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Evaluation of Drug Responses to Human β_2AR Using Native Mass Spectrometry

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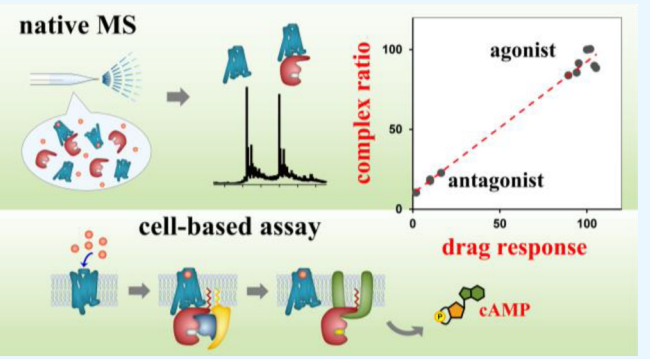
ABSTRACT: We aimed to develop a platform to rapidly investigate the responses of agonists and antagonists to G-protein-coupled receptors (GPCRs) using native mass spectrometry (MS). We successfully observed the ligand-bound human β_2 adrenergic receptor (hβ_2AR); however, it was challenging to quantitatively discuss drug efficacy from MS data alone. Since ligand-bound GPCRs are stabilized by the Gα subunit of G proteins on the membrane, mini-G_s and nanobody80 (Nb80) that can mimic the Gα interface of the GPCR were utilized. Ternary complexes of hβ_2AR, ligand, and mini-G_s or Nb80 were prepared and subjected to native MS. We found a strong correlation between the hβ_2AR−mini-G_s or −Nb80 complex ratio observed in the mass spectra and agonist/antagonist efficacy obtained using a cell-based assay. This method does not require radioisotope labeling and would be applicable to the analysis of other GPCRs, facilitating the characterization of candidate compounds as GPCR agonists and antagonists.



INTRODUCTION

Signal transduction in human cells begins with the signal received at the receptor in the plasma membrane. G-protein-coupled receptors (GPCRs) are membrane receptors that receive a variety of signals, including hormones and neurotransmitters.[1] Ligand-bound GPCRs are activated to form a complex with trimeric G protein ($\alpha\beta\gamma$) located inside the plasma membrane, promoting the release of guanosine diphosphate (GDP) bound to the α-subunit of G protein (Gα). After dissociation of the GDP molecule, guanosine triphosphate (GTP) promptly binds to Gα, which triggers conformational changes in the G protein; GTP-binding to Gα leads to dissociation of the G-protein complex and activates both the Gα and G$\beta\gamma$ complexes (G$\beta\gamma$). Activated Gα and G$\beta\gamma$ interact with their respective target proteins in the plasma membrane, resulting in successive signal transduction. Since GPCRs are involved in various signal transduction processes in the cell, they are major targets for drug development.[2,3] To date, more than 800 human GPCRs have been identified, and of approximately 350 non-olfactory GPCRs, 165 proteins have been validated as drug targets.[3−5] In contrast, approximately 200 GPCRs have not yet been explored in clinical trials.[5]

Therefore, it is important to establish a convenient biophysical method that can examine the efficacy of agonist and antagonist candidates for GPCRs. We applied native mass spectrometry (native MS)[6−9] to develop a platform that can rapidly and conclusively identify the intrinsic efficacy of agonists and antagonists. Since GPCRs are extremely unstable in the absence of lipid bilayer membranes, it is challenging to isolate and observe intact ions of the GPCR−ligand complexes using native MS. For native MS, ligand-bound GPCR samples are generally prepared in the micellized form and then subjected to analysis under mild conditions to avoid denaturation, whereas detergents should be removed completely before mass determination. To date, only a few ligand-bound GPCRs have been successfully observed using native MS; these include the purinergic receptor $P2Y_1$ complexed with its natural ligand adenosine diphosphate/adenosine triphosphate (ADP/ATP), or a synthetic ligand that forms electrostatic interactions with $P2Y_1$[10,11] and glucagon-bound glucagon receptor (GCGR).[12] These studies indicate that it is difficult to retain a ligand that binds to the target membrane protein via hydrophobic interactions during MS analysis. Yen et al. applied native MS to indicate agonist-binding to turkey β_1 adrenaline receptor (tβ_1AR) by utilizing a mini-G protein and/or nanobody, which mimics the interface of the Gα subunit to GPCR.[13] In the mass spectra of the ternary complexes of

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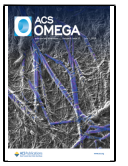

tβ_1AR, agonist, and the mini-G protein and/or nanobody, only the ions corresponding to the GPCR−mini-G and GPCR−nanobody complexes, in which the ligand was not retained, were observed. Similarly, Gavriilidou et al. utilized native MS to investigate the basal activity and activation mechanism of the tβ_1AR in the presence of an agonist or an inverse agonist.[14] It was demonstrated that higher agonist concentrations facilitated the formation of the transducing complex, whereas higher inverse agonist concentrations disrupted the complex. Expanding on this method, Wu et al. developed a platform that can distinguish agonist/antagonist by observing the cross-linked GPCR−mini-G and GPCR−nanobody complexes using MALDI-TOFMS.[15] Recently, Yen et al. investigated the biased signaling of a GPCR using native MS by forming a complex with a mini-G protein and/or nanobody.[16]

In the present study, we developed a method that can rapidly estimate the efficacy of agonists and antagonists of the human β_2 adrenaline receptor (hβ_2AR). β_2 adrenaline receptor (β_2AR) is a GPCR responsible for relaxing the smooth muscle of bronchioles, blood vessels, and other organs by binding adrenaline, a signal transducer. β_2AR is activated by the binding of adrenaline and forms a complex with the G protein, leading to the activation of adenylyl cyclase, which catalyzes the synthesis of cyclic adenosine monophosphate (cAMP). Thus, the concentration of cAMP is an indicator of the efficacy of ligand binding to and activation of β_2AR. To this end, hβ_2AR-Δ was prepared[17] and analyzed using native MS. hβ_2AR-Δ is a stabilized mutant of hβ_2AR, in which N- and C-terminal flexible segments are truncated, and several sites that do not affect the function are mutated. We first examined the intact hβ_2AR-Δ−ligand complexes using native MS under conditions optimized to be as mild as possible. Next, we prepared an engineered minimal Gαs protein, mini-G_s, and specific nanobody80 (Nb80) for β_2AR to form a complex with each ligand-bound hβ_2AR-Δ; these two engineered proteins had been identified to form a specific complex with tβ_1AR by the previous studies using native MS[14,16] and MALDI-TOFMS of the cross-linked complexes.[15] Native MS was performed for the ternary complex of hβ_2AR-Δ, ligand, and mini-G_s or Nb80 to analyze the efficacies of agonists and antagonists for signal transduction by referring to the results of the cell-based assays.[18,19]

MATERIALS AND METHODS

Materials. All chemical reagents were purchased from Nacalai Tesque (Kyoto). [G1]-ether-C12 (G1) was synthesized as previously reported.[18] Agonists and antagonists of hβ_2AR used in this study are listed in Table S1.

Protein Expression and Purification. hβ_2AR-Δ, mini-G_s, and Nb80 were obtained by expression culture and purification. Details of the experiments are described in the Supporting Information.

Sample Preparation for Mass Spectrometry. The sample was prepared using 7.3−10 μM purified hβ_2AR-Δ. For ligands except isoproterenol, a final concentration of 2 mM of ligand was added to the sample to make the ligand 100 times in excess of isoproterenol present in the sample and incubated at 4 °C for 16 h.

For hβ_2AR-Δ−ligand complexes, the ligand-bound hβ_2AR-Δ was buffer-exchanged into MS buffer (200 mM ammonium acetate (pH 7.4)) containing 20 μM ligand, 0.02% DMSO, and 0.03% [G1]-ether-C12 (G1) using a centrifugal device (Micro Bio-Spin 6, Bio-Rad, Hercules).

To prepare the hβ_2AR-Δ−Nb80 and hβ_2AR-Δ−mini-G_s complexes, Nb80 or GDP-bound mini-G_s was mixed with hβ_2AR-Δ at a 1:1 ratio 10 min before applying the sample to a centrifugal device. The samples complexed with Nb80 were replaced with MS buffer containing 20 μM ligand and 0.03% G1, and the samples complexed with GDP-bound mini-G_s were replaced with MS buffer containing 20 μM ligand, 0.03% G1, and 10 μM GDP. Measurement was immediately performed.

Mass Spectrometry. MS measurements were performed on SYNAPT G2 HDMS (Waters, Milford, MA) equipped with a nano ESI source in triplicate for each sample. The samples were deposited in borosilicate capillaries with an inner diameter of ∼2 μm prepared in-house. To perform an electrospray, a 0.127 mm diameter Pt wire (Sigma-Aldrich, St. Louis, MO) was dipped in the sample solution and 0.68−0.7 kV of capillary voltage was applied. The cone and collision voltages were set to 10 V and 15−60 V, respectively. The source temperature was held at 40 °C, the Ar gas flow rate was set at 3.0 mL/min, and the backing pressure in the source region was set to 5.2−5.6 mbar.

Calculation of Abundance Ratios and % Complex (Iso). Abundance ratios (%) of free hβ_2AR-Δ (P) and the complex (PE) were calculated using the following equations

$$\mathrm{P} = \frac{\sum_m [\mathrm{P}^{m+}]}{\sum_n [\mathrm{PE}^{n+}] + \sum_m [\mathrm{P}^{m+}]} \times 100 \quad (1)$$

$$\mathrm{PE} = \frac{\sum_n [\mathrm{PE}^{n+}]}{\sum_n [\mathrm{PE}^{n+}] + \sum_m [\mathrm{P}^{m+}]} \times 100 \quad (2)$$

Free hβ_2AR-Δ and the engineered protein (mini-G_s or Nb80) are denoted as P and E, respectively. $[\mathrm{P}^{m+}]$ is the peak height of m+-charged ion of hβ_2AR-Δ, including the Zn^{2+} adduct. $[\mathrm{PE}^{n+}]$ is the peak height of the n+-charged ion of the complex of hβ_2AR-Δ and the engineered protein (mini-G_s or Nb80) containing the Zn^{2+} adduct. The peaks of the complex with mini-G_s include GDP-bound and GDP-unbound mini-G_s peaks.

The % complex (Iso) was determined from the obtained PE and $\mathrm{PE_{Iso}}$ (abundance ratio of the complex in the presence of isoproterenol (%)) using the following equation

$$\%\ \text{complex (Iso)} = \frac{\mathrm{PE}}{\mathrm{PE_{Iso}}} \times 100 \quad (3)$$

The average and standard deviation (S.D.) values of the "% complex (Iso)" for each agonist/antagonist were calculated using the peak height observed in the three independent mass spectra.

RESULTS AND DISCUSSION

Observation of the hβ_2AR−Ligand Complex. To avoid denaturation in the absence of membranes, hβ_2AR-Δ was purified not in an apo-form but in a form bound to isoproterenol, a potent agonist, micellized with dodecylmaltoside (DDM).[17] Since the possible phosphorylation sites remained in the sequence, the purified hβ_2AR-Δ was treated with phosphatase and phosphate groups were removed (Figure S1). Prior to native MS, the solvent was exchanged with 200 mM ammonium acetate containing 0.03% G1,[20] an oligoglycerol detergent specifically designed for the native MS of membrane proteins. Because detergent G1 forms micelles and

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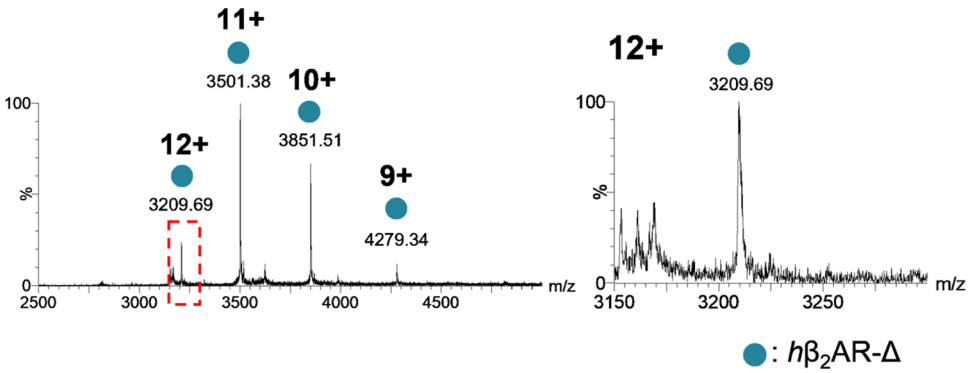

Figure 1. ESI mass spectra of the hβ_2AR-Δ−ligand complex. Mass spectra of hβ_2AR-Δ in the presence of isoproterenol at 60 V CE. (Left) Mass spectrum in the range of m/z 2500−5000; (right) expanded spectrum for 12+ charged ion, corresponding to the area enclosed by a red dashed line in the left panel. Only hβ_2AR-Δ without isoproterenol was observed.

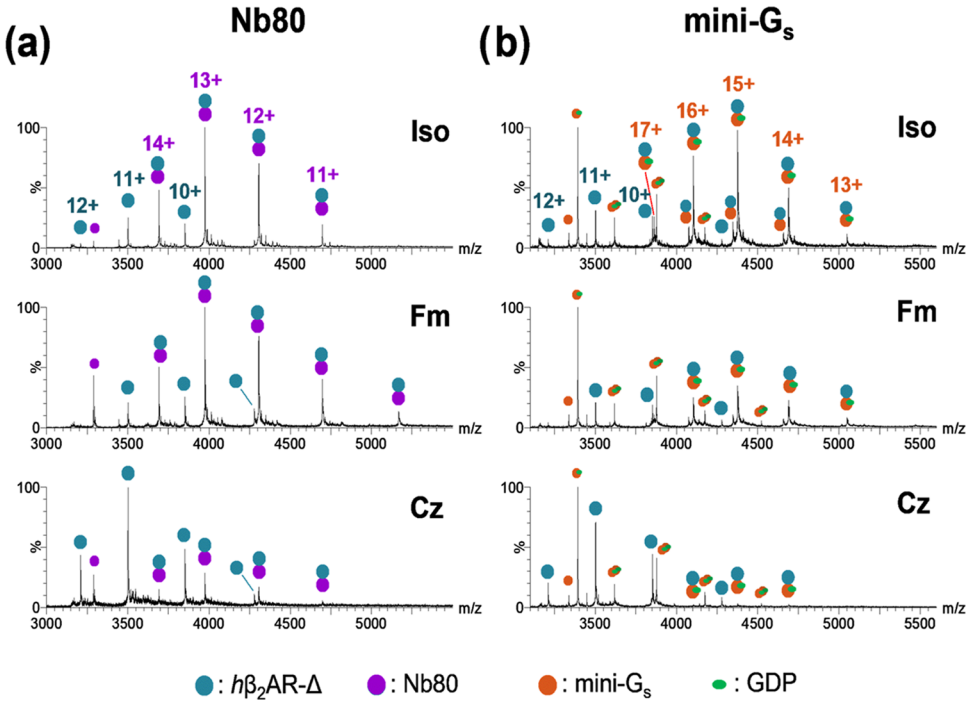

Figure 2. ESI mass spectra of hβ_2AR-Δ−mini-G_s and hβ_2AR-Δ−Nb80 complexes. Mass spectra of the complexes of ligand-bound hβ_2AR-Δ and Nb80 (a) or mini-G_s (b). The complexes were formed in the presence of the agonists, isoproterenol (Iso) (top) and formoterol (Fm) (middle), and the antagonist, carazolol (Cz) (bottom). The greenish-blue, purple, and orange circles correspond to hβ_2AR-Δ, Nb80, and mini-G_s, respectively. GDP is denoted by the green oval.

softly wraps membrane proteins, the collision energy (CE) required for dissociation is relatively low,[20] and it is expected that only the micelles bound to the membrane proteins will be stripped off and that the drug will remain during the MS measurement. Figure 1 shows the mass spectra of hβ_2AR-Δ in the presence of isoproterenol micellized with G1. Upon applying 60 V of CE, detergents were completely removed and multiply charged ions of hβ_2AR-Δ were clearly observed; however, the ions of isoproterenol-bound hβ_2AR-Δ were not observed. This may be because isoproterenol was more easily removed from the micellized complex than the detergents, G1, and the residual DDM. Since DDM requires a higher CE than G1, we replaced the detergent with G1 in the sample preparation for native MS, but it is possible that DDM was not completely replaced with G1 during the buffer exchange process by centrifugation. Despite this drawback, it is likely that the residual DDM stabilized the complex and reduced the denaturation of hβ_2AR-Δ, resulting in the observation of intense hβ_2AR-Δ signals with a high signal-to-noise ratio (S/N) (Figure 1).

After examining the measurement conditions in detail, we observed the ligand-bound hβ_2AR-Δ signals with a low S/N (Figure S2). Upon reducing the CE to 15 V, the peaks were considerably broadened, but mild dissociation of the detergents was achieved for the micellized protein, leading to the observation of multiply charged ions of isoproterenol-bound hβ_2AR-Δ (Figure S2d). Some population of isoproterenol-bound hβ_2AR-Δ was associated with G1 molecules that were not completely removed at 15 V CE. By elevating the CE voltage, the peak width decreased but no ion of isoproterenol-bound hβ_2AR-Δ was observed at ≥20 V of CE (Figure S2a−c). The binding of hβ_2AR-Δ to another agonist, formoterol, and an antagonist, carazolol, was then examined. After ligand exchange, the peaks of formoterol- and carazolol-bound

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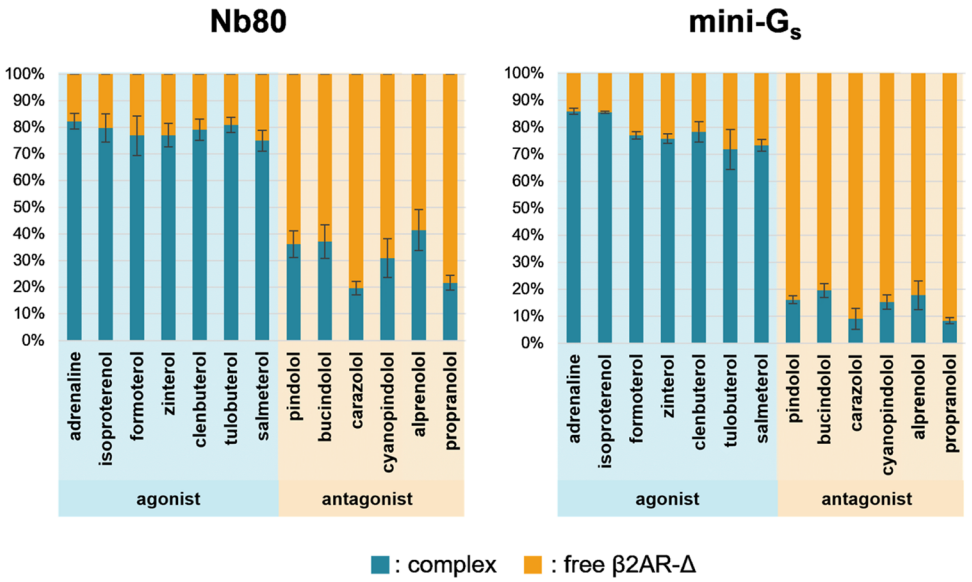

Figure 3. Abundance ratios of complexes versus free hβ_2AR-Δ. Abundance ratios of hβ_2AR-Δ−mini-G_s or hβ_2AR-Δ−Nb80 complexes (blue bar) versus free hβ_2AR-Δ (orange bar) for each ligand (Table S1) obtained from the mass spectra (Figures S3 and S4). Error bars indicate standard deviations in three independent experiments. The abundance ratio of the complex for the agonist-bound hβ_2AR-Δ was distinctly higher than that for the antagonist-bound hβ_2AR-Δ and was more pronounced for mini-G_s-bound complexes.

hβ_2AR-Δ (Figure S3) were similarly observed in the mass spectra. The hβ_2AR-Δ−ligand interaction was weaker than the hβ_2AR-Δ−detergent interaction; thus, the peaks of the ligand-bound forms were considerably broadened by the association of many G1 and residual DDM molecules. As previously reported, it is found to be difficult to retain the ligand−GPCR complexes bound by hydrophobic interactions, but weak signals of the complex ions were observed in the spectra under extremely mild conditions. Considering that all ligands observed in complex with GPCRs in native MS to date have been highly polar small molecules, such as nucleic acid derivatives,[10,11] or a peptide hormone,[12] it is noteworthy that we successfully observed β_2AR complexed with less polar small molecules, isoproterenol, formoterol, and carazolol. Although we successfully observed ligand-bound hβ_2AR-Δ, it was difficult to analyze the drug affinity and efficacy of GPCR using these spectra due to a low S/N.

The binding affinity of agonist/antagonist for β_2AR is relatively high compared to general protein−drug interactions, as shown in Table S1. β_2AR antagonists in particular bind strongly to the receptor in aqueous solutions at the nM level of dissociation constant (K_d). In general, it is not very difficult to observe the ions of a protein−drug complex with a K_d of nM by native MS. However, observation of the β_2AR−ligand complex was not successful. This may be due to the impact of stripping the micelles in the gas phase after desolvation, which disrupted the structure of β_2AR and dissociated the ligand. Consequently, to discuss the efficacy of β_2AR agonist/antagonist, we investigated experimental methods to stabilize the β_2AR−ligand complex even in the gas phase.

Observation of hβ_2AR-Δ−mini-G_s and hβ_2AR−Nb80 Complexes. The engineered Gα subunit of the G protein, which includes mini-G_s and Nb80, can stabilize the activated GPCRs via ligand binding, which was verified by the structures of GPCR−Gs, −mini-G_s, and −Nb80 complexes.[21−24] Since there was little difference in receptor structure between the GPCR−mini-G_s and GPCR−Gs complexes,[23] we then examined native MS results of the ternary complex of hβ_2AR-Δ, ligand, and mini-G_s or Nb80, which corresponds to the activated GPCR complex, by referring to previous studies.[14,15] Seven agonists and six antagonists (Table S1) were examined to determine whether they can form ternary complexes observable by native MS. After ligand exchange, GDP-bound mini-G_s or Nb80 were added to the ligand-bound hβ_2AR-Δ, and the solvent was exchanged with ammonium acetate containing G1 for mass spectrometry. Native MS experiments were performed triplicate for each sample to confirm experiment reproducibility.

Figure 2 shows the representative mass spectra of the complexes of ligand-bound hβ_2AR-Δ and Nb80 or mini-G_s for the agonist (isoproterenol and formoterol) and antagonist (carazolol). Observed and theoretical mass values are summarized in Table S2. Mass spectra were obtained at 60 V CE to observe clear signals of the protein complexes without peak broadening; it was difficult to obtain the mass spectra of the ternary complex, which allows direct confirmation of the ligand attachment. In the mass spectra, the ions of the hβ_2AR-Δ−Nb80 or −mini-G_s complexes and free hβ_2AR-Δ, Nb80, and mini-G_s were clearly observed, but those of the ligand-bound ternary complexes were not.

To observe the ternary complex of hβ_2AR-Δ, ligand, and Nb80 or mini-G_s in native MS requires more severe measurement conditions than ligand-bound hβ_2AR-Δ alone, due to its larger molecular size. In contrast, maintaining the ligand in the complex requires measurements at low collision energies, but at low collision energies, the observed complex ions contain many detergent molecules, broadening the complex peaks. To solve this problem, various sample preparation and measurement conditions were examined, but it was not possible to observe the ternary complex ions.

The mass spectra in Figure 2 showed different relative intensities for the free hβ_2AR-Δ and hβ_2AR-Δ−Nb80 or −mini-G_s complexes for each ligand (also in Figures S4 and S5), as summarized in Figure 3. The relative ratio of the hβ_2AR-Δ−Nb80/mini-G_s complex for the agonist-bound hβ_2AR-Δ was distinctly higher than that for the antagonist-

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bound hβ_2AR-Δ, which was especially evident for the mini-G_s bound complex. Extensive analysis of the mass spectra of the hβ_2AR-Δ−Nb80/mini-G_s complexes in the presence of an agonist or antagonist will be described later.

Native MS Analysis of Ligand-Binding Response. We analyzed whether there was a correlation between our native MS results and the biological responses obtained using cell-based assays. In cell-based assays, the concentration of cAMP, the synthesis of which is triggered by the activation of β_2AR, is used as the biological response; [^{3}H]-cAMP is employed for highly sensitive analysis.[18] To compare these two data sets, the % isoproterenol abundance ratios of the complex from native MS and the % isoproterenol maximal response in the cell-based assay were used. The % isoproterenol abundance ratio of the complex, % complex (Iso), was calculated in the presence of each ligand using the relative ratio of the complex by setting the value obtained in the presence of isoproterenol as 100% (Table 1). The mass spectra of the hβ_2AR-Δ−Nb80/mini-G_s complexes showed satellite peaks for the multiply charged ions of the complexes (Figure S6), suggesting that endogenous Zn^{2+} ions were bound to the complex, as reported in previous studies.[16,25] The relative intensities of the satellite peaks of the hβ_2AR-Δ−mini-G_s complex ions were higher than those of the hβ_2AR-Δ−Nb80 complex ions. In the calculation of the % complex (Iso) values, these satellite peaks were also considered. In the cell-based assay, the maximal response was obtained by measuring the maximum increase in cAMP concentration triggered by the binding of ligand to β_2AR; this value obtained with each ligand was converted to a relative value, % max response (Iso), by setting the value obtained in the presence of isoproterenol as 100% (Table 1).[18] A positive correlation was observed between % complex (Iso) and % max response (Iso) for both the hβ_2AR-Δ−mini-G_s and hβ_2AR-Δ−Nb80 complexes (Figures 4 and S7). In the case of hβ_2AR-Δ−mini-G_s, the % complex (Iso) and % max response (Iso) values were very close and appeared to be strongly correlated (Figure 4a).

Table 1. % Complex (Iso) and % Max Response (Iso)[a]

		% complex (Iso)		
		mini-G_s	Nb80	% max response (Iso)[18]
agonist	adrenaline	100.4	103.1	101.9
	isoproterenol	**100.0**	**100.0**	**100.0**
	formoterol	90.0	96.4	104.3
	zinterol	88.5	96.6	105.3
	clenbuterol	91.5	99.1	95.3
	tulobuterol	83.9	101.4	89.3
	salmeterol	85.7	93.9	94.1
antagonist	pindolol	18.7	45.3	9.9
	bucindolol	22.8	46.6	16.2
	carazolol	10.5	24.6	1.9
	cyanopindolol	17.8	38.7	9.9
	alprenolol	20.7	51.9	NA[b]
	propranolol	9.7	27.2	NA[b]

[a]From the results shown in Figure 2b, the relative value of the % isoproterenol abundance ratio of the complex (% complex (Iso)) was calculated for each ligand using the abundance ratio of the complex, by setting the complex abundance ratio for isoproterenol as 100%. % isoproterenol maximal responses (% max response (Iso)) for each ligand obtained from the cell-based assay are shown.[18] Reprinted in part with permission from ref 18. Copyright 2010 John Wiley and Sons. [b]For alprenolol and propranolol, % isoproterenol maximal responses have not been reported in previous studies.[18,19]

When looking at the correlations between % complex (Iso) and % max response (Iso) for agonists and antagonists independently, the antagonists (n = 4) showed a very strong positive correlation for both Nb80 (R = 0.918) and mini-G_s (R = 0.992) (Figures S7b and 4b), although the number of examined antagonists (n = 4) was not large enough to assert the correlation for the antagonists. For agonists (n = 7), no correlation was observed with Nb80 (R = −0.142), whereas a certain level of correlation was observed with mini-G_s (R = 0.476) (Figures S7b and 4b). The % max responses (Iso) of two antagonists, alprenolol and propranolol, were not reported in the previous studies,[18,19] but can be estimated from the plots in Figure 4.

To determine the reason for the difference in the trend between the hβ_2AR-Δ−mini-G_s and hβ_2AR-Δ−Nb80 complexes in the presence of antagonists, we investigated the receptor structures in the complexes with Gs and Nb80.[21,22] In the agonist-bound β_2AR−Gs and β_2AR−Nb80 complexes, the receptor structure of the extracellular side of agonist-bound β_2AR was highly conserved, but a small difference was found in the structure of the cytoplasmic ends of transmembrane helices 5 (TM5) and 6 (TM6) of β_2AR. In the β_2AR complex with Gs, the end of TM6 moved outward by 3 Å, and two helical turns were extended for TM5 compared with those in the complex with Nb80.[21] When the receptor structures in the agonist-bound β_2AR−Gs and β_2AR−Nb80 complexes were compared to the antagonist-bound β_2AR structure, TM6 was positioned outward by 14 and 11 Å, respectively.[21,22] These data indicate that Gs requires a larger space than that required by Nb80, enabling strict recognition of agonist-activated β_2AR, that is, binding to antagonist-bound β_2AR is unfavorable for Gs but would be acceptable for Nb80. Accordingly, a higher correlation between the % complex (Iso) and % maximal response (Iso) for the hβ_2AR-Δ−mini-G_s complex is reasonable.

Notably, most of the hβ_2AR-Δ−mini-G_s complex and free mini-G_s observed in the mass spectra retained a GDP molecule (Figures 2b and S4), as reported in the X-ray structure of human adenosine A_{2A} receptor−mini-G_s complex.[23] In contrast, GDP was readily dissociated from the tβ_1AR−mini-G_s complex in the native mass spectra in a previous study.[16] Our successful observation of the hβ_2AR-Δ−mini-G_s complex may be due to the fact that GDP concentration was maintained at 5 μM or more throughout the mini-G_s purification and preparation procedures for MS measurement to avoid the removal of GDP and reduction in the binding affinity to hβ_2AR-Δ. In addition, we set the native MS parameters to be as mild as possible. As a result, the GDP-bound forms of the mini-G_s protein and hβ_2AR-Δ−mini-G_s complex were predominantly observed in the samples. These sample preparation and MS measurement conditions are thought to avoid the removal of GDP from mini-G_s and facilitate the quantitative evaluation of the efficacy of agonists and antagonists via native MS.

CONCLUSIONS

Since GPCRs are extremely unstable in the absence of a plasma membrane, it is not easy to observe intact ligand-bound hβ_2AR-Δ using native MS, wherein the measurement is carried out in the gas phase. By optimizing the MS parameters, we were able to observe ligand-bound hβ_2AR-Δ, but the signal

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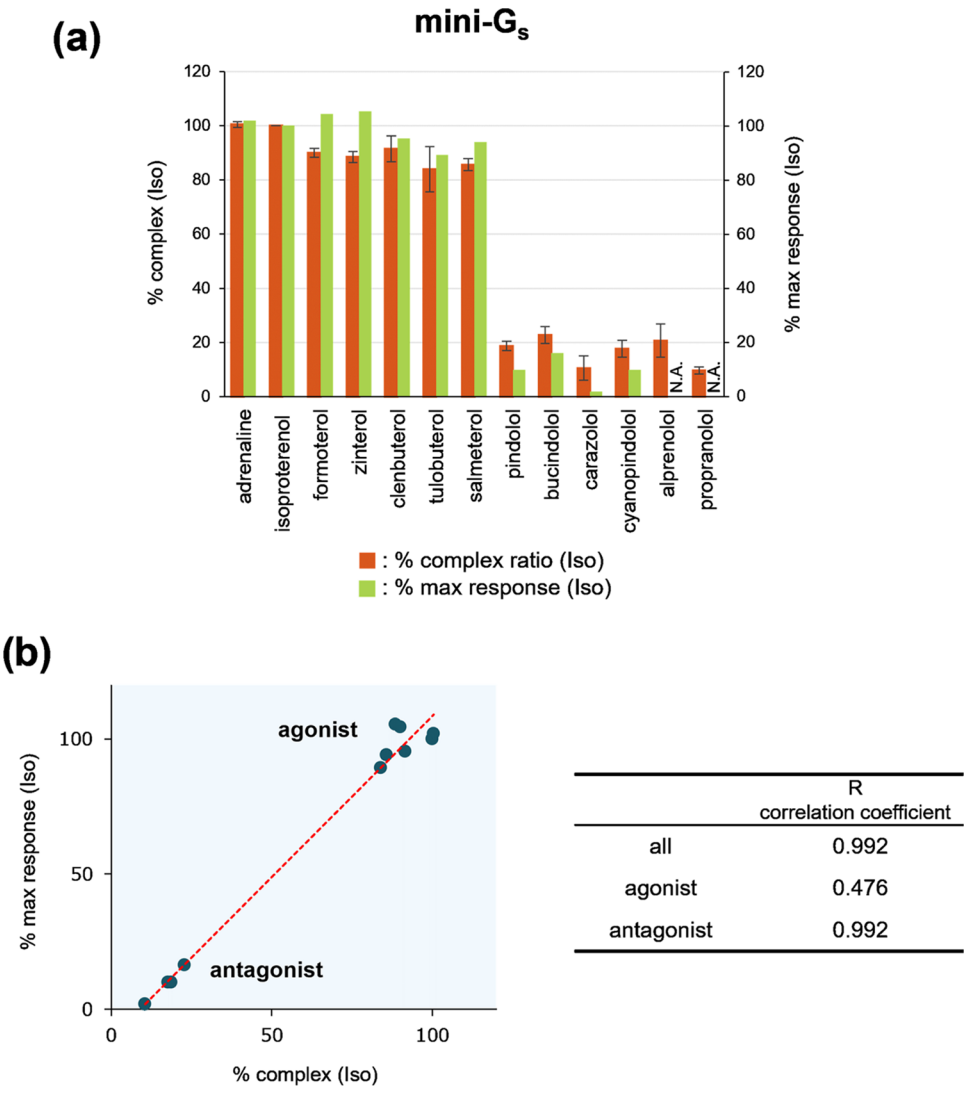

	R correlation coefficient
all	0.992
agonist	0.476
antagonist	0.992

Figure 4. Correlation between the % complex (Iso) of mini-G_s-bound complexes from native MS and % max response (Iso) from the cell-signaling assay.[18] (a) The % complex (Iso) (brown bar) and the % isoproterenol maximal response (% max response (Iso)) from the cell-signaling assays (green bar), as listed in Table 1, are shown for each agonist and antagonist. Error bars indicate standard deviations in three independent experiments. For alprenolol and propranolol, % isoproterenol maximal responses have not been reported in previous studies.[18,19] (b) Correlation between % complex (Iso) for mini-G_s and % max response (Iso) (left) and correlation coefficient (R) for all ligands, agonist (n = 7), and antagonist (n = 4) (right) are shown. Reprinted in part with permission from ref 18. Copyright 2010 John Wiley and Sons.

intensity and S/N were not sufficient to quantitatively discuss drug efficacy. By stabilizing the ligand-bound hβ_2AR-Δ with proteins that mimic the Gα-subunit of G proteins, we could observe the hβ_2AR-Δ−mini-G_s and −Nb80 complexes using native MS. In addition, a strong correlation was observed between the relative ratio of the complex observed in the mass spectra and the drug efficacy observed in the human cell-based assays. Since we performed native MS experiments on human β_2AR-Δ, a true target protein that is unstable in the absence of cell membrane, it was possible to directly compare our results with those of human cell-based assays. With this platform, it was possible to estimate the efficacy of the agonists and antagonists to hβ_2AR-Δ from the "% complex (Iso)" value obtained via native MS of the hβ_2AR-Δ−mini-G_s in the presence of the ligands. The present study showed that agonist/antagonist efficacies can be reliably analyzed by native MS using human GPCRs, rather than GPCRs from other species. Our native MS method is rapid and requires a small amount of sample. Furthermore, this method, unlike cell-based assays, does not require radioactive reagents for the measurement; this is a good advantage of our method. In future, if optimal detergents for observing unstable membrane protein complexes are developed, it will be possible to detect intact ligand-bound ternary complexes. Although the ternary complexes have not been observed in the spectra at present, this strategy could be applied to the estimation of the efficacy of candidate compounds of agonists/antagonists of other GPCRs and is promising for accelerating drug development targeting GPCRs.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.3c02737.

Experimental procedures for protein preparation; list of agonists and antagonists (Tables S1); observed and theoretical masses of hβ_2AR-Δ and its complexes with

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mini-G_s and Nb80 (Table S2); ESI mass spectra of hβ_2AR-Δ before and after dephosphorylation (Figure S1); ESI mass spectra of isoproterenol-bound hβ_2AR-Δ at various CE (Figure S2); ESI mass spectra of formoterol- and carazolol-bound hβ_2AR-Δ at 15 V CE (Figure S3); ESI mass spectra of hβ_2AR-Δ−mini-G_s and −Nb80 complexes in the presence of an agonist/antagonist (Figures S4 and S5); ESI mass spectra of hβ_2AR-Δ−mini-G_s, showing Zn^{2+} adduct (Figure S6); and correlation between % complex (Iso) and % max response for hβ_2AR-Δ−Nb80 (Figure S7) (PDF)

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Author Contributions

S.A. and M.T. conceived the project with advice from I.S. M.T., S.I., and T.K. carried out experiments. K.S. contributed to the chemical synthesis of the specific detergent for the MS experiments. M.T. and S.A. analyzed data. S.A., M.T., and T.K. wrote the manuscript. All authors have approved the final version of the manuscript.

Notes

The authors declare no competing financial interest.

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