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Detection of G protein-coupled receptor-mediated cellular response involved in cytoskeletal rearrangement using surface plasmon resonance

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ABSTRACT

G protein-coupled receptors (GPCRs) form a superfamily of cell surface receptors that play fundamental roles in physiology and pathophysiology. Although GPCRs have been the most successful targets for drug discovery, there still remain many orphan GPCRs, which provides opportunities for development of novel drugs. Here, we introduce a new method for evaluation of GPCR activation utilizing a surface plasmon resonance (SPR) sensor. Cells expressing GPCRs were cultured directly on an SPR sensor chip and stimulated with GPCR ligands, resulting in SPR responses that were dependent on the type of G alpha subunits coupling with receptors. Namely G_i- and/or $G_{12/13}$-coupled receptors evoked SPR responses but G_s- or G_q-coupled ones did not. Analyses on the intracellular signal pathways revealed that small G protein Rho/Rac-mediated actin rearrangement plays an important role in the signal transduction pathways leading to the SPR responses. An SPR response was also evoked by insulin-like growth factor-1, which stimulates Rac-dependent stress fiber formation via its receptor-tyrosine kinase. Thus, this method provides a unique opportunity for real-time monitoring of cellular responses involved in cytoskeletal rearrangements, and may be useful in ligand/drug discovery for certain types of receptor, such as G_i- and $G_{12/13}$-coupled receptors.

1. Introduction

G protein-coupled receptors (GPCRs), a superfamily of transmembrane receptors, play fundamental roles in physiology and pathophysiology by mediating a wide variety of biological processes in response to various agonists, including photons, odorants, amines, peptides, proteins, nucleotides and lipids (Fredriksson et al., 2003). GPCRs are potential targets for clinical therapeutics and drug discovery. At present, about 100 GPCRs, whose ligands have not been identified, are called orphan GPCRs. De-orphaning of these GPCRs is expected to lead to the discovery of novel bioactive molecules and better understandings of physiological or pathological processes.

Heterotrimeric G proteins act as molecular switches that transduce conformational changes in GPCRs to activate intracellular effectors. Alpha subunits of heterotrimeric G protein are classified into subfamilies based on intracellular signaling pathways: G_s, G_i, G_q and $G_{12/13}$. G_s activates adenylyl cyclase and increases intracellular cAMP level, whereas G_i inhibits adenylyl cyclases. G_q activates phospholipase C and increases intracellular calcium concentration. $G_{12/13}$ activates Rho guanine nucleotide exchange factor (RhoGEF), and induces the formation of actin stress fibers (Offermanns, 2003). Ligand screening of orphan GPCRs has been performed based on changes of intracellular cAMP or calcium concentration and receptor internalization. However, there still remain many orphan GPCRs, which encouraged us to develop new screening methods based on different principles.

Surface plasmon resonance (SPR) is an optical sensing technique that is based on the phenomenon of attenuated total reflection and has been developed for real-time analysis of molecule–molecule interactions. It has been reported that SPR is also able to detect cellular responses in antigen-stimulated mast cells (Hide et al., 2002) and in EGF-stimulated keratinocytes (Yanase et al., 2007). These SPR responses were assumed to reflect changes in overall cytoskeletal rearrangements, though the underlying mechanisms are unknown.

In this study, we determined whether an SPR sensor could be utilized to detect cellular responses mediated by GPCRs in living cells directly cultured on the sensor chip. We also analyzed intracellular signaling pathways leading to the SPR responses. Our results indicate that the SPR responses reflect actin cytoskeletal rearrangements downstream of G_i and/or $G_{12/13}$.

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2. Materials and methods

2.1. Materials

Nutrient mixture F-12 HAM, fetal bovine serum, Trizma base, glucagon, lysophosphatidic acid (LPA), cytochalasin D (cyto D), Ki16425 and Alexa Fluor 546 phalloidin were purchased from Sigma–Aldrich Japan. Sphingosine-1-phophate (S1P) was purchased from Biomol; insulin-like growth factor-1 (IGF-1) from PeproTech; JTE013 from Tocris; bovine serum albumin (BSA) from Serologicals; cAMP-Screen system from Applied Biosystems; Fura 2-AM from Dojindo; F127 from Molecular Probes; Lipofectamine 2000, opti-MEM I medium and neomycin from Invitrogen; and anti-G_{13} antibody from Santa Cruz. All other chemicals were from Wako.

2.2. Plasmid DNA for expression of GPCR

Coding sequences of muscarinic acetylcholine receptor (M1, M2 and M3), dopamine receptor (D1 and D2), leukotrienes B_4 receptor (BLT1), platelet-activating factor receptor (PAFR) and glucagon receptor (GCGR) were amplified by PCR and ligated with pcDNA3 vector (Invitrogen).

2.3. Cell preparation

Chinese hamster ovary (CHO)-K1 cells were maintained in Ham's F-12 medium containing 10% fetal bovine serum. Cells were transfected with plasmid DNA using Lipofectamine 2000 reagent. Stable CHO cell clones that express GPCRs were established by limiting dilution and selection with 1 mg/ml G418 (Invitrogen). The expression of GPCRs was confirmed by RT-PCR, and by ligand-evoked changes in intracellular calcium and/or cAMP concentration using established protocols (Yokomizo et al., 1997). Activated Rac was detected using an EZ-Detect™ Rac1 Activation Kit (PIERCE) according to the manufacturer's instructions.

2.4. SPR assay

SPR assays were performed using a dual-channel SPR670-MACS system (Moritex) in which a semiconductor laser acts as a source with a fixed wavelength of 670 nm in conjunction with a photodiode sensor to measure reflection light. This system provides a sensitivity of 0.001°/s. SPR sensor chips (Moritex, gold-layered chip, 13 mm × 20 mm × 0.7 mm) were sequentially washed with acetone, 70% ethanol and phosphate-buffered saline (PBS) at room temperature under sterilized conditions. Cells were directly seeded onto the SPR sensor chips without any chemical treatment on the surface of the chips, then placed in 35 mm culture dishes at a density of 1×10^6 cells/dish in Ham's F-12 medium containing 10% fetal calf serum and cultured overnight. After incubation in HEPES–Tyrode's–BSA buffer (HTB buffer, 25 mM HEPES–NaOH, pH 7.4, 140 mM NaCl, 2.7 mM KCl, 1.0 mM $CaCl_2$, 12 mM $NaHCO_3$, 5.6 mM D-glucose, 0.37 mM NaH_2PO_4, 0.49 mM $MgCl_2$ and 0.1% fatty acid-free BSA) for 30 min, the cells were mounted on the SPR system and further washed with HTB buffer at a flow rate of 30 μl/min through the flow channels until the baseline signal became stable. SPR measurements were performed using two flow channels; ligands for GPCRs dissolved in HTB buffer were added to one channel (the sensing channel) and the corresponding vehicle to the other (the reference channel). SPR responses are shown as maximum changes in resonance angle in the sensing channel minus those in the reference channel unless otherwise noted. Usually, the cells on the SPR sensor chip were able to be stimulated several times at intervals of approximately 30 min.

2.5. Measurement of intracellular calcium concentration

CHO cells were loaded with 5 μM Fura 2-AM in HTB buffer containing 1.25 mM probenecid and 0.02% Pluronic F127 for 1 h at 37 °C, and washed with HTB buffer. Changes in intracellular calcium concentrations upon ligand stimulation were monitored with a FLEX-station scanning fluorometer system (Molecular Devices).

3. Results

3.1. SPR responses in CHO-K1 cells expressing exogenous GPCRs

We examined ligand-induced SPR responses in CHO cells stably expressing each GPCR. The results are summarized in Table 1. The typical SPR responses in these CHO cells are shown in Fig. S1 (in the Supporting Information). Clear SPR responses were observed in M2 and D2 receptors (G_i-coupled), and PAFR and BLT1 (G_q- and G_i-coupled), whereas no SPR response was observed in other G_q- or G_s-coupled GPCRs. These results suggest that SPR responses can be evoked by signals mediated via G_i-coupled receptors.

Table 1
Summary of SPR responses in CHO cells.

Receptor	Ligand tested	G protein	Ca^{2+}	cAMP	SPR
M1	Carbachol	G_q	300 ± 20 nM[a]	Not done	No response[b]
M2	Carbachol	G_i	Not done	3 ± 2 μM[a,c]	5 ± 2 μM[a]
M3	Carbachol	G_q	100 ± 20 nM[a]	Not done	No response[b]
D1	Dopamine	G_s	Not done	3 ± 2 nM[a]	No response[d]
D2	Dopamine	G_i	20 ± 10 nM[a]	30 ± 10 nM[a,c]	30 ± 10 nM[a]
BLT1	Leukotriene B_4	G_i, G_q	5 ± 1 nM[e]	Not done	0.3 ± 0.2 nM[e]
PAFR	PAF	G_i, G_q	0.04 ± 0.02 nM[e]	Not done	0.1 ± 0.1 nM[e]
GCCR	Glucagon	G_s	Not done	10 ± 5 nM[e]	No response[d]
LPA1	LPA	G_i, G_q, $G_{12/13}$	No response[d]	No response[c,d]	5 ± 2 nM[a]
S1P2	S1P	G_i, G_q, $G_{12/13}$	No response[d]	No response[c,d]	4 ± 2 nM[a]
IGF-1R	IGF-1	Not applicable	Not done	Not done	7 ± 3 nM[a]

SPR responses in CHO cells are summarized with the results of calcium and cAMP assays. The responses of CHO cells stably expressing exogenous human GPCRs were analyzed after the application with each ligand for 2 min. The responses of endogenous receptors (LPA1, S1P2 and IGF-1R) expressing in CHO cells were also analyzed. PAF, platelet-activating factor; LPA, lysophosphatidic acid; S1P, sphingosine-1-phophate; IGF-1, insulin-like growth factor-1.

[a] Mean ± S.D. of EC_{50} value from triplicate measurements.
[b] No response up to 300 nM.
[c] Inhibitory effect on the 10 μM forskolin-induced cAMP accumulation.
[d] No response up to 100 nM.
[e] Mean ± S.D. of EC_{50} value from quadruplicate measurements.