

Structural mechanisms of PIP₂ activation and SEA0400 inhibition in human cardiac sodium-calcium exchanger NCX1

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NCX1 is an **important** cardiac Ca²⁺/Na⁺ exchanger whose activity is tightly regulated. This manuscript describes the structural basis of activation by the lipid PIP₂ and inhibition by binding of a small molecule to NCX1. These results provide key insights into NCX1 regulation and cellular Ca²⁺ signaling, but the evidence presented is still **incomplete**.

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Abstract

Na⁺/Ca²⁺ exchangers (NCXs) transport Ca²⁺ across the plasma membrane in exchange for Na⁺ and play a vital role in maintaining cellular Ca²⁺ homeostasis. Our previous structural study of human cardiac NCX1 (HsNCX1) reveals the overall architecture of the eukaryotic exchanger and the formation of the inactivation assembly by the intracellular regulatory domain that underlies the cytosolic Na⁺-dependent inactivation and Ca²⁺ activation of NCX1. Here we present the cryo-EM structures of HsNCX1 in complex with a physiological activator phosphatidylinositol 4,5-bisphosphate (PIP₂), or pharmacological inhibitor SEA0400 that enhances the inactivation of the exchanger. We demonstrate that PIP₂ binding stimulates NCX1 activity by inducing a conformational change at the interface between the TM and cytosolic domains that destabilizes the inactivation assembly. In contrast, SEA0400 binding in the TM domain of NCX1 stabilizes the exchanger in an inward-facing conformation that facilitates the formation of the inactivation assembly, thereby promoting the Na⁺-dependent inactivation of NCX1. Thus, this study reveals the structural basis of PIP₂ activation and SEA0400 inhibition of NCX1 and provides some mechanistic understandings of cellular regulation and pharmacology of NCX family proteins.

Introduction

Sodium-calcium exchangers (NCX) are transporters that control the flux of Ca^{2+} across the plasma membrane and play a vital role in maintaining cellular calcium homeostasis for cell signaling (Berridge et al., 2003; Blaustein and Lederer, 1999; Clapham, 2007; DiPolo and Beauge, 2006; Ottolia et al., 2021; Philipson and Nicoll, 2000). NCXs facilitate the exchange of 3 Na^+ for 1 Ca^{2+} in an electrogenic manner, primarily responsible for extruding Ca^{2+} from the cytoplasm. However, this process can be reversed to permit Ca^{2+} entry, depending on the chemical gradients of Na^+ and Ca^{2+} , as well as the membrane potential (Blaustein and Lederer, 1999; Blaustein and Russell, 1975; Hilgemann et al., 1991; Kang and Hilgemann, 2004; Kimura et al., 1986; Matsuoka and Hilgemann, 1992; Rasgadoflores and Blaustein, 1987; Reeves and Hale, 1984). Three NCX isoforms (NCX1-3) are present in mammals, with each isoform bearing multiple splice variants expressed in distinct tissues, thereby modulating numerous fundamental physiological events (Dyck et al., 1999; Kofuji et al., 1994; Lee et al., 1994; Linck et al., 1998; Lytton, 2007; Philipson and Nicoll, 2000; Quednau et al., 2004). Dysfunctions of NCXs are associated with a plethora of human pathologies including cardiac hypertrophy, arrhythmia, and postischemic brain damage (Blaustein and Lederer, 1999; Matsuda et al., 1997; Pott et al., 2011; Watanabe et al., 2006). The cardiac variant NCX1.1 has been extensively studied, with its function playing a central role in cardiac excitation and contractile activity (Bridge et al., 1990; Kimura et al., 1987; Ottolia et al., 2013; Scranton et al., 2024; Shigekawa and Iwamoto, 2001).

The eukaryotic NCX consists of a transmembrane (TM) domain with 10 TM helices and a large intracellular regulatory domain between TMs 5 and 6 (Dong et al., 2024; Hilgemann, 1990; Matsuoka et al., 1993; Philipson and Nicoll, 2000; Ren and Philipson, 2013; Sharma and O'Halloran, 2014; Xue et al., 2023). The TM domain is responsible for the ion exchange function in NCX. It consists of two homologous halves (TMs 1-5 and TMs 6-10) with TMs 2-3 and TMs 7-8 forming the core of the TM domain and hosting the residues that coordinate the transported Na^+ and Ca^{2+} (Liao et al., 2012; Liao et al., 2016). The large regulatory domain contains two calcium-binding domains (CBD1 and CBD2). Ca^{2+} binding at CBDs enhances NCX activity and rescues it from the Na^+ -dependent inactivation, a process that manifests as slow decay of the exchange current due to elevated levels of cytosolic Na^+ ions (Hilge et al., 2006; Hilgemann, 1990; Hilgemann et al., 1992a; Hilgemann et al., 1992b; Matsuoka and Hilgemann, 1992, 1994; Matsuoka et al., 1995; Matsuoka et al., 1993; Ottolia et al., 2009). A stretch of residues known as XIP region (eXchanger Inhibitory Peptide) at the N terminus of the regulatory domain plays a pivotal role in the Na^+ inactivation process (Li et al., 1991; Matsuoka et al., 1997).

Several other cellular cues can also modulate NCX1 activity, including phosphatidylinositol 4,5-bisphosphate (PIP_2) in the membrane. PIP_2 has been shown to stimulate the NCX1 activity by reducing the Na^+ -dependent inactivation and the XIP region was suggested to participate in the PIP_2 activation (He et al., 2000; Hilgemann and Ball, 1996; Hilgemann et al., 1992a; Yaradanakul et al., 2007). In addition, several small molecule NCX inhibitors have been developed to provide valuable tools for studying the physiological and pharmacological properties of NCXs, among which compound SEA0400 is a highly potent and selective NCX1 inhibitor that promotes the Na^+ -dependent inactivation of the exchanger (Bouchard et al., 2004; Iwamoto et al., 2004; Lee et al., 2004; Matsuda et al., 2001; Tanaka et al., 2002; Watanabe et al., 2006; Watano et al., 1996).

We previously determined the human cardiac NCX1 structure in an inward-facing inactivated state in which XIP and the β -hairpin between TMs 1 and 2 form a TM-associated four-stranded β -hub and mediate a tight packing between the TM and cytosolic domains, resulting in the formation

of a stable inactivation assembly that blocks the TM movement required for ion exchange function (Xue et al., 2023 [DOI](#)). The study also provides a mechanistic insight into how cytosolic Ca^{2+} binding at CBD2 destabilizes the inactivation assembly and activates the exchanger (Xue et al., 2023 [DOI](#)). To expand our understanding of NCX modulation by PIP_2 lipid and small molecule inhibitors, we present the cryo-EM structures of human cardiac NCX1 in complex with PIP_2 or SEA0400, revealing the structural basis underlying PIP_2 activation and SEA0400 inhibition of NCX1.

Results

PIP_2 activation of NCX1

Phosphatidylinositol 4,5-bisphosphate (PIP_2) has been shown to modulate NCX1 activity by reducing the Na^+ -dependent inactivation of the exchanger (He et al., 2000 [DOI](#); Hilgemann and Ball, 1996 [DOI](#); Hilgemann et al., 1992a [DOI](#); Yaradanakul et al., 2007 [DOI](#)). To characterize the effect of PIP_2 on HsNCX1, we expressed the exchanger in *Xenopus laevis* oocytes and recorded the outward exchanger currents using the giant patch technique in the inside-out configuration with or without applying porcine brain PIP_2 (Fig. 1 [DOI](#), and Methods). The recording was performed with 12 μM free cytosolic $[\text{Ca}^{2+}]_i$ (bath) and the outward NCX1 current was elicited by the rapid replacement of 100 mM Cs^+ with 100 mM Na^+ in the bath solution. In the patches without applying PIP_2 , the outward exchanger currents quickly decay and reach a steady state due to Na^+ -dependent inactivation (Fig. 1A [DOI](#)). Introducing 10 μM PIP_2 at the steady state progressively increases the current that plateaus at about two-fold of the peak current measured before PIP_2 addition (Fig. 1A [DOI](#)). After PIP_2 washout, the outward current remains at the pre-removal level without obvious inactivation, indicating high-affinity PIP_2 binding and its positive modulation of NCX1 by both potentiating the peak current and reducing the Na^+ -dependent inactivation. Intriguingly, the commonly used shorter chain PIP_2 substitute (PIP_2 diC8) does not have the equivalent activation effect on NCX1 and the exchanger remains susceptible to Na^+ -dependent inactivation when recorded in the presence of 10 μM PIP_2 diC8 (Fig. 1B [DOI](#)). However, PIP_2 diC8 still binds and stimulates both the peak and steady currents of the exchanger. This stimulation effect is abolished after PIP_2 diC8 washout, indicating a lower affinity of short-chain PIP_2 than that of the long-chain native PIP_2 .

Structural insight into PIP_2 binding in NCX1

To reveal the structural mechanism of PIP_2 activation, we tried to obtain the EM structure of HsNCX1 in the presence of the long-chain porcine brain PIP_2 . However, the exchanger becomes highly dynamic, yielding a low-resolution EM map with an overall shape similar to a cytosolic Ca^{2+} -activated NCX1 whose β -hub-mediated inactivation assembly is destabilized and cytosolic domain (CBD1 and CBD2) is detached from the TM domain (Xue et al., 2023 [DOI](#)) (Fig. S1). We suspect the long-chain PIP_2 exerts the same activation effect on NCX1 as high cytosolic Ca^{2+} by destabilizing the inactivation assembly. As NCX1 retains its Na^+ -dependent inactivation property in the presence of the short-chain PIP_2 , we reasoned that the PIP_2 diC8-bound NCX1 likely remain in an inward-facing inactivated state in high Na^+ low Ca^{2+} condition and its structure would still reveal how PIP_2 binds in NCX1. We therefore determined the EM structure of NCX1 in complex with PIP_2 diC8 at 3.5Å (Fig. 2 [DOI](#), Fig. S2, Table S1, and Methods), which indeed adopts an inward-facing conformation with intact inactivation assembly. The density of the IP_3 head group from the bound PIP_2 diC8 is well-defined in the EM map. The acyl chains, however, are flexible and could not be resolved in the structure (Fig. S2). The lipid is attached to the cytosolic sides of TMs 4 and 5 with its head group positioned at the C-terminal end of TM5 (Fig. 2A [DOI](#)). Four positively charged residues including K164 and R167 from the N-terminus of TM4 and R220 and K225 from the C-terminus of TM5 participate in the electrostatic interactions with the head group.

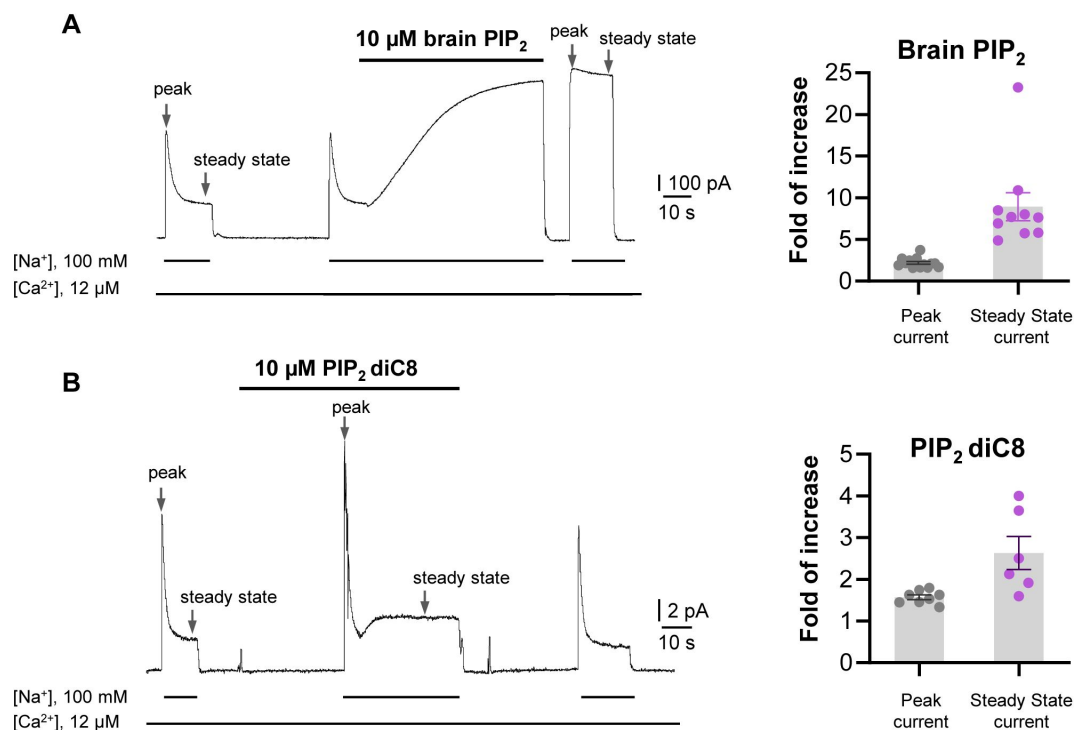


Fig. 1.

PIP₂ enhances HsNCX1 activity.

(A) Representative outward currents recorded from oocytes expressing the human NCX1 before and after application of long-chain brain PIP₂. Currents were activated by replacing cytosolic Cs⁺ with Na⁺. Application of 10 μM brain PIP₂ enhanced HsNCX1 current and abolished the Na⁺-dependent inactivation irreversibly. Perfusion time of PIP₂ is indicated above traces while lines below traces indicate solution exchange. Arrows mark the peak and steady currents used to measure the fold of increase upon PIP₂ application. The fold of current increase was calculated by comparing the peak or steady-state current before and after PIP₂ application.

(B) Representative outward currents recorded before and after application of short-chain PIP₂ diC8 (10 μM). PIP₂ diC8 was perfused from the cytosolic side before HsNCX1 activation (in the presence of Cs⁺ for 30 s) and during transport (in the presence of Na⁺). Both peak and steady-state currents of HsNCX1 are enhanced by PIP₂ diC8 and the effect is reversible. The Na⁺-dependent inactivation remains in the presence of PIP₂ diC8.

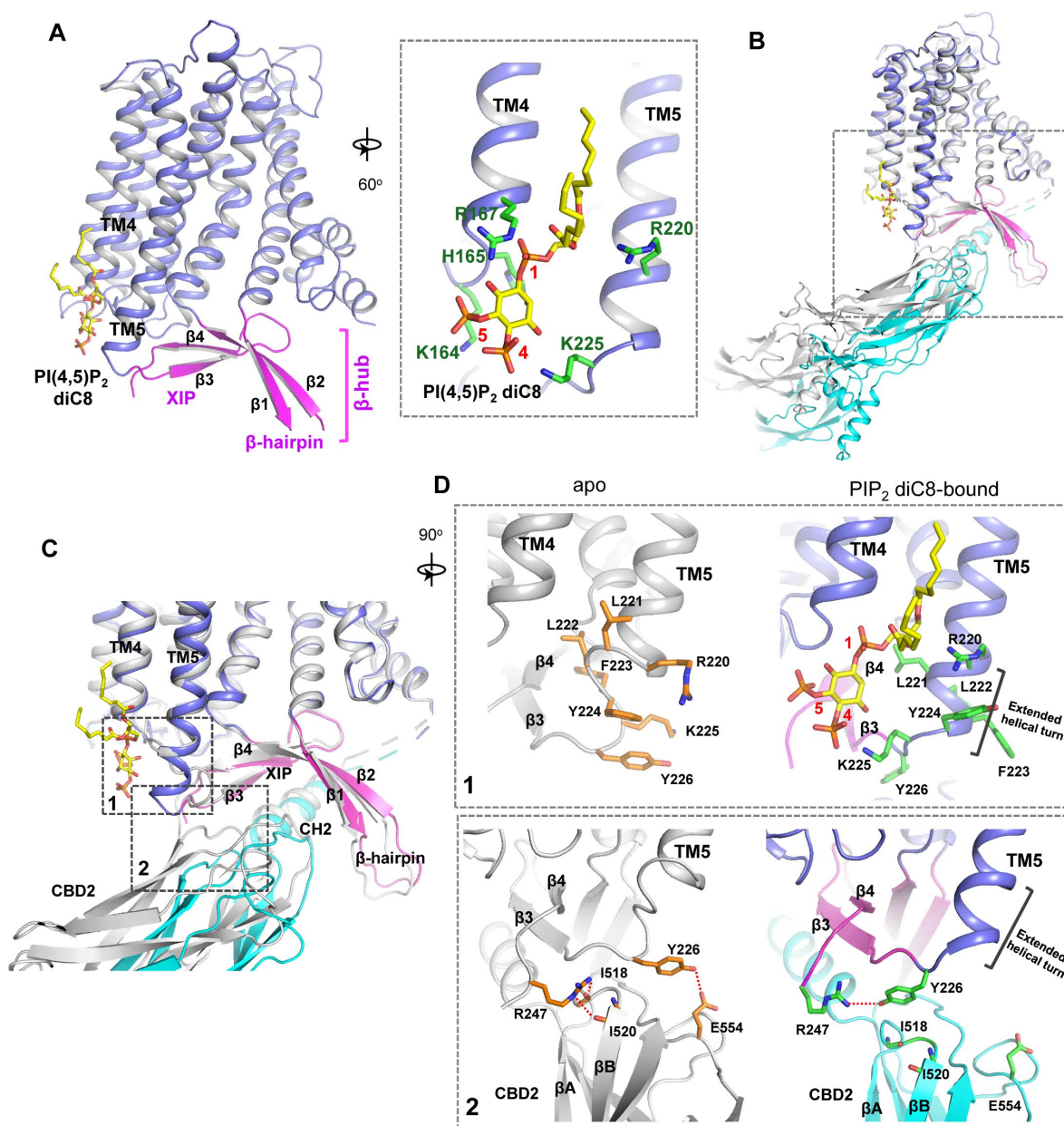


Fig. 2.

PIP₂ binding in NCX1.

(A) Structure of the TM and β-hub regions of PIP₂ diC8-bound NCX1 with a zoomed-in view of the lipid binding site.

(B) Structural comparison between apo (grey) and PIP₂ diC8-bound (color) NCX1.

(C) Zoomed-in view of the structural comparison (boxed area in (B)). The two major conformational changes occur in the boxed regions with the two areas SEA0400 binding site and the schematic diagram detailing the interactions between NCX1 residues and SEA0400.

(D) Zoomed-in views of the two conformational changes between apo (left in grey) and PIP₂ diC8-bound (right in color) state. Top: conformational change 1 at the C-terminus of TM5. Bottom: conformational change 2 at the interface between XIP and CBD2.

PIP₂ diC8-induced conformational changes in NCX1

Two major conformational changes occur in NCX1 upon PIP₂ diC8 binding (**Fig. 2B-D**). The first change occurs at the C-terminus of TM5 which ends at R220 and is connected to the 2-stranded XIP β -sheet (β 3 and β 4) via a 6-residue loop in the apo structure. When PIP₂ binds, the TM5 helix extends by one helical turn. This loop-to-helix transition significantly changes the locations of these connecting loop residues and their side-chain orientations to accommodate PIP₂, enabling K225 to reorient and interact with the IP₃ head group (**Fig. 2D**, top panel). The second conformational change is the partial detachment of the CBD2 domain from XIP upon PIP₂ binding, resulting in a downward swing of cytosolic CBD domains (CBD1 and CBD2) as a rigid body (**Fig. 2B & C**). This CBD2 detachment is a result of the first PIP₂-induced conformational change at TM5 that disrupts part of the interactions between CBD2 and XIP as follows: In the apo inactivated state, Y226 and R247, the two termini residues of the 2-stranded XIP β -sheet, form H-bonds with several CBD2 residues including E554 sidechain and backbone carbonyls of I518 and I520 (**Fig. 2D**, bottom panel); The loop-to-helix transition of TM5 upon PIP₂ binding leads to a dramatic rotation of Y226 that allows it to move closer to and directly interact with R247, resulting in the loss of H-bonding interactions between XIP and CBD2 (**Fig. 2D**, bottom panel); In addition, the rotation of Y226 also causes a direct collision with CBD2 if it remains closely attached to XIP. Thus, the PIP₂-induced TM5 movement, particularly the reorientation of Y226, abolishes some local interactions between CBD2 and XIP and also pushes CBD2 away from XIP, causing the partial detachment of CBD2. However, the short-chain PIP₂ only partially destabilizes rather than completely disassembles the inactivation assembly as the CH2 helix of CBD2 still engages in extensive interactions with the C-shaped β -hub.

To test if the PIP₂-interacting residues play a critical role in the native long-chain PIP₂ activation, we performed mutagenesis to those positively charged residues, including K164A, R167A, R220A, and K225A single mutants. We also generated an R220A/K225A double mutant as these two residues undergo PIP₂-induced conformational change at TM5. While all mutants remain susceptible to current potentiation upon brain PIP₂ application as seen in the wild-type NCX1, the PIP₂-potentiation effects on peak and steady-state currents are weakened in some mutants, most notably in R220A and R220A/K225A (**Fig. 3A-C**). Interestingly, these two mutants also have reduced Na⁺-dependent inactivation as demonstrated by their higher fractional activity (ratio between steady-state and peak currents) before applying PIP₂ (**Fig. 3D**). The retained PIP₂ activation on these mutants implies that the longer acyl chain from native PIP₂ plays a more dominant role in its interaction and activation of NCX1. While we expect the long-chain PIP₂ to bind in the same location as PIP₂ diC8, it is unclear how it exacerbates the destabilization effect on the inactivation assembly. The long acyl chain of native PIP₂ may engage in some interactions with the TM module of NCX1 that are not present in the short-chain lipid, rendering a higher affinity binding and more profound destabilization effect on the inactivation assembly than PIP₂ diC8.

Structure of NCX1 in complex with SEA0400 inhibitor

SEA0400 is known to potently inhibit cardiac NCX1 by facilitating the inactivation of the exchanger (Bouchard et al., 2004; Iwamoto et al., 2004; Lee et al., 2004). To reveal the structural mechanism of its inhibition, we determined the structure of HsNCX1 in complex with SEA0400 at 2.9 Å (**Fig. 4**, Fig. S3, Table S1, and Methods). The SEA0400-bound NCX1 structure adopts an inward-facing, inactivated state identical to the apo NCX1 structure obtained at high Na⁺, nominal Ca²⁺-free condition (Xue et al., 2023) (**Fig. 4A** and Fig. S4). SEA0400 binds at the TM domain in a pocket enclosed by the internal halves of TMs 8 (8a segment), 2 (2ab segments), 4, and 5 (**Fig. 4B**). The pocket has a lateral fenestration in the middle of the membrane (**Fig. 4B**), which provides a portal for the SEA0400 entrance. The pocket is sealed off at the cytosolic side by E244 from the XIP β -sheet. As demonstrated in our previous study, XIP and the linker β -hairpin (β 1 and

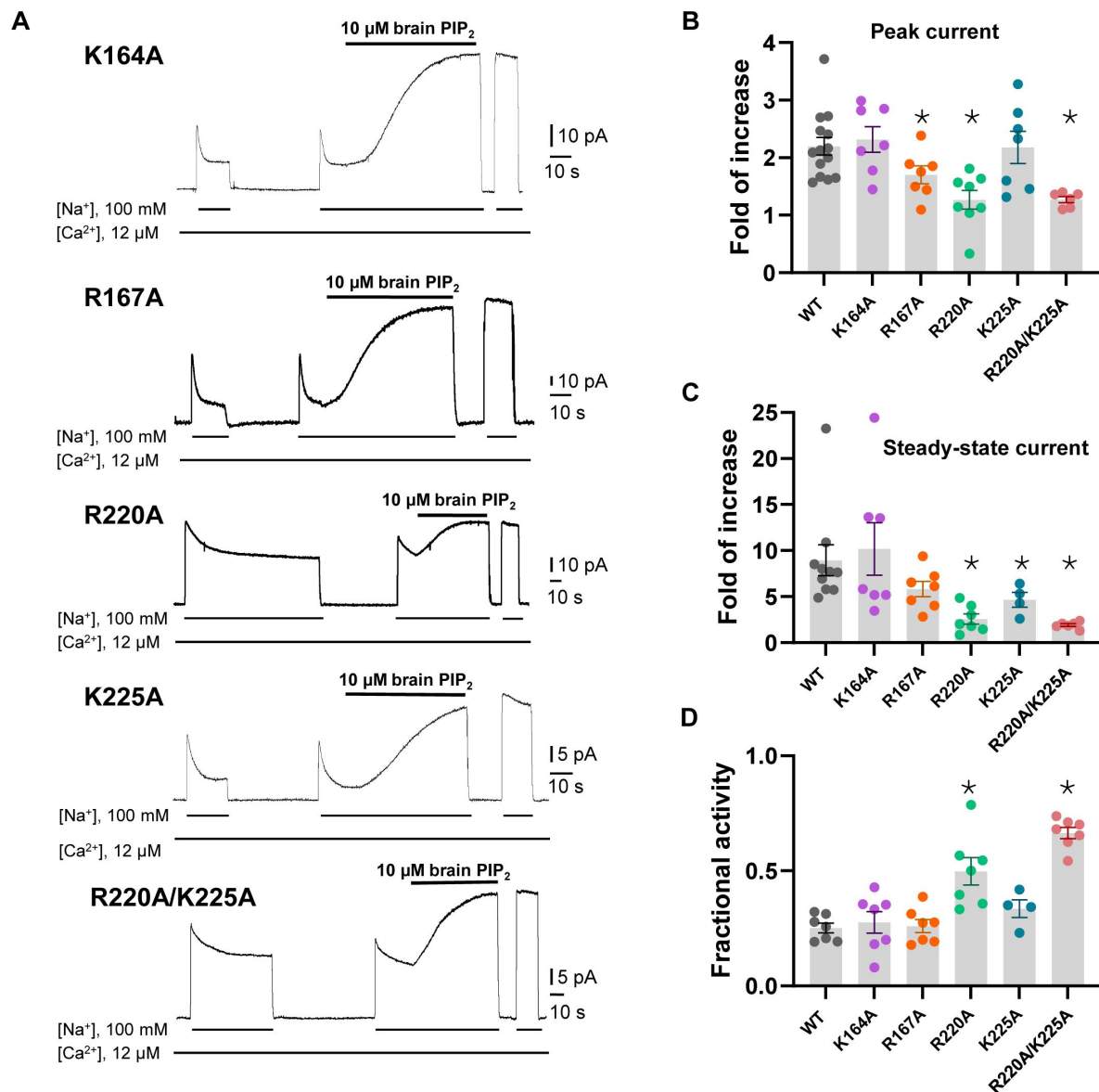


Fig. 3.

Mutagenesis at the PIP_2 binding.

(A) Representative HsNCX1 currents recorded of single mutants before and after perfusion of 10 μM brain PIP_2 to the cytosolic side of the patch.

(B&C) Summary graphs demonstrating the effects of PIP_2 on the enhancement of peak (B) and steady-state currents (C). Potentiation (fold of increase) was measured by comparing the current magnitude before and after PIP_2 application. Mutants R167A, R220A, and K225A showed decreased response to PIP_2 , while the double mutant R220A/K225A response to PIP_2 at steady state decreased by ~70% (Fold of increase WT=8.9 $\pm$ 1.6, n=10 vs R220A/K225A=2.4 $\pm$ 0.5, n=7). Data points are mean $\pm$ s.e.m. (* p < 0.1).

(D) The extent of Na⁺-dependent inactivation was measured as the ratio between steady state and peak currents (fractional activity) and values for WT and the indicated mutants are shown. Mutants R220A and R220A/K225A displayed significantly higher fractional activity values when compared to WT, indicating that the Na⁺-dependent inactivation was less pronounced in these mutant exchangers.

β 2) between TMs 1 and 2ab form a β -hub that stabilizes the exchanger in an inactivated state. This β -hub has to be disassembled in an activated NCX1 and XIP is expected to be detached from the TM domain, which would lead to the opening of the SEA400-binding pocket to the cytosol and provide a cytosolic portal for the release of the inhibitor release as further discussed below. **Fig. 4C** summarizes the interactions between SEA0400 and NCX1 and demonstrates that SEA0400 fits perfectly in the pocket, making extensive contact with surrounding residues. Mutations of some key interacting residues, such as F213 and G833, have been shown to compromise the inhibitor binding (Iwamoto et al., 2004). The same SEA0400 binding was also demonstrated in the recent study of human NCX1.3 and mutations at some pocket-forming residues mitigate the SEA400 inhibition of the exchanger (Dong et al., 2024).

Inhibition mechanism of SEA0400

The TM domain of NCX1 shares a similar overall architecture to the archaea exchanger NCX_Mj (Liao et al., 2012). The structural comparison between the TM domains of the inward-facing NCX1 and the outward-facing NCX_Mj reveals the conformational changes that occur during ion exchange, providing structural insight into the SEA0400 inhibition mechanism (**Fig. 5A**). The inward-outward transition mainly involves the sliding motion of TMs 1 and 6 and the bending movement of TMs 2ab and 7ab (Liao et al., 2012; Liao et al., 2016; Marinelli and Faraldo-Gomez, 2023; Xue et al., 2023). As TM2ab directly interacts with SEA0400 in the inward-facing state, its bending movement towards the outward conformation would cause a direct collision with the inhibitor (**Fig. 5A**). Thus, the binding of SEA0400 stabilizes the exchanger in the inward-facing state and blocks the conformational change from the inward to the outward state. The Na^+ -dependent NCX1 inactivation occurs when the exchanger is in a Na^+ -loaded, inward-facing state with low cytosolic $[\text{Ca}^{2+}]$ (Hilgemann, 2020; Hilgemann et al., 1992b; Matsuoka and Hilgemann, 1994). As demonstrated in our previous study, only in this state, the β -hub can form and readily interact with the cytosolic CBD domains, generating the inactivation assembly that locks TMs 1 and 6 and prevents the TM module from transporting ions (Xue et al., 2023). Thus, SEA0400 promotes NCX1 inactivation by stabilizing NCX1 in the inward-facing conformation which facilitates the formation of the inactivation assembly. The formation of the inactivation assembly also reciprocally stabilizes SEA0400 binding as the XIP of the assembly interacts with the TM domain and seals off the inhibitor binding pocket from the cytosolic side (**Fig. 5B**). Under conditions in which the inactivation assembly cannot form in NCX1, such as chymotrypsin treatment or forward exchange mode (Na^+ influx/ Ca^{2+} efflux), the removal or detachment of XIP from the TM domain would generate a cytosolic open portal for SEA0400 release and effectively reduce its binding affinity (**Fig. 5C**) (Bouchard et al., 2004; Lee et al., 2004). Indeed, cysteine scanning mutagenesis studies have shown that the pocket-forming G833 residue is accessible to intracellular sulfhydryl reagents in the chymotrypsin-treated inward-facing NCX1, confirming the cytosolic exposure of the SEA400-binding pocket upon XIP removal (John et al., 2013). This cytosolic opening of the SEA400 pocket explains the low efficacy of SEA0400 inhibition in NCX1 when the Na^+ -dependent inactivation is absent.

Summary

In this study, we provide mechanistic underpinnings of cellular regulation and pharmacology of NCX family proteins by revealing the structural basis of PIP_2 activation and SEA0400 inhibition in HsNCX1. Both compounds modulate NCX1 activity by reducing or enhancing the physiologically relevant Na^+ -dependent inactivation process that occurs when the exchanger is in an inward-facing, Na^+ -loaded state with high $[\text{Na}^+]$ and low $[\text{Ca}^{2+}]$ on the cytosolic side (Hilgemann et al., 1992b; Scranton et al., 2024). In this inactivation state, XIP and β -hairpin can assemble into a TM-associated four-stranded β -hub that mediates a tight packing between the TM and cytosolic domains, resulting in the formation of an inactivation assembly that blocks the TM conformational changes required for ion exchange function (Dong et al., 2024; Xue et al., 2023). PIP_2 or SEA0400 binding changes the stability of the inactivation assembly in NCX1, resulting in a

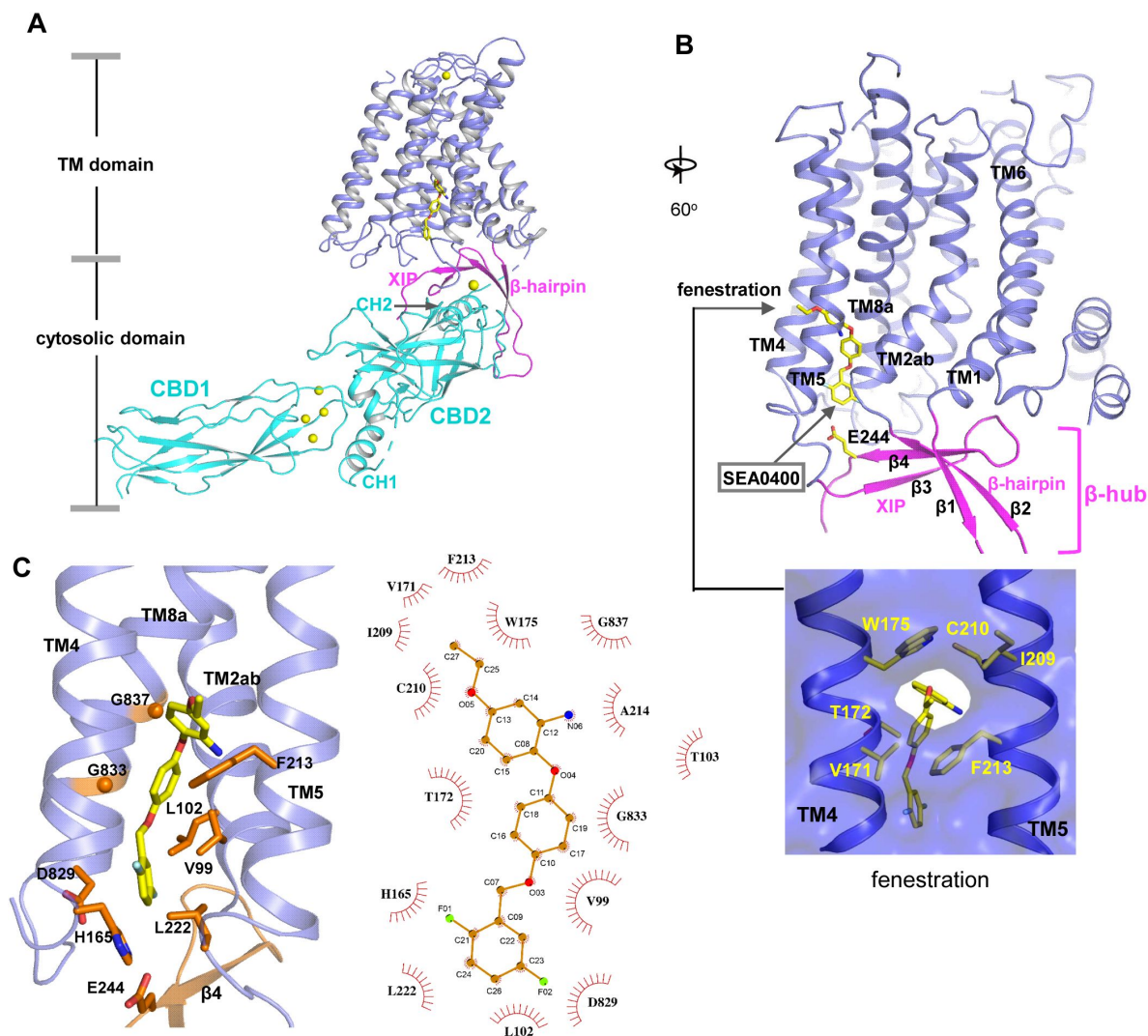


Fig. 4.

SEA0400 binding in NCX1.

(A) Overall structure of human NCX1 in complex with SEA0400 obtained in high Na^+ and low Ca^{2+} conditions. Yellow spheres represent the bound Ca^{2+} in CBD1 and XIP.

(B) Cartoon representation of the TM domain and β -hub of the complex with surface-rendered view of the fenestration in the middle of the membrane. The β -hub is assembled by β -hairpin (β 1 and β 2) and XIP (β 3 and β 4).

(C) Zoomed-in view of SEA0400 binding site and the schematic diagram detailing the interactions between NCX1 residues and SEA0400.

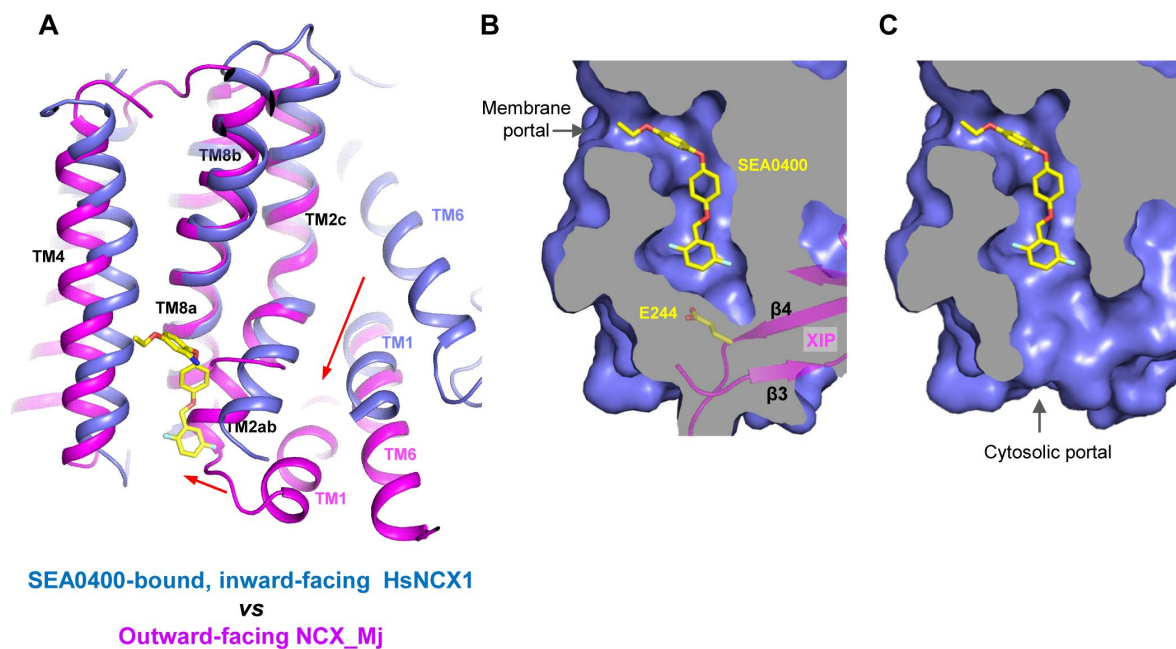


Fig. 5.

Structural mechanism of SEA0400 inhibition.

(A) Structural comparison at the core part of the TM domain between the SEA0400-bound, inward-facing HsNCX1 and the outward-facing NCX_Mj (PDB 3V5U). Red arrows mark the sliding movement of TMs 1 and 6 and the bending of TM2ab from inward to outward conformation.

(B) Surface-rendered views of the SEA0400-binding pocket sealed off from the cytosolic side by E244 from XIP in the inactivated state.

(C) Removal of XIP would generate a cytosolic portal that facilitates the release of SEA0400.

reduction or potentiation of inactivation. Although short-chain PIP_2 cannot fully recapitulate the NCX1 activation effect from long-chain native PIP_2 , the structure of PIP_2 diC8 NCX1 allows us to define the lipid-binding site and the lipid-induced conformational changes at the interface between CBD2 and XIP that destabilize the inactivation assembly. The SEA0400-bound NCX1 structure presented here, along with the recent study by Dong et al. (Dong et al., 2024 [DOI](#)), suggests that the drug can directly inhibit NCX1 by blocking the inward-outward conformational change at TM2ab. SEA0400 binding also traps NCX1 in an inward-facing state, facilitating the formation of the inactivation assembly in which the interaction between XIP and the TM domain can in turn stabilize SEA0400 binding by sealing the drug-binding pocket from the cytosol. When XIP is removed as in the chymotrypsin-treated NCX1 or when the inactivation assembly cannot form as in the NCX1 at forward exchange mode, the SEA0400-binding pocket becomes exposed to the cytosol, mitigating the inhibition efficacy of SEA0400 (Bouchard et al., 2004 [DOI](#); Lee et al., 2004 [DOI](#)).

Methods

Protein expression and purification

The expression and purification of the HsNCX1 (cardiac isoform NCX1.1, indicated as HsNCX1 or NCX1 throughout the manuscript) were carried out as described previously (Xue et al., 2023 [DOI](#)). Truncated HsNCX1 ($\Delta 341$ -365aa) containing a C-terminal Strep-tag was cloned into a pEZT-BM vector and baculoviruses were produced in Sf9 cells (Morales-Perez et al., 2016 [DOI](#)). For protein expression, cultured Expi293F GnTI-cells were infected with the baculoviruses at a ratio of 1:20 (virus: GnTI-, v/v) for 10 hours. 10 mM sodium butyrate was then introduced to boost protein expression level and cells were cultured in suspension at 30°C for another 60 hours and harvested by centrifugation at 4,000 g for 15 mins. All purification procedures were carried out at 4°C. The cell pellet was resuspended in lysis buffer (25 mM HEPES pH 7.4, 300 mM NaCl, 2 $\mu\text{g/ml}$ DNase I, 0.5 $\mu\text{g/ml}$ pepstatin, 2 $\mu\text{g/ml}$ leupeptin, 1 $\mu\text{g/ml}$ aprotinin, and 0.1 mM PMSF) and homogenized by sonication. NCX1 was extracted with 2% (w/v) N-dodecyl- β -D-maltopyranoside (DDM, Anatrace) supplemented with 0.2% (w/v) cholesteryl hemisuccinate (CHS, Sigma Aldrich) by gentle agitation for 2 hours and supernatant collected by centrifugation at 40,000 g for 30 mins was incubated with Strep-Tactin affinity resin (IBA) for 1 hour. The resin was then collected on a disposable gravity column (Bio-Rad) and washed with 30 column volumes of buffer A (25 mM HEPES pH 7.4, 200 mM NaCl, 0.06% digitonin). NCX1 was eluted in buffer A supplemented with 50 mM biotin and further purified by size-exclusion chromatography on a Superdex200 10/300 GL column (GE Healthcare). For the generation of NCX1-Fab 2E4 complex, NCX1 was incubated with purified Fab in a molar ratio of 1:1.2 (NCX1: Fab 2E4) for 2 hours and further purified by size-exclusion chromatography in buffer A. The peak fractions were collected and concentrated to ~5-6 mg/ml for cryo-EM analysis. To prepare the protein samples in complex with various compounds, 0.9 mM SEA0400, 0.47 mM $\text{PI}(4,5)\text{P}_2$ diC8 or 0.42 mM brain $\text{PI}(4,5)\text{P}_2$ were added to the protein samples 2 hours before grid preparation.

Expi293F GnTI-cells were purchased from and authenticated by Thermo Fisher Scientific. The cell lines were tested negative for mycoplasma contamination.

Cryo-EM sample preparation and data acquisition

HsNCX1-Fab 2E4 samples (~5-6 mg/ml) in various conditions were applied to a glow-discharged Quantifoil R1.2/1.3 300-mesh gold holey carbon grid (Quantifoil, Micro Tools GmbH, Germany), blotted for 4.0 s under 100% humidity at 4 °C and plunged into liquid ethane using a Mark IV Vitrobot (FEI). For the SEA0400-bound NCX1-Fab 2E4, raw movies were acquired on a Titan Krios microscope (FEI) operated at 300 kV with a K3 camera (Gatan) at 0.83 Å per pixel and a nominal defocus range of -0.9 to -2.2 μm . Each movie was recorded for about 5 s in 60 subframes with a

total dose of $60 \text{ e}^-/\text{\AA}^2$. For other samples, raw movies were acquired on a Titan Krios microscope operated at 300 kV with a Falcon 4i (Thermo Fisher Scientific) at $0.738 \text{ \AA}$ per pixel and a nominal defocus range of -0.8 to $-1.8 \text{ \mu m}$. Each movie was recorded for 4 s with a total dose of $60 \text{ e}^-/\text{\AA}^2$.

Image processing

Cryo-EM data were processed following the general scheme described below with some modifications to different datasets (Figs. S1-S3). First, movie frames were motion-corrected and dose-weighted using MotionCor2 (Zheng et al., 2017 [DOI](#)). The CTF parameters of the micrographs were estimated using the GCTF program (Zhang, 2016 [DOI](#)). After CTF estimation, micrographs were manually inspected to remove images with bad defocus values and ice contamination. Particles were picked using program Gautomatch (Kai Zhang, <https://www.mrc-lmb.cam.ac.uk/kzhang/> [DOI](#)) or crYOLO (Wagner et al., 2019 [DOI](#)) and extracted with a binning factor of 3 in RELION (Scheres, 2012 [DOI](#); Zivanov et al., 2018 [DOI](#)). Extracted particles were subjected to 2D classification, ab initio modeling, and 3D classification. The particles from the best-resolving 3D class were then re-extracted with the original pixel size and subjected to heterogeneous 3D refinement, non-uniform refinement, CTF refinement, and local refinement in cryoSPARC (Punjani et al., 2017 [DOI](#)). The quality of the EM density maps for the transmembrane (TM) and cytosolic domains was further improved through focused refinement, allowing for accurate model building for a major part of the protein. For the dataset of NCX1 in complex with brain PI(4,5)P₂, the maps of apo inactive NCX1 (PDB 8SGJ) and Ca²⁺-bound active NCX1 (PDB 8SGT) are used as references for heterogeneous refinement. Due to the highly dynamic nature of the protein samples, the particles sorted into the active state produce a map with very low resolution. All resolution was reported according to the gold-standard Fourier shell correlation (FSC) using the 0.143 criterion (Henderson et al., 2012 [DOI](#)). Local resolution was estimated using cryoSPARC.

Model building, refinement, and validation

The EM maps of HsNCX1 in the SEA0400-bound and PI(4,5)P₂ diC8-bound states show high-quality density and model building is facilitated by previous apo NCX1 structure (PDB 8SGJ) (Xue et al., 2023 [DOI](#)). Models were manually adjusted in Coot (Emsley et al., 2010 [DOI](#)) and refined against maps using the phenix.real_space_refine with secondary structure restraints applied (Adams et al., 2010 [DOI](#)). The final NCX1 structural model contains residues 17-248, 370-467, 482-644, 652-698, 707-718, and 738-935. The EM map of HsNCX1 in complex with brain PI(4,5)P₂ is relatively poor. The Ca²⁺-bound activated NCX1 structure (PDB 8SGT) is directly docked into the EM map without adjustment.

The statistics of the geometries of the models were generated using MolProbity (Chen et al., 2010 [DOI](#)). All the figures were prepared in PyMol (Schrödinger, LLC.), Chimera (Pettersen et al., 2004 [DOI](#)), and ChimeraX (Goddard et al., 2018 [DOI](#)).

Electrophysiological experiments

The wild-type HsNCX1 and its mutants were cloned into a pGEMHE vector and expressed in oocytes for electrophysiological recordings. RNA was synthesized using mMessage mMachine (Ambion) and injected into *Xenopus laevis* oocytes as described in (John et al., 2018 [DOI](#)). Oocytes were kept at 18°C for 4-7 days. Outward HsNCX1 currents were recorded using the giant patch technique in the inside-out configuration. The external solution (pipette solution) contained the following (mM): 100 CsOH (cesium hydroxide), 10 HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid), 20 TEOAH (tetraethyl-ammonium hydroxide), 0.2 niflumic acid, 0.2 ouabain, 8 Ca(OH)₂ (calcium hydroxide), pH = 7 (using MES, (2-(*N*-morpholino) ethanesulfonic acid)); bath solution (mM): 100 CsOH or 100 NaOH (sodium hydroxide), 20 TEOAH, 10 HEPES, 10 EGTA(ethylene glycol-bis(β-aminoethyl ether)-*N,N,N',N'*-tetra acetic acid) or HEDTA (*N*-(2-

Hydroxyethyl) ethylenediamine-N,N',N'-triacetic acid) and different $\text{Ca}(\text{OH})_2$ concentrations to obtain the desired final free Ca^{2+} concentrations, pH = 7 (using MES). Free Ca^{2+} concentrations were calculated according to WEBMAXc program and confirmed with a Ca^{2+} electrode.

HsNCX1 currents were evoked by the rapid replacement of 100 mM Cs^+ with 100 mM Na^+ , using a computer-controlled 20-channel solution switcher. As HsNCX1 does not transport Cs^+ , there is no current and only upon application of Na^+ does the exchange cycle initiate. Data were acquired at 4 ms/point and filtered at 50 Hz using an 8-pole Bessel filter. Experiments were performed at 35°C and at a holding potential of 0 mV. The effects of the Na^+ -dependent inactivation were analyzed by measuring fractional currents calculated as the ratio of the steady-state current to the peak current (fractional activity). All *P* values were calculated using an unpaired, two-sided Welch's *t*-test.

PI(4,5) P_2 diC8 (Phosphatidylinositol 4,5-bisphosphate diC8, Echelon Bioscience) and brain PI(4,5) P_2 (L- α -phosphatidylinositol-4,5-bisphosphate, Brain, Porcine, Avanti Polar Lipids) were dissolved in water and kept as stock at -20 °C. Immediately prior to recordings, PI(4,5) P_2 was diluted in the bath solution to 10 μM final concentration and perfused cytosolically for the indicated time.

Data availability

The cryo-EM density maps of the human NCX1 have been deposited in the Electron Microscopy Data Bank under accession numbers EMD-40456 for the SEA0400-bound state and EMD-60921 for the PI(4,5) P_2 diC8-bound state, respectively. Atomic coordinates have been deposited in the Protein Data Bank under accession numbers 8SGI for SEA0400-bound structure and 9IV8 for the PI(4,5) P_2 diC8-bound structure.

Supplementary figures and tables

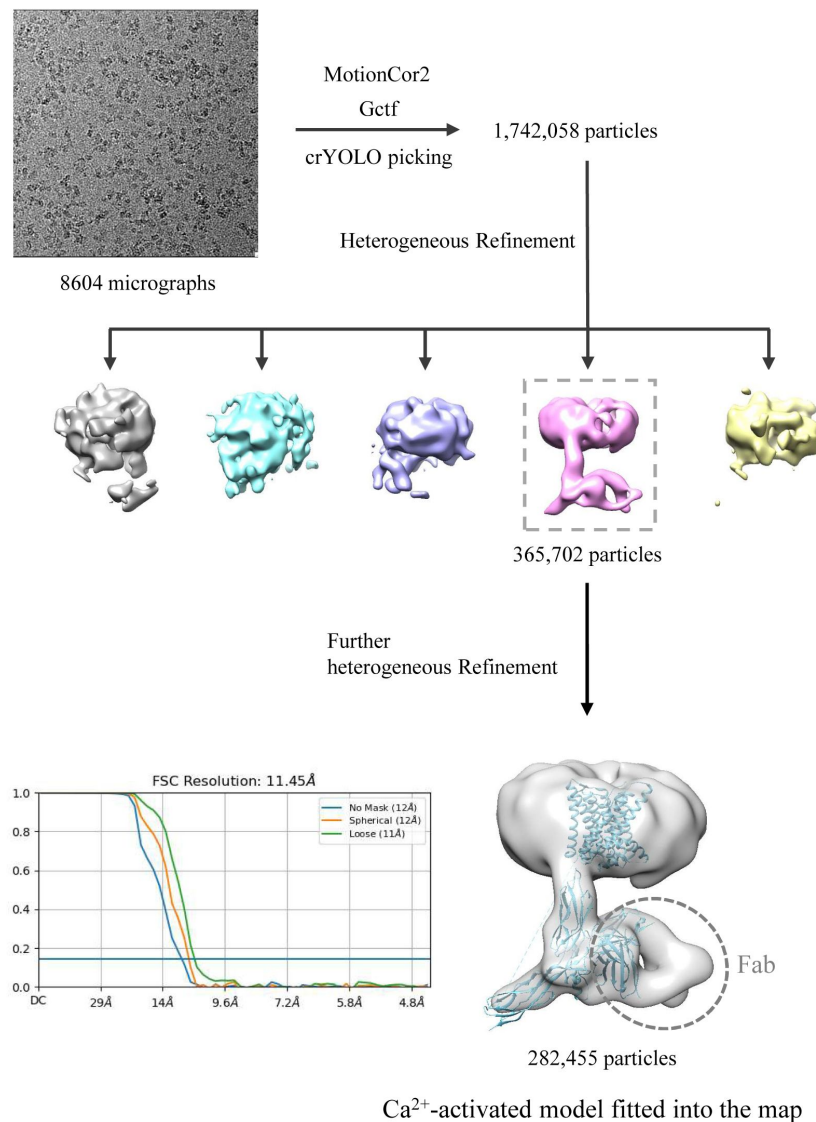


Fig. S1.

Cryo-EM data processing of HsNCX1 in the presence of long-chain porcine brain PIP₂.

The structural model of Ca²⁺-activated HsNCX1 from a previous study (PDB 8SGT) was directly fitted into the low-resolution EM-map (~11.5 Å). The Fab fragment from a monoclonal antibody against NCX1 was used as a fiducial marker to facilitate the single-particle alignment.

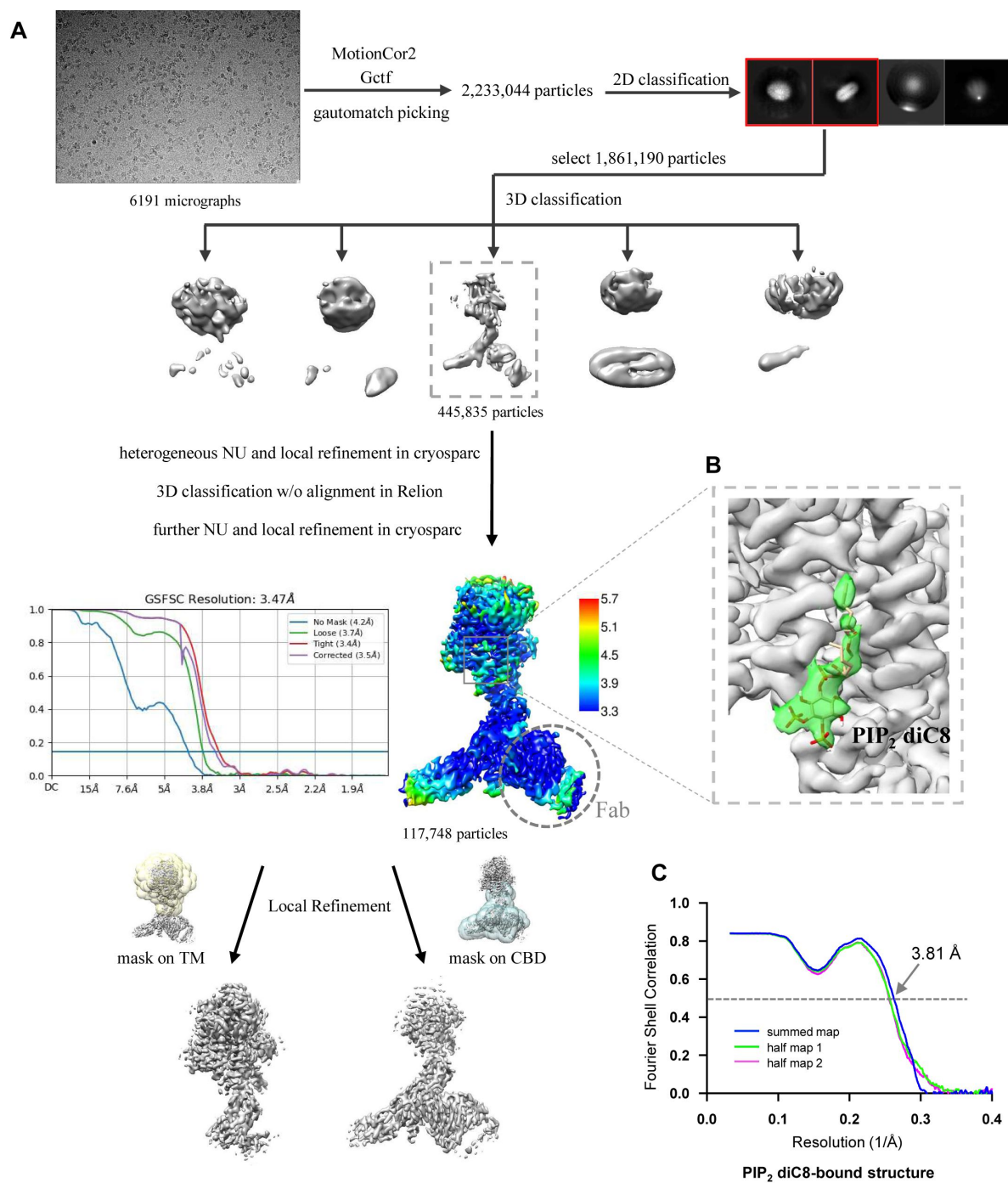


Fig. S2.

Structure determination of HsNCX1-PIP₂ diC8 complex.

- (A) Cryo-EM data processing of HsNCX1 in complex with short-chain PIP₂ diC8. The Fab fragment from a monoclonal antibody against NCX1 was used as a fiducial marker to facilitate the single-particle alignment.
- (B) Zoomed-in view of density map of the bound PIP₂ diC8 contoured at the threshold level of 0.43 using ChimeraX.
- (C) The Fourier shell correlation (FSC) curves for cross-validation between the maps and the models.

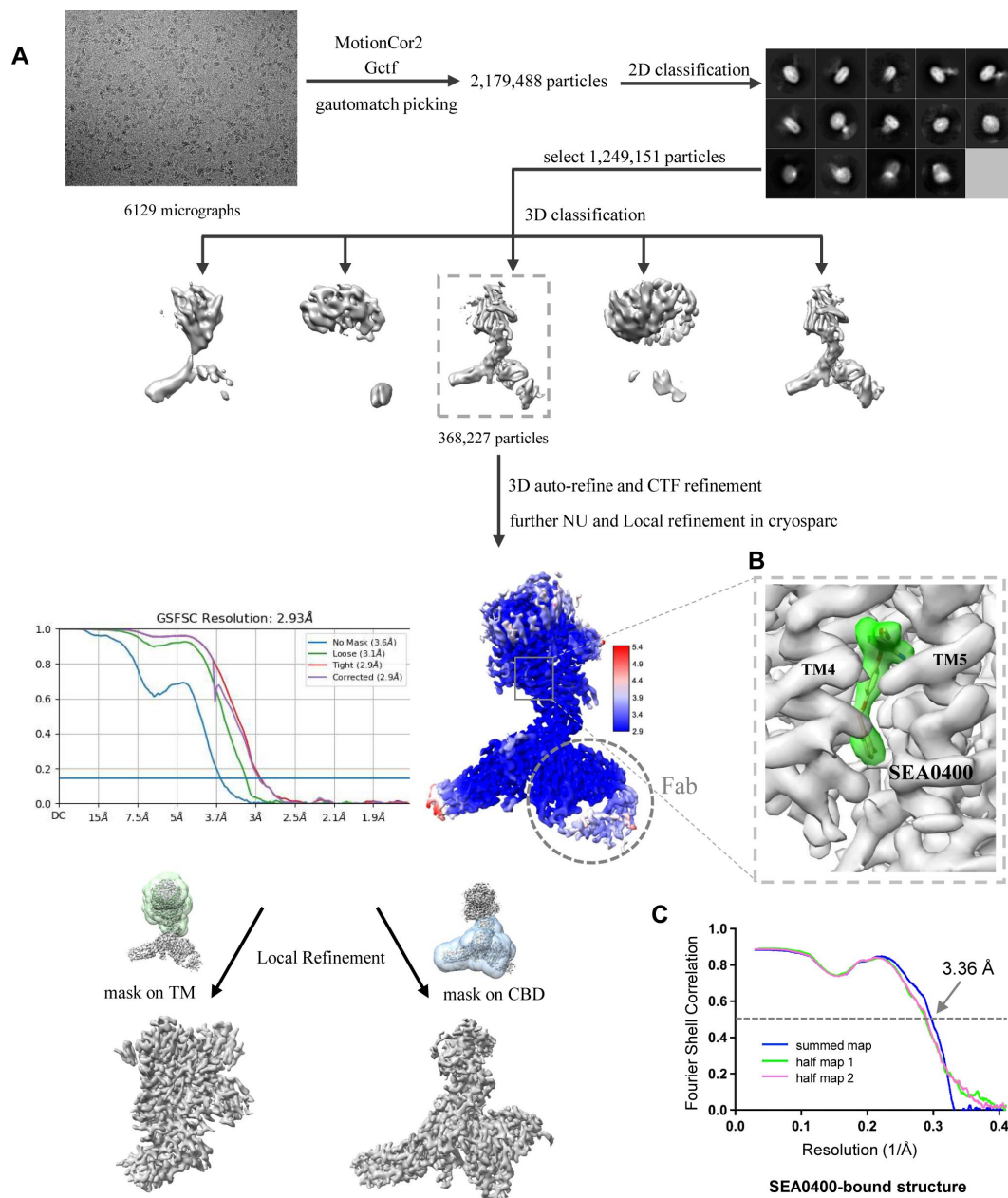


Fig. S3.

Structure determination of HsNCX1-SEA0400 complex.

- (A) Cryo-EM data processing scheme of HsNCX1 in complex with SEA0400 inhibitor. The Fab fragment from a monoclonal antibody against NCX1 was used as a fiducial marker to facilitate the single-particle alignment.
- (B) Zoomed-in view of density map of the bound SEA0400 inhibitor contoured at the threshold level of 0.52 using ChimeraX.
- (C) The Fourier shell correlation (FSC) curves for cross-validation between the maps and the models.

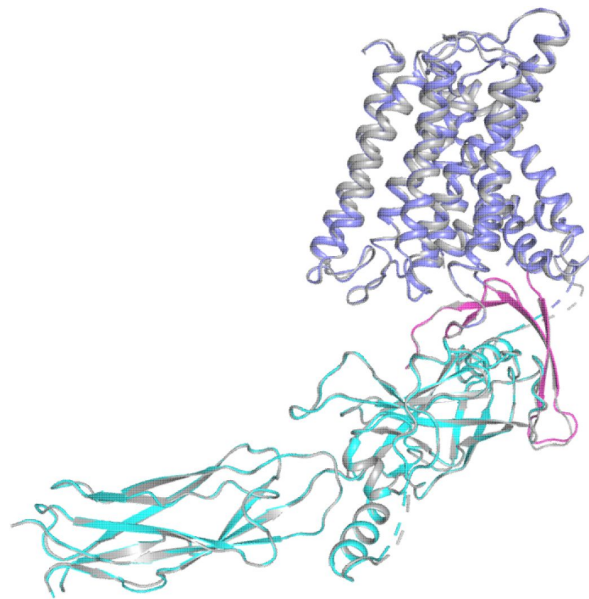


Fig. S4.

Structural comparison between the apo (grey) and SEA0400-bound (color) HsNCX1.

Sample preparation conditions	25 mM Hepes pH 7.4, 200 mM NaCl 0.9 mM SEA0400	25 mM Hepes pH 7.4, 200 mM NaCl, 0.47 mM PI(4,5)P ₂ diC8
	SEA0400-bound state (EMD-40456, PDB 8SGI)	PI(4,5)P₂ diC8-bound state (EMD-60921, PDB 9IV8)
Data collection and processing		
Magnification	105k	105k
Voltage (kV)	300	300
Electron exposure (e ⁻ /Å ²)	60	60
Defocus range (μm)	-0.9 - -2.2	-0.9 - -2.2
Pixel size (Å)	0.83	0.84
Symmetry imposed	C1	C1
Initial particle images (no.)	1,249,151	2,233,044
Final particle images (no.)	368,227	117,748
Map resolution (Å)	2.93	3.47
FSC threshold	0.143	0.143
Refinement		
Initial model used (PDB code)	8SGJ	8SGJ
Model resolution (Å)	3.36	3.81
FSC threshold	0.5	0.5
Model composition		
Non-hydrogen atoms	7667	5947
Protein residues	982	750
Ligands	3: Na 6: Ca 1: H ₂ O 1: SEA0400	5: Ca 1: PI(4,5)P ₂ diC8
B factors (Å ²)		
Protein	66.21	50.47
Ligands	58.55	106.20
R.m.s. deviations		
Bond lengths (Å)	0.005	0.004
Bond angles (°)	0.696	0.679
Validation		
MolProbity score	1.36	1.28
Clashscore	5.24	5.22
Poor rotamers (%)	0	0
Ramachandran plot		
Favored (%)	97.62	98.24
Allowed (%)	2.38	1.76
Disallowed (%)	0	0

Table S1.

Cryo-EM data collection and model statistics.

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Additional information

Author contributions

J.X. prepared the samples and performed data acquisition, image processing, and structure determination; M.O., S.J., N.A., and W.Z. performed electrophysiology recording; Y.J. supervised the work; all authors participated in research design, data analysis, discussion, and manuscript preparation.

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Reviewer #1 (Public review):

This study uses structural and functional approaches to investigate the regulation of the Na/Ca exchanger NCX1 by an activator, PIP2, and an inhibitor, SEA0400.

State-of-the-art methods are employed, and the data are of high quality and presented very clearly. The manuscript combines two rather different studies (one on PIP2; and one on SEA0400) neither of which is explored in the depth one might have hoped to form robust conclusions and significantly extend knowledge in the field.

The novel aspect of this work is the study of PIP2. Unfortunately, technical limitations precluded structural data on binding of the native PIP2, so an unnatural short-chained analog, di-C8 PIP2, was used instead. This raises the question of whether these two molecules, which have similar but very distinctly different profiles of activation, actually share the same binding pocket and mode of action. In an effort to address this, the authors mutate key residues predicted to be important in forming the binding site for the phosphorylated head group of PIP2. However, none of these mutations prevent PIP2 activation. The only ones that have a significant effect also influence the Na-dependent inactivation process independently of PIP2, thus casting doubt on their role in PIP2 binding, and thus identification of the PIP2 binding site. A more extensive mutagenic study, based on the di-C8 PIP2 binding site, would have given more depth to this work and might have been more revealing mechanistically.

The SEA0400 aspect of the work does not integrate particularly well with the rest of the manuscript. This study confirms the previously reported structure and binding site for SEA0400 but provides no further information. While interesting speculation is presented regarding the connection between SEA0400 inhibition and Na-dependent inactivation, further experiments to test this idea are not included here.

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Reviewer #2 (Public review):

The study by Xue et al. reports the structural basis for the regulation of the human cardiac sodium-calcium exchanger, NCX1, by the endogenous activator PIP2 and the small molecule inhibitor SEA400. This well-written study contextualizes the new data within the existing literature on NCX1 and the broader NCX family. This work builds upon the authors' previous study (Xue et al., 2023), which presented the cryo-EM structures of human cardiac NCX1 in both inactivated and activated states. The 2023 study highlighted key structural differences between the active and inactive states and proposed a mechanism where the activity of NCX1 is regulated by the interactions between the ion-transporting transmembrane domain and the cytosolic regulatory domain. Specifically, in the inward-facing state and at low cytosolic calcium levels, the transmembrane (TM) and cytosolic domains form a stable interaction that results in the inactivation of the exchanger. In contrast, calcium binding to the cytosolic domain at high cytosolic calcium levels disrupts the interaction with the TM domain, leading to active ion exchange.

In the current study, the authors present two mechanisms explaining how both PIP2 stimulates NCX1 activity by destabilizing the protein's inactive state (i.e., by disrupting the interaction between the TM domain and the cytosolic domain) and how SEA400 stabilizes this interaction, thereby acting as a specific inhibitor of the system.

The first part of the results section addresses the effect of PIP2 and PIP2 diC8 on NCX1 activity. This is pertinent as the authors use the diC8 version of this lipid (which has a shorter acyl chain) in their subsequent cryo-EM structure due to the instability of native PIP2. I am not an electrophysiology expert; however, my main comment would be to ask whether there is sufficient data here to characterise fully the differences between PIP2 and PIP2 diC8 on NCX1 function. It appears from the text that this study is the first to report these differences, so perhaps this data needs to be more robust. The spread of the data points in Figure 1B is possibly a little unconvincing given that only six measurements were taken. Why is there one outlier in Figure 1A? Were these results taken using the same batch of oocytes? Are these technical or biological replicates? Is the convention to use statistical significance for these types of experiments?

I am also somewhat skeptical about the modelling of the PIP2 diC8 molecule. The authors state, "The density of the IP3 head group from the bound PIP2 diC8 is well-defined in the EM map. The acyl chains, however, are flexible and could not be resolved in the structure (Fig. S2)."

However, the density appears rather ambiguous to me, and the ligand does not fit well within the density. Specifically, there is a large extension in the volume near the phosphate at the 5' position, with no corresponding volume near the 4' phosphate. Additionally, there is no bifurcation of the volume near the lipid tails. I attempted to model cholesterol hemisuccinate (PDB: Y01) into this density, and it fits reasonably well - at least as well as PIP2 diC8. I am also concerned that if this site is specific for PIP2, then why are there no specific interactions with the lipid phosphates? How can the authors explain the difference between PIP2 and PIP2 diC8 if the acyl chains don't make any direct interactions with the TM domain? In short, the structures do not explain the functional differences presented in Figure 1.

The side chain densities for Arg167 and Arg220 are also quite weak. While there is some density for the side chain of Lys164, it is also very weak. I would expect that if this site were truly specific for PIP2, it should exhibit greater structural rigidity - otherwise, how is this specific?

Given this observation, have the authors considered using other PIP2 variants to determine if the specificity lies with PI4,5P2 as opposed to PI3,5P2 or PI3,4P2? A lack of specificity may explain the observed poor density.

I also noticed many lipid-like densities in the maps for this complex. Is it possible that the authors overlooked something? For instance, there is a cholesterol-like density near Val51, as well as something intriguing near Trp763, where I could model PIP2 diC8 (though this leads to a clash with Trp763). I wonder if the authors are working with mixed populations in their dataset. The accompanying description of the structural changes is well-written (assuming it is accurate).

I would recommend that the authors update the figures associated with this section, as they are currently somewhat difficult to interpret without prior knowledge of NCX architecture. My suggestions include:

- Including the density for the PIP2 diC8 in Figure 2A.
- Adding membrane boundaries (cytosolic vs. extracellular) in Figure 2B.
- Labeling the cytosolic domains in Figure 2B.
- Adding hydrogen bond distances in Figure 2A.
- Detailing the domain movements in Figure 2B (what is the significance of the grey vs. blue structures?).

The section on the mechanism of SEA400-induced inactivation is strong. The maps are of better quality than those for the PIP2 diC8 complex, and the ligand fits well. However, I noticed a density peak below F02 on SEA400 that lies within the hydrogen bonding distance of Asp825. Is this a water molecule? If so, is this significant?

Furthermore, there are many unmodeled regions that are likely cholesterol hemisuccinate or detergent molecules, which may warrant further investigation.

The authors introduce SEA400 as a selective inhibitor of NCX1; however, there is little to no comparison between the binding sites of the different NCX proteins. This section could be expanded. Perhaps Fig. 4C could include sequence conservation data.

Additionally, is the fenestration in the membrane physiological, or is it merely a hole forced open by the binding of SEA400? I was unclear as to whether the authors were suggesting a physiological role for this feature, similar to those observed in sodium channels.

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Reviewer #3 (Public review):

NCXs are key Ca^{2+} transporters located on the plasma membrane, essential for maintaining cellular Ca^{2+} homeostasis and signaling. The activities of NCX are tightly regulated in response to cellular conditions, ensuring precise control of intracellular Ca^{2+} levels, with profound physiological implications. Building upon their recent breakthrough in determining the structure of human NCX1, the authors obtained cryo-EM structures of NCX1 in complex with its modulators, including the cellular activator PIP2 and the small molecule inhibitor SEA0400. Structural analyses revealed mechanistically informative conformational changes induced by PIP2 and elucidated the molecular basis of inhibition by SEA0400. These findings underscore the critical role of the interface between the transmembrane and cytosolic domains in NCX regulation and small molecule modulation. Overall, the results provide key insights into NCX regulation, with important implications for cellular Ca^{2+} homeostasis.

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