it difficult to compare the importance of Type I PDZ ligands for GPCR activity with common end-point assays. For example, intracellular Ca^{2+} or IP_3 generation is typically used to examine $\text{G}_{\alpha q}$ -coupled receptors, whereas alterations in cytosolic cAMP levels are used to quantify drug efficacy when examining $\text{G}_{\alpha s}$ - and $\text{G}_{\alpha i}$ -coupled receptors. As a result, a multitude of GPCR reporter systems have been created to circumvent this issue [26], yet none share the major advantage of the label-free DMR assays, in that a single functional readout (DMR) can be obtained for any GPCR examined, regardless of the signal-transduction cascade to which it is coupled.

The major finding of this study was that agonist-stimulated DMR responses of at least 9 GPCRs were significantly modified by truncation of the PDZ ligand. Interestingly, no definitive trend was identified based on known characteristics of each GPCR (i.e. G_{α} subtype, agonist, PDZ ligand sequence), demonstrating the complexity and uniqueness of each GPCR signaling cascade. Nevertheless, these observations provide an invaluable resource for understanding individual GPCR responses.

Given that the PDZ ligand is a well-known protein-protein interaction domain, it is likely that PDZ-domain containing proteins have a direct role in binding and modifying the pharmacodynamic properties of the GPCR. We and others have identified multiple GPCR:PDZ protein interactions that regulate GPCR activity. For example, ADRB1 interacts with multiple PDZ proteins, including MAGI2 and PSD95 , both of which have been shown to regulate ADRB1 internalization [27–29]. As shown in this study, the ADRB1 PDZ ligand is required for an agonist-induced DMR response, which may suggest that the response is partially comprised of receptor internalization or dependent upon it. Of note, three additional GPCRs that are poorly understood, namely CXCR1 , MCHR2 , and S1PR2 , also require an intact PDZ ligand for DMR response. Thus, it is plausible that the PDZ ligands in these receptors also regulate receptor internalization, which is important for the function of these receptors. It would be of interest to delineate and compare CXCR1 , MCHR2 and S1PR2 downstream functional responses with the more thoroughly characterized ADRB1 signal transduction cascade.

In addition to receptors that require an intact PDZ ligand for a DMR response, we also identified at least five GPCRs (HTR2B , SSTR5 , SSTR2 , LPAR2 , and GALR1) where the PDZ domain inhibits agonist responses. For example, somatostatin-stimulated DMR responses are minimal in cells expressing WT SSTR5 , whereas the response is increased at

least 3-fold in the absence of the PDZ domain. In our previous proteomic analysis of SSTR5 [12], we were unable to identify PDZ proteins associated with unstimulated or steady-state levels of SSTR5, suggesting that PDZ proteins may be recruited to the receptor upon activation by agonist, which then act to inhibit downstream signaling events. Conversely, in the absence of a PDZ ligand, it is possible that SSTR5 is now able to internalize and proceed with downstream signaling events. In support of this, previous studies demonstrate the PDZ ligand of SSTR5 is not required for plasma membrane targeting, but multiple PDZ proteins, including SNX27, PIST/GOPC and SLC9A3R1/NHERF1, regulate its subcellular trafficking, endocytosis, and recycling to the membrane following agonist stimulation and washout [30, 31].

Finally, we did not detect any noticeable differences in DMR responses between WT and PDZ for 15 GPCRs containing a Type I PDZ ligand. At least two possible explanations exist. First, it is entirely feasible that the PDZ ligand is dispensable for GPCR activation in specific receptors. Second, as we only analyzed these GPCRs in HEK293T cells, it is possible that the expression levels of putative PDZ proteins are insufficient to regulate GPCR activation. In our previous analysis of ADRA1D, we observed weak activation by phenylephrine for both WT and PDZ ADRA1D. However, overexpression of SCRIB and/or syntrophins enhanced the DMR response, nearly 5 fold higher with SCRIB alone [13]. Furthermore, others have shown that DMR response profiles are cell type dependent [6], adding another layer of complexity to GPCR activation and function.

While it is clear that DMR responses comprise G-protein dependent signaling [4–6], our results suggest that it is much more complex, with hints of receptor trafficking and recycling having significant contributions to agonist-induced responses, and possibly G protein-independent signaling events. Future studies that directly assess the relative contributions of each component of GPCR signaling to the DMR responses will provide robust datasets that enhance our general understanding of GPCR biology.

Conclusion

This study demonstrates the utility of label free DMR assays to study complex functional responses stimulated by modular GPCR oligomers in living cells, critical features in the future of drug discovery. Historically, most drugs targeting GPCRs bind to the orthosteric site, in that they compete with the endogenous ligand for binding. A major hurdle in this approach involves imparting ligand selectivity while ensuring adequate bioavailability of investigational small molecules/peptides; chemical similarities of ligand binding pockets in closely related GPCR subtypes makes achieving this task arduous and costly, and as a result, high risk. Thus, the next era of pharmacology may very well involve targeting discrete protein-protein interaction domains within a specific GPCR macromolecular complex. This innovative approach may enhance medicinal therapeutic index and improve clinical outcomes by honing in on discrete signaling events, while reducing off-target modulation of highly homologous GPCR subtypes. To achieve this goal, functional assays, like label-free DMR, that provide a real-time, holistic, consolidated vantage point of all GPCR signaling events with cell-type specificity will be required.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

ADRA1D	α_{1D} adrenergic receptor
ADRA2B	α_{2B} -adrenergic receptor
ADRB1	β_1 -adrenergic receptor
ADRB2	β_2 -adrenergic receptor
C3AR1	Complement Component 3a Receptor 1
CXCR1	Chemokine Receptor 1
CXCR2	Chemokine Receptor 2
CXCR3	Chemokine Receptor 3
CXCR5	Chemokine Receptor 5
GALR1	Galanin Receptor 1
HRH3	Histamine Receptor H3
HTR2A	5-Hydroxytryptamine (Serotonin) Receptor 2A
HTR2B	5-Hydroxytryptamine (Serotonin) Receptor 2B
HTR2C	5-Hydroxytryptamine (Serotonin) Receptor 2C
LPAR2	Lysophosphatidic Acid Receptor 2
MCHR2	Melanin-Concentrating Hormone Receptor 2
P2RY1	Purinergic Receptor P2Y1
P2RY12	Purinergic Receptor P2Y12
S1PR2	Sphingosine-1-Phosphate Receptor 2
SSTR1	Somatostatin Receptor 1
SSTR2	Somatostatin Receptor 2
SSTR3	Somatostatin Receptor 3
SSTR4	Somatostatin Receptor 4
SSTR5	Somatostatin Receptor 5

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Fig. 1. SNAP-PAGE of WT and Δ PDZ-GPCRs

N-terminal SNAP-tagged GPCRs were transfected into HEK293T cells, lysed, incubated with BG 782 and run on PAGE. Full length (WT) and C-terminal Type I PDZ ligand truncated (Δ PDZ) GPCRs were analyzed.

Fig. 2. Propranolol functional affinity for antagonizing isoproterenol-stimulated DMR responses in HEK293T cells expressing ADRB1

a, DMR responses stimulated by 3 μM isoproterenol in the absence and presence of increasing concentrations of the β-adrenergic receptor antagonist propranolol in β1-adrenergic receptor (ADRB1) transfected HEK293T cells. **b,** Isoproterenol-stimulated DMR concentration-response curves in the absence and presence of propranolol. **c,** Schild plot analysis of data in (B). Data are the mean ± SEM of n = 4.

Fig. 3. Epic DMR responses diminished by removal of the GPCR C-terminal PDZ ligand
 Epic DMR responses in HEK293T cells expressing WT (a) or Δ PDZ (b) β 1-adrenergic receptor (ADRB1); WT (c) or Δ PDZ (d) chemokine type I receptor (CXCR1). Data are the mean $\pm$ SEM (n = 4). ISO = isoproterenol; IL-8 = interleukin-8.

Fig. 4. Agonist efficacies abrogated by C-terminal PDZ ligand truncation

Concentration-response curves for agonist-stimulated DMR responses in HEK293T cells expressing WT or PDZ β 1-adrenergic receptor (ADRB1) (a), chemokine subtype 1 receptor (CXCR1) (b), Melanin-Concentrating Hormone Receptor 2 (MCHR2) (c) or Sphingosine-1-Phosphate Receptor 2 (S1PR2) (d). ISO = isoproterenol; IL-8 = interleukin-8; 17 ALA-MCH = [Ala 17]-melanin concentrating hormone; S1P = sphingosine-1-phosphate. Data are the mean $\pm$ SEM (n = 4).

Fig. 5. Epic DMR responses enhanced by removal of the GPCR C-terminal PDZ ligand
Epic DMR responses in HEK293T cells expressing WT (a) or Δ PDZ (b) 5-hydroxytryptamine type 2B receptor (HTR2B); WT (c) or Δ PDZ (d) somatostatin receptor 5 (SSTR5). 5-HT = 5-hydroxytryptamine; SST = somatostatin. Data are the mean $\pm$ SEM (n = 4).

Fig. 6. Agonist efficacies enhanced by C-terminal PDZ ligand truncation
 Concentration-response curves for agonist-stimulated DMR responses in HEK293T cells expressing WT or Δ PDZ 5-hydroxytryptamine type 2B receptor (HTR2B) (a), somatostatin receptor 5 (SSTR5) (b), somatostatin receptor 2 (SSTR2) (c), or lysophosphatidic acid receptor 2 (LPAR2) (d) GPCRs. 5-HT = 5-hydroxytryptamine; SST = somatostatin; LPA = lysophosphatidic acid. Data are the mean $\pm$ SEM (n = 4).

Table 1
SNAP cell surface expression of WT and PDZ GPCRs

Cell surface expression of wild type (WT) and PDZ-ligand truncated (PDZ) GPCRs was quantified in HEK293T cells using SNAP cell impermeable substrate BG782. Data are expressed as % of HTR2A CSE. Data were analyzed with GraphPad Prism and are expressed as mean $\pm$ SEM (n = 3–4).

GPCR gene name	WT CSE (%WT HTR2A)	PDZ CSE (%WT HTR2A)
ADRA1D	1.39 $\pm$ 0.645	3.57 $\pm$ 0.99
ADRA2B	7.26 $\pm$ 0.91	6.87 $\pm$ 0.97
ADRB1	22.72 $\pm$ 4.41	25.03 $\pm$ 2.76
ADRB2	9.77 $\pm$ 0.97	11.21 $\pm$ 2.27
HTR2A	100 $\pm$ 1.85	75.44 $\pm$ 6.12
HTR2B	30.72 $\pm$ 6.73	34.86 $\pm$ 5.77
HTR2C	22.71 $\pm$ 4.40	25.02 $\pm$ 2.76
SSTR1	22.88 $\pm$ 2.96	21.08 $\pm$ 4.05
SSTR2	28.53 $\pm$ 7.94	29.01 $\pm$ 5.85
SSTR3	58.46 $\pm$ 6.51	46.26 $\pm$ 12.16
SSTR4	33.7 $\pm$ 3.50	32.80 $\pm$ 2.63
SSTR5	19.98 $\pm$ 3.82	27.69 $\pm$ 8.92
CXCR1	56.27 $\pm$ 2.89	53.88 $\pm$ 3.43
CXCR2	23.28 $\pm$ 2.20	25.68 $\pm$ 4.90
CXCR3	15.48 $\pm$ 6.10	14.7 $\pm$ 3.27
CXCR5	20.1 $\pm$ 1.02	17.6 $\pm$ 4.41
GALR1	29.11 $\pm$ 1.41	23.06 $\pm$ 2.22
HRH3	37.99 $\pm$ 5.99	52.90 $\pm$ 6.03
P2RY1	86.61 $\pm$ 3.83	78.81 $\pm$ 8.78
P2RY12	17.88 $\pm$ 3.19	20.93 $\pm$ 2.08
MCHR2	3.38 $\pm$ 7.79	0.532 $\pm$ 8.36
C3AR1	5.65 $\pm$ 0.96	1.16 $\pm$ 0.39
LPAR2	58.82 $\pm$ 4.74	68.05 $\pm$ 6.39
S1PR2	11.49 $\pm$ 0.67	14.81 $\pm$ 7.79

Table 2
Pharmacological properties of agonists targeting WT and PDZ GPCRs

Agonist stimulated DMR concentration response curves were constructed for wild type (WT) and PDZ-ligand truncated (PDZ) GPCRs, and used to calculate potencies (pEC_{50}). Maximal agonist DMR responses (Max) and time when maximal response reached (Time To Peak, TTP) in seconds are shown. All data were analyzed with GraphPad Prism and are expressed as mean $\pm$ SEM ($n = 2-4$). Max DMR of PDZ GPCRs significantly different than WT GPCR Max DMR are denoted with ** (student's t-test, $p < 0.05$). ND = not determined.

GPCR	Agonist	WT pEC_{50}	Max (DMR)	TTP (sec)	PDZ pEC_{50}	Max (DMR)	TTP (sec)
ADRA2B	Clonidine	-7.31 ± 0.17	74.40 ± 2.54	11.10	-7.15 ± 0.10	90.10 ± 6.31	9.51
ADRB1	Isoproterenol	-8.42 ± 0.15	255.12 ± 3.74	31.67	-9.09 ± 0.12	$48.17 \pm 8.08^{**}$	61.30
ADRB2	Isoproterenol	-8.01 ± 0.15	251.48 ± 15.45	28.37	-7.65 ± 0.26	219.84 ± 5.73	30.37
HTR2A	5-hydroxytryptamine	-6.84 ± 0.07	118.10 ± 10.50	14.10	-6.78 ± 0.13	106.10 ± 3.96	14.70
HTR2B	5-hydroxytryptamine	-7.02 ± 0.17	80.60 ± 12.92	60.0	-7.34 ± 0.13	$213.20 \pm 15.52^{**}$	60.0
HTR2C	5-hydroxytryptamine	-8.04 ± 0.25	90.30 ± 9.89	5.90	-7.83 ± 0.14	106.61 ± 12.89	39.21
SSTR1	Somatostatin	-8.98 ± 0.21	35.01 ± 2.47	4.17	-8.50 ± 0.44	32.48 ± 2.57	5.87
SSTR2	Somatostatin	-9.29 ± 0.16	41.06 ± 2.27	5.37	-8.91 ± 0.15	$75.62 \pm 7.34^{**}$	5.57
SSTR3	Somatostatin	-8.84 ± 0.16	60.58 ± 1.29	5.07	-8.97 ± 0.11	62.07 ± 2.52	4.97
SSTR4	Somatostatin	-8.65 ± 0.30	38.76 ± 3.73	5.60	-8.83 ± 0.22	$54.74 \pm 2.07^{**}$	5.60
SSTR5	Somatostatin	-9.39 ± 0.17	31.09 ± 3.49	3.90	-8.46 ± 0.16	$155.86 \pm 10.81^{**}$	5.60
CXCR1	Interleukin-8	-8.29 ± 0.07	69.69 ± 1.88	3.60	-7.08 ± 0.45	$9.48 \pm 1.53^{**}$	3.80
CXCR2	Interleukin-8	-8.53 ± 0.11	34.89 ± 2.08	3.20	-8.45 ± 0.13	37.50 ± 2.20	3.50
CXCR3	Interleukin-8	ND	10.17 ± 2.00	11.70	ND	18.3 ± 3.16	15.60
CXCR5	Interleukin-8	-10.34 ± 0.16	23.22 ± 1.80	2.50	-9.97 ± 0.12	$40.70 \pm 2.54^{**}$	2.30
GALR1	Galanin 1-30	-9.98 ± 0.12	67.70 ± 3.45	4.07	-5.67 ± 0.09	$93.60 \pm 2.08^{**}$	3.97
HRH3	Histamine	-7.26 ± 0.16	46.97 ± 3.71	7.30	-7.74 ± 0.18	60.19 ± 5.07	6.30
P2RY1	UTP	-5.39 ± 0.15	116.19 ± 2.22	4.87	-5.69 ± 0.19	118.68 ± 10.27	5.27
P2RY12	UTP	-5.47 ± 0.07	170.04 ± 2.93	5.40	-5.70 ± 0.08	$192.60 \pm 6.35^{**}$	5.20
MCHR2	Ala17-MCH	-6.27 ± 0.12	92.70 ± 4.57	60.0	-8.70 ± 0.61	$19.16 \pm 7.57^{**}$	60.0
C3AR1	Benzeneacetamide	ND	36.90 ± 1.82	4.50	ND	38.77 ± 2.59	3.50
LPAR2	Lysophosphatidic acid	-9.89 ± 0.92	19.70 ± 8.56	10.55	-8.17 ± 0.10	$110.73 \pm 2.72^{**}$	27.07
S1PR2	Sphingosine-1-phosphate	-8.42 ± 0.36	-40.54 ± 3.76	17.61	-8.10 ± 0.85	$-10.16 \pm 4.91^{**}$	16.31