GSK1702934A and M085 directly activate TRPC6 via a mechanism of stimulating the extracellular cavity formed by the pore helix and transmembrane helix S6

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Transient receptor potential canonical (TRPC) channels, as important membrane proteins regulating intracellular calcium (Ca\(^{2+}\)) signaling, are involved in a variety of physiological and pathological processes. Activation and regulation of TRPC are more dependent on membrane or intracellular signals. However, how extracellular signals regulate TRPC6 function remains to be further investigated. Here, we suggest that two distinct small molecules, M085 and GSK1702934A, directly activate TRPC6, both through a mechanism of stimulation of extracellular sites formed by the pore helix (PH) and transmembrane (TM) helix S6. In silico docking scanning of TRPC6 identified three extracellular sites that can bind small molecules, of which only mutations on residues of PH and S6 helix significantly reduced the apparent affinity of M085 and GSK1702934A and attenuated the maximal response of TRPC6 to these two chemicals by altering channel gating of TRPC6. Combing metadynamics, molecular dynamics simulations, and mutagenesis, we revealed that W679, E671, E672, and K675 in the PH and N701 and Y704 in the S6 helix constitute an orthosteric site for the recognition of these two agonists. The importance of this site was further confirmed by covalent modification of amino acid residing at the interface of the PH and S6 helix. Given that three structurally distinct agonists M085, GSK1702934A, and AM-0883, act at this site, as well as the occupancy of lipid molecules at this position found in other TRP subfamilies, it is suggested that the cavity formed by the PH and S6 has an important role in the regulation of TRP channel function by extracellular signals.

TRPC (transient receptor potential canonical) channels are known as receptor-operated, nonselective cation channels (1–3). TRPCs have been considered as potential therapeutic targets in various pathological conditions, such as cardiovascular dysfunction (4), endocrine (5), neuronal (6) and chronic kidney diseases (7), pain (8), and cancer (9). M085, also known as 4o, is a potent and selective TRPC3/6/7 activator with an EC\(_{50}\) value (the concentration yielding half maximum response) of 4.66 ± 0.03 μM, measured by patch-clamp rather than calcium imaging method (10). Other TRPC3/6/7 activators include GSK1702934A (11), which has been used to study cardiac contractility and arrhythmogenesis in the heart (12). SKF-96365, as a general TRPC antagonist, was used in the study of cardiovascular disease (13, 14). BI 749327, a selective inhibitor of TRPC6, reduces the ventricular dilation and fibrosis caused by hemodynamic stress abnormalities (15). The pyrazole compound Pyr3 selectively inhibits TRPC3 channels and prevents stent-induced arterial remodeling (16, 17). Piperazine-derived compounds PPZ1 and PPZ2 activate TRPC3/6/7 and trigger Ca\(^{2+}\) signaling to promote neurite outgrowth and neuronal survival in the absence of serum (18). The study of subtype-specific agonists and antagonists of TRPC could facilitate the development of new drug therapies, such as the treatment of glomerulonephritis (19), renal fibrosis (20), pulmonary arterial hypertension (21), and certain types of tumors (22, 23).

Activation of the TRPC channel occurs mainly downstream of GPCRs–G\(_{q/11}\)–PLC\(_{\beta}\) and the receptor tyrosine kinase coupled to PLC\(_{\gamma}\). PLC stimulates the hydrolysis of PIP\(_2\), leading to the production of IP\(_3\) and DAG, and IP\(_3\)-dependent Ca\(^{2+}\) release from the endoplasmic reticulum, which causes the intracellular Ca\(^{2+}\) store depletion and eventual activation of the channel (24). TRPC6 channels, on the other hand, can be directly activated by DAG, the hydrolysis product of phospholipase PLC (25). Although an increasing number of intracellular signals have been found to activate TRPC6 in recent years (25–27), the very recent cryo-electron microscopy (cryo-EM) structure of TRPC6 complexed with AM-0883, a
Activation of TRPC6 by GSK1702934A and M085

A novel agonist disclosed for the first time in this study (28), showed that stimulation of extracellular sites is also capable of directly activating TRPC6, which provides direct evidence for the existence of an extracellular orthosteric site.

Indeed, intracellular signaling regulatory molecules as new drug candidates or research tools must first pass through the barrier of the cell membrane. Therefore, activation or modulation of TRPC6 from outside the cell can offer great advantage for drug development of this important subtype of TRP superfamily. Although the open structure of TRPC6 has revealed one of the extracellular orthosteric sites (28), it remains unclear whether there are other extracellular sites that are essential for the activation/regulation of TRPC6. Are the known agonists dependent on stimulation of the DAG binding site upon entry into the cell (28, 29), or do they activate TRPC6 directly extracellularly? In addition, it is unclear whether GSK1702934A and M085 activate TRPC6 independently of the pocket formed between the pore helix (PH) and transmembrane (TM) helix S6 (AM-0883’s site) and whether there are novel modes of extracellular regulation of TRPC6.

To explore these questions, here, via a combination of mutagenesis, covalent modification on TRPC mutant, whole-cell and single channel recordings, surface expression analysis, conventional molecular dynamics (CMD) simulations, metadynamics (MetaD), and free energy reconstruction, we found that although chemical structures of GSK1702934A and M085 are very different, they both activate TRPC6 via a mechanism of stimulating the extracellular site formed by the PH and TM helix S6, similar to the mechanism of AM-0883 revealed by the open structure of TRPC6 (28). Our findings also imply that among several small molecule binding pockets in the extracellular domain (ECD), the pocket formed by PH and S6 may be the only extracellular orthosteric site that can be activated by small-molecule agonist rather than large biomolecules (e.g., toxins).

Results

M085- and GSK1702934A-mediated activations of TRPC6 are dependent on extracellular orthosteric sites

To investigate the activation mechanism of M085 and GSK1702934A, we synthesized both compounds (see Experimental procedures), and the synthesized M085 and GSK1702934A were able to activate mouse TRPC6 (mTRPC6) at EC₅₀ = 3.80 ± 0.67, 0.78 ± 0.16 μM, respectively (Fig. 1, B and D). Meanwhile, to more accurately predict small molecule–protein interactions, subsequent molecular simulations were based on the cryo-EM structure of human TRPC6 (hTRPC6) rather than the homology model of mTRPC6. hTRPC6-EGFP plasmid suffers from relatively low expressing efficiency in HEK293 cells, low mutagenesis success rate, and it

Figure 1. M085 and GSK1702934A-mediated direct activations of TRPC6 are dependent on extracellular orthosteric sites. A, chemical structures of M085 and GSK1702934A. B, representative current traces of mTRPC6 channel induced by different concentrations of M085 and GSK1702934A, respectively. C, pore helix and S6 sequence alignments of mTRPC6 and hTRPC6. D, concentration–activation curves of mTRPC6-WT and hTRPC6-WT. Each solid line was a fit of the Hill equation to the M085-and GSK1702934A-dependent activation (mean ± S.D., n = 3–4). E and F, typical current traces (E) and pool data (F) of M085 and GSK1702934A recorded in inside-out or outside-out macro-patch configurations. Data are presented as mean ± S.D. (n = 3). **p < 0.01 compared with control group, Student’s t test.
is not easy to perform electrophysiological recordings. Therefore, we adopted the following strategy: molecular simulations were based on the cryo-EM structure of hTRPC6, while mutagenesis, electrophysiological recording as well as membrane expression of mutants were validated on mTRPC6. Two points support the rationality of this strategy: 1. The sequence alignment of mTRPC6 and hTRPC6 indicates that they are highly homologous and that the amino acid sequences of the putative binding sites (see below) for these two structurally distinct small molecules are identical (Fig. 1, A and C); 2. The EC\textsubscript{50} values of hTRPC6 activated by M085 and GSK1702934A are 2.94 ± 0.70 and 0.45 ± 0.03 μM, respectively, which turn out to be consistent with the corresponding EC\textsubscript{50} on mTRPC6 (Fig. 1D).

We then examined whether the binding sites of M085 and GSK1702934A were present extracellularly or intracellularly by using the outside-out and inside-out configurations. Currents for M085 (100 μM) and GSK1702934A (100 μM) could be recorded with the outside-out configuration, while in the inside-out configuration no currents were recorded (Fig. 1, E and F), indicating that M085 and GSK1702934A-mediated activation of TRPC6 through their orthosteric sites are likely to be in the ECD of TRPC6. This result also implies that the cell membrane permeability of these two chemicals is also relatively low.

**Several possibilities for the interaction of M085 and GSK1702934A with the ECD of TRPC6**

We performed a scanning of small molecule binding pockets based on the cryo-EM structure of hTRPC6 and identified nine pockets including S1 to S9, of which S1, S2, and S3 are extracellular sites (Fig. 2, A and B). We focused on interactions of S1 to S3 with M085 and GSK1702934A (Fig. 2C), where the binding pocket S1 consists of amino acids hR464, hT488, hF491, and hY658 (the superscript \textsuperscript{h} indicates hTRPC6 numbering); S2 is composed of hE672, hF675, and hW680 on the pore helix (PH), and hN702, hY705, hY706, and hV710 on the S6 helix; S3 consists of mL684, hS686, hY688, and hK689. In silico docking revealed that M085 and GSK1702934A have good recognition by S1 to S3, but M085 and GSK1702934A acting on S3 are more likely to exhibit an inhibitory effect because the bound small molecule could occupy the ion permeation pathway. S1 and S2 are more likely to be binding sites for agonists.

To confirm the possibility of S1 to S3, we mutated the corresponding sites in mTRPC6. Because M085 and GSK1702934A activate the mTRPC6 channel with the EC\textsubscript{50} of approximately 5 μM and 1 μM and saturation concentrations of approximately 10 μM and 3 μM, respectively, we used the ratio of M085 activation current (I\textsubscript{10} μM/\textsubscript{I5} μM) and the ratio of GSK1702934A activation current (I\textsubscript{1} μM/\textsubscript{I5} μM) to carry out a preliminary simplified screening of the effect of different mutants in these potential binding sites on the apparent affinity of M085 and GSK1702934A. The result showed that compared with the wild-type (WT) mTRPC6 (M085-I\textsubscript{10} μM/\textsubscript{I5} μM = 1.65 ± 0.21; GSK1702934A-I\textsubscript{1} μM/\textsubscript{I5} μM = 1.87 ± 0.22), the mutants \textsuperscript{m}R463K (the superscript \textsuperscript{m} indicates mTRPC6 numbering), \textsuperscript{m}T487L, \textsuperscript{m}F490W, and \textsuperscript{m}Y657W in the S1 pocket (M085-I\textsubscript{10} μM/\textsubscript{I5} μM = 1.54 ± 0.17, 1.26 ± 0.22, 1.91 ± 0.12, and 1.68 ± 0.24 for \textsuperscript{m}R463K, \textsuperscript{m}T487L, \textsuperscript{m}F490W and \textsuperscript{m}Y657W, respectively; GSK1702934A-I\textsubscript{1} μM/\textsubscript{I5} μM = 1.65 ± 0.01, 1.00 ± 0.08, 1.94 ± 0.12 and 1.97 ± 0.12 for \textsuperscript{m}R463K, \textsuperscript{m}T487L, \textsuperscript{m}F490W, and \textsuperscript{m}Y657W, respectively), and mutants \textsuperscript{m}V687L, \textsuperscript{m}K688R, \textsuperscript{m}L684V and \textsuperscript{m}N686T in the S3 pocket (M085-I\textsubscript{10} μM/\textsubscript{I5} μM = 1.13 ± 0.26 and 1.80 ± 0.22 for \textsuperscript{m}V687L and \textsuperscript{m}K688R respectively; GSK1702934A-I\textsubscript{1} μM/\textsubscript{I5} μM = 0.54 ± 0.07 and 1.39 ± 0.31 for \textsuperscript{m}V687L and \textsuperscript{m}K688R respectively) did not significantly alter the current ratio of M085 and GSK1702934A (Fig. 2, D and E), suggesting that these two structurally distinct small molecules may have the same mechanism of action. Combined with the observations of inside-out- and outside-out- macro-patch and site-directed mutagenesis, S2 is likely to be the key site for the activation of M085 and GSK1702934A in mTRPC6.

**Dynamic interactions of M085 and GSK1702934A with the site formed by the pore helix and TM helix S6 of TRPC6**

Before further delving into the mechanism of M085/GSK1702934A interaction with TRPC6, both unbiased (conventional molecular dynamics, CMD) and biased (molecular-dynamics, MetaD) conformation sampling approaches were used to evaluate the interaction of S2 with these two compounds from a dynamic perspective, which can effectively improve the accuracy of mode derived from in silico docking.

MetaD, a method based on free-energy reconstruction and acceleration of rare events (30), has been used to study chemical reaction processes, protein–small molecule interactions (31), and optimal pathway for conformational changes in macromolecules (32–34), with the help of which the stretching of agonists (M085 and GSK1702934A) from the putative binding site and all binding modes between agonists and hTRPC6 can be evaluated extensively. During this process, the relative binding free energy for each binding mode could be measured simultaneously. MetaD simulations revealed the best binding modes for the interaction of M085 and GSK1702934A with hTRPC6 (Fig. 3, A and B). The optimal pose with low-binding free-energy exhibits almost the same interaction pattern of hTRPC6 and GSK1702934A as shown in the cryo-EM structure of hTRPC6 in complex with AM-0883: GSK1702934A is tightly locked by hydrogen bonding (H-bond) to the side chains of hW680 and hN702 (corresponding to \textsuperscript{m}W679 and \textsuperscript{m}N701 of mTRPC6, respectively) and cation–π interactions with \textsuperscript{h}K689 and \textsuperscript{h}E679 (identical to \textsuperscript{m}K697 and \textsuperscript{m}E679, respectively) and hTRPC6 as well as mimicking the H-bond with hY658 (corresponding to \textsuperscript{m}Y657) in mTRPC6. The optimal pose of GSK1702934A is stabilized by extensive hydrogen bonds and multiple H-bonds with hTRPC6. The optimal pose of M085 is not very different from that of GSK1702934A.
Almost the same interaction of M085 with site S2 was revealed by MetaD, where M085 is tightly locked by H-bond to the side chains of hW680 and hN702, and cation-π with hF675 (Fig. 3B).

We also further evaluated the rationality of above modes using CMD simulations. Protein–ligand interactions were monitored throughout the MD simulations and these interactions can be classified into four types: H-bonds,
hydrophobic, ionic, and water bridges (Fig. 4, A and B). A timeline representation of the interaction and contact of hTRPC6 with M085 and GSK1702934A was also summarized (Fig. 4, C and D). hTRPC6 interacting with GSK1702934A was very stable in CMD simulations, during which hF675, hF679, hW680, hN702, and hY705 (corresponding to mF674, mF678, mW679, mN701, and mY704 in mTRPC6, respectively) interacted with GSK1702934A via hydrophobic, H-bond, cation-π, or water-bridge contacts (Fig. 4, B and D). hE672, hF675, hW680, hE701, hN702, and hY705 (corresponding to mE671, mF674, mW679, mE700, mN701, and mY704 of mTRPC6) were also able to find similar interactions with M085 (Fig. 4, A and C). The ligand torsion diagram (Fig. 4, E and F) summarized the conformational evolution of each rotatable bond in the ligand throughout the simulated trajectory, where the two-dimensional schematic of the ligand was shown with color-coded rotatable bonds. The dial diagram and the bar diagram of the same color were shown for each twist of the rotatable bond. The dial (or radial) diagram depicted the conformation of the twist throughout the simulation. The start of the simulation was at the center of the radial plot, and the evolution of time was plotted radially outward. This analysis showed that agonist binding to TRPC6 was effective in limiting the conformational fluctuations of these two small molecules (except for the methoxy group of M085), especially for GSK1702934A, which may account for the better affinity of GSK1702934A compared with M085 (Fig. 4, E and F).

**Mutations of residues in the orthosteric site significantly reduce the apparent affinity of M085/GSK1702934A**

Compared with M085 activation of WT TRPC6 channels (EC\textsubscript{50} = 3.80 ± 0.67 μM), mE671C, mE672D, mK675R, mN701Q, mY704W, mK697R, and mV709A (EC\textsubscript{50} = 23.32 ± 6.86, 25.06 ± 5.64, 6.90 ± 2.36, 55.71 ± 19.15, 26.67 ± 4.04, 14.12 ± 1.95 and 6.95 ± 2.13 μM for mE671C, mE672D, mK675R, mN701Q, mY704W, mK697R, and mV709A corresponding to hE672C, hE673D, hK676R, hN702Q, hY705W, hK698R, and hV710A of hTRPC6, respectively) significantly reduced the affinity of M085 and shifted the EC\textsubscript{50} curve to the right. Similarly, compared with GSK1702934A activation of WT TRPC6 (EC\textsubscript{50} = 0.78 ± 0.16 μM), mE671C, mE672D, mK675R, mN701Q,
MY704W, mK697R, and mV709A (EC50 = 6.63 ± 0.40, 1.80 ± 0.33, 4.76 ± 0.41, 5.37 ± 0.30, 2.93 ± 0.74, 1.19 ± 0.14, and 2.61 ± 0.26 μM, respectively) also remarkably reduced the GSK1702934A apparent affinity and right shifted the concentration–response curve. Meanwhile, we found that mutant mF640W (corresponding to hF641W) had the effect of enhancing the apparent affinity of M085 (EC50 = 0.84 ± 0.27 μM) and GSK1702934A (EC50 = 0.27 ± 0.05 μM) and shifted the EC50 curve to the left (p < 0.01, n = 3–4) (Fig. 5B). Thus, the subunit interface between the PH and S6 might be the orthosteric site responsible for both M085 and GSK1702934A-mediated channel activations.

Additionally, these important residues in hTRPC6 (M085-EC50 = 50.16 ± 6.65, 22.13 ± 5.46, 44.00 ± 5.75, 54.50 ± 6.53, and 35.30 ± 6.15 μM for hE672C, hE673D, hK676R, hN702Q, and hY705W, respectively; GSK1702934A-EC50 = 4.47 ± 0.37, 1.80 ± 0.33, 4.76 ± 0.41, 5.37 ± 0.30, 2.93 ± 0.74, 1.19 ± 0.14, and 2.61 ± 0.26 μM, respectively) also remarkably reduced the GSK1702934A apparent affinity and right shifted the concentration–response curve.
2.04 ± 0.60, 4.08 ± 1.04, 3.07 ± 0.81, and 3.92 ± 0.37 μM for hE672C, hE673D, hK676R, hN702Q, and hY705W, respectively) were also found to be significantly reduced in the apparent affinity of M085/GSK1702934A compared with WT hTRPC6 channels (EC50 = 2.94 ± 0.70 and 0.45 ± 0.03 μM for M085/GSK1702934A in hTRPC6, respectively; Fig. 5, C and D), which is consistent with mTRPC6. Therefore, it should be reasonable that all mutagenesis, electrophysiological recordings, and protein surface expression analysis were performed on mTRPC6, while in silico docking, MetaD and CMD simulations were carried out in the cryo-EM structures of hTRPC6.

Figure 5. Single amino acid substitutions at the orthosteric site formed by the pore helix and S6 significantly reduce the apparent affinity of M085/GSK1702934A. A and B, concentration–activation curves of mTRPC6-WT and its mutants induced by M085 (A) and GSK1702934A (B). C and D, concentration–activation curves of hTRPC6-WT and its mutants induced by M085 (C) and GSK1702934A (D). Data points were presented as mean ± S.D. (n = 3–4) and fitted with Hill equation.

Mutations of residues in the orthosteric site do not alter the surface expression of mTRPC6 but significantly reduce the maximal agonistic effect and alter the channel gating of M085 and GSK1702934A

Compared with WT mTRPC6 currents induced by saturated M085 (I max = 75.11 ± 14.20 pA/pF, 30 μM M085) and GSK1702934A (I max = 95.64 ± 14.62 pA/pF, 10 μM GSK1702934A), mE671C, mE672D, mK675R, mN701Q, mY704W, and mK697R significantly decreased maximal currents of these two agonists (M085-I max = 24.09 ± 4.53, 10.28 ± 1.19, 8.26 ± 1.12, and 25.58 ± 1.86 pA/pF from mE671C, mE672D, mK675R, mN701Q, mY704W, and mK697R, respectively, 100 μM M085; GSK1702934A-I max = 27.38 ± 4.69, 11.72 ± 1.02, 1.43 ± 0.20, 0.86 ± 0.59, 6.88 ± 2.31, and 8.53 ± 2.36 pA/pF from mE671C, mE672D, mK675R, mN701Q, mY704W, and mK697R respectively, 100 μM GSK1702934A; Fig. 6, A and B), indicating that amino acids at the interface of the PH and S6 helix are important for the function of mTRPC6, and any change in their charge or size of these residues may lead to abnormalities in the function of mTRPC6. In addition, small changes in side chain size of two residues, mF678W and mW679F, had caused complete loss of function in mTRPC6 (Fig. 6 A). To further illustrate this point, we also added alanine-substitution mutants to residues located in PH and S6 that interact strongly with GSK1702934A or M085 (Fig. 4, A–D), i.e., mF674A, mW679A, mE700A, mN701A, and mY704A (corresponding to hF675A, hW680A, hE701A, hN702A, and hY705A) (Figs. 3 and 4, A–D), all of which resulted in the disappearance of GSK1702934A/M085 current (Fig. 6 A).
Activation of TRPC6 by GSK1702934A and M085

To examine whether the reduction in the maximum current of agonist response of key residues might be due to altered protein surface expression, the surface biotinylation and Western blotting were used to determine the surface and total protein levels. The protein surface expression levels of mE671C, mE672D, mK675R, mF678W, mW679F, mN701Q, mY704W, mK697R, mF674A, mW679A, mE700A, mN701A, and mY704A were comparable to that of WT TRPC6 (Fig. 6, C–F), suggesting that these substitutions reduced the agonistic potency of M085 and GSK1702934A in TRPC6 perhaps through altered channel gating rather than reduced receptor expression. In particular, the side chain of mW679 formed H-bond contacts with M085 and GSK1702934A, which is essential for their bindings (Fig. 3), but we could not assess EC50 changes because mutations at this site resulted in a loss of agonistic effect. Normal surface expression of mW679F and mW679A (Fig. 6, C–F) demonstrated that the loss of function of this mutant (Fig. 6, A and B) is caused more by altering conformation required for the agonist recognition and/or channel gating of TRPC6. Similar results could also be observed in mF674A, mE700A, mN701A, and mY704A (Fig. 6, A, B, E and F). These results suggest that the key amino acids predicted by in silico docking, MetaD and MD simulations are important recognition residues for M085 and GSK1702934A.

Unitary currents of M085 and GSK1702934A were recorded in an outside-out configuration in HEK293 cells expressing TRPC6. At -60 mV clamp voltage, both M085 (100 μM) and GSK1702934A (1 μM) induced full channel opening with a

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**Figure 6.** Single residue replacements at the orthosteric site do not alter the membrane expression of mTRPC6, but significantly reduce the maximum agonistic effect of M085 and GSK1702934A. A and B, representative current traces (A) and pooled data (B) of TRPC6-WT and its mutants induced by saturated M085 and GSK1702934A. All data are expressed as mean ± S.D. Each mutant was repeated at least three times for independent experiments. *p < 0.01 versus WT, one-way ANOVA with Bonferroni post hoc test (F(11, 31) = 87.89, p < 0.0001) and (F(11, 31) = 49.88, p < 0.0001) for M085 and GSK1702934A, respectively. C and D, representative Western blotting (C) and mean values (D) of the membrane and total expressions of mTRPC6-WT and its mutants. At least four experiments were performed for WT and each mutant. *p < 0.05, **p < 0.01 versus WT, one-way ANOVA with Bonferroni post hoc test (F(7, 31) = 1.490, p = 0.2075) and (F(7, 31) = 0.9172, p = 0.2690) for total and surface expressions, respectively. E and F, representative Western blots (E) and means (F) of membrane and total expression of mTRPC6-WT and mutants in which alanine substitutions on key residues produced strong contacts with M085 and GSK1702934A during MD simulations. At least four experiments were performed for WT and each mutant. *p < 0.05, **p < 0.01 versus WT, one-way ANOVA with Bonferroni post hoc test (F(6, 33) = 2.122, p = 0.0771) and (F(6, 33) = 0.9614) for total and surface expressions, respectively.
Occupancy of the orthosteric site via covalent modification on mE671C abolishes M085/GSK1702934A-mediated TRPC6 activation

To further explore the critical role of the putative subunit interface between the S6 and PH in agonist recognition, we replaced mE671 with cysteine with the aim of impairing M085/GSK1702934A binding by covalently occupying this orthosteric site. 5,5′-dithiobis (2-nitrobenzoic acid) (DTNB) is used here to study the effect of covalent occupancy of the binding site, whose thiobenzene (2-nitrobenzoic acid) (TNB) moiety could be covalently attached to free cysteine via a disulfide bond (36, 37) (Fig. 8A). For WT TRPC6 channels, DTNB had no effect on GSK1702934A- and M085-mediated activations of mTRPC6 (Fig. 8C), which is consistent with the lack of distribution of free cysteine residues in the extracellular region of the natural receptor.

As mentioned above (Fig. 5, A and B), the mE671C mutant reduced the affinity of M085/GSK1702934A and shifted the concentration–response curve to the right (Fig. 8B), indicating its important role in the orthosteric site. Interestingly, M085- or GSK1702934A-mediated activation in mTRPC6_E671C was completely inhibited after DTNB (2 mM)-treatment, and application of dithiothreitol (DTT, 10 mM) to remove the TNB moiety from the receptor partially “recovered” the current of M085/GSK1702934A (Fig. 8, C and D), suggesting that the specific covalent modification on mE671C may interrupt the recognition of the ligand. Thus, the putative subunit interface between S6 and PH is involved in the recognition of M085 and GSK1702934A.

Discussion

In this study, we demonstrate that two structurally distinct TRPC6 agonists, M085 and GSK1702934A, both act at an extracellular site formed by PH and S6, which might be the only orthosteric site that can accept extracellular small-molecule agonists during TRPC6 sequence evolution. We used both outside- and inside-out electrophysiological recording patterns to demonstrate that the binding site for M085 and GSK1702934A exists extracellularly rather than intracellularly (Fig. 1, E and F). When M085 (or GSK1702934A) was in silico docked to hTRPC6, three potential extracellular binding pockets (S1, S2, and S3) were identified, and mutation screening revealed that two of them (S1 and S3) were not the binding pockets of M085 and GSK1702934A, while S2 was most likely the binding pocket of GSK1702934A and M085 (Fig. 2, D and E). We further evaluated the rationality and stability of the interaction of TRPC6 with these two compounds in the S2 pocket by combining CMD and MetaD simulations (Figs. 3 and 4). Meanwhile, mutations of amino acids in the S2 pocket could significantly reduce the apparent affinity of M085/GSK1702934A (Fig. 5).

The reduced agonistic effect was not caused by a reduction in the membrane expression of TRPC6 (Fig. 6, C–F). In addition, mutations of residues in the orthosteric site reduced the channel opening probability and conductance of mTRPC6 (Fig. 7, E–L). After DTNB’s covalent occupancy in mE671C, currents of M085 and GSK1702934A were completely suppressed and were partially restored after DTT reduction (Fig. 8C). All these data suggest that the site formed by PH and S6 is critical for the extracellular activation of M085 and GSK1702934A.

Our results also coincide with some observations from other TRP subfamilies or with corresponding sites in other TRPC subtypes. For example, previous studies have identified a similar site near the subunit interface between the S6 and PH, which has been identified as a binding site for the agonist of TRPML1 (ML-SA1) (38). Meanwhile, studies of TRPC5 have identified this region as the binding site for the both the endogenous lipid and the exogenous inhibitor Pico145 (39). In TRPC4, it has been shown that the phosphate head group of phospholipids (PA) interacts with main chain atom and side chains of T599 (in the S6 helix), W573 and Q569 (in the pore helix). In addition, DAG activation of TRPC6 is likely to stimulate at the lipid-binding site close to the pore region of the channel and therefore promotes channel opening (40). Recent structural analysis of the open state of TRPC6 has also shown that the small-molecule AM-0883 can bind to this site as well (28).

Three structurally distinct agonists, M085, GSK1702934A, and AM-0883 (10, 28), all act on this binding pocket, implying that this site may be the only orthosteric site that could recognize extracellular small-molecule agonists during evolution of TRPC sequences. Certainly, we cannot exclude the possibility that large-molecular-weight toxins act on the large extracellular domain of TRPC6 and thus lead to channel activation. For example, the agonist of TRPV1, double junctional toxin-DkTx, binds to the extracellular domain of the TRPV1 channel. Its two junctions bind at two equivalent positions on two adjacent subunits of this tetrameric channel, straddling between the two subunits, while DkTx’s junctions are connected by a segment of the linker that keeps the TRPV1 channel in the open state (41). In contrast, from the perspective that small molecules are limited in size and can only bind in restricted regions, this site formed by PH and S6 may be the only orthosteric site for TRPC6 to be activated by extracellular small molecules.
Figure 7. Mutations of the orthosteric site alter the unitary current of TRPC6 induced by M085 and GSK1702934A. A–D, unitary currents recorded from outside-out patches held at −60 mV in responses to 100 μM M085 (A and B) and 1 μM GSK1702934A (C and D) for mTRPC6-WT. E–H, unitary currents recorded from outside-out patches held at −60 mV in responses to 100 μM GSK1702934A (E and F) and 100 μM M085 (G and H) for the N701Q. I–L, unitary currents recorded from outside-out patches held at −60 mV in responses to 100 μM GSK1702934A (I and J) and 100 μM M085 (K and L) for the W679F. Full opening (O) and closing (C) are indicated by black and yellow lines, respectively. y axis (count) denotes the ratio of the number of events to the number of bins (the bin number is set to 320). All results were repeated at least three times.
The gating process of TRPC channels became clearer after structural determinations of different subtypes of the TRPC subfamily (42–44). hTRPC6’s open structure in complex with AM-0883 revealed the mechanism of TRPC6 channel activation by AM-0883 (28), while AM-085, M085, and GSK1702934A act at almost the same position, we infer that the gating mechanism of M085 and GSK1702934A activating TRPC6 is similar to that of AM-0883. During the gating of TRPC6, the binding of agonists (M085 and GSK1702934A) could not only cause structural rearrangement of the agonist binding pocket, but also push the extracellular region of S6 away from the pore and push S6 helix to the cytoplasmic side. Since S6 surrounds to S1 to S4 from the adjacent subunit, the movement of S6 is accompanied by the simultaneous tilting and downward movement of S1 to S4 as well as S4 to S5 linkers. From the cytoplasmic side, agonist binding is accompanied by a counterclockwise rotation around the central ion-permeable pathway, which eventually moves the intracellular portion of S1 to S4 a certain distance and opens the pore channel.

Taken together, this study not only advances our understanding of the contribution of PH and S6 to TRPC6 activation by providing a model to explain how the widely used agonists M085 and GSK1702934A bind to the receptor, but also reveals that this region might be the only extracellular small molecule agonist recognition site in TRPC. Notably, the sequences of the PH and S6 in TRPC3/6/7 are highly conserved, which poses a challenge for the development of TRPC3/6/7-specific small molecules. Our study will also be able to evaluate strategies to regulate TRPC function by stimulating the pocket formed between PH and S6 using new small molecules.

**Experimental procedures**

**Drugs, cell culture, and mutagenesis**

M085 and GSK1702934A were synthesized in our laboratory (see below). The plasmids for mTRPC6 and hTRPC6 were a gift from Xi Zhu. Each mutant was constructed by the KOD-Plus-Mutation Kit (Toyobo). Individual mutants were verified by DNA sequencing and then expressed in HEK293 cells. HEK293 cells were cultured in DMEM medium containing...
10% fetal bovine serum, 1% penicillin/streptomycin, and 1% glutamine at 37 °C with 5% CO₂ and 95% air. Transfection of TRPC6 plasmids was performed with Hilymax (Dojindo Laboratories) according to the manufacturer's protocol. Electrophysiological measurements were performed 24 to 48 h after transfection on HEK293 cells.

Chemical synthesis of GSK1702934A

GSK1702934A was prepared according to previous descriptions (12, 45, 46). Briefly, to a solution of dimethyl formamide (DMF, 30 mmol) in dry toluene (30 ml), POCl₃ (30 mmol) was added at room temperature with stirring for 30 min, followed by addition of cycloheptanone (20 mmol) in toluene and further stirred for 3 h (monitored by TLC). The reaction mixture was then cooled in an ice bath, followed by addition of dimethyldithiocarbonate (DDC, 20 mmol) and 30% aqueous KOH (20 ml) until the solution becomes basic. The solution was then heated at 90 °C for 45 min (monitored by TLC), cooled to room temperature and extracted with petroleum ether, and washed with water until the washings were neutral to litmus. The combined extracts were dried (Na₂SO₄), and the solvent was evaporated under reduced pressure to give crude product S₁ ((Z)-2-(methylthio)cyclohept-1-enecarbaldehyde), which was used for next reaction without purification.

To a solution of S₁ (1.99 g, 11.7 mmol) in ethanol 50 ml was added ethyl thioglycolate (1.41 g, 11.7 mmol) and anhydrous potassium carbonate (1.62 g, 11.7 mmol) at room temperature. The mixture was heated to reflux for 1 h (monitored by TLC), then cooled to room temperature, and evaporated the solvent. The residue was added with water and extracted with ethyl acetate. The combined extracts were dried (Na₂SO₄), and the solvent was evaporated under reduced pressure. The crude product was purified by column chromatography over silica gel using ethyl acetate-petroleum ether (5:95) as eluents. The compound S₂ (ethyl 5,6,7,8-tetrahydro-4H-cyclohepta[b]thiophene-2-carboxylate) was obtained in 71% yield (1.85 g, 8.3 mmol). ¹H NMR (400 MHz, Chloroform-d) δ 7.47 (s, 1H), 4.31 (q, J = 7.1 Hz, 2H), 2.87 to 2.79 (m, 2H), 2.74 to 2.65 (m, 2H), 1.87 (qd, J = 5.9, 5.0, 1.8 Hz, 2H), 1.74 to 1.67 (m, 2H), 1.67 to 1.59 (m, 2H), 1.36 (t, J = 7.1 Hz, 3H).

A solution of S₂ (0.78 g, 3.5 mmol) in EtOH (30 ml) was treated with a solution of NaOH (1 M in water, 7 ml) and stirred at 85 °C for 1 h. The mixture was cooled to room temperature and acidified with 1 M HCl (30 ml). The resulting precipitate was filtered and washed with petroleum ether. The S₃ (5,6,7,8-tetrahydro-4H-cyclohepta[b]thiophene-2-carboxylic acid) was obtained as white solid (0.61 g, 89%). To a solution of S₃ (0.35 g, 1.8 mmol) in SOCl₂ (15 ml) was added DMF 2 drops. The mixture was heated to reflux for 1 h, then cooled to room temperature, and evaporated under vacuum to afford quantitatively corresponding acid chloride S₄.

To a mixture of 4-(2-keto-1-benzimidazolinyl)piperidine (0.39 g, 1.8 mmol) and trimethylamine (0.25 ml, 1.8 mmol) in CH₂Cl₂ (15 ml) added S₄ (in CH₂Cl₂ 5 ml) by dropwise in an ice bath and stirred for 6 h (monitored by TLC). After completion of reaction, the mixture was washed thoroughly with brine. The organic layer was dried over anhydrous Na₂SO₄ and then concentrated to give crude product of GSK1702934A under reduced pressure. The residue was purified by silica gel chromatography to give the desired product (0.56 g, 79%) as a white solid. ¹H NMR (400 MHz, DMSO-d₆) δ 10.91 (s, 1H), 7.33 to 7.09 (m, 2H), 6.99 (dd, J = 6.0, 3.6 Hz, 3H), 4.48 (ddq, J = 12.4, 8.2, 4.0 Hz, 3H), 3.09 (q, J = 11.1, 7.9 Hz, 2H), 2.83 to 2.70 (m, 2H), 2.70 to 2.58 (m, 2H), 2.32 (qd, J = 12.5, 4.1 Hz, 2H), 1.79 (td, J = 10.3, 9.0, 5.0 Hz, 4H), 1.57 (dp, J = 21.7, 5.0 Hz, 4H).

Activation of TRPC6 by GSK1702934A and M085
Chemical synthesis of M085

To a solution of piperidine-4-carboxylic acid (2.58 g, 20 mmol) in water (20 ml) was added Na₂CO₃ (2.52 g, 24 mmol), and then was added ethyl chloroformate (2.56 g, 24 mmol) in THF (20 ml). The reaction mixture was stirred at room temperature until completion and then concentrated in vacuo. Water (30 ml) was added into the mixture and then was adjusted to pH 2 using 2 M HCl. The aqueous layer was extracted with EtOAc (3 × 15 ml). The combined organic layers were washed with brine, dried over anhydrous Na₂SO₄, filtered, and concentrated in vacuo. The residue dissolved in THF (8 ml) was added N,N-carbonyldiimidazole (4.21 g, 26 mmol). The reaction mixture was stirred at room temperature until completion and then concentrated in vacuo.

The residue dissolved in THF (8 ml) was added N, N'-carbonyldiimidazole (4.21 g, 26 mmol). The reaction mixture was stirred at room temperature until completion and then concentrated in vacuo.

To a solution of ethyl potassium malonate (6.8 g, 40 mmol) in THF (100 ml)/CH₃CN (50 ml) was added anhydrous MgCl₂ (3.8 g, 40 mmol) and DMAP (244 mg, 2 mmol) at 0 °C. The above residue dissolved in THF (10 ml) was added into the mixture, and then was added Et₃N (3.03 g, 40 mmol). The reaction mixture was stirred at room temperature until completion, and then was added 1 M HCl (150 ml). The aqueous layer was extracted with Et₂O (3 × 100 ml). The combined organic layers were washed with brine, dried over anhydrous Na₂SO₄, filtered, and concentrated in vacuo. The residue was purified by flash chromatography to give the yellow oil (4.39 g, 81% for three steps).

¹H NMR (300 MHz, CDCl₃) δ 4.27 to 4.03 (m, 5H), 3.48 (s, 1H), 3.38 (s, 1H), 2.84 (t, J = 11.5 Hz, 1H), 2.67 to 2.59 (m, 1H), 2.07 (s, 1H), 1.85 (d, J = 11.9 Hz, 1H), 1.61 to 1.49 (m, 1H), 1.29 to 1.21 (m, 6H). ESI-MS m/z 272.1 [M + H]+.

4-(4-Fluorophenyl)-3-methyl-1H-pyrazol-5-amine

To a solution of 4-fluorophenylacetonitrile (0.65 g, 4.8 mmol) in DMF (5 ml) was added 1,1-dimethoxy-N,N-dimethylethylamine (0.53 g, 4 mmol). The reaction mixture was stirred at 100 °C until completion and then concentrated in vacuo. Hydrazine monohydrochloride (0.41 g, 6 mmol), EtOH (8 ml) and
Activation of TRPC6 by GSK1702934A and M085

water (5 ml) were added into the reaction mixture and then stirred at 80 °C until completion. Saturated NaHCO₃ solution (30 ml) was added, and the aqueous layer was extracted with EtOAc (3 × 10 ml). The combined organic layers were washed with brine, dried over anhydrous Na₂SO₄, filtered, and concentrated in vacuo. The residue was recrystallized with PE/EtOAc to give a white solid (0.82 g, 90% for two steps).

1H NMR (500 MHz, DMSO-d₆) δ 7.38 (dd, J = 8.6, 5.7 Hz, 2H), 7.21 (t, J = 8.9 Hz, 2H), 2.17 (s, 3H). ESI-MS m/z 192.1 [M + H]⁺.

3-Methyl-4-(4-(trifluoromethyl)phenyl)-1H-pyrazol-5-amine

Following the prepare procedure of 4-(4-fluorophenyl)-3-methyl-1H-pyrazol-5-amine, 3-methyl-4-(4-(trifluoromethyl)phenyl)-1H-pyrazol-5-amine was prepared from 4-trifluoromethylphenylacetonitrile as a white solid in 88% yield.

1H NMR (500 MHz, DMSO-d₆) δ 7.82 (d, J = 8.0 Hz, 2H), 7.64 (t, J = 8.4 Hz, 2H), 2.31 (s, 3H). ESI-MS m/z 242.1 [M + H]⁺.

Ethyl 4-(3-(4-fluorophenyl)-7-hydroxy-2-methylpyrazolo[1,5-a]pyrimidin-5-yl)piperidine-1-carboxylate (M085)

To a solution of 4-(4-fluorophenyl)-3-methyl-1H-pyrazol-5-amine (3.82 g, 20 mmol) in AcOH (20 ml) was added ethyl 4-(3-ethoxy-3-oxopropanoyl) piperidine-1-carboxylate (7.99 g, 24 mmol). The reaction mixture was stirred at 130 °C until completion and then concentrated in vacuo. 1 M NaOH was added into the mixture and filtered to give a white solid (6 g, 75% yield).

1H NMR (500 MHz, DMSO-d₆) δ 7.91 (dd, J = 8.6, 5.8 Hz, 2H), 7.21 (t, J = 8.9 Hz, 2H), 5.46 (s, 1H), 4.18 to 4.00 (m, 4H), 2.90 (brs, 2H), 2.65 to 2.57 (m, 1H), 2.50 (s, 3H), 1.87 to 1.84 (m, 2H), 1.65 to 1.59 (m, 2H), 1.25 to 1.19 (m, 3H). 13C NMR (125 MHz, DMSO-d₆) δ 165.4, 160.1, 158.4, 158.2, 154.6, 148.9, 147.2, 131.7, 128.4, 128.4, 114.6, 114.4, 101.8, 90.0, 60.5, 43.7, 43.4, 31.1, 15.0, 14.5. ESI-MS m/z 421.3 [M + Na]⁺.

In silico docking

The cryo-EM structure of hTRPC6 (PDB 5YX9) was prepared using the Protein Preparation Wizard (47) after filling the missing loop using Modeller (48) with the structure of PDB 6CUD as the template. The small-molecule conformations of M085 and GSK1702934A were prepared using LigPrep (49). The docking program Glide (50) was employed to dock M085 or GSK1702934A into the potential binding sites. The grid for the protein was set to 30 Å, and the extra precision (XP) docking mode was selected in in silico docking. For Induce-FitDocking, all procedures including protein preparation, refinement, grid generation, and docking were carried out using the default parameters. The threshold for rejecting minimized poses was 0.5 kcal/mol. Docking scores and M085/GSK1702934A-residue interaction interaction scores were summarized, sorted, and then plotted using Maestro (51, 52).

Conventional molecular dynamic (CMD) simulations and metadynamics (MetaD) simulations

According to our previous description (51, 53), all MD simulations were performed at a constant number of particles, constant pressure and temperature, and periodic boundary conditions using program DESMOND, which uses a particular “neutral territory” method to efficiently exploit a high degree of computational parallelism (54). The default OPLS-2005 force field was used for hTRPC6, ions, and M085/GSK1702934A. The complexes of hTRPC6/M085 and hTRPC6/GSK1702934A were used as the initial structures for MD simulations (54). Using the large 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphatidylcholine (POPC, 300 K) bilayer provided in DESMOND’s System Builder (54), a suitable membrane system was generated in which the TM domain of hTRPC6 could be embedded. The hTRPC6/POPC system is dissolved in simple point charge (SPC) water molecules. Counter ions were then added to compensate for the net negative charge of the system. NaCl (150 mM) was added to the simulation box to represent the background salt under physiological conditions. The DESMOND default relaxation protocol was applied to each system prior to the simulation run. (1) 100 ps simulations in the NVT ensemble with Brownian kinetics using a temperature of 10 K with solute heavy atoms constrained; (2) 12 ps simulations in the NVT ensemble using a Berendsen thermostat with a temperature of 10 K and small-time steps with solute heavy atoms constrained; (3) 12 ps simulations in the NPT ensemble using a Berendsen thermostat and barostat for 12 ps simulations at 10 K and 1 atm, with solute heavy atoms constrained; (4) 12 ps simulations in the NPT ensemble using a Berendsen thermostat and barostat at 300 K and 1 atm with solute heavy atoms constrained; (5) 24 ps simulations in the NPT ensemble using a Berendsen thermostat and barostat at 300 K and 1 atm without constraint. After equilibration, the MD simulations were performed for 100 to 500 ns. Long-range electrostatic interactions were computed using a Smooth Particle Mesh Ewald method. The trajectory recording interval was set to 200 ps and other default parameters were used during CMD simulation runs. The Simulation Interaction Diagram (SID) module in DESMOND was used for exploring the interaction analysis between M085 (or GSK1702934A) and hTRPC6, and the r.m.s (root-mean-square) fluctuations (RMSF) analysis of protein and ligand.
MetaD simulation (55–57) was also conducted by program DESMOND (54) with a constant number of particles, pressure (1 bar) and temperature (300 K) and periodic boundary conditions by using Nose–Hoover chain Thermostat and Martyna–Tobias–Klein Barostat methods. The height and width of the Gaussian and the interval were set to 0.12 kcal/mol, 0.05 Å and 0.09 ps, respectively. Before the simulation, the built systems were relaxed by using the protocol of DESMOND in the following sequence: (1) NVT ensemble with Brownian dynamics for 100 ps at 10 K with small time step, and solute heavy atom restrained; (2) NVT ensemble using Berendsen thermostat for 12 ps at 10 K with small time step, and solute heavy atom restrained; (3) NPT ensemble using a Berendsen thermostat and barostat for 12 ps at 10 K and 1 atm with solute heavy atom restrained; (4) NPT ensemble using a Berendsen for 12 ps at 300 K and 1 atm with solute heavy atom restrained; (5) NPT ensemble using a Berendsen for 24 ps at 300 K and 1 atm with no restraints. After the relaxation, the metadynamics runs were protracted for ~30,000 to 60,000 ps until they showed free diffusivity along the defined collective variables (CVs), which indicates the convergence of metadynamics runs. This criterion was chosen according to the guidelines of assessing the accuracy of metadynamics runs (57). The surface sum of free energy and Gaussians were calculated using the metadynamics analysis tool in DESMOND (34, 51).

All simulations were run on DELL T7910 graphic working station (with NVIDIA Tesla K40C-GPU). Preparation, analysis, and visualization were performed on a 12-CPU CORE DELL T7500 graphic working station.

Electrophysiology
As we described previously (53, 58), whole-cell recordings were performed at room temperature (23 °C ± 2 deg. C) using a voltage clamp under a conventional whole-cell configuration. The pipettes were pulled from glass capillaries through two-stage puller (Narishige PC-10) and filled in a general intra-cellular solution containing (in mM): 110 CsCl, 10 HEPES, 10 BAPTA, 1 MgCl2, 4.77 CaCl2, pH adjusted to 7.2 with HCl (400 mM free Ca2+) (10), and a resistance varied between 3 and 5 MΩ. Membrane currents were measured with an Axon 200B patch-clamp amplifier (Molecular Devices). Current signals were sampled at 10 kHz and filtered at 2 kHz and analyzed by pClamp 10 (Molecular Devices). The bath solution containing (in mM): 140 NaCl, 5 KCl, 2 CaCl2, 1 MgCl2, 10 glucose, and 10 HEPES, pH adjusted to 7.4 with Tris-base (10). During gap-free recordings, the membrane potential was held at −60 mV and the M085 or GSK1702934A solution was applied with the Y-tube. Single-channel recordings or macro-patch using outside-out or inside-out configurations were carried out in HEK293 cells at room temperature (23 °C ± 2 deg. C) 24 to 48 h after transfection and recording electrodes were drawn from borosilicate glass (World Precision Instruments, Inc). The borosilicate glass was polished to produce resistance about 8 to 12 megohms and the holding potential was set at −60 mV. The external solution and internal solutions are the same as the whole-cell recordings. Currents were recorded at 50 kHz with 2 kHz filter and a low-pass filtered at 300 Hz, using the AxonPatch 200B amplifier in conjunction with pClamp 10.0 software (Molecular Device).

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Cell surface biotinylation and western blotting were performed as per the previously described method (58). Briefly, mTRPC6 or its mutants were expressed in HEK-293 cells and washed in chilled PBS, then bathed in solution containing sulfo-NHS-LC-biotin, and the reaction was stopped by incubating the cells with glycine (20 mM) in PBS. Cells were then collected and lysed with RIPA buffer. The biotinylated proteins were separated from the intracellular protein fraction by agarose resin attached to NeutrAvidin, followed by incubation overnight and subsequent centrifugation. The resin was then washed and the bound proteins were eluted with boiling SDS sample buffer, while 10% volume of the supernatant was diluted as the total protein fraction. The remaining lysate was incubated with NeutrAvidin agarose resin at 4 °C overnight, and then the resin was washed several times with chilled PBS. Protein samples for biotinylation assays were analyzed by Western blotting. All samples and protein markers were separated on SDS-polyacrylamide gels (10%) and transferred to polyvinylidene difluoride (PVDF) membranes. The samples were incubated with anti-EE-tag (1:1000) or anti-GAPDH (1:1000) antibodies overnight at 4 °C, followed by appropriate HRP-conjugated secondary antibodies to EE-tag or GAPDH, and finally exposed to ECL solution (Thermo) and an automated chemiluminescence-fluorescence image analysis system (Tanon 5200, Multi) for 1 to 3 min for visualization. Analysis of protein expression was repeated by at least four independent experiments.

Data analysis
The results are expressed as the means ± SD. Statistical significance between different groups was determined using the Student’s t test or Bonferroni’s multiple comparisons test (ANOVA, more than two groups), where p < 0.05 (*) or p < 0.01 (**) was considered significant. By measuring currents in response to different concentrations of M085 and GSK1702934A to obtain the concentration–response relationships for M085 and GSK1702934A activation of wild-type or mutated channels, respectively. Unless otherwise noted, data were obtained from at least three batches of different transfected cells. The data were fitted to the Hill equation, \( I/I_{\text{max}} = 1/[1 + (EC_{50}/[M085 \text{ or GSK1702934A}])]^n \), where \( I \) is the normalized current at a given concentration of M085 or GSK1702934A, \( I_{\text{max}} \) is the maximum normalized current, \( EC_{50} \) is the concentration of M085/GSK1702934A yielding a current that is half of the maximum, and \( n \) is the Hill coefficient.

Data availability
All data are contained within the manuscript.

Author contributions—Y.-T. L. and Y. Y. conceptualization; J. W., Y.-T. L., and Y. Y. data curation; P.-L. Y., Y.-T. L., and Y. Y. formal analysis; W.-H. W., Y.-T. L., and Y. Y. funding acquisition; P.-L. Y.,...
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Conflict of interest—The authors declare that they have no conflicts of interest with the contents of this article.

Abbreviations—The abbreviations used are: CMD, conventional molecular dynamics; cryo-EM, cryo-electron microscopy; DTNB, 5,5′-dithiobis (2-nitrobenzoic acid); ECD, extracellular domain; MetaD, metadynamics; PH, pore helix; TM, transmembrane; TNB, thiobenzene (2-nitrobenzoic acid); TRPC, transient receptor potential canonical; WT, wild-type.

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