論文の内容の要旨

Quantitative evaluation of inhibitory potency of inhibitors for Formyl Peptide Receptor 1 and elucidation of the inhibition mechanism using fluorescence microscopy techniques

（FPR1 阻害剤の阻害能定量評価と蛍光顕微鏡法を用いた阻害機序の解明）

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Introduction

G protein-coupled receptors (GPCRs) are involved in a wide range of diseases with their high ligand specificity. Almost half of the commercial drugs are designed to target at GPCRs. Upon ligand stimulation, GPCRs activate an intracellular heterotrimeric protein, guanine nucleotide-binding protein (G protein), and control the concentration of downstream signaling molecules, including cyclic AMP, Ca^{2+}, and phospholipids. The process is repeated for signal amplification until a scaffold protein called β-arrestin quenches the activation of GPCRs. Based on biochemical detections of the signal events, numerous methods have been established to quantify drug efficacies upon GPCRs. In the thesis of my master degree, I established two high-throughput assay systems and identified ten potential inhibitors for an inflammation-related GPCR, Formyl Peptide Receptor 1 (FPR1), aiming to develop a novel drug for a severe drug eruption, Stevens Johnson Syndrome. However, elucidation of various signal events, in addition to the calcium signaling and the β-arrestin signaling, is essential for efficient drug discovery because ligands and inhibitors can induce their own activation patterns of GPCRs. Thus, the first aim of the doctor thesis was to establish additional assay systems for FPR1 to quantify G protein signaling, ligand binding, and cAMP signaling. Recent studies suggested that oligomer formation of FPR1 might be involved in their activation, but assessment of the oligomeric patterns of FPR1 in living cells is still challenging due to its dynamic and heterologous nature. To reveal the full spectrum of the signal cascades triggered by the newly found inhibitors, I developed analytical methods to quantify the frequency and the duration of interactions among GPCRs and G proteins by using live-cell imaging techniques.

Principle

The direct binding of the potential inhibitors to FPR1 was confirmed by competitive inhibition of fluorescent FPR1 ligand, detected with flow cytometry. The cAMP signaling was quantified with commercial bioluminescence probe. The G protein signaling was assessed by monitoring structural changes of G proteins with a FRET probe, in which two subunits of G protein were labeled with cyan and yellow fluorescent proteins (CFP and YFP), respectively (Fig. 1a). In a basal state, excitation of CFP resulted in transfer of energy to nearby YFP and emission of red-shifted fluorescence. In contrast, activated G proteins dissociated into two subunits and YFP emission decreased. Collectively, the FPR1-induced activation of G protein was quantified by changes in the FRET efficiency.

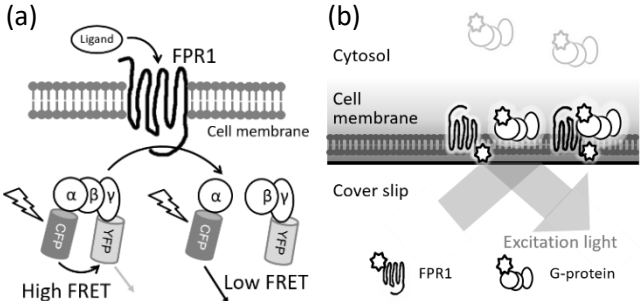

Figure 1. (a) The schematic diagram of the FRET probe. The G protein activation caused their dissociation, leading to a loss of energy transfer from CFP to YFP. (b) The principle of the TIRF observation. Only the cell surface was excited with laser light and cytosolic fluorophores were not visualized.

Next, single molecule dynamics of FPR1 and G protein was investigated with Total Internal Reflection Fluorescence (TIRF) microscopy. An evanescent field was produced at the interface between the cover slip and the sample by the excitation light of TIRF microscopy. Plasma membrane-localizing proteins were selectively visualized with extremely low background, which enabled single molecule detection (Fig. 1b). FPR1 and G protein were genetically fused with SNAP-tag and Halo-tag, which were then specifically labeled with far-red and red fluorescent dyes, SeTau647 and TMR, respectively. Raw images were systematically processed to remove shot noise, identify fluorescent spots, and quantify their fluorescent intensities and temporal locations.

Results

1. Quantitative assessment of G protein signaling with the FRET probe.

The FRET probe and FPR1 were expressed in HEK293 cells and monitored with epi-fluorescence microscopy. An increase in CFP fluorescence and a concomitant decrease in YFP signal indicated the loss of the energy transfer (Fig. 2a), representing activation of FPR1 in response to ligand stimulation. Reproducible and reversible responses indicate that the probe was suitable for quantitative assessment of G protein signaling. The plot of FRET changes for a wide range of the ligand concentration represents a reasonable dose-response curve for wildtype FPR1 (Fig. 2b), verifying the probe. The comparable dose-response curve for SNAP-tagged FPR1 indicates that the N-terminal modification did not affect functionality of FPR1. The results suggest that the inhibitory capacity of the candidate compounds should be assessed with the method. Consequently, the G protein activation induced by FPR1 was quantitatively assessed with the FRET probe.

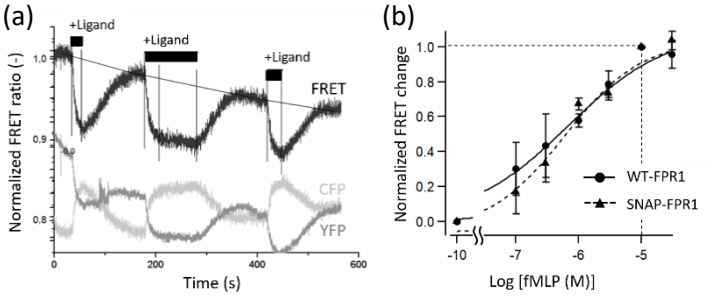

Figure 2. (a) The representative FRET responses for ligand stimulation. FRET changes were calculated based on an interpolated basal line (solid line) to reduce the effect of photobleaching. (b) The dose-response curves of WT and SNAP-tagged FPR1. FRET changes were normalized by the values at 10 μM fMLP (Log[fMLP] = -5) for each sample. n≧2, Bars: Mean±s.e.m.

2-a. Evaluation of oligomerization states of FPR1 with single molecule imaging.

The single molecule dynamics of FPR1 and G protein were simultaneously observed with TIRF microscopy. Both proteins were detected as fluorescent spots diffusing on cell membrane (Fig. 3a). A sudden disappear, instead of a gradual fade, suggests that the spot is constituted by a single protein (Fig. 3b). To reveal the temporal patterns of the oligomer formation of FPR1, fluorescence intensities of the FPR1 spots were calculated (Fig.4a). The broad distribution of fluorescence intensity suggested the formation of FPR1 monomers, dimers and larger oligomers even in their basal states. Based on the fluorescence intensity, the fluorescent spots were categorized as either small or large oligomers and their populations were quantified (Fig. 4b). The result shows that the large oligomer fraction was increased after ligand stimulation (Fig. 4b), suggesting that the ligand stabilized the large oligomers of FPR1. Consequently, the ligand-induced change in the equilibria between small and large oligomers of FPR1 was detected with single molecule imaging.

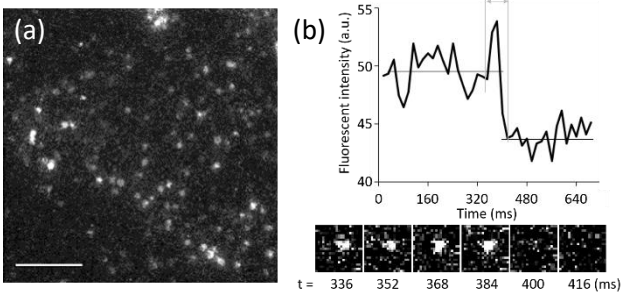

Figure 3. (a) A typical single molecule image. Fluorescent spots in a single cell are visualized in the figure. Scale bar: 5 μm. (b) The temporal change in fluorescent intensity of a FPR1 spot. The time lapse images of the FPR1 spot are shown below. Frame rate: 16 ms. Image size: 1.6×1.6 μm.

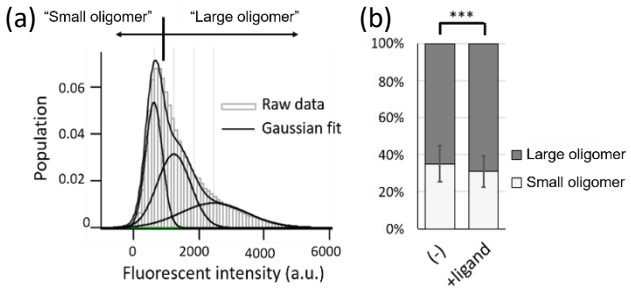

Figure 4. (a) The distribution of fluorescent intensity of FPR1 spots in basal state. The median of the first and the second peaks was defined as a threshold to discriminate small and large oligomers. (b) The fraction of small and large oligomers. Statistical significance was assessed by Student's two-tailed paired *t*-test. n=20, *** p<0.001.

2-b. Time course of the interaction between FPR1 and G protein.

Interaction between FPR1 and G protein was assessed by monitoring duration of colocalization events of the spots (Fig. 5a). Cumulative probability curves of duration of the colocalization events showed an increase in the long-lived colocalization events after ligand stimulation (Fig. 5b). The representative values of the duration time were calculated by fitting the probability curves to single exponential curves. The results showed that the colocalization duration between FPR1 and G protein was significantly increased by ligand stimulation (Fig. 5c). Hence, we evaluated the interacting duration of FPR1 and G protein and detected an elongation of the interacting time for activated FPR1.

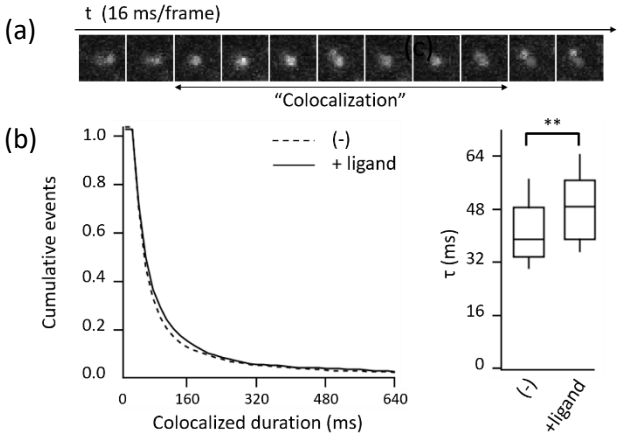

Figure 5. (a) A typical image sequence of transient colocalization. (b) The cumulative population curves of colocalization duration. After ligand stimulation, the number of long-lived (~160 ms) colocalization slightly increased. (c) The time constants of the colocalization. The τ values were calculated by fitting the population curves with single exponential curves. ($y = \mathrm{A} \times \exp(-x/\tau) + \mathrm{B}$). ** p<0.01.

2-c. Relation between oligomerization of FPR1 and FPR1-G protein interaction.

To clarify the relation between the large oligomer formation of FPR1 and the elongation of the G protein interaction upon ligand stimulation, the colocalization durations of small and large oligomers of FPR1 were assessed and quantified (Fig. 6). FPR1 small oligomers showed more transient interactions with G proteins than large oligomers both in the presence and absence of the ligand (Fig. 6b). The effect of the ligand stimulation was only found in an increased interaction time of the large oligomer fraction (Fig. 6b). Therefore, the results suggest that FPR1 activation caused an elongation of the interacting time between G proteins and oligomeric FPR1.

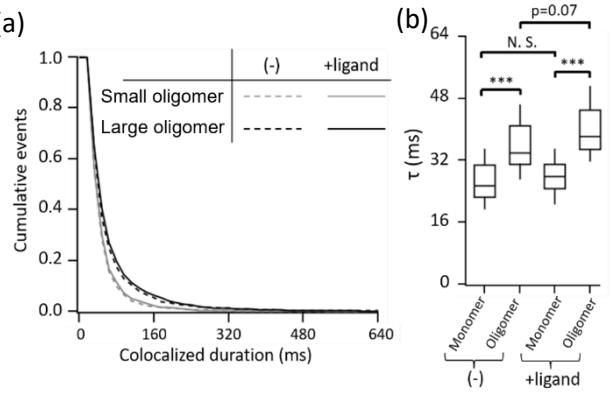

Figure 6. (a) The population curves of colocalization time for small and large oligomer of FPR1. Based on the criterion shown in Fig. 4a, the spots were first categorized as small oligomers or large oligomers, and then the population curves were constructed. (b) The time constants of the colocalization. The τ values were calculated by fitting the population curves with single exponential curves. ($y = \mathrm{A} \times \exp(-x/\tau) + \mathrm{B}$) *** p<0.001. N.S.: not significant

Conclusion

I established ligand binding assay, bioluminescence cAMP assay, and G protein FRET assay system for quantitative assessment of signaling patterns of FPR1 activation. Moreover, I developed a single molecule imaging system to reveal the stoichiometry of FPR1 and its interaction with G proteins during ligand stimulation. An increase in the large oligomer fraction of FPR1 and an elongation of the interaction with G proteins were detected in response to the ligand stimulation. Further analysis suggested that the stable interaction was mainly caused by the large oligomer of FPR1. Therefore, the large oligomer fraction of FPR1 and their interaction kinetics with G proteins would be novel indicators of FPR1 activation.