Calcium-release-activated calcium (CARC) channels are one of the major pathways of calcium entry in non-excitable cells. Despite a decade or two of research, its regulatory mechanism is not yet thoroughly understood. The slow progress is due to the complexity of its pores (i.e., Orai) on one hand and the difficulty in capturing its regulatory complex on the other hand. As a result, possible gating mechanisms have often been speculated by exploring the structure and properties of constitutive open mutants. However, there is much debate about how they can truly reflect the gating of CRAC channels under physiological conditions. In the present study, we combined molecular dynamics simulations with free energy calculations to study three dOrai mutants (G170P, H206A, and P288A), and further calculated their current-voltage curves. Results show that these constructs adopt different approaches to maintain their conductive state. Meanwhile they have unique pore structures and distinctive rectification properties and ion selectivity for cations compared to wild-type pores. We conclude that although the mutants may partially capture the gating motion characteristics of wild-type pores, the information obtained from these mutants is likely not a true reflection of CRAC channel gating under physiological conditions.

Key words: Calcium-release-activated calcium channel, Gating mechanism, Molecular dynamics simulations, Mutant, Free energy profile
that is activated by the Ca\(^{2+}\) sensor stromal interaction molecule (STIM). The CRAC channel is characterized by high Ca\(^{2+}\) selectivity and low conductance [3].

Orai is the pore subunit of the CRAC channel located on the plasma membrane (PM). The Drosophila Orai (dOrai) crystal structure, with the resolution of 3.35 Å, in the closed state provides an early exploration of its three-dimensional architecture [4], which adopts a hexameric configuration. It has four transmembrane helices (TM1–TM4) plus a cytoplasmic C-terminal extension (TM4-ext) [5]. The TM1 in Orai1 can be roughly divided into five parts (FIG. 1). The Ca\(^{2+}\)-accumulating region (CAR, D110, D112, and D114) at the extracellular entrance of the pore facilitates the attraction of Ca\(^{2+}\) cation and raises the local Ca\(^{2+}\) concentration [6]. The putative selectivity filter (SF) is a ring structure formed by six negatively charged E106 residues, a Ca\(^{2+}\) binding site that can regulate cation selectivity [7]. The hydrophobic region (L95, G98, F99, and V102) is the narrowest part of the pore [8], with a radius of \(\sim 3.8\) Å in the closed state [9]. The basic region (R83, K87, and R91) is considered as an electrostatic barrier for cation permeation [10] and also stabilizes Orai1 opening [11]. The extended transmembrane Orai1 N-terminal region (ETON) may have been involved in contacting with STIM [12]. STIM serves as a luminal Ca\(^{2+}\) sensor within the endoplasmic reticulum (ER). It experiences changes in conformation and oligomerization state upon the Ca\(^{2+}\) depletion in the ER luminal environment to expose a conserved STIM1 Orai activating region (SOAR). At the ER-PM junction, the SOAR domains tether and further activate Orai1. However, given that the average size of ER-PM junctions in adherent HeLa cells is 100–200 nm [13], it is difficult to study the Orai-STIM1 complex using structural biological methods. Therefore, to date, the understanding of how STIM binds to Orai and the gating mechanism of Orai is still limited [14].

The study of mutants, especially those that constitute conductivity in the absence of STIM1, by electrophysiological and molecular biology techniques and structural biology approaches, provides an alternative means to explore the gating mechanism of Orai.

G98 on TM1 of Orai1 (equivalent to G170 in dOrai) was proposed to serve as a gating hinge, switching Orai channel conformation between the closed and the open states [15]. The G98A mutant inhibits channel activation, whereas the G98D/P mutant causes constitutive channel activation and dilates the pore from top to bottom. However, G98 has both gain-of-function (GOF) and loss-of-function (LOF) mutations, where the G98C [15, 16] and G98R [17] are LOF mutants, and the G98D/P [15, 16] and G98S [18] are GOF mutants. The dual effect of these constructs makes the hinge mechanism elusive.

In the wild-type (wt) Orai1 pore, F99 (equivalent to F171 in dOrai) is a residue in the middle of the hydrophobic region with its side chain pointing to the pore axis. Current density measurements show that replacing F99 with hydrophilic residues causes Orai1 activation, while substitution with hydrophobic residues maintains its closed state even in the existence of STIM [16]. Molecular dynamics (MD) simulations show that, compared with the dOrai-wt pore, moderate rotation (\(\sim 20^\circ\)) of the TM1 helix and the resulting pore opening by moving the residues at position 171 away from the pore axis was observed in the dOrai-F171V/Y mutants. Therefore, other than the hinge mechanism [15], the TM1 rotation mechanism for pore activation has been proposed [16].

Mutations on V102 in Orai1 (equivalent to V174 in dOrai), a critical residue along the permeation path in the pore and just below the SF E178, have been extensively studied [18]. After replacing the valine residue in turn with the other 19 natural amino acids, it was found that the V102A mutant (and some others) led to a STIM1-independent constitutive Orai1 activation, as the bulky sidechain was replaced by a smaller one. Results from molecular dynamics (MD) simulations further revealed that this mutation increases pore hydration and decreases the free energy barrier for cation permeation without introducing a notable conformational change in the pore [10].

A relatively low resolution (6.7 Å) X-ray structure of dOrai-H206A mutant (equivalent to H134A in Orai1) was reported in 2018 [19]. The hydrophobic region of the Orai channel widens markedly in that construct, though the sidechain cannot be identified. The conducting is attributed to the expansion of the pore-forming TM1, as the sidechain of alanine is smaller than that of histidine on TM2. Independent MD simulations also found that the H206A mutant experiences a slight increase in the pore size at the hydrophobic regions, but the rotation of TM1 was not observed [20]. Very recently, the structure of the dOrai-H206A mutant was further refined using cryo-electron microscopy (cryo-
FIG. 1 The Orai pore and mutants. (A) Shown are two of the six subunits of the pore in diagonal positions, each with four transmembrane helices TM1–TM4. Subunits and pore-lining residues are shown in the cartoon and the CPK modes, respectively. Residues are colored according to their position and properties, and their equivalent numbers in Orai1 are shown in parentheses. (B) Three mutations at different TM helices of a subunit studied in this work. The dOrai-V174A mutant is also shown for comparison. The location where the mutation occurs is shown in the space-filling mode.

EM) at a 3.3 Å resolution [21]. It clearly shows that the F171 residue moves along with the rigidly outward motion of TM1, thereby increasing the exposure G170 residue (G98 in Orai1) to the central pore and breaking the hydrophobic packing. They concluded that the pore opening depends on the rigid body movement of TM1–TM4 helices rather than the rotation of the TM1 helix. Therefore, a pore-dilation model was proposed to explain the gating [21].

The dOrai-P288L mutant (equivalent to P245L in Orai1) structure, in which the wild-type pore is mutated on TM4, was determined by X-ray crystallography and cryo-EM [22]. There is no rotation of TM1 occurring in this structure and no significant expansion of the pore size in the hydrophobic region. Instead, this construct reveals an allosteric regulation from the peripheral TM4 helices to the pore-forming TM1, leading the basic region of the TM1 to twist its cytosolic side outwards and accommodating more anions to facilitate Ca$^{2+}$ permeation. Notably, several years before this experimental evidence was available, we had already used MD simulations to reveal the active role of counterions during the cation permeation through the Orai pore. Specifically, we found that Cl$^-$ ions accumulate in the basic region and potentially flow extracellularly to meet cations, form anion/cation pairs, and aid cation passage by reducing the energy expenditure of cations through the gating region of the pore [23].

The dOrai-L210F (Orai1-L138F) was found to yield a constitutively open pore in the absence of STIM1 [24]. Subsequent MD simulations show that TM2 in this mutant experienced a notable rotation so that the packing of residue at position 210 with TM1 was disrupted, thus allowing for the expansion of both the N- and C-terminus of TM1 [25]. They supported the pore-dilation mechanism for channel activation.

Although we have learned a lot about the mutants (and some more that have not been mentioned here [14]) that are constitutively open in the STIM-free condition, however, the relevance of this information to wild-type Orai gating under physiological conditions is unclear. For example, based on the lanthanide-acceptor energy transfer (LRET) measurements, Hogan et al. proposed that the Orai-V102X constructs cannot accurately reflect the channel gating under physiological conditions [26]. In addition, the microscopic processes associated with the latter binding to STIM and being further activated remain poorly defined, and the Orai-STIM complex still lacks structural information. Therefore, a systematic study of some representative mutants is necessary.

Previously, we used MD simulations to obtain a putative open state structure model of the dOrai-wt [27]. Our model suggests a twist-to-open mechanism to illustrate the activation of the Orai-wt, where the gating involves both the pore rotation and expansion. This model is strongly supported by evidence, including the free energy profile of the cation permeation through the pore, the rearrangement of key residues caused by the gating motion, the characteristic current-voltage ($I$-$V$) profile of the pore showing the inward rectification of the pore as well as the Ca$^{2+}$ selectivity, and relevant information from the literature such as, the reorientation of the residue at position 99 in the Orai1-F99C-V102A
mutant [16] or at position 102 in the Orai1-V102C mutant [26] upon binding with STIM, as well as the breaking of the tightly packed TM4-extension segments between neighboring subunits during the gating [28].

In this work, we further investigated the structure and dynamics of different mutants employing MD simulations in this work. Specifically, three constitutively open dOrai mutants, G170P on TM1 [15], H206A on TM2 [20], and P288A on TM4 [29] (equivalent to G98P, H134A, and P245A in Orai1, respectively), were studied. We compared them with the experimental observations and the putative open state model [27]. Although all three mutants are conducting, they each have their own characteristics. Seemingly, none of the information obtained from these mutants (including dOrai-V174A which we have previously studied [10, 23]) can characterize the full extent of the gating machinery of wild-type Orai, as evidenced from their distinct conformational events and calculated characteristic reversal potentials.

II. COMPUTATIONAL DETAILS

A. System setup

We followed the protocols used in previous works [10, 23, 27]. Briefly, six system models were constructed based on the crystal structure of the dOrai (PDB ID: 4HKR). Single point mutation was made using VMD mutator plugin [30] to generate the G170P, H206A, and P288A constructs. The structures were then embedded within a fully hydrated 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC) bilayer with 150 mmol/L NaCl.

B. MD simulations

Each system was treated with energy minimization, and then was equilibrated under harmonic restraints. The force constant of harmonic restraints started from 100 kcal/mol and gradually decreased to zero. Then MD simulations were performed for each system at 298 K with the isothermal-isobaric ensemble using NAMD 2.12 [31]. The CHARMM36 force field parameters [32] and the TIP3P water model [33] were used. Finally, each production simulation was conducted for 900–1000 ns.

C. Free energy calculations

Due to the well-known problems of the present force parameters of Ca$^{2+}$, we used Na$^+$ as the cation, as we did before [10, 23, 27]. The permeation free energy profile of cation through the dOrai-H206A pore was determined by using the adaptive biasing force (ABF) method in NAMD 2.12 [34].

The reaction coordinate (RC) was defined as the position of the cation in the pore (along the membrane normal direction), where RC=0 is the center of the lipid bilayer. The ABF calculation involved 23 windows with a bin width of 4 Å. A force constant of 100 kcal/mol was imposed at the boundary of each window. The total trajectory was 3.623 ms for the dOrai-H206A mutant to ensure that the free energy calculation was convergent.

D. Current-voltage (I-V) curve

The pore current of the pore in each construct was calculated using the Poisson-Nernst-Planck (PNP) model, which has been widely used to simulate the macroscopic transportation properties of ion channels [35]. More details about the PNP model can be found in our previous work [27]. Here, ten representative configurations of each construct were evenly extracted from MD simulations and used to generate the I-V curves by solving the steady-state PNP equations. The current was calculated at 200, 100, 50, 20, 10, 0, −10, −20, −50, −100, and −200 mV, respectively, for each snapshot. Finally, the calculated currents were averaged from different structures for each voltage.

III. RESULTS AND DISCUSSION

A. Stabilities of mutants

After sub-millisecond scale MD simulations, we analyzed the structural stability of three mutants, G170P, H206A, and P288A. The root-mean-square deviation (RMSD) of backbone atoms started from 100 kcal/mol and gradually decreased to zero. Then MD simulations were performed for each system at 298 K with the isothermal-isobaric ensemble using NAMD 2.12 [31]. The CHARMM36 force field parameters [32] and the TIP3P water model [33] were used. Finally, each production simulation was conducted for 900–1000 ns.

DOI:10.1063/1674-0068/cjcp2111231 ©2021 Chinese Physical Society
FIG. 2 RMSD of different constructs obtained from MD simulations. All the calculations used the backbone structure of the closed dOrai-wt crystal structure (4HKR.pdb) as the reference, except H206A27KR5 (light purple) and P288A6AKI (light orange), which used the backbone of the crystal structures of the corresponding mutants (7KR5.pdb for dOrai-H206A, and 6AKI.pdb for dOrai-P288L) as the reference. The data for WTopen, WTclosed, and V174A are adopted from our previous work [10, 27].

to the dOrai-wt is due to the fact that their initial structures were generated using homology modeling based on the structure template of dOrai-wt, and the pore underwent some degree of conformational change after structural relaxation in the present work.

B. The mutants use different ways to stay open

Data from electrophysiological experiments and structural biology indicate that all three mutants (G170P, H206A, and P288A) are in the conducting state. We calculated the pore sizes of different constructs obtained from the MD simulations (FIG. 3). Comparison with the dOrai-wt in the closed state [10] shows that the three mutants studied in this work, as well as the dOrai-wt in the putative open state structure [27], and the constitutively open dOrai-V174A mutant [10, 23] studied in previous work, have somewhat enlarged pore diameters but change in distinct ways.

The G170P construct, a mutation at the pore-lining helix TM1, is expected to introduce direct structure perturbation on the pore, and therefore alters the channel conductance and ion selectivity, as proline is a residue that produces helix bending [36]. Experimentally, the Orai1-G98P was reported to be a constitutively open channel without Ca$^{2+}$ selectivity [15]. Our MD simulations show that the G170P mutation in dOrai does induce hinge motion of TM1 by bending the TM1 helices and partially damages the rigidity of the selectivity filter structure at E178 (FIG. 4). The kink angle of TM1 in G170P is 24.4°±9.2°, which is consistent with statistical results of average kink angle of 21°±11° from the survey of 199 TM helices with proline residue at the central position [37]. In contrast, the averaged kink angle in the wild type dOrai-wt is 9.1° in crystal [4] and 14.0°±7.0° in MD simulations [10]. The kinking of the pore-lining helices TM1 leads to substantial expansion of the pore at the C-terminal side as well as the gating region (FIG. 4). Pore dilation at E178, the putative SF, is likely to affect the Ca$^{2+}$ binding at this region by promoting nonselective cation permeability and therefore accounts for the loss of Ca$^{2+}$ selectivity in Orai1-G98P.

Then, we looked at a mutant H206A, a mutation at TM2 that neighbors TM1. In dOrai-wt, TM1, TM2 and TM3 from the same subunit form a helix bundle. The imidazole ring of the H206 side chain orients to the center of the bundle and is tightly wrapped by S165 and L168 from TM1 and G255, L256, and F259 from TM3, where they form a close-packed hydrophobic core in the center. In contrast, the smaller side chain in the H206A mutant leaves space between TM1 and TM2 (FIG. 4). Consequently, TM1 has more freedom to move away from the central axis in response to the packing deficiency, and the induced kink angle of TM1 is 18.0°±4.3°. Consequently, pore dilation oc-

FIG. 3 The pore size profiles of different constructs obtained from MD simulations. As a comparison, the pore size of the closed dOrai-wt generated from MD simulations is also shown in each panel (grey dashed line). The shadow represents the fluctuation of the data. The data for WTopen, WTclosed, and V174A are adopted from our previous work [10, 27].

DOI:10.1063/1674-0068/cjcp2111231 ©2021 Chinese Physical Society
The distinct gating motions in three mutants (in color) with respect to the closed dOrai-wt (in grey). (A) the dOrai-G170P, with the mutation occurring on TM1. The kinking induced by proline residue causes pore expansion. (B) the dOrai-H206A, with the mutation occurring on TM2. The kinking induced by introducing proline residue causes pore expansion. The decrease in residue size triggers the arrangement of packing between TM1 and TM2, resulting in pore expansion. (C) the dOrai-P288A, with the mutation occurring on TM4. The reduced kinking of TM4 leads to the propagation of conformational change to the pore-forming TM1 and therefore opens the pore.

In contrast to the three mutants mentioned above, which all caused significant conformational changes, the dOrai-V174A we studied in previous work leads to only minor conformational changes, as evidenced by a slight increase in the pore size at the entrance to the hydrophobic region below the SF. This mutation increases the pore hydration and, therefore, increases the freedom of the pore water to aid in cation transport [10, 23].

C. The mutants show a less-confined pore environment

Ca$^{2+}$ cation ion transfer in the channel requires the assistance of water molecules [23], which are subject to confinement effects within the nano-scale Orai channel and thus affect ion transport [38, 39]. Our previous studies have shown that the orientation distribution of water in the pore can indirectly characterize the channel's activation. Specifically, there are three distinct distribution domains of water molecules within the pore: the SF, the hydrophobic region, and the basic region (FIG. 5). In the closed dOrai-wt, the water molecules within the pore tend to be arranged in an orderly fashion, while in the open state (including the putative open dOrai-wt as well as constitutively conducting construct dOrai-V174A), the water molecules are more likely to change their orientation (especially in the hydrophobic region), thus assisting the passage of cations [10].

A similar scenario is presented for the three mutants studied in this work: the relatively large pore size...
FIG. 5 Distribution of pore water orientation in different constructs obtained from MD simulations. The concerted water dipole is stronger in the closed dOrai-wt than in different open states, including open dOrai-wt and dOrai-mutants, suggesting that waters in the open pore have more freedom to reorient, thus facilitating ion transport through the pore. The data for WT\textsubscript{closed}, WT\textsubscript{open}, and V174A are adopted from our previous work [10, 27].

D. The mutant is open for cation permeation

We further computed the free energy of the cation passing through the pore. Due to the substantial computational cost, we only selected the dOrai-H206A system to carry out ABF calculations. An entire 3.623 μs trajectories were accumulated to ensure the convergence of the generated free energy profile for this system.

Because the pore size of H206A is significantly larger than that in the closed dOrai-wt, the highest energy barrier throughout the permeation process is only \(~6\) kcal/mol, mainly from the basic region (FIG. 6). In contrast, the hydrophobic region in dOrai-H206A, the main gating region in dOrai-wt, shows no barrier to ion passage. Similarly, our previous calculations show that the free energy barrier for cation passage through the dOrai-V174A is also significantly reduced compared to the closed dOrai-wt, and the major barrier (\(~8\) kcal/mol only) is shifted to the basic region. Interestingly, the putative open dOrai-wt shows a different feature compared to the mutants: though the barrier
TABLE I. The reversal potential of different constructs reported in the literature by electrophysiological experiments.

<table>
<thead>
<tr>
<th>Structure</th>
<th>Reversal potential/mV</th>
<th>STIM1</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>20 Ca</td>
<td>DVF</td>
<td></td>
</tr>
<tr>
<td>WT</td>
<td>&gt;80 43.3±3.3</td>
<td>Without</td>
<td>[41]</td>
</tr>
<tr>
<td></td>
<td>+50</td>
<td>With</td>
<td>[41]</td>
</tr>
<tr>
<td></td>
<td>&gt;65</td>
<td>With</td>
<td>[42, 43]</td>
</tr>
<tr>
<td>V174A (V102A)</td>
<td>21.5±3.3 5.84±1.66</td>
<td>Without</td>
<td>[18, 44]</td>
</tr>
<tr>
<td></td>
<td>61.7±6.0 48.2±1.3</td>
<td>With</td>
<td>[18]</td>
</tr>
<tr>
<td></td>
<td>&gt;50</td>
<td>With</td>
<td>[44]</td>
</tr>
<tr>
<td>G170P (G98P)</td>
<td>0</td>
<td>Without</td>
<td>[15]</td>
</tr>
<tr>
<td>H206A (H134A)</td>
<td>32</td>
<td>Without</td>
<td>[45]</td>
</tr>
<tr>
<td></td>
<td>49.1±4.2</td>
<td>Without</td>
<td>[8]</td>
</tr>
<tr>
<td></td>
<td>59.9±3.3</td>
<td>With</td>
<td>[8]</td>
</tr>
<tr>
<td></td>
<td>40</td>
<td>With</td>
<td>[20]</td>
</tr>
<tr>
<td>P288A (P245A)</td>
<td>36.9±1.4</td>
<td>Without</td>
<td>[22]</td>
</tr>
</tbody>
</table>

height is also only ~8 kcal/mol, similar to the mutants and lower than that in the closed one, a broad free energy barrier is formed by the hydrophobic region and part of the basic region.

In any case, the free energy barriers in mutants H206A, V174A, and the putative open dOrai-wt are significantly lower than that of the closed dOrai (~11 kcal/mol), indicating that it is suitable for ion passage. However, the differences in the free energy profiles between the first three suggest that cation permeation processes through these constructs are distinct.

E. The mutants have characteristic electrophysiological properties

We calculated the $I-V$ curve of different mutants. The data for the putative open dOrai-wt are also shown for comparison (FIG. 7). The calculated $I-V$ curves show the inward rectification of the pore, which is a typical property of Orai1 channel to preferentially conduct current in the inward direction [40].

We further compared the calculated reversal potentials of each construct with their experimental values (Table I), and found a good correlation between them ($R^2$ of 0.682). For example, the Orai1-wt is highly Ca$^{2+}$ selectivity in the presence of STIM1, reflected by its relatively large reversal potential, as reported to be in the range of ~80 mV [41] to ~50 mV [8, 29, 33, 41, 46–48] from different experiments. Our calculated value on the putative open dOrai-wt structures with the PNP model is ~64 mV. On the other hand, the Orai1-V102A (equivalent to V174A in dOrai) was identified to disrupt the ion selectivity, and its experimental reversal potential is close to 0 [18]. Our computed data for dOrai-V174A is only ~20 mV, which is consistent with the experiments in terms of trend. In addition, the calculated reversal potentials for dOrai-G170P (~20 mV), dOrai-H206A (~45 mV), and dOrai-P288A (~58 mV) also correlate well with the experimental values. Thus, the above results indicate that the mutant structures
we obtained by calculation reproduce their electrophysiological properties well, and further demonstrate that the conducting states of the mutants are somewhat different from the wild-type case.

IV. CONCLUSION

In the present work, we systematically explored the structure, cation permeation, and electrophysiological properties of three constitutively open mutants, G170P, H206A, and P288A of dOrai, the pore of the CRAC channel. We found that G170P directly impacts the central pore, H206A regulates the pore by changing their packing, and P288A propagates the structural dynamics between the distal segments. This suggests that even though these mutants partially capture the features of the wild-type Orai in the open state, their conformational changes could be different from the gating motion of the wild-type pore triggered by STIM under physiological conditions. We further extend this argument to a more general conclusion that the G98P, H134A, and P245A mutants are not faithful models for the gating of wild-type Orai1 triggered by STIM1.

V. ACKNOWLEDGMENTS

This work was supported by the National Natural Science Foundation of China (No.21771435, and No.22073110), the Natural Science Foundation of Jiangsu Province (No.BK20190056), and the Fundamental Research Funds for the Central Universities (021514380018). Parts of the calculations were performed using computational resources on an IBM Blade cluster system from the High-Performance Computing Center (HPCC) of Nanjing University.