Cryo-EM structure of an activated VIP1 receptor-G protein complex revealed by a NanoBiT tethering strategy

Jia Duan1,2,7, Dan-dan Shen3,4,7, X. Edward Zhou5,7, Peng Bi3,4,7, Qiu-feng Liu1, Yang-xia Tan1,2,6, You-wen Zhuang1,2, Hui-bing Zhang3,4, Pei-yu Xu1,2, Si-Jie Huang1,2,6, Shan-shan Ma1,2, Xin-heng He1,2, Karsten Melcher5, Yan Zhang3,4✉, H. Eric Xu1,2,6✉ & Yi Jiang1,2✉

Vasoactive intestinal polypeptide receptor (VIP1R) is a widely expressed class B G protein-coupled receptor and a drug target for the treatment of neuronal, metabolic, and inflammatory diseases. However, our understanding of its mechanism of action and the potential of drug discovery targeting this receptor is limited by the lack of structural information of VIP1R. Here we report a cryo-electron microscopy structure of human VIP1R bound to PACAP27 and Gs heterotrimer, whose complex assembly is stabilized by a NanoBiT tethering strategy. Comparison with other class B GPCR structures reveals that PACAP27 engages VIP1R with its N-terminus inserting into the ligand binding pocket at the transmembrane bundle of the receptor, which subsequently couples to the G protein in a receptor-specific manner. This structure has provided insights into the molecular basis of PACAP27 binding and VIP receptor activation. The methodology of the NanoBiT tethering may help to provide structural information of unstable complexes.
vasoactive intestinal polypeptide receptors, also known as VIP receptors, including VIP1R and VIP2R, belong to the class B1 of G protein-coupled receptors. Upon activating by vasoactive intestinal peptide (VIP), an endogenous, 28-amino acid neuropeptide, a VIP receptor couples to Gs heterotrimer, resulting in the stimulation of adenyl cyclase. In addition to VIP, VIP receptors also bind to other neuropeptides called pituitary adenylate cyclase-activating peptides (PACAPs) with similar affinity. Two forms of PACAP are known, the 27 amino acid long PACAP27 and the 38 amino acid long PACAP38, of which PACAP27 is a C terminally truncated variant of PACAP38, and shows particularly high homology (~68%) to VIP. The PACAP peptides have been in the spotlight of extensive basic and applied research, and have been linked to for over 40 different pathological conditions with clinical relevance.

VIP1R is widely distributed in the CNS, most abundantly in the cerebral cortex and hippocampus2,3, where it plays diverse and important roles with functions in the control of circadian rhythms, learning, memory, anxiety and responses to stress, and brain injury. VIP1R is also expressed in a number of peripheral tissues, including liver, lung, and intestine4–6, and in T lymphocytes7. The development of drugs acting on VIP receptors may lead to new treatments for sleep disorders, stroke, neurodegenerative disorders, and age-related memory impairment.

Extensive efforts have been made to discover the roles of the VIP1R system and to take advantage of VIP and PACAP analogs in therapeutic applications. Understanding the mechanism of peptide recognition and signal transduction by VIP1R has been aided by insights from several functional data from mutagenesis, photoaffinity labeling10, molecular modeling11,12, and limited structure information of VIP2R extracellular domain (ECD) (PDB code: 2X57) and VIP peptide13. Several of VIP and PACAP peptide analogs have been studied for their potential therapeutic applications1. A high-resolution structure of a full-length VIP receptor is needed for both mechanistic research as well as drug discovery targeting this GPCR system.

The resolution revolution of cryo-EM has made a significant impact on GPCR structural biology14. The atomic resolution or near-atomic resolution GPCR–G protein complex structures solved by cryo-EM have revealed structural details of ligand recognition and signal transduction by this superfamily of cell surface receptors. Various methods have been developed to improve the stability of GPCR-signal transducer complexes, such as the use of thermo-stabilizing mutations15, nanobodies, and antibody fragments16,17, to facilitate structural studies. However, poor sample stability remains the bottleneck in structural studies of GPCR complexes. In this work, we have developed a method to stabilize the interaction between VIP1R and the Gs heterotrimer by bringing the two proteins into close proximity through a NanoBiT tethering approach. This method greatly improved the stability and homogeneity of the PACAP27–VIP1R–Gs protein complex, allowing structural determination of human VIP1R in complex with PACAP27 and Gs heterotrimer. We also demonstrate that the NanoBiT tethering method can be applied to other GPCR–G protein complexes.

Results

The tethered NanoBiT stabilize GPCR–G protein complexes. NanoBiT system is one of the protein-fragment complementation methods based on split luciferase, which is originally developed to monitor protein-protein interactions18. When the NanoBiT is dissected between residues 156 and 157, it can be split into a large component containing 156 amino acid residues named large BiT (LgBiT), and a 13-amino acid peptide called small BiT (SmBiT, Supplementary Fig. 1a). By engineering the sequence of SmBiT, a series of peptides with various equilibrium dissociation constants were created, among which peptide 86 (HiBiT) (VSGWRLFKKIS) has the most potent binding affinity, with five orders of magnitude (~1 nM to ~200 μM) greater than that of the wild-type (WT) peptide 114 (VTGYRLFEEIL) (Supplementary Fig. 1b)18. The fragments of the LgBiT and SmBiT are genetically fused to a pair of interacting proteins. The interaction of fusion partners leads to structural complementation of LgBiT with SmBiT, generating a functional NanoBiT enzyme with a detectable luminescent signal.

Inspired by the complementation principle of NanoBiT, we fused the SmBiT peptide 86 at the C-terminus of the Gβ subunit to bind the LgBiT that was attached to the C-terminus of the truncated receptor (VIP1R 31–437), thus providing an additional linkage to stabilize the interface of helix 8 of VIP1R and the Gβ subunit of the G protein (Fig. 1a, Supplementary Fig. 2; see “Methods”). The flexible C-terminus of VIP1R serves as the natural linker to connect LgBiT. The WT VIP1R and different lengths of C-terminus truncated VIP1R at L437, G424, and K417 were screened for assembly of the complex. When the receptor was truncated to L437, the components can be assembled into the VIP1R(31–437)–VIP1R(31–437)–Gs complex with an equal proportion, suggesting a better assembly efficacy for the complex. Thus, unless otherwise specified, VIP1R refers to VIP1R(31–437), which is used in structure determination and functional analyses. Compared to WT VIP1R, the truncated receptor exhibited a comparable response to PACAP27–induced activation. The LgBiT fusion to the truncated VIP1R or cotransfection of the truncated receptor with Gβ-HiBiT does not affect PACAP27–induced VIP1R activation (Supplementary Fig. 1c). Combined with Nb35, which is used to stabilize the complex between Gαs and Gβ10, the NanoBiT tethering method can enhance the stability of the VIP1R–Gs complex and facilitate the structure study of this GPCR complex.

To investigate the effect of the NanoBiT tethering method on stabilization of the PACAP27–VIP1R–Gs complex, SDS-PAGE analysis, gel filtration chromatography, dynamic light scattering (DLS), and negative staining was performed. The SDS-PAGE analysis showed that all components of the VIP1R–Gs complex were present with the NanoBiT tethering (Supplementary Fig. 1d). Gel filtration chromatography also revealed that the complex with the NanoBiT tethering had a much more uniform distribution than the WT VIP1R–Gs complex, indicating that the NanoBiT tethering method contributed additional stability to the VIP1R–Gs complex (Fig. 1b).

We further used DLS to evaluate complex homogeneity and thermostability. A peak around a radius of ~10 nm corresponds to the monomeric complex of VIP1R–Gs complex, while the peak at ~100 nm represents protein aggregation. Our data show that the NanoBiT tethering improved the monodispersity of the VIP1R complex with a 3.3-fold increase of monomer/aggregation ratio (Fig. 1c). The relatively smaller radius size and narrower radius size distribution also suggest that the NanoBiT tethering complex was more compact and homogeneous than the WT complex, while the protein aggregation onset temperature (T onset), a marked temperature point indicating protein denaturation and aggregation, remained unchanged (Supplementary Fig. 1e). The negative staining images displayed that particle morphology and integrity of the NanoBiT-tethered complex have been improved relative to the less consistent particles of the WT complex, indicating improved homogeneity and integrity of the NanoBiT-tethered sample (Fig. 1d).

We further investigated whether NanoBiT tethering conferred a similar stabilization effect on other GPCR–G protein complexes. CCR7, a class A GPCR that couples to Gi protein, was chosen as a representative receptor. The NanoBiT tethering method significantly improved the homogeneity of the complex,
leading to high homogeneity and integrity of negatively stained complex particles, which is in agreement with its effect on the V1PR–Gs complex. The NanoBiT tethering also increased the thermostability of the CCR7-Gi complex, as evidenced by an increase of $T_{\text{onset}}$ by ~10 °C (Supplementary Fig. 3).

Taken together, we developed a strategy to stabilize the GPCR–G protein complex by direct linking of a GPCR with its G protein through NanoBiT protein-fragment complementation. Using this method, we were able to obtain a stable PACAP27–V1PR–Gs complex for cryo-EM studies.

**Structure determination of V1PR bound to PACAP27 and Gs.**

The structure of the PACAP27–V1PR–Gs complex was determined from 131,263 particles to a resolution of 3.2 Å (Supplementary Fig. 4 and Supplementary Table 1). The density is clear for the V1PR TM bundle, the bound peptide PACAP27, the heterotrimeric Gs, and Nb35. Like many other GPCR–G protein complexes, density is missing for the α-helical domain of the Gαs. In addition, the ECD of V1PR was not resolvable with this limited dataset, perhaps reflecting its highly dynamic and conformationally flexible property when bound to PACAP27. This is consistent with the highly dynamic nature of ECD in class B GPCRs when bound to activating ligands as the ECD structures were not well resolved in most of other active class B GPCR–G protein complexes20–22. The complex structure of PACAP27–V1PR–Gs was built with the recently published PTH1R–Gs complex structure (PDB: 6NBH)23 as an initial model. The final structure contains all residues of PACAP27 (residues 1–27), the GαsRas-like domain, Gβγ subunits, Nb35, and the V1PR residues from A1291.26b to Q4098.64b (class B GPCR numbering in superscript24) (Fig. 2). The majority of amino acid side chains were well resolved in the final model, which were refined against the EM density map (Supplementary Fig. 5). Thus, the complex structure can provide detailed information on the interface between Gαs and the receptor, as well as the binding interface between PACAP27 and helix bundle core of the receptor.

The TMD of the V1PR receptor is surrounded by an annular detergent micelle mimicking the natural phospholipid bilayer.
With the help of the receptor ligand in a specific pocket. The TMD peptide-binding pocket of VIP1R is similar to that of PAC1R with pocket volumes of 3261 and 3246 Å³, respectively. As these two receptors share peptidic ligand PACAP with a similar affinity, the TMD peptide-binding pocket of VIP1R and PAC1R are smaller than those of class B GPCRs solved to date (Supplementary Fig. 6c and Supplementary Table 3).

A comparison of the structures of PACAP27–VIP1R–Gs with the newly released PACAP38–PAC1R–Gs complex (PDB: 6P9Y) will help to clarify the recognition mechanism of VIP1R and PAC1R by PACAP peptide. For VIP1R, the first peptide residue H1 not only makes extensive hydrophobic contacts with several nonpolar residues from the ligand pocket (V2263.40b, F2303.44b, W2945.36b, I3015.43b, and the backbone of K2985.37b), but also forms a hydrogen bond with Q2233.37b (Fig. 3d, Supplementary Table 3). Alanine mutations in Q2233.37b and W2945.36b reduced PACAP27-mediated VIP1R activation, supporting the fact that H1 of the peptide is critical for peptide-induced receptor activation (Supplementary Table 4). For PAC1R, H1 interacts with highly conserved hydrophobic residues with VIP1R. However, the hydrogen bond interacts with residue at 3.37 (H2343.37b) is absent (Fig. 3d, Supplementary Table 3). Compared to H1, S2 faces a significant different residue environment in these two receptors. S2 additionally hydrogen-bonded with R1992.60b and Y2413.44b in PAC1R compared to VIP1R, making the PACAP38 inserted deeper into the TMD core of PAC1R (Fig. 3e, Supplementary Table 3). D3 forms hydrogen bond with R1882.60b, and also hydrophobic contacts with F2223.36 and L3747.43. These interactions are highly conserved between VIP1R and PAC1R (Fig. 3d, Supplementary Table 3). Mutations of the peptide residue D3 or the receptor residue R1882.60b and F2223.36 impaired ligand-induced receptor activation (Supplementary Table 4). G4, I5, and F6 from the peptide ligand are surrounded by hydrophobic pocket residues of VIP1R from TM1 (Y1391.36b, V1421.39b, Y1461.43b), TM2 (L1992.71b), TM5 (W2945.36b), and TM7 (M3707.39b and L3747.43b), as well as from ECL2 (I289ECL2) (Fig. 3f, Supplementary Table 3). Mutations of hydrophobic residues Y1461.43b, L1992.71b, and W2945.36b to alanines significantly decreased the PACAP27-induced VIP1R activation, indicating that G4, I5, and F6 may be involved in VIP1R activation (Supplementary Table 4). These interactions also highly conserved between PACAP38 and cognate residues in PAC1R (Supplementary Table 3).

Compared to the six N-terminal residues of PACAPs, peptide residues from T7 to R14 exhibit different binding modes to VIP1R and PAC1R. Besides identical hydrogen bonds between S11 in PACAPs and D ECL2 in two receptors, other polar interactions (D8 and I289ECL2, Y13 and D1321.29, as well as T1361.33 for VIP1R, and S9 and K3787.35, S11 and Y211ECL1, R12 and D301ECL2 for PAC1R) are unique (Fig. 3g, h, Supplementary Table 3). Mutations of I289ECL2 to Ala decreased PACAP27...
conserved (Fig. 3a), these two peptides may interact with VIP1R. The peptide sequences from PACAP27 and VIP are highly determining the affinity of VIP supported the fact that H1, D3, F6, R12, and R14, identical in a peptide-specific manner. The previous alanine scanning analysis of VIP supported the fact that H1, D3, F6, R12, and R14 are also supposed to be involved in PACAP27-mediated activation of VIP1R, R12 of PACAP27 seems not to form any substantial interaction with residues in VIP1R binding pocket, indicating a distinct VIP1R binding mode for these two peptides.

The structural studies on VIP1R binding pocket also provide a clue on the potential recognition mechanism of VIP1R by VIP, a peptidic ligand shares highly conserved sequences and bound VIP1R with similar affinities compared to PACAP27. Although the peptide sequences from PACAP27 and VIP are highly conserved (Fig. 3a), these two peptides may interact with VIP1R in a peptide-specific mode. The previous alanine scanning analysis of VIP supported the fact that H1, D3, F6, R12, and R14, identical amino acids at cognate positions of PACAP27, are important for determining the affinity of VIP to VIP1R. Although H1, D3, F6, R12, and R14 are also supposed to be involved in PACAP27-mediated activation of VIP1R, R12 of PACAP27 seems not to form any substantial interaction with residues in VIP1R binding pocket, indicating a distinct VIP1R binding mode for these two peptides.

The structural-based mutagenesis analysis also provides a potential explanation of VIP selectivity for VIP1R over PAC1R. Structurally, G4 in PACAP closely contacts W5.36b in VIP1R. When mutating G4 of PACAP to Ala, the cognate amino acid of VIP, a more significant steric constraint, is generated between the newly mutated A4 and W306 5.36b of PAC1R compared to W296 5.36b in VIP1R, which may restrict the binding of VIP to PAC1R and lead to a lower selectivity for VIP for PAC1R than VIP1R (Supplementary Fig. 7). This structure feature is coincident with the fact that when replacing A4-V5 dipeptide of PACAP38 with VIP, the cognate amino acid of PACAP 5.36b in VIP1R, this may restrict the binding of VIP to PAC1R and lead to a lower selectivity for VIP for PAC1R than VIP1R (Supplementary Table 4).

Fig. 3 Comparison of the binding mode of PACAPs to VIP1R and PAC1R. a Sequence alignment of the VIP1R peptide ligands VIP, PACAP27, and PACAP38. b The binding mode of PACAP27 to VIP1R, showing that PACAP27 adopts α-helical conformation and interacts with all TM helices of VIP1R except TM4. c The cross-section view of the PACAP27 binding pocket in the TM bundle of VIP1R. Structural comparisons of PACAP binding pockets in VIP1R and PAC1R. Residues interact with peptide amino acids H1 and D3 (d), S2 (e), G4, I5, and F6 (f), as well as amino acids from T7 to R14 (g, h) are shown as sticks. The hydrogen bonds between PACAP27 and residues of VIP1R are marked as black dotted lines, and the hydrogen bonds between PACAP38 and residues of VIP1R are shown as red dotted lines. PACAP27 is colored in orange, and VIP1R in green. PACAP38 is shown in cyan, and PAC1R (PDB code: 6P9Y) in light blue.
VIP by G4-15 in PACAP, the new VIP analog obtains the ability to bind and activate PAC1R. Similarly, PACAP27 abolished its propensity to bind PAC1R when its G4-15 sequence was substituted for A4-V5 in VIP31.

Together, these observations provide a rationale for understanding VIP1R recognition by PACAP27 and VIP1R-targeted ligand discovery.

**Activation of VIP1R by PACAP27.** The structural hallmark of class B GPCR activation is the much more pronounced outward shift of TM6 than that in class A GPCRs, which is accompanied by the formation of a sharp kink in the middle of the TM6 induced and stabilized by ligand binding. The N-terminal residues H1 and S2 from PACAP27 pack directly against the C-terminus of TM6, and disrupt the helical conformation of the conserved PxxG motif (P3486.47b−L3496.48b−F3506.49b−G3516.50b), and create a ~90° sharp kink at the middle of TM6 (Fig. 4a). The kink conformation of TM6 is stabilized by polar interactions between P3486.47b and F3506.49b with the side chains of Q3807.49b and N3085.50b, respectively, (Fig. 4b). It is notable that Q3807.49b also forms polar interaction with Y3546.53b, a residue at the C-terminal end of the TM6 kinked conformation (Fig. 4b, Supplementary Fig. 9). Previous experiments showed that point mutations of VIP1R residues R1882.60b, N2293.43b, and Q3807.49b severely affect the binding of VIP and VIP-mediated cAMP production32, in agreement with our structural data. Interestingly, in many published class B GPCR active structures, these polar network residues are not in close contact with peptide ligands, except for VIP1R, PAC1R, and PTH1R. We observed that residue R1882.60b of VIP1R forms a charge interaction with D3 of PACAP27. The corresponding residue in PAC1R, R1992.60b, forms direct polar interactions with N-terminal S2 and D3 of PACAP38. A similar interaction can also be observed between R2332.60b of PTH1R and E4 of LA-PTH (Supplementary Fig. 9). These polar interactions between the peptide and the receptor serve as the structural basis of ligand-induced receptor activation.

Our PACAP27-bound VIP1R–Gs complex structure also exhibits broken HETY and TM2−6−7−helix 8 polar networks, which are caused by the outward movement of the intracellular segment of TM6 that takes away TM6 residues T3436.42b and Y3546.53b, respectively, from these two networks (Fig. 4d, e). VIP1R contains the conserved HETY motif, which is known to mediate inter-helix interactions of TM2−6−7−helix 8 polar networks in GPCR, PTH1R, and CRFR1. Disruption of this inter-helix interactions has resulted in constitutively active class B GPCRs receptors33−35. We, therefore, speculate that TM2−3−6−7 polar networks may also be required for maintaining an inactive conformation, and the breakage of these polar networks may represent the active conformations of VIP1R. Indeed, mutations that disrupt this polar network in VIP1R have resulted in the constitutively active receptor35−37.

Taken together, despite the different sequence and physicochemical environment of VIP1R in ligand binding pocket, VIP1R shares a common activation mechanism with other class B GPCRs, which is characterized by a set of conserved residues involved in ligand-induced conformational changes in the receptor helix bundle as well as residues involved in G protein coupling. The polar networks in the helix bundle core, the central polar network, HETY, and TM2−6−7−helix 8 networks, required
in maintaining the inactive conformation of the receptor, undergo ligand-induced conformational changes that rearrange the network residues to facilitate the ligand binding and to stabilize the active conformation of the receptor.

Gs heterotrimer coupling by VIP1R. The overall assembly of the receptor with Gs is remarkably similar to many other class B GPCRs solved to date, with several unique features of receptor-specific interactions with the Gs heterotrimer.\(^\text{23,25-28}\) The outward moved cytoplasmic end of TM6 and concomitantly shifted TM5 form a cytoplasmic cavity together with TM2, 3, and 7 to accommodate the α5 helix of Gas. This interface serves as a crucial contact between the receptor and Gs heterotrimer. Additional contacts are observed between extended helix 8 of the receptor and the Gβ subunit of the Gs heterotrimer. ICL3, although invisible in our complex structure, also makes important contributions because residues I328-S331 in the central part of ICL3 are crucial for efficient binding of VIP1R to Gas.\(^\text{36,37}\)

Structural alignment of our PACAP27–VIP1R–Gs complex with other class B GPCR–Gs protein complex structures solved to date by superimposing their receptor TM domains reveals different orientations of the Gs heterotrimers with rigid body rotations around the axis of the Gβ subunit (Supplementary Fig. 10a). The structural similarities in the Gs heterotrimer may be influenced by the use of Nb35, which has been used in all the structures of Gs-buried surface area of VIP1R than those of other class B GPCR–Gs complexes (Supplementary Fig. 10b and Supplementary Table 2). This is consistent with the fact that the VIP1R–Gs complex is not sufficiently stable for cryo-EM studies without NanoBiT tethering.

Discussion

Here, we report a near-atomic resolution structure of PACAP27-bound VIP1R in complex with Gs, determined by cryo-EM. For successful structure determination, we stabilized the assembly between PACAP27-bound VIP1R and Gs heterotrimer using a developed NanoBiT tethering method. The structure has provided a rationale to understand how PACAP27 interacts with the transmembrane bundle of VIP1R and provides the basis of ligand binding specificity. Structural comparison with other class B GPCRs shed light on the basis of PACAP27 binding as well as a common mechanism of ligand-induced receptor activation and coupling to downstream Gs heterotrimer. As VIP receptors have been identified as potential therapeutic targets for metabolic, inflammatory, and neuronal diseases,\(^\text{36,37}\) this structure presents key information for the rational design of peptides or small molecule compounds to target VIP receptors. In addition, we expect that NanoBiT tethering method can be used to stabilize not only GPCR–G protein complexes but also other unstable macro-molecular complexes for structural determination.
Methods

Constructs. Human VIP1R (residues 31–437) was cloned into pFastBac with an N-terminal FLAG tag followed by a His6 tag, as well as LgBiT at the C-terminus using homologous recombination (CloneExpress One Step Cloning Kit, Vazyme). The primers used in this study are shown in Supplementary Table 5. The native signal peptide was replaced with the prolactin precursor sequence to increase the protein expression. A dominant-negative bovine Gαi (DNGαi) construct was generated by site-directed mutagenesis to incorporate mutations S54N, G226A, E268A, N271K, K274D, R280K, T284D, and I285T to decrease the affinity of nucleotide-binding and increase the stability of Gαi complex28. Rat Gβ1 was cloned with an N-terminal His6 tag and a C-terminal SmBiT connected with a 15 residues linker. All three G protein components together with bovine Gγ2 were cloned into a pFastBac vector, respectively.

Insect cell expression. VIP1R(31–437)–LgBiT fusion, DNGαi, Gβ1–SmBiT fusion, and Gγ2 were coexpressed in S9 insect cells (Invitrogen) using the Bac-to Bac baculovirus expression system (Thermo Fisher). Cell cultures were grown in ESF 921 serum-free medium (Expression Systems) to a density of 3 × 10^6 cells ml^-1 and then infected with baculovirus expressing VIP1R(31–437)–LgBiT fusion, DNGαi, Gβ1–SmBiT fusion, and Gγ2, respectively, at the ratio of 1:1:1:1. The cells were collected by centrifugation at 1000 × g (Thermo Fisher, H12000) for 20 min after infection for 48 h, and then kept frozen at −80 °C until use.

Expression and purification of Nb35. Nanobody-35 (Nb35) with a C-terminal His6 tag was expressed in the periplasm of E. coli strain BL21. Cultures of 1 L cells were grown to OD600 = 1.0 at 37 °C in TB media containing 0.1% glucose, 2 mM MgCl2, and 100 µg ml^-1 ampicillin. Then, 1 mM IPTG was added to the medium to induce protein expression for another 4.5 h at 37 °C. Cells were harvested by centrifugation and lysed in ice-cold buffer (50 mM Tris pH 8.0, 12.5 mM EDTA, and 0.125 M sucrose), then centrifuged to remove cell debris. Nb35 was purified from the affinity chromatography, followed by size-exclusion chromatography using a HiLoad 16/600 Superdex 75 column, and finally spin concentrated to −2.5 mg ml^-1.

PACAP27-VIP1R-Gs complex formation and purification. Cell pellets from 2 L culture were thawed and lysed in 20 mM HEPES, pH 7.4, 100 mM NaCl, 10% glycerol, 0.25 mM TCEP, 5 mM MgCl2, and 5 mM CaCl2, supplemented with EDTA-Free Protein Inhibitor Cocktail (Sigma). The VIP1R-Gs complex was formed in membranes by the addition of 10 µM PACAP27 (Supernpette), 10 µg ml^-1 Nb35, and 25 µM NaCl^-1 apyrase and incubation for 1.5 h at room temperature. Cell membranes were collected by ultracentrifugation at 64,000 × g for 35 min. The membranes were then resuspended and solubilized in buffer containing 20 mM HEPES, pH 7.4, 100 mM NaCl, 0.5% v/v Triton-X 100, 0.02% w/w CHS, 0.002% w/v LMNG, 0.001% w/v glycodeoxigenin (GDN, Anatarace), and 0.0002% w/v CHS, 5 µM PACAP27, and 25 µM Nb35 -1 apyrase for 3 h at 4 °C. The supernatant was collected by centrifugation at 80,000 × g for 40 min and then incubated with 3 mL pre-equilibrated Nickel-NTA resin for 2 h at 4 °C. After washing the resin loaded into a gravity flow stacking column and washed with ten column volumes of 20 mM HEPES, pH 7.4, 100 mM NaCl, 40 mM imidazole, 10% glycerol, 0.25 mM TCEP, 2 mM MgCl2, 2 mM CaCl2, 0.01% (w/v) LMNG, 0.01% glycodeoxigenin (GDN, Anatarace) and 0.002% (w/v) CHS, 5 µM PACAP27 and eluted with five column volumes of the same buffer plus 250 mM imidazole. The final yield of the purified complex was determined against the electron microscopy map. The model was docked into the electron microscopy density map using Chimera44, followed by iterative manual adjustment and rebuilding in COOT45. Real space refinement was performed using phenix.real_space_refine from Phenix program package46. The resulting model was validated using MolProbity47. Structural figures were prepared in Chimera (https://www.cgl.ucsf.edu/chimera/) and PyMOL (https://pymol.org/2/). The final refinement statistics are provided in Supplementary Table 8.

cAMP accumulation assay. The full-length VIP1R(31–437) and VIP1R mutants were cloned into pcDNA6.0 vector (Invitrogen) with a FLAG tag at its N-terminus (see Supplementary Table 5 for a list of primers used in this study). CHO-K1 cells (ATCC, #CCL-61) were cultured in Ham’s F-12 Nutrient Mix ( Gibco) supplemented with 10% (v/v) fetal bovine serum. Cells were maintained at 37 °C in a 5% CO2 incubator with 100,000 cells per well in a 12-well plate. Cells were grown overnight and then transfected with 1 µg VIP1R constructs by FuGENE® HD transfection reagent (DNA/FuGENE® HD ratio of 1:3) in each well. After 24 h, the transfected cells were seeded onto 384-well microtiter plates (3000 cells per well). cAMP accumulation was measured using the LANCE ™ cAMP kit (PerkinElmer) according to the manufacturer’s instructions with different concentrations of peptides. Fluorescence signals were then measured at 620 and 665 nm by an Envision multilabel plate reader (PerkinElmer). Data presented are means ± SEM of at least three independent experiments.

Detection of surface expression of VIP1R mutants. The VIP1R mutants were cloned into pcDNA6.0 vector (Invitrogen) with a FLAG tag at its N-terminus. The cell seeding and transfection followed the same method as cAMP accumulation assay. After 24 h of transfection, cells were washed once with PBS and digested with 0.2% (w/v) EDTA in PBS. Cells were blocked with PBS containing 5% (w/v) BSA for 15 min at room temperature and then incubated with primary anti-FLAG antibody (diluted with PBS containing 5% BSA at a ratio of 1:300, Sigma) for 1 h at room temperature. Thereafter, cells were washed three times with PBS containing 1% (w/v) BSA before incubating with anti-mouse Alexa-488-conjugated secondary antibody (diluted with PBS containing 5% BSA at a ratio of 1:1000, Invitrogen) at 4 °C in the dark for 1 h. After another three times wash, cells were resuspended, and fluorescence intensity was quantified in a BD Accuri C6 flow cytometer system (BD Biosciences) at excitation 488 nm and emission 519 nm. Approximately 10,000 cellular events per sample were collected and data were normalized to WT.
Dynamic light scattering. DSL sample was prepared at about 0.2–1.0 mg mL\(^{-1}\) and equilibrated for 5 min before loading 10 µL onto the DynaPro NanoStar (Wyatt Technology). For thermostability assay, the intensity was read with a thermal ramp from 25 to 75 °C with a ramp rate of 2 °C min\(^{-1}\). All data acquisition and analysis were performed by the Dynamics software.

Reporting summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability

Data supporting the findings of this manuscript are available from the corresponding authors upon reasonable request. A reporting summary for this article is available as a Supplementary Information file. Source data are provided with this paper. Density map and structure coordinate have been deposited to the Electron Microscopy Database and the Protein Data Bank with the accession number of EMD-21249, PDB6VN7 for the PACAP27–VIP1R–Gs complex.

Received: 30 January 2020; Accepted: 17 July 2020; Published online: 17 August 2020

References

1. Denes, V., Geck, P., Mester, A. & Gabriel, R. PITuitary adenylate cyclase-activating polypeptide: 30 years in research spotlight and 600 million years in service. J. Clin. Med. 8, 1488 (2019).
2. Ishihara, T., Shigemoto, R., Mori, K., Takahashi, K. & Nagata, S. Functional expression and tissue distribution of a novel receptor for vasoactive intestinal polypeptide. Neuron 8, 811–819 (1992).
3. Usdin, T. B., Bonner, T. I. & Mezei, E. Two receptors for vasoactive intestinal polypeptide with similar specificity and complementary distributions. Endocrinology 135, 2662–2670 (1994).
4. Ichikawa, S., Sreedharan, S. P., Owen, R. L. & Goetzl, E. J. Immunochemoical localization of type I VIP receptor and NK-1-type substance P receptor in rat lung. Am. J. Physiol. 268, L584–L588 (1995).
5. Sreedharan, S. P., Huang, J. X., Cheung, M. C. & Goetzl, E. J. Structure, expression, and chromosomal localization of the type I human vasoactive intestinal peptide receptor gene. Proc. Natl Acad. Sci. USA 92, 2939–2943 (1995).
6. Kaltreider, H. B. et al. Upregulation of neuropeptides and neuropeptide Pevepeptide PACAP27 the Protein Data Bank with the accession number of EMD-21249, PDB6VN7 for the PACAP27–VIP1R–Gs complex.
7. Dal Maso, E. et al. The molecular control of calcitonin receptor signaling. ACS Pharmac. Trans. Sci. 2, 31–51 (2019).
8. Rasmussen, S. G. et al. Development of an antibody fragment that stabilizes GPCR/G-protein receptor mutants. J. Biol. Chem. 283, 213–221 (2008).

Acknowledgements

The cryo-EM data were collected at the Center of Cryo-Electron Microscopy, Zhejiang University. This work was partially supported by the National Natural Science Foundation of China (31700796 to Y.J. and 81922071 to Y.Z.); the National Science & Technology Major Project “New Key Drug Creation and Manufacturing Program” (2018ZX0911002 to Y.J.); Shanghai Municipal Science and Technology Major Project
Author contributions
J.D. designed the expression constructs, purified the PACAP27–VIP1R–Gs complex, prepared the final samples for negative stain and data collection toward the structures, and participated in figure and manuscript preparation; D.-d.S. and P.B. performed specimen screening by negative-stain EM, cryo-EM grid preparation, cryo-EM data collection, and map calculations; X.E.Z. built and refined the structure models, analyzed the structures, and wrote the manuscript; Q.f.-L., Y.-x.T., H.-b.Z., P.-y.X., S.-j.H., S.-s.M., Y.-w.Z., and X.-h.H. performed the experiments; K.M. analyzed the structures, and wrote the manuscript; Y.J. prepared the bulk of figures, performed the structural analysis, and wrote the manuscript; Y.J. and H.E.X. conceived the project, initiated collaborations with Y.Z., and supervised J.D.; and Y.Z. supervised the EM studies.

Competing interests
The authors declare no competing interests.

Additional information
Supplementary information is available for this paper at https://doi.org/10.1038/s41467-020-17933-8.