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Invited Paper

G protein-assisted optimization of GPCR-activation based (GRAB) sensors

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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 Gα subunit to engineer new GRAB sensors. We found that the conformational changes mediated by mini-G proteins interacting with GPCRs, or Gα C-terminal peptides interacting with GPCRs could be harnessed to regulate fluorescence outputs of a GPCR fused circular permuted GFP (cpGFP). In addition, inter-molecular fusion of Gα C-terminal peptides significantly suppressed ectopic activation of G protein signaling in a GRAB acetylcholine sensor. Finally, we showed Gα 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, Gα 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 cpGFP[1-4]. GPCR Activation-Based (GRAB) sensors enable detection of various neurotransmitters, including acetylcholine[1] (GACh), dopamine[2] ($GRAB_{DA}$) and norepinephrine[3] ($GRAB_{NE}$). 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 $GRAB_{DA}$[2], dLights[4] and $GRAB_{NE}$[3], 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[1]. 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 studied[5-7]. Upon ligand binding, activated GPCRs will promote the nucleotide exchange on heterotrimeric G proteins, which are composed of the α, β and γ subunits[8]. 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 studies[9-11] or are able to recognize activated GPCRs in cells[12,13]. 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 proteins[14-16], 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 Gα 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 GPCRs[17-19]. Therefore, Gα C terminal-peptides provide an additional generic G protein mimic to insulate GPCR-G protein signaling. Here, we explored mini-G proteins and Gα C-terminal peptides as tools to insulate GRAB sensors-G protein signaling. We further tested whether ligand-dependent interaction of Gα 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 described[1-3]. 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 described[1-3]. Briefly, HEK293T cells were cultured in incubators at 37℃ with 5% CO_2, 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 ℃ 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-Gs[14,15] protein (named $GRAB_{Epi}$-mGs or $GRAB_{Epi}$, 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 adrenoreceptor agonist) from 0.1 nM to 10 μM, the fluorescence intensity increased progressively in $GRAB_{Epi}$-expressing cells (Fig. 1b), yielding an apparent EC_{50} of 83 nM (Fig. 1c), similar with the wild-type β2AR[21]. 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 $GRAB_{Epi}$-mGs expressing cells was observed (Fig. 1e and 1f). Dose-dependent responses of this $GRAB_{Epi}$-mGs sensor to ISO revealed a ~ 7-fold lower apparent EC_{50} compared with $GRAB_{Epi}$ (12 nM vs. 83 nM, Fig. 2f). The higher affinity dosage-dependency, but opposite fluorescence signal changes in $GRAB_{Epi}$-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.

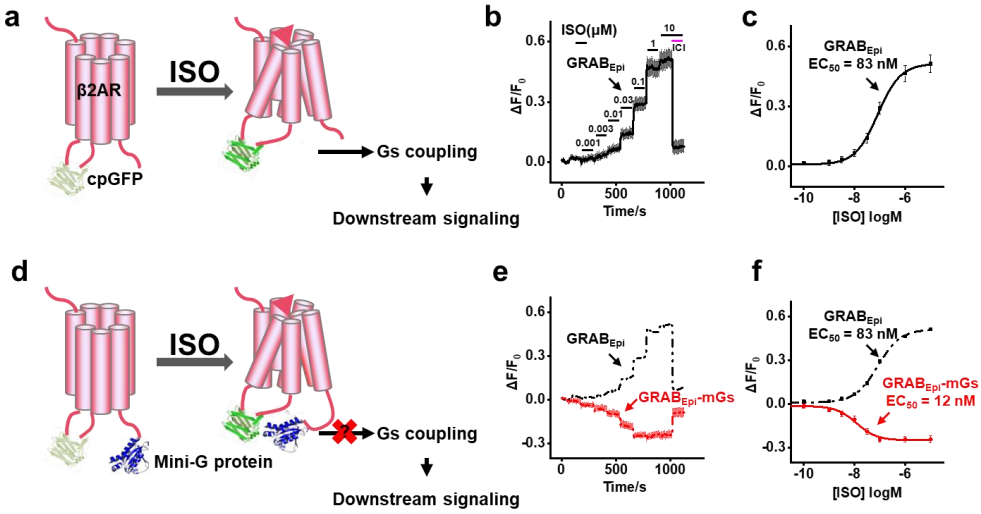

Figure 1. Design a GRAB epinephrine sensor based on the β2AR and mini-Gs protein. (a, d) Schematic drawing shows the principle of the $GRAB_{Epi}$ (a) and $GRAB_{Epi}$-mGs (d). (b, e) Averaged responses of $GRAB_{Epi}$- (b) and $GRAB_{Epi}$-mGs- (e) expressing HEK293T cells to isoproterenol (ISO) application. Note that the responses are blockade by β2AR's specific antagonist ICI 118,551 (ICI). (c, f) Dose-dependent response of the $GRAB_{Epi}$ (c) and $GRAB_{Epi}$-mGs (f) with apparent EC_{50} of 83 nM and 12 nM, respectively. Data are represented as mean ± SEM.

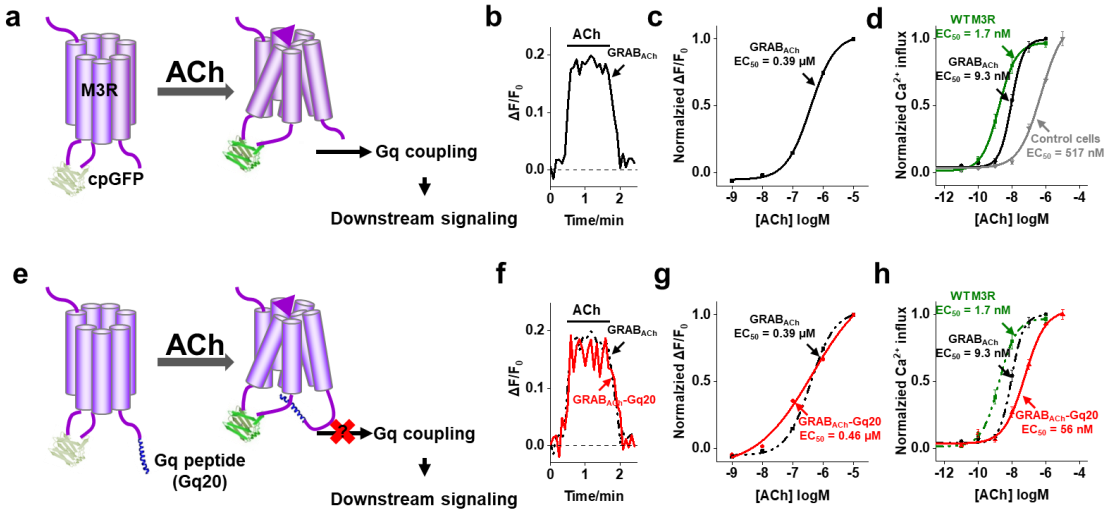

Figure 2. Fusion of a Gq peptide to the C-terminus of $GRAB_{ACh}$ significantly reduced the downstream G protein coupling. (a, e) Schemes show the design of $GRAB_{ACh}$ and $GRAB_{ACh}$-Gq20. (b, f) Averaged responses of $GRAB_{ACh}$ and $GRAB_{ACh}$-Gq20 to the ACh application in HEK293T cells. (c,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

Compared to mini-G proteins, Gα 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[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 $GRAB_{ACh}$ sensor (also called GACh1.0 in the previous work[1]) (Fig. 2a and 2e).

The chimeric $GRAB_{ACh}$-Gq20 showed comparable response (Fig 2b and 2f) and similar apparent EC_{50} (Fig 2c and 2g) to ACh as the $GRAB_{ACh}$, 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 $GRAB_{ACh}$ and modified $GRAB_{ACh}$-Gq20 sensors. Similar as previous published GACh2.0[1], the coupling efficiency of $GRAB_{ACh}$ was ~ 7-fold lower compared to wild-type M3R (apparent EC_{50} = 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_{50} compared to $GRAB_{ACh}$ (apparent EC_{50} = 56 nM vs. 9.3 nM, Fig. 2h). Taken together, our data suggests that intra-molecular Gα peptide fusion could reduce the downstream G protein coupling without altering the GRAB sensor's response and affinity.

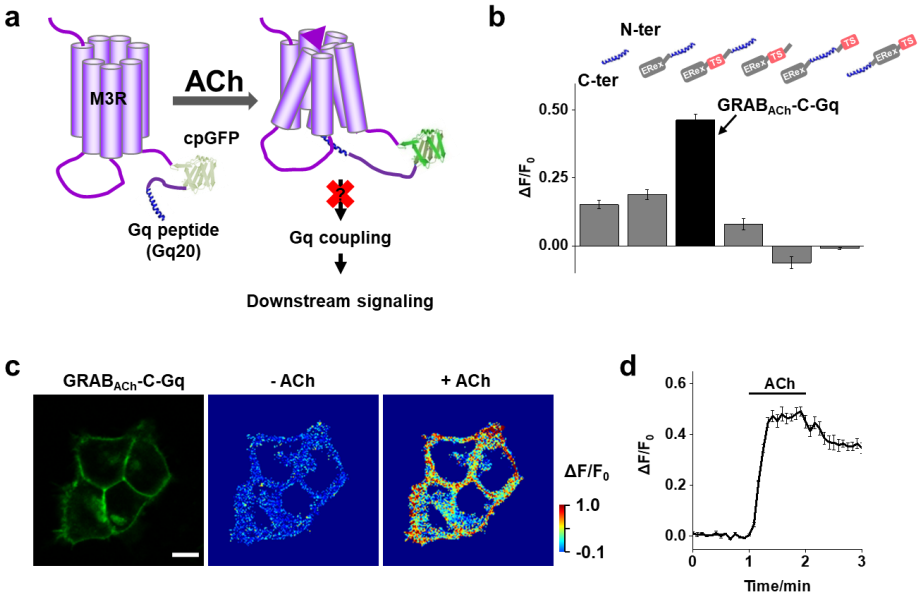

Figure 3. Gq peptide assisted the development of new $GRAB_{ACh}$ sensors. (a) Schematic representation of the fusion of cpGFP and G protein peptide to the C-terminus of M3R. (b) Quantification of maximum fluorescence responses for each variant. Note that addition of the ER export sequence (ERex) and 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 $GRAB_{ACh}$-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)[12] 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 $GRAB_{ACh}$-C-Gq. The $GRAB_{ACh}$-C-Gq showed excellent plasma membrane expression (Fig. 3c) and increased fluorescence responses ($\Delta F/F_0$ ~ 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 $GRAB_{ACh}$-C-Gq to the C-terminus of selected GPCRs (Fig. 4a). Similar as the $GRAB_{ACh}$-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).

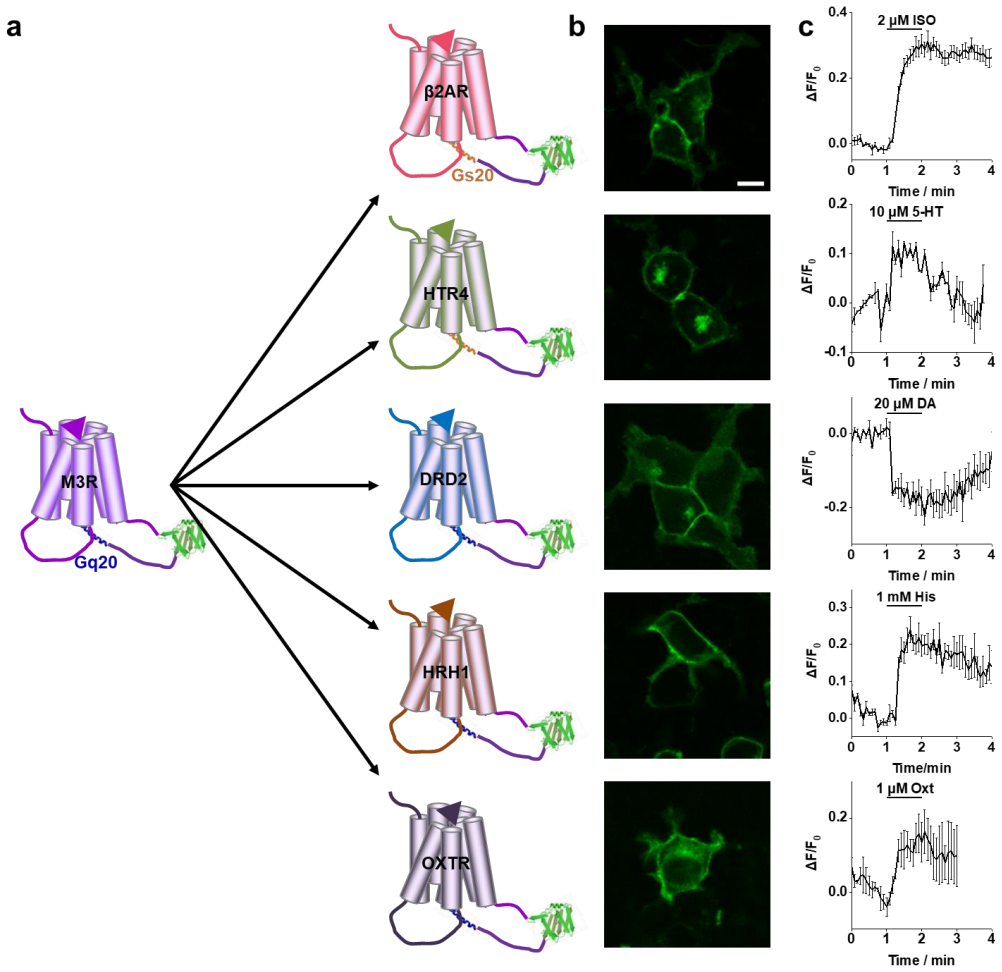

Figure 4. Versatility of G-protein peptide mediated cpGFP conformation change strategy to develop additional GRAB sensors. (a) Schemes showing the expansion strategy by swapping the C terminal of $GRAB_{ACh}$-C-Gq20 to other GPCRs. (b) Plasma membrane expression of new GRAB sensors in HEK293T cells. (c) Averaged responses of new GRAB sensors in HEK293T cells to the application of corresponding ligands. Scale bar = 10 μm; data are represented as mean ± SEM.

4. CONCLUSIONS

In conclusion, intra-molecular fusion of G protein derives, namely mini-G proteins or Gα 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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