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ABSTRACT

In the brain, neurotransmitters or neuromodulators play pivotal roles in chemical synaptic transmission and consequently, monitoring their dynamics, especially in vivo, is critical for understanding their physiological- or pathophysiological roles at molecular, cellular, and circuit levels during behaviors and/or during diseases. We recently developed genetically-encoded GPCR-activation based (GRAB) sensors capable of reporting dynamics of acetylcholine, dopamine and norepinephrine with rapid kinetics, chemical- and cell-specificity in multiple organisms in vivo. Here, we explored the usage of G protein derivatives, either mini-G proteins or C-terminal peptides of Ga subunit to engineer new GRAB sensors. We found that the conformational changes mediated by mini-G proteins interacting with GPCRs, or Ga C-terminal peptides interacting with GPCRs could be harnessed to regulate fluorescence outputs of a GPCR fused circular permutated GFP (cpGFP). In addition, inter-molecular fusion of Ga C-terminal peptides significantly suppressed ectopic activation of G protein signaling in a GRAB acetylcholine sensor. Finally, we showed Ga C-terminal peptides fusion strategy could be applied to generate various GRAB sensors for small molecular compounds or neuropeptides.

Keywords: Neurotransmitter, GRAB sensor, GPCR, G protein, mini-G protein, Ga C terminal-peptide

1. INTRODUCTION

Neurotransmitters or neuromodulators are bioactive molecules that mediate or shape chemical synaptic transmission between neurons. Despite their critical functions, their dynamics and regulations in the nervous system are poorly understood. Addressing these important biological questions requires new tools that enable measuring the dynamics of these key molecules with precise chemical specificity, single-cell spatial resolution, and physiologically relevant temporal resolution, ideally in an in vivo system. Very recently, we and others have developed a series of genetically-encoded fluorescent probes based on ligand stabilized GPCR conformational changes which couple to fluorescence emission changes of cpGFP1–4. GPCR Activation-Based (GRAB) sensors enable detection of various neurotransmitters, including acetylcholine1 (GACH), dopamine2 (GRABDA) and norepinephrine3 (GRABNE). Because GRAB sensors used GPCRs as neurotransmitter/neuromodulator sensing modules, a potential caveat regarding using GRAB sensors is their coupling with downstream molecules, e.g., G proteins, which may inadvertently affect target cells’ physiology. Although the presence of a fluorescent protein cpGFP largely reduced downstream signaling of G proteins and arrestin for GRABDA, dLights4 and GRABNE5, some of the GRAB sensors may still retain partial signaling, for example, GACH could still couple to G protein, albeit with 7-fold less potency comparing with a wild-type GPCR. It is therefore useful to devise generic strategies that can be used to further insulate GRAB sensors from G protein coupling.

GPCR-G protein interactions have been extensively studied6,7. Upon ligand binding, activated GPCRs will promote the nucleotide exchange on heterotrimeric G proteins, which are composed of the α, β and γ subunits8. Therefore, to insulate GPCR-G protein signaling, it is conceivable that a surrogate non-signaling G protein or a G protein mimic could be

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provided in cis. The resulted intramolecular engagement of G protein or G protein mimic would be expected to compete out G proteins in trans because of the high local concentration. Indeed, G protein mimic proteins, e.g. single-chain camelid antibodies (nanobodies) have been developed and are able to replace G proteins for structural studies⁹⁻¹¹ or are able to recognize activated GPCRs in cells¹²,¹³. However, because each nanobody needs to be individually tuned and optimized for stabilization of distinct activated GPCRs, this approach might not be easily scalable. Alternatively, recent development of mini-G proteins¹⁴,¹⁶, which are small and stable engineered G proteins that could sufficiently couple GPCRs in the absence of Gβγ subunits, provides a viable solution. Moreover, short C-terminal peptides derived from various Ga proteins, not able to catalyze GDP/GTP exchange, are well established as the critical components that could be sufficient to bind to and stabilize the activated conformation of GPCRs¹⁷,¹⁹. Therefore, Ga C-terminal peptides provide an additional generic G protein mimic to insulate GPCR-G protein signaling. Here, we explored mini-G proteins and Ga C-terminal peptides as tools to insulate GRAB sensors-G protein signaling. We further tested whether ligand-dependent interaction of Ga C-terminal peptides with GPCRs could be exploited to develop new GRAB sensors.

2. MATERIALS AND METHODS

2.1 Molecular biology

Molecular cloning was conducted as previous described¹⁻³. Briefly, plasmids used in this study were constructed using Gibson Assembly. All sequences were verified using Sanger sequencing at the sequencing platform at the School of Life Sciences of Peking University. All GRAB sensors were cloned into the pDisplay vector (Invitrogen), with an IgK leading sequence inserted at the N-terminus of GPCRs.

2.2 Cell cultures and transfection

GRAB sensors were characterized in HEK293T cells as previous described¹⁻³. Briefly, HEK293T cells were cultured in incubators at 37°C with 5% CO₂, were plated on 12-mm glass coverslips in 24-well plates and grown to 60-80% confluence before transfection. Transfection was performed by incubating the cells with a mixture containing 1-mg DNA and 3-mg PEI for 4-6 h.

2.3 Fluorescence imaging

HEK293T cells expressing GRAB sensors were imaged 24-48 h after transfection. Cal-590 AM (AAT Bio) loading cells were incubated at 37°C for ~ 1 hour before imaging. Fluorescence imaging was performed under an inverted Ti-E A1 confocal microscope (Nikon) with a 40X /1.35 NA oil immersion objective. A 488-nm laser and a 561-nm laser were used to collect GFP and RFP/Cal590 signals, respectively.

3. RESULTS AND DISCUSSIONS

We first tested whether a C-terminus fusion of a mini-G protein to a GPCR could still be capable of interacting with GPCR intra-molecularly in a ligand dependent manner, and therefore would prevent ectopic G protein signaling (Fig. 1). We compared a GRAB sensor based on β2 adrenergic receptor (β2AR)²⁰, with- or without a C-terminus fusion of mini-Gs¹⁴,¹⁵ protein (named GRABEps-mGs or GRABEps, respectively). Of note, a pre-optimized cpGFP was inserted into the third intracellular loop (Fig. 1a). When applying gradually increased concentration of isoproterenol (ISO, a non-selective β adrenoceptor agonist) from 0.1 nM to 10 μM, the fluorescence intensity increased progressively in GRABEps-expressing cells (Fig. 1b), yielding an apparent EC₅₀ of 83 nM (Fig. 1c), similar with the wild-type β2AR²¹. ISO-induced fluorescence signals could be blocked by co-application of a selective β2AR antagonist ICI 118,551 (ICI) (Fig. 1b). Interestingly, a progressive decrease in fluorescence signals (or off-responses) upon ISO treatment in GRABEps-mGs expressing cells was observed (Fig. 1e and 1f). Dose-dependent responses of this GRABEps-mGs sensor to ISO revealed a ~ 7-fold lower apparent EC₅₀ compared with GRABEps (12 nM vs. 83 nM, Fig. 2f). The higher affinity dosage-dependency, but opposite fluorescence signal changes in GRABEps-mGs expressing cells suggest that fusion of a mini-G protein is functional and could engage with β2AR intra-molecularly. Thus, mini-G protein fusion provides a useful way to insulate GRAB sensors from inter-molecular ectopic activation of G protein coupling.
Figure 1. Design a GRAB epinephrine sensor based on the β2AR and mini-Gs protein. (a, d) Schematic drawing shows the principle of the GRABEpi (a) and GRABEpi-mGs (d). (b, e) Averaged responses of GRABEpi- (b) and GRABEpi-mGs- (e) expressing HEK293T cells to isoproterenol (ISO) application. Note that the responses are blocked by β2AR’s specific antagonist ICI 118,551 (ICI). (c, f) Dose-dependent response of the GRABEpi (c) and GRABEpi-mGs (f) with apparent EC\textsubscript{50} of 83 nM and 12 nM, respectively. Data are represented as mean ± SEM.

Figure 2. Fusion of a Gq peptide to the C-terminus of GRAB\textsubscript{ACh} significantly reduced the downstream G protein coupling. (a, e) Schemes show the design of GRAB\textsubscript{ACh} and GRAB\textsubscript{ACh-Gq20}. (b, f) Averaged responses of GRAB\textsubscript{ACh} and GRAB\textsubscript{ACh-Gq20} to the ACh application in HEK293T cells. (c, g) Dose-dependent responses of GRAB\textsubscript{ACh} and GRAB\textsubscript{ACh-Gq20} with apparent EC\textsubscript{50} of 0.39 μM and 0.46 μM, respectively. (d, h) The ACh concentration-dependent \textsuperscript{45}Ca\textsuperscript{2+} response measured using Cal-590 dye and calculated apparent EC\textsubscript{50} of M3R-, GRAB\textsubscript{ACh} and GRAB\textsubscript{ACh-Gq20}-expressing HEK293T cells. Data are represented as mean ± SEM.

Compared to mini-G proteins, Gq C-terminal peptides are much smaller (~20 amino acids vs. >200 amino acids for mini-G protein) and their affinity to various GPCRs are well characterized\textsuperscript{17}. Their small size and tunable affinity, in principle, offer a very convenient and versatile approach to construct insulated chimeric GRAB sensors. We firstly fused a 20-amino-acid Gq peptide (Gq20) to a GRAB\textsubscript{ACh} sensor (also called GACh1.0 in the previous work\textsuperscript{1}) (Fig. 2a and 2e).
The chimeric GRAB<sub>ACh</sub>-Gq20 showed comparable response (Fig 2b and 2f) and similar apparent EC<sub>50</sub> (Fig 2c and 2g) to ACh as the GRAB<sub>ACh</sub>, suggesting the fusion of Gq peptide did not affect the ACh dependent fluorescent output signals of the GRAB sensor. We next examined the downstream Gq-dependent calcium signaling of GRAB<sub>ACh</sub> and modified GRAB<sub>ACh</sub>-Gq20 sensors. Similar as previous published GACH2.0<sup>1</sup>, the coupling efficiency of GRAB<sub>ACh</sub> was ~ 7-fold lower compared to wild-type M3R (apparent EC<sub>50</sub> = 9.3 nM vs. 1.7 nM, Fig. 2d). Fusion of the Gq20 peptide further reduced the coupling efficiency, with ~ 6-fold lower apparent EC<sub>50</sub> compared to GRAB<sub>ACh</sub> (apparent EC<sub>50</sub> = 56 nM vs. 9.3 nM, Fig. 2h). Taken together, our data suggests that intra-molecular Gq peptide fusion could reduce the downstream G protein coupling without altering the GRAB sensor’s response and affinity.

![Diagram](image-url)

**Figure 3.** Gq peptide assisted the development of new GRAB<sub>ACh</sub> sensors. (a) Schematic representation of the fusion of cpGFP and G protein peptide to the C-terminus of M3R. (b) Quantification of maximum fluorescence responses for each variant. Note that addition of the ER export sequence (ERex) and the trafficking signal (TS) in the C-terminus of Gq peptide showed the highest response to ACh application. (c, d) The plasma membrane expression and pseudocolor image showing averaged response to ACh application of the best sensor GRAB<sub>ACh</sub>-C-Gq showing in (b). Scale bar = 10 μm; data are represented as mean ± SEM. Above results that adding a Gq peptide reduced the downstream G protein coupling of GRAB sensors suggest that the C-terminus fused Gq peptide undergoes a significant conformational change upon GPCR activation. We therefore hypothesized inserting a cpGFP in between this Gq peptide and the C-terminus of GPCR could respond to this conformational change and result in sensitive fluorescent signal changes. To test this possibility, we engineered the GRAB sensor by simultaneously inserting the cpGFP and the G protein peptide into the C-terminus of a GPCR, M3R (Fig. 3a). We further found that addition of an ER export motif (ERex) and the trafficking signal (TS)<sup>12</sup> at the C-terminus, but not at the N-terminus of the Gq20 peptide improved the membrane trafficking (data not shown) and maximum responses (Fig. 3b) and we named this sensor GRAB<sub>ACh</sub>-C-Gq. The GRAB<sub>ACh</sub>-C-Gq showed excellent plasma membrane expression (Fig. 3c) and increased fluorescence responses (ΔF/F<sub>0</sub> ~ 50%) to saturating concentration of ACh perfusion in HEK293T cells (Fig 3d).

Next, to test the generalizability of cpGFP insertion into C-terminus strategy, we applied this approach to a number of GPCRs that sense various neuromodulators including epinephrine (Epi), serotonin (5-HT), dopamine (DA), and histamine (His), as well as neuropeptides, e.g. oxytocin (Oxt). A subset of GRCRs including β2AR, HTR4, DRD2, HRH1, and OXTR were selected as ligand binding modules. We grafted the C-terminus of GRAB<sub>ACh</sub>-C-Gq to the C-terminus of selected GPCRs (Fig. 4a). Similar as the GRAB<sub>ACh</sub>-C-Gq, we optimized these chimeras by: 1) adding/not adding ERex and/or TS sequences and 2) testing different G protein peptides. After optimization, all sensors trafficked well to the plasma membrane (Fig. 4b) and showed selective fluorescence changes (mostly increase, except DRD2 fusion) to corresponding ligands (Fig. 4c).
In conclusion, intra-molecular fusion of G protein derives, namely mini-G proteins or Ga C-terminal peptides could be harnessed either to insulate GRAB sensors from G protein coupling, or to build new GRAB sensors capitalizing their ligand-dependent binding to GPCRs. Firstly, when adding a mini-Gs protein to the GRAB_Epi, we obtained evidence that mini-G protein is still functional and undergoes intra-molecular interaction with the parental GPCR, thereby capable of reducing ectopic G protein signaling. Secondly, a C-terminus fusion of Gq20 peptide to GRAB_ACh was sufficient to significantly reduce the downstream G protein coupling, while maintaining GRAB_ACh’s affinity and response amplitude to ACh application. Lastly, when a cpGFP is inserted between a G protein peptide and a GPCR’s C-terminus, it is capable of emitting different green fluorescence in a ligand-dependent manner and thereby reports the presence of a number of important small neural chemicals or neuropeptides. We believe that the G protein-assisted engineering strategy described here could facilitate the development of more robust GRAB sensors for a diverse array of neuromodulators.
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