Dear Editor,

G-protein-coupled receptors (GPCRs) modulate cytoplasmic signaling in response to extracellular stimuli, and are important therapeutic targets in a wide range of diseases. Differential ligands binding to receptor promote different conformations of GPCR–G-protein complex, which can adopt diverse active states. Such ligand-directed biased agonism is now an important focus in drug discovery. Therefore, structure determination of GPCR–G-protein complexes in variable activation states is important to elucidate the mechanisms of signal transduction, and to facilitate drug discovery.

The β2-adrenergic receptor (β2AR), a canonical class A GPCR, is activated by adrenaline and norepinephrine. Recent years, many agonists have been synthesized to stimulate the activation of β2AR, and some of these ligands have been developed for the clinical treatment of asthma and chronic obstructive pulmonary diseases. Since the first crystal structure of β2AR bound with the inverse agonist carazolol was reported, several crystal structures of the β2AR bound with different agonists have been determined. However, only structure of the BI167107–β2AR–Gs complex was determined to date, which represented the real active-state of β2AR. Whether the observed β2AR–Gs interactions in the complex upon BI167107 binding provide a general rule for signal transductions from the binding of different agonists to cyclic adenosine monophosphate (cAMP) accumulation requires further validation, and also remains a major concern for the pharmacological understanding of β2AR and further drug development.

Formoterol is a selective, long-acting agonist of β2AR, which is unique as it both has a long-acting bronchodilator effect (>12 h) and exhibits a fast onset of action (1–3 min from inhalation), suggesting that it is effective both as maintenance and reliever medication. Herein, the cryo-EM structure of the formoterol-bound β2AR–Gs complex was determined with an overall resolution of 3.8 Å. Formoterol was reported to have a weaker affinity than BI167107 in β2AR binding, and also has lower β2AR activation potency than BI167107 (Fig. 1a). Therefore, comparisons between the structure of the formoterol–β2AR–Gs complex and the previously reported structure of the BI167107–β2AR–Gs complex will provide insights into the conformational responses of the β2AR upon binding to agonists with different potency.

First, we optimized the previously reported β2AR construct and obtained an engineered construct with improved expression in the sf9 insect expression system (Supplementary Fig. S1). The formoterol–β2AR–Gs complex in lauryl maltose neopentyl glycol (LMNG) detergent micelles was visualized using a Titan Krios microscope. After imaging and initial two-dimensional classification, three-dimensional classification yielded a final map at a global resolution of 3.8 Å (Fig. 1b; Supplementary Figs. S2, S3 and Table S1). The cryo-EM density map of the formoterol–β2AR–Gs complex exhibits well-resolved side chains, allowing rotamer placements for most amino acids (Fig. 1b; Supplementary Fig. S4). As revealed in Fig. 1c, the agonist formoterol is clearly identified in the orthosteric-binding site on the formoterol–β2AR–Gs complex.
extracellular side of β₂AR. The extensive receptor–Gs interface in the complex is mainly formed by the α5 helix in the Gαs-Ras domain, which extends into the transmembrane core of the receptor from the intracellular side. When compared the structure of formoterol-bound β₂AR from cryo-EM complex with that of carazolol-bound β₂AR in an inactive state (PDB: 2RH1), remarkable differences were observed for TM5, TM6 and ICL2 (Fig. 1d), suggesting that formoterol-bound β₂AR is in an active state.
When focusing on the structural details of the orthosteric-binding pocket, we found that the catecholamine moiety of formoterol formed hydrogen bonds with Ser203<sup>5.42</sup> and Ser207<sup>5.46</sup> in TM5 (Fig. 1f, g; Supplementary Fig. S4). The alkyamine and the β-OH in the middle of formoterol formed polar interactions with Asp113<sup>3.32</sup>, Asn312<sup>7.39</sup> and Tyr316<sup>7.43</sup> in TM7. Moreover, formoterol formed hydrophobic interactions with receptors through V117<sup>3.36</sup>, F193<sup>5.32</sup>, F289<sup>5.51</sup>, F290<sup>5.52</sup>, and Y308<sup>7.35</sup>, stabilizing the orthosteric agonist-binding pocket in the active-state (Fig. 1g). cAMP accumulation assay revealed that mutation of the hydrophobic amino acids F193A, F289A, F290A, and Y308A in the formoterol-binding pocket decreased the potency of formoterol (Fig. 1h). Moreover, alanine substitution of residues D113, S203, S207, N312, and Y316 significantly impaired cAMP signaling (Fig. 1h). All of these results confirmed that residues involved in interactions between the ligand and β<sub>2</sub>AR play important roles in the formoterol-mediated cAMP signaling pathway.

When compared the cryo-EM structure of formoterol-bound β<sub>2</sub>AR with the crystal structure of BI167107-bound β<sub>2</sub>AR (PDB: 3SN6), significant differences were observed for extracellular regions, which contains the orthosteric ligand-binding pocket of the β<sub>2</sub>AR. Specifically, the extracellular top of TM1 extracellular region in formoterol-bound receptor moves outward by 3.2 Å when measured at the Cα carbon of Val34. ECL3, which connects TM6 and TM7, was also observed to extend slightly into the extracellular side (3.7 Å when measured at the Cα carbon of Asn301). Another notable difference observed between the two active-state β<sub>2</sub>AR structures was the short α-helix inside ECL2, which was observed to move upward by 4.1 Å when measured at the Cα carbon of Asn183 (Fig. 1e). It is worth noting that, when compared the crystal structure of BI167107-bound β<sub>2</sub>AR to the cryo-EM structure of BI167107-bound β<sub>2</sub>AR (PDB: 6N13), the ligand-binding pocket in the extracellular region is exactly the same (Supplementary Fig. S6). Thus, the structural differences observed between the cryo-EM structure of formoterol-bound β<sub>2</sub>AR and the crystal structure of BI167107-bound β<sub>2</sub>AR are not due to the steric restraints in the crystal lattice, but owing to the binding of different agonists. Taken together, these structural differences at the extracellular side of the receptors endow β<sub>2</sub>AR–formoterol with a slightly larger ligand-binding pocket. There are a total of ten amino acid residues that interact with formoterol in the orthosteric agonist-binding pocket, including five hydrophobic residues and five hydrophilic residues (Fig. 1g), compared with a total of 13 amino acid residues that interact with BI167107<sup>5.9</sup> (Supplementary Fig. S5b). The decreased number of interacting residues between these two complexes might contribute to the lower affinity of formoterol versus that of BI167107<sup>5.9</sup>. Noteworthy, the side chains of both S204<sup>5.43</sup> and N293<sup>6.55</sup> rotate away from the formoterol molecule, which excludes the interactions stabilizing the binding between agonist and β<sub>2</sub>AR (Fig. 1e). Considering these observations, we speculate that the lower binding affinity of formoterol is mainly caused by the enlarged ligand-binding pocket and the reduced interactions between receptor and agonist due to changes of S204<sup>5.43</sup> and N293<sup>6.55</sup>.

In the formoterol–β<sub>2</sub>AR–Gs complex, the most extensive contacts between the G-protein and the β<sub>2</sub>AR are formed by the α5 helix of the Gas-Ras domain, which inserts into the intracellular central cavity of the β<sub>2</sub>AR transmembrane domain, consequently leading to a 14 Å outward movement of TM6. Briefly, the interfaces are mediated mainly by extensive hydrophobic interactions (i) between the α5 helix of Gas and ICL2, TM3, TM5, TM6 and TM7 of β<sub>2</sub>AR, and (ii) between the αN helix, αN–P1 loop of Gas, and ICL2 of β<sub>2</sub>AR (Fig. 1i; Supplementary Fig. S7). As shown in Fig. 1i, the imidazole ring of H41 in...
the αN helix and the phenyl ring of F376 in the α5 helix from Gαs protein in the formoterol–β2AR–Gs complex rotate away from the hydrophobic pocket compared with that in the BI167107–β2AR–Gs complex, which might attenuate the hydrophobic interactions between the αN helix, αN/β1 loop of Gαs and ICL2 of β2AR (Fig. 1i). Since the hydrophobic pocket between β2AR and Gαs protein is crucial for GDP release and is probably necessary for the stabilization of the nucleotide-free β2AR–Gs complex, the decreased hydrophobic interaction in the formoterol-bound β2AR–Gs structure might have an impact on subsequent signal transduction. Moreover, the side chain of R380 in Gαs protein from the formoterol–β2AR–Gs complex has a notable rotation away from TM3 relative to that in the BI167107–β2AR–Gs complex. The side chain rotation increases the distance between R380 in Gαs protein and T136 in β2AR, hence making it impossible to maintain the corresponding polar interaction found in the BI167107–β2AR–Gs complex. A new interface absent in the structure of the BI167107–β2AR–Gs complex was observed between the Gβ protein and ICL1 of β2AR, which is mediated by the charge interaction between residue R63 in β2AR and residue D312 in the Gβ protein (Fig. 1i). To be noted, a similar interface was observed in the interaction between Gβ and class F GPCRs or between Gβ and helix 8 of the class B GPCRs. Taken together, in comparison to the structure of BI167107-bound β2AR–Gs, the attenuated hydrophobic interaction between αN–β1 loop of Gαs and ICL2 of the receptor, combined with the disappeared polar interaction between T136 in TM3 and R380 in α5 helix, might decrease the coupling interaction between β2AR and the Gαs–Ras domain. This is consistent with the observed lower G-protein activation potency of formoterol versus BI167107 (Fig. 1a). Thus, structural comparison between the formoterol- and BI167107-bound β2AR–Gs complexes provides insights into conformational differences that are responsible for the distinct cAMP accumulation potency of different agonists.

Owing to the intrinsic flexibility, the density of the α-helical domain (αHD) could not be well-resolved, and the αHD was, therefore, excluded from the high-resolution map of the formoterol–β2AR–Gs complex. Superposition of the three Gαs-Ras domains from our cryo-EM structure of the formoterol–β2AR–Gs complex, a previously reported crystal structure of the BI167107–β2AR–Gs complex and the crystal structure of the Gαs-GTPyS complex (PDB:1AZT) revealed pronounced conformational differences for the α5 helix, which displaced toward the receptor in the two agonist-bound β2AR–Gs complexes versus that in the Gαs–GTPyS complex (Fig. 1j). In Gαs proteins, β6–α5 loop and β1–α1 loop (P loop) in the Gαs-Ras domain were reported to interact directly with the guanine ring and the diphosphate of nucleotide. As nucleotide exchange is the essential step in cAMP accumulation during the signal transduction of the activated GPCR, conformational changes of these loop regions will directly affect the potency of GPCR. As shown in Fig. 1j, both P loop and β6–α5 loop in formoterol–β2AR–Gs displaced outward from the nucleotide-binding site, when compared with those of BI167107–β2AR–Gs. We suggest that the displacement of the P loop and β6–α5 loop from the nucleotide-binding site may attenuate their interaction with the guanine ring and diphosphate in GTP, further decreasing the catalytic efficacy of Gαs-Ras toward GTP. This might in turn be responsible for the observed lower potency of β2AR binding to formoterol than that to BI167107 (Fig. 1a).

In summary, here we report the cryo-EM structure of β2AR–Gs complexes with the high-affinity full agonist formoterol. When compared with the BI167107-bound β2AR–Gs complex, structural differences were observed at the extracellular side of the receptors, which endow formoterol-bound β2AR with a slightly larger ligand-binding pocket. Besides, the side chains of S204 and N293 in formoterol-bound β2AR rotate away from the ligand-binding pocket, which reduces the interaction between formoterol and β2AR. We suggest that these structural differences might be responsible for different affinities and activation potency of agonists formoterol and BI167107, and thus residues involved in these structural differences might be potential targets for new agonist design and drug development. Moreover, the influence of attenuated interactions between the Gαs-Ras domain and β2AR will be transduced to the nucleotide-binding pocket, ultimately leading to a lower GTP-binding affinity and hydrolytic activity of Gαs. The decreased interactions between the Gαs-Ras domain and β2AR observed in our structure of the formoterol–β2AR–Gs complex might in turn be partially responsible for the lower affinity of β2AR for formoterol, when compared with that of BI167107–β2AR–Gs complex structure. These findings enrich our understanding of ligand-binding interactions and cAMP accumulation potency, enabling the exploration of new avenues for the development of innovative drugs targeting β2AR.

Density maps and structure coordinates have been deposited to the Electron Microscopy Database and the Protein Data Bank with accession numbers EMD-30249 and 7BZ2.

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Author contributions
S.L. and Y. Zhang developed sample preparation protocols. F.Y. collected cryo-EM data and solved the structure. Y. Zhou, P.S., P.L. and W.S. performed functional assays. W.F. and S.L. assisted with structural analysis. L.Z., P.S., S.L. and C.T. supervised the project and co-wrote the manuscript.

Conflict of interest
The authors declare that they have no conflict of interest.

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