Identification of a Different Agonist-Binding Site and Activation Mechanism of the Human P2Y<sub>1</sub> Receptor

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The human P2Y<sub>1</sub> receptor (P2Y<sub>1</sub>R) is a purinergic G-protein-coupled receptor (GPCR) that functions as a receptor for adenosine 5′-diphosphate (ADP). An antagonist of P2Y<sub>1</sub>R might potentially have antithrombotic effects, whereas agonists might serve as antidiabetic agents. On the basis of the antagonist-bound MRS2500-P2Y<sub>1</sub>R crystal structure, we constructed computational models of apo-P2Y<sub>1</sub>R and the agonist-receptor complex 2MeSADP-P2Y<sub>1</sub>R. We then performed conventional molecular dynamics (cMD) and accelerated molecular dynamics (aMD) simulations to study the conformational dynamics after binding with agonist/antagonist as well as the P2Y<sub>1</sub>R activation mechanism. We identified a new agonist-binding site of P2Y<sub>1</sub>R that is consistent with previous mutagenesis data. This new site is deeper than those of the agonist ADP in the recently simulated ADP-P2Y<sub>1</sub>R structure and the antagonist MRS2500 in the MRS2500-P2Y<sub>1</sub>R crystal structure. During P2Y<sub>1</sub>R activation, the cytoplasmic end of helix VI shifts outward 9.1 Å, the Ser146<sub>3.47</sub>-Tyr237<sub>5.58</sub> hydrogen bond breaks, a Tyr237<sub>5.58</sub>-Val262<sub>6.37</sub> hydrogen bond forms, and the conformation of the χ<sub>1</sub> rotamer of Phe269<sub>6.44</sub> changes from parallel to perpendicular to helix VI. The apo-P2Y<sub>1</sub>R system and the MRS2500-P2Y<sub>1</sub>R system remain inactive. The newly identified agonist binding site and activation mechanism revealed in this study may aid in the design of P2Y<sub>1</sub>R antagonists/agonists as antithrombotic/antidiabetic agents, respectively.

The members of the G-protein-coupled receptor (GPCR) superfamily, the largest family of cell-surface receptors<sup>1</sup>, translate chemical information from extracellular signals into interpretable stimuli, thus resulting in intracellular biological responses. The activation of GPCR causes conformational changes in the transmembrane helices, thereby triggering downstream signalling through partners, such as G proteins or β-arrestins, on the intracellular side of the membrane. GPCRs are thought exist in an equilibrium between inactive and active conformations. Agonists stabilize the active conformation, and antagonists stabilize the inactive conformation of these receptors<sup>2</sup>. In addition, GPCRs provide therapeutic targets for a diverse set of human diseases<sup>3,4</sup> and are the targets of more than 40% of modern drugs<sup>5</sup>.

The P2Y<sub>1</sub> (P2Y<sub>1</sub>R) and P2Y<sub>12</sub> (P2Y<sub>12</sub>R) receptors are human purinergic GPCRs and are two of the eight members of the human P2YR family<sup>6</sup>. Both P2Y<sub>1</sub>R and P2Y<sub>12</sub>R can be activated in platelets by the endogenous agonist adenosine 5′-diphosphate (ADP). The activation of each of these receptors facilitates platelet aggregation and plays a vital role in thrombosis formation<sup>7,8</sup>. Accordingly, P2Y<sub>12</sub>R is one of the most important clinical targets for antithrombotic drugs<sup>9</sup>. P2Y<sub>1</sub>R is expressed in a number of different tissues, such as the heart, blood vessels, brain, skeletal muscle and smooth muscles<sup>10</sup>. Recent preclinical data have suggested that antagonists of P2Y<sub>1</sub>R and P2Y<sub>12</sub>R provide equivalent antithrombotic efficacy, whereas a P2Y<sub>12</sub>R antagonist shows potential for decreasing the risk of bleeding<sup>7</sup>. Hence, antagonists of P2Y<sub>12</sub>R might potentially serve as attractive antithrombotic compounds. However, adenosine 5′-triphosphate (ATP) stimulates pancreatic insulin release via a glucose-dependent mechanism involving P2Y<sub>1</sub>R, thus indicating that agonists of P2Y<sub>1</sub>R might have potential as antidiabetic agents<sup>11</sup>.

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agonist-binding site from that of the ADP-P2Y 1R system in the cMD simulations of Yuan et al.36. In the 2MeSADP-P2Y 12R structure, MRS2500 binds P2Y 1R within the seven transmembrane helical bundle through electrostatic interactions with phosphate groups. The binding of 2MeSADP involves an inward shift of the extracellular part of helix VI and VII towards the center of the seven transmembrane helical bundle. Recently, the crystal structure of antagonist MRS2500-bound P2Y 1R has been reported by Zhang et al. (PDBID: 4XNW)17. In this structure, MRS2500 binds to the extracellular vestibule of P2Y 1R, a pocket composed of residues mainly from the N-terminal, ECL2 and extracellular side of helices VI and VII. However, the crystal structure of agonist-bound P2Y 1R has not been resolved. Therefore, it is essential to study the conformational dynamics of P2Y 1R after binding with agonists/antagonists and the activation mechanism of this receptor by using alternative tools.

The conformational dynamics induced by agonist/antagonist and activation mechanism of GPCRs have been extensively studied recent years by many computational chemists using conventional molecular dynamics (cMD) simulations36–38. cMD allows studies on timescales of tens to hundreds of nanoseconds, or several microseconds at most; however, many biological processes (e.g., the activation process of GPCR) occur on longer timescales of up to milliseconds or more39. To overcome the challenge of long timescales and to explore the portions of the energy landscape that are separated by high barriers from the initial minimum, the McCammon group has developed accelerated molecular dynamics (aMD) by introducing a bias potential into cMD36–38. In aMD, the system's potential is modified with a bias boost potential, and the height of local barriers is decreased; thus, the calculation evolves much faster than that in cMD36–38. aMD has been successfully used to study many GPCR systems39-44 and aMD simulations at hundreds of nanoseconds have been shown to capture events that occur on millisecond scale39,45.

While this manuscript was in preparation, Yuan et al. published long-timescale cMD simulations on P2Y 1R using Schrödinger Desmond software36. In their simulations, the agonist ADP was first placed 15 Å from the orthosteric site of P2Y 1R, After 6 × 2 μs cMD simulations, ADP was found to bind to the extracellular vestibule of P2Y 1R, a similar site to that of the antagonist MRS2500. The activation of P2Y 1R was characterized by the breaking of an extracellular ionic lock (Asp204 for Arg310 for the Asp/Glu salt bridge) and the formation of a water channel through the seven transmembrane helical bundle.

Here, we performed cMD and aMD simulations for 2MeSADP-P2Y 1R, apo-P2Y 1R and MRS2500-P2Y 1R embedded in a lipid bilayer/water environment to investigate the conformational dynamics after binding with agonists/antagonists and the P2Y 1R activation mechanism. Our calculations identified a different agonist-binding site from that of the ADP-P2Y 1R system in the cMD simulations of Yuan et al.36. The newly identified agonist-binding site in our simulations is consistent with previous mutagenesis data4, 6, 12, 13, 17, 37, 38. In our aMD simulations, the activation of P2Y 1R is characterized by (i) the outward shift of helix VI cytoplasmic end of approximately 9.1 Å; (ii) the breaking of the Ser149-Tyr237 hydrogen bond; (iii) the formation of a Tyr237-Val262 hydrogen bond; and (iv) a χ1 rotamer change of Phe269 for parallel to perpendicular to helix VI. In contrast, the apo-P2Y 1R system and the MRS2500-P2Y 1R system remain in the inactive state.

Results and Discussion

The binding mode of 2MeSADP in P2Y 1R. Like other GPCRs, P2Y 1R can exist in multiple distinct states (e.g., active state or inactive state), and apo-P2Y 1R typically exhibits basal activity. GPCRs exhibit an equilibrium between inactive and active conformations. Agonists stabilize the active conformation, whereas antagonists stabilize the inactive conformation. Therefore, we studied the conformational dynamics of P2Y 1R in the apo form and in the presence of the agonist 2MeSADP and the antagonist MRS2500 to capture the characteristics of the different P2Y 1R states.

The currently available structure of P2Y 1R is the antagonist MRS2500-bound form (PDBID: 4XNW)17. In this structure, MRS2500 binds to the extracellular vestibule of P2Y 1R, a pocket composed of residues mainly from the N-terminal, ECL2 and extracellular side of helices VI and VII.

Initially, we docked 2MeSADP to the MRS2500 binding site (depicted in Supplementary Figure S1). Figure S1 shows that 2MeSADP binds with P2Y 1R in the same orientation as that of MRS2500. The aromatic adenine ring of 2MeSADP interacts with the hydroxynaphthalenyl group of Tyr303 through π-π stacking. The amino group and the N1 in adenine of 2MeSADP interact with the amide group of Asn283 through hydrogen bonds. The negatively charged pyrophosphates interact with the positively charged amine groups of Arg128 and Arg310 through electrostatic interactions and with the phenolic hydroxyl groups of Tyr110 and Tyr306 through hydrogen bonds. The residues in the 2MeSADP binding site (Supplementary Figure S1) are consistent with previous mutagenesis data4, 6, 12, 13, 17, 37, 38 (Table 1). Recently, Yuan et al.36 have published long-timescale cMD simulations of the agonist ADP bound with P2Y 1R. Their simulations showed that ADP binds with P2Y 1R in a similar site to that of the antagonist MRS2500 in the MRS2500-P2Y 1R crystal structure.17. Our identified site for the agonist 2MeSADP (Figure S1) is the same as the simulated ADP binding site36 and that of the antagonist MRS2500, as determined by crystallography17.

However, the mutagenesis of residues His132, Thr136, Thr222 and Lys280 decreased the P2Y 1R binding affinity of 2MeSADP4, 6, 12, 13, 17, 37, 38 (Table 1). These residues are located much deeper than the antagonist MRS2500 binding site in the available P2Y 1R crystal structure. However, these residues do overlap with the agonist 2MeSADP binding site in the 2MeSADP-P2Y 12R crystal structure (PDB ID: 4PXZ)36. These observations suggest the possibility of a new potential agonist-binding site distinct from the MRS2500 site in P2Y 1R. However, the deep cavity in the MRS2500-P2Y 1R crystal structure is too small to accommodate 2MeSADP. The ECL2
extends deep into the ligand-binding pocket and blocks 2MeSADP from accessing the deep cavity. Therefore, we cut a portion of ECL2, mutated two residues in the deep cavity and induced fit docked 2MeSADP to the deep cavity of P2Y1R. Then, the 2MeSADP-P2Y1R structure was reconstructed by adding the missing residues in ECL2 and mutating back in the cavity. We then identified a new agonist-binding site in the 2MeSADP-P2Y1R structure. Figure 1 shows a comparison of the 2MeSADP and the MRS2500 binding sites between 2MeSADP-P2Y1R and MRS2500-P2Y1R. Recent long-timescale cMD simulations by Yuan et al.36 have shown that ADP binds to P2Y1R in a similar site to that of the antagonist MRS2500 in the MRS2500-P2Y1R crystal structure. However, it can be seen in Figure 1 that the newly identified binding site of the agonist 2MeSADP is deeper than that of ADP in the simulated ADP-P2Y1R structure and the antagonist MRS2500 in the MRS2500-P2Y1R crystal structure. The specific binding mode of 2MeSADP in P2Y1R is depicted in Figure 2 and the interactions between 2MeSADP and P2Y1R over time are shown in Supplementary Figure S2. The aromatic adenine ring of 2MeSADP

| Mutated residue | Position | Kd or EC50 value | Reference |
|-----------------|----------|-----------------|-----------|
| Y110F           |          | 2.63            | 17        |
| R128A           |          | 3.29            | >100000   | 38        |
| H132A           |          | 3.33            | 10        | 38        |
| Y136A           |          | 3.37            | 10        | 38        |
| T205A           | ECL2     |                | 10        |
| T221A           |          | 5.42            | 13        | 38        |
| T222A           |          | 5.43            | 13        | 38        |
| H280A           |          | 5.45            | 13        | 38        |
| N283A           |          | 5.68            | N5        | 17        |
| R287A           |          | 5.62            | 11654     | 38        |
| Y303F           |          | 5.72            | 1.24      | 17        |
| Y306F           |          | 5.73            | >300      | 17        |
| R310A           |          | 5.89            | 329       | 38        |

Table 1. The effects of the mutated residue on the Kd or EC50 value loss compared with those of wild-type P2Y1R. N5: negligible specific binding for [3H]2MeSADP in the mutant P2Y1R.}

Figure 1. Comparison of the agonist/antagonist-binding sites between (A) 2MeSADP-P2Y1R and (B) MRS2500-P2Y1R. The P2Y1R structure is shown in cartoon and is colored in silver, 2MeSADP and MRS2500 are shown in sphere and colored in cyan and yellow, respectively.
interacts with the imidazole group of His132 and with the ε-amino group of Lys280 through π-π and π-cation stacking. In addition, the amino group in the adenine of 2MeSADP forms a hydrogen bond with the hydroxyl group of Thr222. The N1 in the adenine of 2MeSADP forms hydrogen bonds with the phenolic hydroxyl group of Tyr136 and the hydroxyl group of Thr221. The negatively charged pyrophosphates interact strongly with several positively charged or polar residues, including Arg128, Arg287, Arg310, Lys280, and Tyr306. The residues shown in the 2MeSADP binding site in Figure 2 have been demonstrated to be important for the binding of this ligand to P2Y1R and show 9 or more fold changes in Kd or EC50 values in previous mutation studies (Table 1). This result indicates that the newly identified 2MeSADP binding site in our simulation is consistent with previous mutagenesis data.

In addition, we performed metadynamics simulation for the 2MeSADP-P2Y1R system and compared the results with those from our aMD simulations. The free energy surface associated with 2MeSADP-P2Y1R interactions along the distance between the COMs of 2MeSADP and the seven transmembrane helical bundles of P2Y1R in the direction perpendicular to membrane (i.e., the Z-direction) is depicted in Figure 3A. Figure 3A shows two minima (A and B). The distance between the COMs of 2MeSADP and P2Y1R in the Z-direction is around 12 to 15 Å and 18 to 19 Å in minima A and B, respectively. In the newly identified 2MeSADP binding site (Figure 2) and the initial site (Figure S1), this distance is 13.1 Å and 19.6 Å, respectively. Moreover, the binding pose in minimum A revealed using the metadynamics simulation aligns well with the newly identified 2MeSADP binding mode in the aMD simulation (Figure 3B). These confirm the reasonability of the newly identified 2MeSADP binding site and the reliability of the aMD method.

Conformational states revealed by PMF analyses. The starting X-ray structure of P2Y1R was for the inactive state. For each system (i.e., 2MeSADP-P2Y1R, apo-P2Y1R, and MRS2500-P2Y1R), we performed a 100-ns cMD simulation and a subsequent 300-ns aMD simulation. In the 100-ns cMD simulations, P2Y1R did not deviate substantially from the starting inactive structure. In the aMD simulations, P2Y1R shows increased conformational dynamics, especially in the 2MeSADP-P2Y1R system. Supplementary Figure S3 shows the RMSFs of the Cα atoms in P2Y1R, as calculated from the 300-ns aMD trajectories of the 2MeSADP-P2Y1R, apo-P2Y1R, and MRS2500-P2Y1R systems. The RMSFs indicated that the ICLs and ECLs show higher conformational fluctuations than the helices. The most significant conformational dynamics in the 2MeSADP-P2Y1R system compared with the apo-P2Y1R system and MRS2500-P2Y1R systems was the fluctuation in the cytoplasmic end of helix VI (residues Leu254 to Pro275, labelled with a pink box in Supplementary Figure S3). This result was consistent with the conventional concept that movements of VI are absolutely essential for GPCR activation.

Figure 2. (A) Binding mode of 2MeSADP in P2Y1R. Hydrogen bonds between 2MeSADP and P2Y1R are represented by dashed lines. (B) Schematic representation of interactions between 2MeSADP and P2Y1R.
The movement of helix VI was monitored on the basis of the helix III-helix VI distance (Cα-Cα distance of Val1533.54 and Leu2546.29). A significantly larger conformational space was sampled in the P2Y1R aMD simulations than in the cMD simulations. To gain insight into the effects of binding with 2MeSADP/MRS2500 on the overall conformational dynamics of P2Y1R, we performed PMF analyses on the basis of the 300-ns aMD trajectories, generating 2D energy landscape maps for the 2MeSADP-P2Y1R, apo-P2Y1R and MRS2500-P2Y1R systems. Figure 4 shows the PMF maps for these systems.

The PMF map reveals three different conformational states (i.e., the inactive, intermediate and active states) of P2Y1R in the 2MeSADP-P2Y1R system (Figure 4A). In contrast, in the apo-P2Y1R and the MRS2500-P2Y1R systems (Figure 4B and 4C), only two P2Y1R states (i.e., the inactive and intermediate states) are identified by using the 300-ns aMD trajectories.

To get solid and statistical conclusion, we performed additional 3 × 300 ns aMD simulations for each of the 2MeSADP-P2Y1R, apo-P2Y1R and MRS2500-P2Y1R systems. The PMF maps calculated from the 3 × 300 ns trajectories for each system show similar results that the 2MeSADP-P2Y1R system (Supplementary Figure S4) equilibrates in the inactive, intermediate and active states, whereas the apo-P2Y1R system (Supplementary Figure S5) and the MRS2500-P2Y1R system (Supplementary Figure S6) only stay in the inactive and intermediate states.

Activation/inactivation mechanisms of P2Y1R after binding with 2MeSADP/MRS2500. As shown in Figure 4, P2Y1R undergoes significant conformational changes after activation. To explore the conformational dynamics induced by the agonist 2MeSADP and the antagonist MRS2500 on P2Y1R, we monitored the dynamic movements of helix VI in the 2MeSADP-P2Y1R, apo-P2Y1R and MRS2500-P2Y1R systems. Figure 5 depicts the time dependence of the bend angle (θ) of helix VI and the representative snapshots extracted from the aMD trajectories of the three systems.

The bend angle of helix VI stretches after 2MeSADP binding (Figure 5B and 5C). The stretch of the helix VI bend angle causes helix VI to shift away from helix III. The bend angles of the inactive, intermediate and active state are approximately 137°, 142° and 156°, respectively. The corresponding helix III-helix VI distance of the inactive, intermediate and active state are approximately 7.6 Å, 10.6 Å and 16.7 Å (Supplementary Figure S7A). In comparison, no significant stretching of the bend angle of helix VI was observed in the apo-P2Y1R system during

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**Figure 3.** (A) Free energy surface associated with 2MeSADP-P2Y1R interactions, as a function of the distance between the COMs of 2MeSADP and the seven transmembrane helical bundles of P2Y1R in the Z-direction. (B) Comparison of the newly identified agonist-binding site and minimum A in Figure 3A. The P2Y1R structure is shown in cartoon and is colored in silver. 2MeSADP in the newly identified agonist-binding site and minimum A are shown in stick and colored in cyan and magenta, respectively.

**Figure 4.** Potential of mean force (PMF) calculated for the helix III-helix VI distance and the RMSD of the NPxxY motif relative to the inactive starting structure for (A) the 2MeSADP-P2Y1R system, (B) the apo-P2Y1R system and (C) the MRS2500-P2Y1R system.
the aMD simulation (Figure 5B and 5D). Moreover, in the MRS2500-P2Y, R system, binding of the antagonist MRS2500 blocks the bend angle of helix VI from stretching and locks P2Y, R in its inactive state. These observations were consistent with a model in which 2MeSADP is a P2Y, R agonist and MRS2500 is a P2Y, R antagonist.

The initial structure of the 2MeSADP-P2Y, R system was the inactive state. During the aMD simulation, P2Y, R passed through the intermediate state and reached an active state. The three different conformational states identified in Figure 4A correspond to the inactive, intermediate and active states of P2Y, R. To identify the microscopic structural characters discriminating the three states during the P2Y, R activation, the snapshots in the aMD trajectory of the 2MeSADP-P2Y, R system were grouped into three clusters: the inactive state (snapshots in 0–40 ns), the intermediate state (snapshots in 55–130 ns) and the active state (snapshots in 180–300 ns). Figure 6 shows the representative structures for each state. The following values were measured for the 2MeSADP-P2Y, R,
apo-P2Y<sub>1</sub>R and MRS2500-P2Y<sub>1</sub>R systems in the 300-ns aMD simulations: the helix III-helix VI distance; the O–O distance between the hydroxyl of Ser<sub>146</sub> and the hydroxyl of Tyr<sub>237</sub>; the O–O distance between the hydroxyl of Tyr<sub>237</sub> and the backbone oxygen of Val<sub>262</sub>; and the χ<sub>1</sub> rotamer (measured by the N-C<sub>α</sub>-C<sub>β</sub>-C<sub>γ</sub>-C<sub>δ</sub> torsion angle) of Phe<sub>269</sub>. The initial condition of the 2MeSADP-P2Y<sub>1</sub>R system is the inactive state. In this state (Figure 6A), the helix III-helix VI distance is 7.6 Å (Supplementary Figure S7A), which is very close to the distance of 7.5 Å found in the crystal structure. The hydroxyl oxygen of Ser<sub>146</sub> and the hydroxyl oxygen of Tyr<sub>237</sub> form a hydrogen bond with an O–O distance of 3.0 Å (Supplementary Figure S7B). The χ<sub>1</sub> rotamer of Phe<sub>269</sub> is approximately −82° (i.e., the gauche− state) and almost parallel to helix VI (Supplementary Figure S7D). P2Y<sub>1</sub>R then transitions to the intermediate state. In the intermediate state (Figure 6B), the helix III-helix VI distance increases to 10.6 Å (Supplementary Figure S7A). In addition, the cytoplasmic end of helix V exhibits high mobility, thus leading to disruption of the hydrogen bond between the two hydroxyl oxygen atoms of Ser<sub>146</sub> and Tyr<sub>237</sub> (Supplementary Figure S7B). The χ<sub>1</sub> rotamer of Phe<sub>269</sub> remains in the gauche− state (Supplementary Figure S7D). After the intermediate stage, the helix III-helix VI distance sharply increases and approaches 16.7 Å (Supplementary Figure S7A). The helix end of helix VI shifts outward by 9.1 Å relative to the inactive structure. This change drives P2Y<sub>1</sub>R to an active state similar to the active X-ray structures of P2Y<sub>1</sub>R and rhodopsin<sup>[43,44]</sup>. In addition, the largely opened G-protein-binding crevice allows for a G-protein to bind to the cytoplasmic surface of P2Y<sub>1</sub>R. In the active state, Tyr<sub>237</sub> reorients its side chain, thus allowing a hydrogen bond to form with the backbone oxygen of Val<sub>262</sub> (Supplementary Figure S7C). The χ<sub>1</sub> rotamer of Phe<sub>269</sub> changes to ±180° (i.e., the trans state) and is almost perpendicular to helix VI (Supplementary Figure S7D).

In the apo-P2Y<sub>1</sub>R/MRS2500-P2Y<sub>1</sub>R system, the helix III-helix VI distance is 7.5/7.8 Å in the beginning of the aMD simulation (0 to 50 ns in Supplementary Figures S8A and 0 to 10 ns in Supplementary Figure S9A), which is very close to the distance in the inactive crystal structure. This distance increases to 11.3/10.3 Å and is maintained for the remaining trajectory. In the aMD simulation, the hydrogen bond between the hydroxyl oxygen of Ser<sub>146</sub> and the hydroxyl oxygen of Tyr<sub>237</sub> is closed, with an O–O distance of 3.4 Å (Supplementary Figures S8B and S9B). The hydroxyl oxygen of Tyr<sub>237</sub> and the backbone oxygen of Val<sub>262</sub> cannot form a hydrogen bond (Supplementary Figures S8C and S9C). The χ<sub>1</sub> rotamer of Phe<sub>269</sub> is always in the gauche− state (Supplementary Figures S8D and S9D). These observations revealed that P2Y<sub>1</sub>R remained in the inactive state during the aMD simulations of the apo-P2Y<sub>1</sub>R and the MRS2500-P2Y<sub>1</sub>R systems.

Thus, the activation/inactivation mechanisms of P2Y<sub>1</sub>R after binding with 2MeSADP/MRS2500 can be summarized as follows. Binding of agonist 2MeSADP to P2Y<sub>1</sub>R leads to a stretching of the bend angle of helix VI by 19°. Consequently, the cytoplasmic end of helix VI shifts outward by 9.1 Å from helix III, thus activating P2Y<sub>1</sub>R for G-protein binding. Tyr<sub>237</sub> reorients, thereby breaking the hydrogen bond with Ser<sub>146</sub> and forms a new hydrogen bond with Val<sub>262</sub>. The χ<sub>1</sub> rotamer of Phe<sub>269</sub> changes from parallel to perpendicular to helix VI. In comparison, the binding of the antagonist MRS2500 blocks the bend angle of helix VI and locks P2Y<sub>1</sub>R in its inactive state.

Conclusion

In the present study, we identified a new agonist-binding site and explored the activation mechanism of P2Y<sub>1</sub>R. The identified 2MeSADP binding site is much deeper than that in the crystal MRS2500-P2Y<sub>1</sub>R structure and the previous simulated ADP-P2Y<sub>1</sub>R structure but partially overlaps with the corresponding 2MeSADP binding site in the 2MeSADP-P2Y<sub>1</sub>R crystal structure. 2MeSADP interacts with His<sub>132</sub>, Lys<sub>280</sub>, Tyr<sub>136</sub>, Thr<sub>221</sub>, Thr<sub>225</sub>, Arg<sub>128</sub>, Arg<sub>287</sub>, Arg<sub>310</sub>, Tyr<sub>306</sub> through π−π stacking, π−cation interaction, hydrogen bonds and salt bridges. This binding mode is consistent with previous mutagenesis data. Binding of the agonist 2MeSADP to P2Y<sub>1</sub>R leads to stretching of the bend angle of helix VI by 19° and a significant outward shifting of the helix VI cytoplasmic end. The activation of P2Y<sub>1</sub>R is also characterized by the breaking of the Ser<sub>146</sub>-Tyr<sub>237</sub> hydrogen bond, the formation of the Tyr<sub>237</sub>-Val<sub>262</sub> hydrogen bond and a χ<sub>1</sub> rotamer change of Phe<sub>269</sub> (from parallel to perpendicular to helix VI). In contrast, binding of the antagonist MRS2500 locks P2Y<sub>1</sub>R in its inactive state. The newly identified agonist-binding site and the activation mechanism P2Y<sub>1</sub>R revealed in this work should provide assistance in the design of potent P2Y<sub>1</sub>R antagonists and agonists, which might be used as antithrombotic and antidiabetic drugs.

Methods

System preparation. Three simulation systems were set up, including 2MeSADP-P2Y<sub>1</sub>R, apo-P2Y<sub>1</sub>R and MRS2500-P2Y<sub>1</sub>R. The MRS2500-P2Y<sub>1</sub>R structure was extracted from Protein Data Bank crystal structures (PDB ID: 4XNW)<sup>[45]</sup>. T4 lysozyme and unnecessary small molecules were removed from this crystal structure. The missing residues were constructed by homology modelling using the Modeller module of CHIMERA<sup>[46]</sup>. The proteinization state for titratable residues at neutral pH were determined using H++<sup>[45]</sup>. The apo-P2Y<sub>1</sub>R structure was prepared by removing MRS2500 from the MRS2500-P2Y<sub>1</sub>R complex. The 2MeSADP-P2Y<sub>1</sub>R structure was constructed by consulting the 2MeSADP-P2Y<sub>1</sub>R crystal structure<sup>[45]</sup>. In addition to the MRS2500-binding site, there was a deeper cavity in the MRS2500-P2Y<sub>1</sub>R structure that partially overlapped with 2MeSADP in the 2MeSADP-P2Y<sub>1</sub>R structure. However, this cavity was too small to accommodate 2MeSADP. The ECL2 domain extended deep into the ligand-binding pocket and blocked the access of 2MeSADP to the deep cavity. Thus, we removed Asp<sub>204</sub>, Thr<sub>205</sub>, Thr<sub>206</sub> and Ser<sub>207</sub> of ECL2 to open the access to this deep cavity. To enlarge the deep cavity to accommodate 2MeSADP, we also mutated Thr<sub>20</sub><sup>232</sup> and Ile<sub>186</sub><sup>232</sup> to alanines. Then, 2MeSADP was docked into this deep cavity by using the Schrödinger Induced Fit Docking protocol<sup>[46]</sup>. Then, the Asp<sub>204</sub>, Thr<sub>205</sub>, Thr<sub>206</sub> and Ser<sub>207</sub> of ECL2 residues were added back by homology modelling using the Modeller module of CHIMERA<sup>[44]</sup> and residues Ala<sub>205</sub><sup>232</sup> and Ala<sub>186</sub><sup>232</sup> were mutated back to threonine and isoleucine.
The CHARMM-GUI Membrane builder was used to construct the membrane-lipid systems. The transmembrane helical bundle of P2Y1R was oriented along the Z-axis of the POPC bilayer and the overlapping lipid molecules were removed. Then, the P2Y1R-bilayer complexes were neutralized at 0.15 M KCl and were solvated in TIP3P water boxes. The final simulation systems of 2MeSADP-P2Y1R, apo-P2Y1R and MRS2500-P2Y1R consisted of 67498, 64172 and 67132 atoms.

Molecular dynamics simulations. The cMD and aMD simulations in the present study were performed using the PMEMD module of AMBER 14. The AMBER FF99SB force field was used for P2Y1R, the general AMBER force field (GAFF) was used for 2MeSADP and MRS2500, and the amber lipid force field LIPID14 was used for POPC. A series of minimizations were carried out for each system (i.e., 2MeSADP-P2Y1R, apo-P2Y1R and MRS2500-P2Y1R). First, the waters were minimized for 10000 steps, and the P2Y1R, ligand and POPCs were constrained with 500 kcal mol\(^{-1}\) Å\(^{-2}\). Second, the waters and the POPCs were minimized for 20000 steps, and P2Y1R and the ligand were constrained with 500 kcal mol\(^{-1}\) Å\(^{-2}\). Third, the whole system was released and minimized for 10000 steps. Then, each system was heated from 0 K to 310 K in 700 ps with a Langevin thermostat, and the P2Y1R, ligand and POPCs were constrained with 10 kcal mol\(^{-1}\) Å\(^{-2}\). Then, each system was equilibrated for 200 ps with 10 kcal mol\(^{-1}\) Å\(^{-2}\) on P2Y1R, the ligand and the POPCs and for 5 ns with no constraints. After equilibration, the 100-ns cMD simulation was carried out in a constant pressure (NPT) ensemble for each system. Long-range electrostatic interactions was treated by using the particle mesh Ewald (PME) algorithm. All of the covalent bonds involving hydrogen atoms were constrained by using the SHAKE algorithm. To capture more obvious conformational changes involved in P2Y1R activation, three independent 300-ns aMD simulations were performed on the 2MeSADP-P2Y1R, apo-P2Y1R and MRS2500-P2Y1R systems by restarting from the last snapshot of the 100-ns cMD simulations.

Metadynamics simulation. The well-tempered metadynamics was performed using the AMBER 14 program and PLUMED 2.4a plugin after the 100 ns cMD simulation of the 2MeSADP-P2Y1R system. We used two collective variables, CV1 and CV2, to investigate the binding of 2MeSADP to P2Y1R. CV1 was the distance between the centers of mass (COM) of 2MeSADP and the seven transmembrane helical bundles of P2Y1R in the direction perpendicular to membrane (Z-direction). CV2 was the distance between the cytoplasmic ends of helix III and helix VI (represented by the Cα-Cα distance between Val153 and Leu254). The metadynamics was activated in CV1 and CV2 by depositing a Gaussian bias term every picosecond with height of 1 kJ mol\(^{-1}\) and width of 0.05 nm. The bias factor was 15, and the temperature was 310 K. To keep the 2MeSADP in contact with P2Y1R, we had enforced the XY component of the distance between COM of 2MeSADP and COM of the seven transmembrane helical bundles of P2Y1R below 3 nm.

Potential of mean force. We used potential of mean force (PMF) analyses and generated 2D energy landscapes to characterize the conformational changes of P2Y1R for each of the three simulated systems. The reaction coordinates of PMF map were the helix III-helix VI distance on the cytoplasmic side and the RMSD of the NPxxY motif. The energy landscape was calculated as:

\[
\Delta G(x, y) = -k_B T \ln p(x, y)
\]

in which \(k_B\) is the Boltzmann constant, \(T\) is the temperature, and \(g(x, y)\) is the normalized joint probability distribution.

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Author Contributions
D. L. designed the research, Y. L., C. Y. and P. L. performed the research; Y. L., C. Y. and D. L. analyzed the data; Y. L., C. Y., D. L. and J. L. were all involved in drafting, review and subsequent approval of the manuscript.

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