

On the Cryo-EM Conformational Observations of Kv2.1 Channels: A Discussion on the Tetrameric Cooperative Hinge-Sliding Model and the Optimal Origami-Windmill Rotational Gating Hypothesis

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Abstract

Mandala & MacKinnon published work in PNAS in 2025, in which cryo-electron microscopy (cryo-EM) was employed to capture multiple conformations of the human Kv2.1 potassium channel. They proposed a hinge-sliding gating scenario featuring stepwise cooperative downward displacement of voltage-sensing domains (VSDs): the pore remains permeable when two VSDs undergo displacement, and pore closure occurs only after all four VSDs are fully positioned. The study incorporated these conformational patterns into the S4 “paddle model” framework and attempted to generalize observations obtained from vesicle systems as the fundamental universal mechanism for voltage-gated potassium channels and even neuronal bioelectrical generation.

This paper fully acknowledges the authenticity of the original cryo-EM conformational data reported in that study. Nevertheless, it demonstrates that the reconstituted vesicle system is an artificially constructed unidirectional hyperpolarization-limit system containing only Kv2.1. The -40 mV and -145 mV potentials within the system are drift potentials generated by outward K^+ efflux along the concentration gradient without counterbalancing sodium ions. The potential-formation logic and ionic balance relationships differ from the physical boundary conditions of physiological action potentials with bidirectional Na^+ - K^+ coupling and oscillating potentials in native squid giant axon membranes. Meanwhile, the classical interpretation of high-frequency axial sliding of S4 encounters mechanical-dissipation difficulties originating from nanoscale viscous damping. The original paper itself contains uncertainties such as insufficient resolution of the closed-state structure and unclear VSD behaviors under strong hyperpolarization.

To distinguish observations from artificial systems from native physiological rules, this paper designs falsifiable control experiments to test whether the stepwise pattern “pore remains open upon displacement of two VSDs” can be reproduced in native excitable membranes. Under existing physical, energetic and physiological constraints, this paper further argues that the origami-windmill impeller rotational gating represents the optimal mechanical solution under the set of constraints. This model intrinsically separates independent ion-entry portals and sphincter-style exit orifices, enabling ordered passage of sodium and potassium ions through distinct pathways without enforced co-entry and co-exit. Such rotational mechanism can further echo the topological logic of DNA double-strand pairing, providing a unified framework for thinking about shared underlying topological rules of coiling, engagement and phase-matching between ion channels and genetic macromolecules. The

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windmill hypothesis offers an alternative path for subsequent experimental verification in investigations of voltage-gated channel mechanisms.

Keywords: Kv2.1 potassium channel; cryo-EM; tetrameric cooperativity; origami-windmill model; sphincter-style exit orifice; ion shunting; squid giant axon; DNA topological pairing

1 Introduction

Voltage-gated potassium channels are core components underlying the generation and propagation of nerve impulses. According to the classical paradigm established by MacKinnon, channel selectivity is achieved by the atomic filter within the pore; voltage sensing relies on axial sliding of the S4 segment along the membrane normal; pore opening and closing are regulated by hinge bending of S6. This paddle model has dominated structural interpretations in this field over the past two decades.

In their 2025 PNAS work, Mandala & MacKinnon captured multiple intermediate conformations of Kv2.1 using cryo-EM and supplemented structural evidence for strong cooperative coupling among tetrameric subunits: the fully open state upon depolarization, permeable intermediate states with partial VSD displacement, and the closed state with all four subunits in place^[1]. The authors sought to explain the functional properties of Kv2.1 via the stepwise-cooperative mechanism of hinge-sliding and further extend it toward general interpretations of neuronal bioelectricity.

Cryo-EM reconstituted vesicles constitute a standard tool for membrane-protein research. The sample preparation, data collection and structural-refinement workflows in that paper are well-standardized, with publicly archived EMDB and PDB depositions; this paper respects the factual observations. The core point of discussion herein is not the structural images themselves, but the logical leap from static conformational snapshots obtained in unidirectional vesicle systems to mechanistic extrapolations for bidirectional action potentials in living neurons. This paper reconstructs the temporal evolution of potentials in vesicle preparations, analyzes mechanical weaknesses of the reciprocating-sliding model, proposes falsifiable verification experiments for native membranes, puts forward the origami-windmill optimal hypothesis under multiple constraints, and relates it to homologous topological ideas of DNA pairing.

2 Re-examining the experimental system: -40 mV and -145 mV inside vesicles represent unidirectional hyperpolarization-limit processes and do not equate to physiological dynamic potentials

2.1 Potential Evolution and Boundary Conditions of the Vesicle System

According to the Materials and Methods of the original paper: Kv2.1 was expressed and purified in HEK293S cells and reconstituted into vesicles mixed with POPC/POPG/cholesterol lipids. Vesicles were pre-loaded with high- K^+ internal solution. Valinomycin was added and incubated on ice for 30 min for insertion into vesicle membranes to mediate K^+ transmembrane diffusion. After incubation for carrier insertion, both intra- and extra-vesicular compartments contained high K^+ with no net ionic flux. External solution was rapidly exchanged to 300 mM NaCl using Zeba desalting columns, leaving very low residual extravesicular K^+ and instantly establishing a large transmembrane K^+ concentration gradient that initiated unidirectional K^+ efflux. Samples were immediately frozen after buffer exchange to capture corresponding conformations^[1].

Valinomycin is a lipid-soluble potassium ionophore without active-pump activity; ion direction is entirely determined by K^+ concentration gradients. Three non-negotiable boundary conditions govern this system:

- (1) Only reconstituted Kv2.1 channels reside in vesicle membranes; no sodium channels, calcium channels or other cation pathways are present. The background leakage rate of hydrated Na^+ across lipid bilayers is extremely low, so Na^+ influx contributes negligibly within the cryo-sampling time window.
- (2) The driving force is exclusively unidirectional outward K^+ flux with constant potential direction. Immediately after buffer exchange the potential approximates 0 mV. As K^+ continuously flows outward, positive intravesicular charge is depleted and the potential drifts persistently toward hyperpolarization.
- (3) Potential evolution follows only a unidirectional descending branch. There exists no depolarizing rebound driven by Na^+ influx, making it impossible to recapitulate the rise-and-fall cyclic behavior of action potentials.

During this drifting process the potential passes -40 mV, corresponding to the reported intermediate permeable conformation: two VSDs shift downward while the pore remains open for sustained K^+ efflux. At -145 mV, close to the Nernst equilibrium potential for K^+ , net K^+ efflux ceases, all four subunits become properly positioned and the pore constricts and closes. By controlling incubation duration and freezing timing, the authors captured distinct snapshots along this unidirectional decay trajectory. These snapshots, however, belong solely to a unidirectional decaying process rather than physiologically bidirectional cyclic potentials.

2.2 Same potential readouts but distinct physical meanings: two systems with different underlying physics

In native excitable membranes of squid giant axons: resting potential ranges from -60 mV to -80 mV. Upon stimulation sodium channels open, Na^+ influx depolarizes the membrane toward $+40$ mV. Subsequently sodium channels inactivate, potassium channels activate, K^+ efflux repolarizes the membrane. The full cycle relies on alternating counterbalancing of Na^+ and K^+ ionic currents^[2]. Each potential value represents a dynamic equilibrium resulting from opposing ionic flows of the two ion species.

By contrast, within the vesicle system only outward K^+ flow exists with no counterbalancing sodium return pathway. Despite identical numerical readings of -40 mV or -145 mV, ionic compositions, driving directions and equilibrium conditions are fundamentally different. Conformation-potential relationships obtained from vesicles cannot be directly transferred to interpret bioelectrical events in living neurons.

3 Intrinsic limitations of the stepwise-cooperative hinge-sliding model: internal consistency does not guarantee universality and hard constraints arise from mechanical dissipation

3.1 Inherent Limitations in the Applicable Scope of the Model

The S4-sliding plus hinge-bending interpretative framework developed by MacKinnon's group exhibits internal logical consistency within its own paradigm and has been widely cited across decades^[3,4]. Nevertheless, two inherent weaknesses persist. First, as acknowledged in the original paper, closed-state structural resolution remains insufficient, and tetrameric VSD cooperative behavior under strong hyperpolarization is unknown. Only intermediate states under moderate hyperpolarization were obtained; structural evidence for extreme-potential regimes is missing, naturally restricting the scope for extrapolation of conclusions. Second, all conformational snapshots were collected along a unidirectional hyperpolarization trajectory; the system cannot achieve depolarization or potential reversal. Reliance merely upon static snapshots along one trajectory to support dynamics for bidirectional action-potential cycles constitutes excessive extrapolation.

3.2 Viscous-damping and energetic constraints for the S4-sliding hypothesis

The paddle-sliding hypothesis requires reciprocating axial motion of S4 segments to match high-frequency neuronal firing ranging from tens to hundreds of hertz. Displacement of S4 is approximately 5 Å, yielding a round-trip travel distance of 10 Å for Stokes-law viscous-drag estimation:

Equivalent spherical radius of S4: $r \approx 0.5$ nm

Lipid-phase viscosity: $\eta \approx 0.1$ Pa·s

Frequency: $f = 100$ Hz, peak velocity: $v = 2df$

The calculated work per sliding cycle is about 1.9×10^{-20} J, with continuous power dissipation around 1.9×10^{-18} W. Individual Na⁺/K⁺-ATPase pumps operate within a power range of $10^{-17} \sim 10^{-16}$ W. Even adopting more optimistic geometric parameters, the order-of-magnitude energy cost from viscous dissipation remains unchanged.

Note: the estimation uses an equivalent-sphere simplification. Even if real S4 geometry reduces drag, the order-of-magnitude constraint holds robustly.

Original-paper structural figures show small global displacements of the S6 backbone, with deformation localized to hinge bending at the PXP motif. To date, all cryo-EM snapshots represent static captures at discrete time points; no direct dynamic imaging demonstrates sustained reciprocating back-and-forth sliding of S4^[1,5]. One must distinguish static unidirectional displacement signals from high-frequency reversing reciprocating motion. Each reversal of reciprocating movement must overcome viscous drag and repeatedly bend the protein backbone, progressively accumulating mechanical stress fatigue. Neurons must operate stably for decades. Repeated millisecond-scale reciprocating deformations would readily induce mechanical failure of protein backbones over long time scales. Dominant existing structural models, including paddle-sliding, flip-gate and traditional pore-plug hypotheses, are all built upon reciprocating deformation and share this fundamental mechanical shortcoming.

4 Experimental proposal for testing the VSD stepwise-cooperativity hypothesis in native squid-giant-axon membranes

The core goal is to test whether the central proposition “pore is permeable upon displacement of two VSDs; pore closes once all four VSDs are positioned” can be reproduced within native excitable-membrane environments. Experimental preparation: isolated squid giant axon^[2]. Two sets of signals are acquired simultaneously: potassium currents recorded under voltage-clamp and dynamic conformational imaging of fluorescently-labeled VSDs (Tab. 1).

Table 1 Experimental design for verifying the VSD stepwise-cooperativity hypothesis in native squid giant-axon membranes

Group	Extracellular solution	Prediction from hinge-sliding model	Falsification expectation	Observed variables
Exp. 1	Artificial seawater with normal $[Na^+]$	During hyperpolarization, potassium pore remains open upon displacement of two VSDs; pore closes once four VSDs are positioned	Pore closes substantially upon displacement of two VSDs in native membranes; stepwise-cooperativity pattern disappears	Fluorescent conformational signals, channel open probability, current time courses
Exp. 2	Low-sodium extracellular solution to weaken Na^+ influx	Vesicle-derived stepwise-cooperativity pattern persists stably	Sodium counter-flux is necessary for the stepwise phenomenon; cooperative signatures are altered under low-sodium conditions	Steady-state potential, conformational time courses, current amplitudes

Judging logic: stable reproduction of the stepwise pattern in native membranes supports physiological reality for the hinge-sliding cooperative mechanism. Disappearance of such stepwise signatures in native membranes would indicate that the phenomenon represents a conditional effect specific to sodium-free unidirectional vesicle systems and cannot be generalized to living neurons.

5 Discussion: inherent limitations of static structural snapshots and extrapolation pitfalls in structural biology

Cryo-EM is a powerful static-structure tool capable of capturing stable conformations at energy minima. It cannot directly record continuous dynamic trajectories between conformations. Chen & Frank (2016) pointed out that static snapshots intrinsically lack temporal information, constituting a general methodological limitation within structural biology^[6].

Lipid-reconstituted vesicle systems offer cleanliness and controllable variables at the cost of stripping away native membrane microenvironments: sodium channels, calcium channels, intracellular cytoskeleton and accessory regulatory membrane proteins are all absent. Rules obtained from artificially simplified systems may well represent system-specific phenomena rather than universal cellular mechanisms. This paper explicitly states: we do not negate the structural observations reported by Mandala et al.^[1]; we oppose direct elevation of conformation-relationship findings from simplified systems to universal mechanisms explaining action potentials.

6 Conclusions

- (1) The stepwise-conformation findings for Kv2.1 from Mandala et al. apply only to sodium-free unidirectional vesicle systems and cannot be directly extrapolated to bidirectional action potentials in squid giant axons^[1,2];
- (2) The S4 reciprocating-sliding hypothesis faces hard physical constraints from viscous-energy dissipation and long-term stress fatigue;
- (3) Under multiple physical constraints, unidirectional-rotation origami-windmill model represents an optimal solution^[7]: separate entry portals plus sphincter-style exits realize sodium-potassium ion shunting without enforced co-entry-co-exit;
- (4) Impeller-coiling and phase-matching topology of the windmill is homologous with DNA double-strand base-pairing mechanisms, forming unified molecular topological logic;
- (5) This hypothesis still lacks direct dynamic-imaging evidence and can be falsified and tested by combined electrophysiology and fluorescent conformational imaging experiments on native squid giant-axon membranes.

7 Alternative mechanical picture: origami-windmill impeller rotational-gating hypothesis

The above conclusions define the boundary of applicability for the stepwise-model. Having questioned one mechanistic framework, it is natural to ask whether there exists a dynamic pathway for voltage-gated potassium-channel gating simultaneously satisfying mechanical, energetic and long-term-physiological-stability constraints.

As shown by the preceding mechanical-dissipation analysis, classical schemes relying on S4 axial reciprocating sliding, repeated hinge bending or periodic protein flipping encounter intractable contradictions. High lipid-bilayer viscosity generates persistent damping losses for reversing reciprocating motion; each reversal imposes opposite stresses on protein subunits, continuously accumulating fatigue damage. For neurons to sustain stable function across decades, millisecond-scale repeated bending and pushing-pulling would progressively damage protein backbones and render long-term steady-state operation difficult. Major existing structural models, including MacKinnon's S4 paddle-sliding model, Yan's flip-gate model and traditional pore-plug hypotheses, are all built upon reciprocating deformation and share this fundamental physical limitation^[3,4].

The origami-windmill rotational-gating hypothesis advanced herein is rooted in the landmark 1998 KcsA potassium-channel atomic-structure determination by MacKinnon — work forming the core foundation for his 2003 Nobel Prize in Chemistry^[3,8]. We accept well-established structural facts established by MacKinnon including tetrameric

pore architecture and potassium-selectivity filters. We merely challenge the S4-axial-sliding dynamic interpretation subsequently derived from those structures and perpetuated in recent Kv2.1 studies, and propose a mechanically superior circumferential-rotation mechanism within that same atomic-structural framework.

Based on the hard constraints outlined above, the origami-windmill impeller-rotation hypothesis is proposed: four Kv-channel subunits assemble into circumferentially-arranged windmill-like impeller blades. Driven by transmembrane electric-field potential energy, the impeller undergoes continuous, smooth unidirectional rotation around the central pore. Aperture modulation is achieved by rotation-phase and tilt-angle of impeller blades. Within the full set of pre-existing constraints, this rotational scheme constitutes an optimal constrained solution. The windmill architecture intrinsically establishes physically independent ion-entry portals and distal sphincter-style exit orifices rather than sharing one single aperture. Sodium and potassium ions travel along distinct pathways with no mandatory co-entry-co-exit of ions. Impeller rotation fulfills dual regulatory roles: blade tilt dynamically selects ions and modulates entry-portal opening; rotation phase synchronously controls sphincter-exit gating timing to decouple ion uptake and release and prevent mixing and counter-flux of ions. By contrast, the hinge-sliding model features tightly coupled entry-exit pathways. To achieve sodium-potassium shunting it must superimpose multiple additional assumptions such as atomic filters and specific binding sites, violating Occam's razor. The windmill model accomplishes voltage sensing, aperture tuning, ion sorting and temporal shunting simultaneously through one continuous rotating structure with fewer assumptions, while satisfying multiple requirements including low mechanical dissipation and fatigue resistance.

Addressing a likely reviewer objection: why employ static cryo-EM snapshots to critique the sliding model yet also invoke static snapshots in support of the rotational hypothesis? There exists no double standard here. The sliding hypothesis requires periodic reciprocating back-and-forth movement to complete open-close cycles; reversal events themselves incur high energy costs and fatigue damage and directly conflict with foregoing mechanical constraints. The windmill hypothesis, by contrast, assumes continuous unidirectional circumferential rotation without reversal. Static images cannot capture dynamic processes directly. We do not infer movement modes solely from individual static structures. Instead we interpret static conformations within the full constraint-space including mechanical dissipation, long-term protein stability, ion-shunting requirements, Occam's razor and topological self-consistency. Static snapshots represent instantaneous frames captured at distinct phases along a rotation cycle.

Such coiling and phase-matching topological logic can be further compared with DNA double-strand-pairing mechanisms. DNA accomplishes molecular recognition via double-helix coiling and base-pair phase matching. Similarly, windmill impellers achieve ion recognition, screening and permeation through blade-coiling configurations and rotational phases. Both share core organizational principles of helical coiling, phase encoding and topological engagement, offering mutually referential unified topological perspectives for molecular behaviors of ion channels and genetic macromolecules.

This hypothesis strictly follows nanomechanics, energy-budget and long-term cellular-homeostasis criteria. It should be emphasized clearly that this paper only presents a self-consistent, constraint-favored interpretative framework. Direct single-molecule dynamic-imaging evidence remains absent. Further validation is needed via time-resolved cryo-EM, high-speed fluorescence imaging and mutagenesis-based electrophysiology. Compared with long-standing reciprocating-deformation hypotheses, the origami-windmill model intrinsically avoids reversal-associated stress-fatigue and reduces energy consumption, matching physiological demands for lifelong stable neuronal operation. It provides a testable alternative idea for mechanistic investigations of voltage-gated-channel function.

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