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## The ion pathway through the opened Na<sup>+</sup>,K<sup>+</sup>-ATPase pump

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P-type ATPases pump ions across membranes, generating steep electrochemical gradients that are essential for the function of all cells. Access to the ion-binding sites within the pumps alternates between the two sides of the membrane<sup>1</sup> to avoid the dissipation of the gradients that would occur during simultaneous access. In Na<sup>+</sup>,K<sup>+</sup>-ATPase pumps treated with the marine agent palytoxin. this strict alternation is disrupted and binding sites are sometimes simultaneously accessible from both sides of the membrane, transforming the pumps into ion channels (see, for example, refs 2, 3). Current recordings in these channels can monitor accessibility of introduced cysteine residues to water-soluble sulphydryl-specific reagents<sup>4</sup>. We found previously<sup>5</sup> that Na<sup>+</sup>,K<sup>+</sup> pump-channels open to the extracellular surface through a deep and wide vestibule that emanates from a narrower pathway between transmembrane helices 4 and 6 (TM4 and TM6). Here we report that cysteine scans from TM1 to TM6 reveal a single unbroken cation pathway that traverses palytoxin-bound Na+,K+ pump-channels from one side of the membrane to the other. This pathway comprises residues from TM1, TM2, TM4 and TM6, passes through ion-binding site II, and is probably conserved in structurally and evolutionarily related P-type pumps, such as sarcoplasmic- and endoplasmicreticulum Ca<sup>2+</sup>-ATPases and H<sup>+</sup>,K<sup>+</sup>-ATPases.

The Na<sup>+</sup>,K<sup>+</sup>-ATPase is a P-type (named for its phosphorylated intermediate) pump that exports three Na<sup>+</sup> ions and imports two K<sup>+</sup> ions per ATP hydrolysed. The ion-binding sites are accessible from the extracellular space in the phosphorylated conformation, called E2P, and from the cytoplasm in the dephosphorylated configuration, E1. But the routes by which ions approach and leave those sites are still not understood6 despite the availability of X-ray crystal structures of sarcoplasmic- and endoplasmic-reticulum Ca<sup>2+</sup>-ATPase (SERCA) P-type pumps in several states<sup>6-11</sup>, although a recent structure of a BeF<sub>3</sub><sup>-</sup>-trapped E2P-like state captured an open luminal pathway<sup>12,13</sup>. However, sensitive electric current recording methods developed for studies of ion channels14 have begun to probe the ion pathway of the Na<sup>+</sup>,K<sup>+</sup> pump<sup>5,15–18</sup>, after its transformation into an ion channel by palytoxin<sup>2</sup> and electrophysiological analyses of reactivity of introduced cysteines to methanethiosulphonate (MTS) reagents4.

Cysteines modified by MTS reagents in palytoxin-bound Na<sup>+</sup>,K<sup>+</sup> pump–channels include those substituted for ion-binding<sup>19–21</sup> acidic residues in the pocket between TM4 (for example E336, equivalent to SERCA E309) and TM6 (for example D813, equivalent to SERCA N796), as well as T806 (equivalent to SERCA P789) at the outermost end of TM6 within the external vestibule floor<sup>5,16,17</sup>. These three positions approximately align (Fig. 1) in extracellular views of the transmembrane domain of the Na<sup>+</sup>,K<sup>+</sup>-ATPase, whether of the recent<sup>21</sup> E2•MgF<sub>4</sub><sup>2−</sup> Na<sup>+</sup>,K<sup>+</sup>-ATPase structure (Fig. 1a), which is an occluded conformation with both cytoplasmic and extracellular pathways shut, or of a model based on the E2P-like SERCA E2•BeF<sub>3</sub><sup>−</sup>

structure<sup>12,13</sup> (Fig. 1b), in which the cytoplasmic pathway is shut but the extra-cytoplasmic pathway is open. However, palytoxin, with Na<sup>+</sup> and ATP present, appears to stabilize an E2P-related Na<sup>+</sup>,K<sup>+</sup> pump conformation<sup>22,23</sup> in which the gates to the binding sites can both be open<sup>3</sup>, a structure not yet visualized (or expected) for any native P-type pump. In occluded structures of SERCA containing two Ca<sup>2+</sup> ions<sup>7,10,11</sup> and of Na<sup>+</sup>,K<sup>+</sup>-ATPase<sup>21</sup> containing two K<sup>+</sup> ions, side chains of residues in TM5 and TM6 help coordinate the bound ion in site I, and TM4 and TM6 side chains help coordinate that in site II. The near alignment of accessible TM4 and TM6 positions (Fig. 1) therefore raises two questions: do ions in the Na<sup>+</sup>,K<sup>+</sup> pump's extracellular pathway flow between TM4, TM6 and TM5 (ref. 17) or between TM4, TM6, TM2 and TM1 (compare with refs 6, 12, 13) (Fig. 1, red question marks); and what pathway(s) do ions take from the binding sites to the cytoplasm?

To answer these questions, we first introduced cysteines, one at a time, at 20 contiguous positions (I778–I797) along TM5 and at 4 more (A798–P801) in the external loop connecting TM5 and TM6, into the Xenopus Na $^+$ ,K $^+$ -ATPase  $\alpha_1$  subunit made ouabain resistant by the mutation C113Y. We co-expressed each cysteine-tagged mutant with the Xenopus  $\beta_3$  subunit in Xenopus oocytes  $^{5,18}$ . After applying 50 nM palytoxin (Fig. 2a–d, black arrowheads) to outside-out membrane patches, to transform all Na $^+$ ,K $^+$  pumps into ion channels (signals from native ouabain-sensitive Xenopus Na $^+$ ,K $^+$  pumps were prevented using 100  $\mu$ M ouabain in all external solutions), we assessed

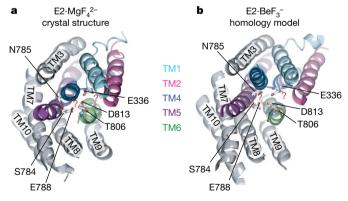


Figure 1 | Alternative routes for ions through the Na<sup>+</sup>,K<sup>+</sup>-ATPase transmembrane domain. Extracellular views of the ten transmembrane helices of the Na<sup>+</sup>,K<sup>+</sup>-ATPase E2·MgF<sub>4</sub><sup>2-</sup> crystal structure<sup>21</sup> (a; Protein Data Bank code 3B8E) and a homology model of the Na<sup>+</sup>,K<sup>+</sup>-ATPase based on the SERCA E2·BeF<sub>3</sub><sup>-</sup> structure<sup>12</sup> (b; Protein Data Bank code 3B9B). Helices are coloured grey except TM1 (pale blue), TM2 (magenta), TM4 (blue), TM5 (purple) and TM6 (green). Red question marks label two possible ion pathways: one between TM5, TM4 and TM6, and the other between TM4, TM1, TM2 and TM6. Key residues in these pathways are labelled.

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reactivity of positively charged, membrane-impermeant, 1 mM 2-trimethylammonium-ethyl-methanethiosulphonate (MTSET $^+$ ; Fig. 2a–d, blue arrows) with each engineered cysteine. Reaction was signalled by alteration of the inward Na current (symmetrical 125 mM Na solutions with -50-mV membrane potential) flowing through pump–channels (Fig. 2). MTSET $^+$  tests were preceded by exposure to  $10\,\mathrm{mM}$  dithiothreitol (grey arrows) to restore any spontaneously oxidized thiols. There was no evidence of MTSET $^+$  reaction with any residue in TM5, but it rapidly decreased current by  $\sim\!25\%$  in construct N799C with a cysteine in the TM5–TM6 loop (Fig. 2a).

We similarly scanned 21 contiguous positions (F99–I119) in TM1, 11 (Y133–V142, T145) in TM2 and 6 (Q120, Q128–L132) in the extracellular TM1–TM2 connecting loop, testing reactivity of each introduced cysteine with 1 mM MTSET $^+$  (Fig. 3). Reactive positions (defined as >10% change in pump–channel current) in TM1 were G100, G101, F102, S103, L106, C113, A116 and Y117 (Fig. 3a–c), those in the TM1–TM2 loop included Q120, Q128, D130 and N131 (Fig. 3c, f), and those in TM2 included Y133 and L134 at the outer end and T145 towards the cytoplasmic end (Fig. 3d–f).

The summarized results from these scans, mapped onto an Na<sup>+</sup>,K<sup>+</sup> pump homology model based on the SERCA E2•BeF<sub>3</sub><sup>-</sup> structure, show, as red sticks, residues in positions where substituted cysteines showed evidence of modification by 1 mM MTSET<sup>+</sup>, and, as yellow sticks, residues in positions where there was no evidence of reactivity (Fig. 4a, b). Our previously reported<sup>5</sup> results on cysteines introduced in TM4 (E321, E336, G337), the TM5–TM6 loop (L802–L804) and TM6 (G805–C811, D813, D817) are included. To fill gaps, we tested 16 additional strategically located positions in TM3 (I290, I294, I297, A301) and TM4 (A322–F325, I327, G328, V331, A332,

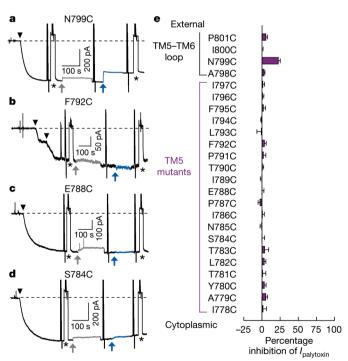


Figure 2 | Effects of MTSET $^+$  on current through palytoxin-bound Na $^+$ ,K $^+$  pump-channels with cysteines in TM5 or the TM5-TM6 loop. a–d, Current at -50 mV in outside-out patches exposed to symmetrical Na $^+$  concentrations. Application of 50 nM palytoxin (black arrowheads) generated inward (negative) current,  $I_{\rm palytoxin}$  (dashed line marks zero total membrane current). Temporary substitution (asterisk) of less permeant tetramethylammonium (TMA $^+$ ) for external Na $^+$  monitored patch integrity. Application of 10 mM dithiothreitol (grey arrows, grey traces) caused a small, reversible, poorly understood current decrease. Then 1 mM MTSET $^+$  (blue arrows, blue traces) was applied until the current became steady. **e**, Summary of mean ( $\pm$ s.e.m.; n, 3-6 patches) percentage inhibition of  $I_{\rm palytoxin}$  by 1 mM MTSET $^+$  at -50 mV for each single-cysteine mutant.

P335, L339, T341, V342): only P335C and L339C mutants showed reactivity with  ${\rm MTSET}^+$ .

The red residues mark out a single, unbroken MTSET<sup>+</sup>-accessible pathway (Fig. 4a, b; Supplementary Figs 1, 2) that runs between TM1, TM2, TM6 and TM4 rather than between TM5, TM4 and TM6 (Fig. 4a; Supplementary Figs 1, 2), passes through site II, and spans the full distance across the membrane (approximate boundaries indicated by lines  $\sim 35$  Å apart in Fig. 4b; see also Supplementary Fig. 2 and Supplementary Movie). Red reactive positions are enveloped in a yellow non-responsive surround (Fig. 4a, b; Supplementary Fig. 2a, b), indicating that the scan was complete and thus fully delimits this principal pathway through the pump. Moreover, as current was practically abolished after MTSET<sup>+</sup> modification of cysteine substitutes at TM1 position L106 (Figs 3a, c, 4c; Supplementary Figs 4a, 7c, g), or position G337 in TM4 (ref. 5) or T806 in TM6 (ref. 5; Supplementary Fig. 3b), the pathway depicted in Fig. 4 (and Supplementary Fig. 2) is probably the sole route for rapid  $(\sim 10^7 \, \text{s}^{-1})$  Na<sup>+</sup> ion flow through palytoxin-bound Na<sup>+</sup>,K<sup>+</sup> pump-channels.

The negative charges of site-II residues E336 (TM4) and D813 (TM6), which are largely conserved in P-type cation pumps, form a cation-selectivity filter<sup>5</sup>. This was proposed to be responsible for the apparent lack of reactivity of a cysteine substituted for nearby G337 with negatively charged 2-sulphonato-ethyl-methanethiosulphonate

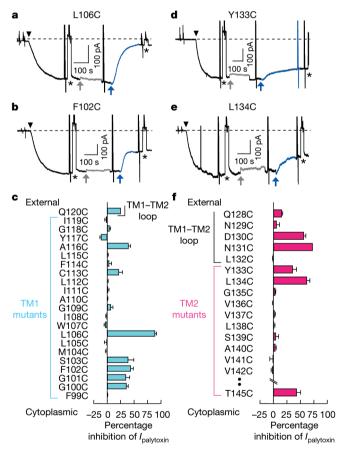


Figure 3 | Effects of MTSET $^+$  on current through palytoxin-bound Na $^+$ ,K $^+$  pump-channels with cysteines in TM1, TM2 or the TM1-TM2 loop. a, b, d, e, Representative current recordings in outside-out patches under the same conditions, and with the same applications of palytoxin, TMA $^+$ , dithiothreitol and MTSET $^+$ , as in Fig. 2. c, f, Summary of percentage inhibition of  $I_{\rm palytoxin}$  by 1 mM MTSET $^+$  at -50 mV for each single-cysteine mutant, given as mean ( $\pm$ s.e.m.; n, 3–11 patches, except for Q120C (n = 2) and N131C (n = 1), both previously shown $^{18}$  to be MTSET $^+$  accessible). C113C indicates data from wild-type, ouabain-sensitive Xenopus Na $^+$ ,K $^+$  pumps tested (in the absence of ouabain) in patches from non-injected control oocytes.

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(MTSES<sup>-</sup>), despite reaction with similarly sized, but positively charged, MTSET<sup>+</sup> (ref. 5). MTSET<sup>+</sup> reaction with cysteines substituted for deeper TM1 residues L106, S103, F102, G101 and G100 (Fig. 4c) decreased current by  $\sim$ 40–90% (Figs 3a–c, 4c). The smaller current decrease, of 20–30%, on reaction with the comparably sized neutral reagent 2-aminocarbonyl-ethyl-methanethiosulphonate (MTSACE; Fig. 4c and Supplementary Fig. 4) is consistent with simple steric interference with Na<sup>+</sup> current flow by the  $\sim 6 \text{ Å} \times 8 \text{ Å}$ adduct. Negatively charged MTSES, however, failed to react, neither altering pump-channel current nor preventing its subsequent decrease by MTSET<sup>+</sup> (Fig. 4c; Supplementary Fig. 4a, c); deep TM2 position T145 (see Fig. 3f) behaved comparably. By contrast, MTSES increased current in pump-channels with cysteines at the more superficial TM1 position A116 and TM1-TM2 loop residues Q128 and D130 (as previously shown<sup>18</sup> for Q120 and N131), the negative adduct electrostatically elevating the local concentration of current-carrying Na+ ions5,18. These results show that MTS reagents had to pass the cation-selectivity filter formed by E336 and D813 to reach every deeper reactive cysteine.

Our findings are all broadly consistent with corresponding locations of target residues in the Na<sup>+</sup>,K<sup>+</sup> pump model based on the SERCA E2•BeF<sub>3</sub><sup>-</sup> structure (Figs 1b, 4a, b; Supplementary Figs 1, 2), supporting its overall applicability. This is despite both the mere 26% amino acid identity between SERCA and Na<sup>+</sup>,K<sup>+</sup>-ATPase in the TM1–TM6 region scanned here, and the fact that the cytoplasmic-side pathway is tightly<sup>12,13</sup> closed in SERCA E2•BeF<sub>3</sub><sup>-</sup> (and also, apparently, in Na<sup>+</sup>,K<sup>+</sup>-ATPase E2•BeF<sub>3</sub><sup>-</sup>; Supplementary Fig. 5), whereas it can demonstrably open in the Na<sup>+</sup>,K<sup>+</sup> pump–channel. That open cytoplasmic access pathway runs between TM1, TM2 and

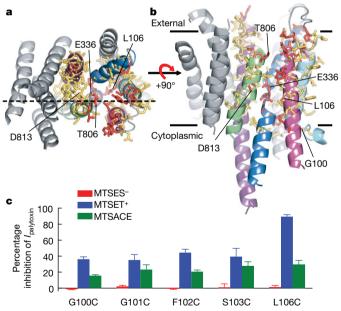


Figure 4 | Structural model and characteristics of ion pathway through the palytoxin-bound Na<sup>+</sup>,K<sup>+</sup>-ATPase. Results (including reactive and non-responsive positions from ref. 5) mapped onto a homology model of the Na<sup>+</sup>,K<sup>+</sup>-ATPase transmembrane domain (helices coloured as in Fig. 1) based on the SERCA E2·BeF<sub>3</sub><sup>-</sup> structure<sup>12</sup>, viewed from the extracellular surface (a) or from the membrane plane (b). Dashed line in a indicates plane of cut in Supplementary Fig. 2a. Red sticks mark reactive positions ( $I_{\text{palytoxin}}$  altered by >10% by MTSET<sup>+</sup>) and yellow sticks mark non-responsive positions. Reaction rate constants for MTSET<sup>+</sup> decreased from  $\geq$ 10<sup>4</sup> M<sup>-1</sup>s<sup>-1</sup> for superficial positions to  $\geq$ 10 M<sup>-1</sup>s<sup>-1</sup> for deep positions (Supplementary Fig. 8). c, Accessibility of cysteines beyond the cation-selectivity filter depends on the charge of the MTS reagent; summary of mean percentage inhibition ( $\pm$ s.e.m.; n, 3–8 patches) of  $I_{\text{palytoxin}}$  at -50 mV by  $\sim$ 2.5-min applications (all 1 mM) of MTSES<sup>-</sup> (red bars), MTSET<sup>+</sup> (blue bars) or MTSACE (green bars).

TM4, beyond the TM1 kink (at G101) seen in E2 structures<sup>10,11</sup> (Fig. 4b; Supplementary Figs 1, 2). MTSET<sup>+</sup>-accessible TM1 positions G101, F102, and L106 (Figs 3, 4) correspond to residues (rat Na<sup>+</sup>,K<sup>+</sup>-ATPase  $\alpha_1$  G94, F95 and L99) important in Na<sup>+</sup> and K<sup>+</sup> binding and occlusion in E2 conformations<sup>25,26</sup>, with L99 (here L106) in particular<sup>26</sup> cooperating with E329 (here E336) to lock exit or entry at site II. The equivalent SERCA TM1 region appears to gate cytoplasmic access for Ca<sup>2+</sup> ions<sup>8,10,11</sup>.

We found no sign of reaction with 1 mM MTSET<sup>+</sup> at any of 20 contiguous TM5 positions (Fig. 2), even though the E788-equivalent TM5 residue appears accessible from the extra-cytoplasmic side in the open<sup>12</sup> SERCA E2•BeF<sub>3</sub><sup>-</sup> structure (Supplementary Fig. 6), and residues equivalent to S784, N785 and E788 help coordinate Rb<sup>+</sup> at site I in the occluded E2•MgF<sub>4</sub><sup>2</sup> Na<sup>+</sup>,K<sup>+</sup> pump when both gates are shut<sup>21</sup>. Given that MTSET<sup>+</sup> reaction at many nearby positions altered pump–channel current (Fig. 4), it is unlikely that TM5 sites reacted without modifying current.

Although we cannot rule out the possibility that distortion of the Na $^+$ ,K $^+$ -ATPase ion pathway by palytoxin made TM5 residues inaccessible, this seems unlikely for several reasons. First, palytoxin action can be readily reversed, and repeated, on the same population of Na $^+$ ,K $^+$  pumps $^3$ . Second, the gates to the ion pathway through palytoxin-bound pump–channels still respond to the Na $^+$ ,K $^+$  pumps physiological ligands $^3$ . Third, positions as deep as the pathway narrowing are accessible to MTSET $^+$  without palytoxin (Supplementary Fig. 3). Fourth, blockers of access channels to ion-binding sites in unmodified Na $^+$ ,K $^+$  pumps similarly impede cation movement in palytoxin-bound pumps $^{23}$ . Fifth, MTSET $^+$ -accessible positions in palytoxin-bound pump—channels map reasonably onto unmodified pump structures (Fig. 4a, b and Supplementary Figs 1, 2; compare with refs. 5, 16, 17) and include sites expected to interact with transported ions $^{19-21,25,26}$ .

We conclude that unfavourable geometry precluded reactivity of MTSET<sup>+</sup> with TM5 positions at site I because they do not lie on the principal ion pathway. This is consistent with the side-chain charge of site-I residues E778 and D817 having little apparent influence on cation selectivity of Na<sup>+</sup>,K<sup>+</sup> pump–channels<sup>5</sup>. It is also consistent with the very slow reaction of E788C with a smaller reagent, namely 1-trimethylammonium-methyl-methanethiosulphonate

(MTSMT<sup>+</sup>; Supplementary Fig. 7); similarly small 2-aminoethylmethanethiosulphonate (MTSEA<sup>+</sup>; compare with ref. 15) is unreliable as it is membrane permeant and slowly reacts with Na<sup>+</sup>,K<sup>+</sup> pumps lacking engineered cysteines (Supplementary Fig. 7).

That  $\sim$ 6 Å wide,  $\sim$ 12 Å long MTSET<sup>+</sup>, MTSES<sup>-</sup> and MTSACE pass through palytoxin-bound Na<sup>+</sup>,K<sup>+</sup> pump-channels corroborates the findings that these channels conduct N-methyl-D-glucamine ions (diameter  $\geq 7 \text{ Å}$ ) only  $\sim 50 \text{ times more slowly than Na}^+$ ions<sup>22</sup>, and that their measured<sup>27</sup> Na<sup>+</sup> flux ratio exponent<sup>28</sup> is  $\sim 1.0$ , implying little interaction between Na<sup>+</sup> ions in a queue along the principal pathway that passes through site II. Occupancy by a second Na<sup>+</sup> ion of site I, off the main pathway but linked to it by a connection narrow enough to preclude reactivity with MTSET<sup>+</sup>, could account for suggested average pump-channel occupancy by two Na<sup>+</sup> ions<sup>23</sup>. In SERCA, lock-in of a Ca<sup>2+</sup> ion in site I by binding of the second Ca<sup>2+</sup> ion<sup>29</sup> in site II, and sequential release of the two Ca<sup>2</sup> ions<sup>30</sup>, are similarly consistent with transported ions negotiating a single common pathway from the cytoplasm to the ion-binding sites in E1 states, and from those sites to the reticulum lumen during release in the E2P state. The present snapshot of an ion pathway right through the Na<sup>+</sup>,K<sup>+</sup> pump affords a structural basis for understanding cation translocation in P-type pumps.

## **METHODS SUMMARY**

Ouabain- and MTS-insensitive *Xenopus* Na<sup>+</sup>,K<sup>+</sup> pumps. *Xenopus* Na<sup>+</sup>,K<sup>+</sup> pumps were made insensitive to ouabain and extracellular MTS reagents by the mutation C113Y (ref. 24) in *Xenopus* Na<sup>+</sup>,K<sup>+</sup>-ATPase  $\alpha_1$  subunits as described previously<sup>18</sup>. Single cysteines were introduced into C113Y Na<sup>+</sup>,K<sup>+</sup>-ATPase  $\alpha_1$  by

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PCR. Complementary DNA in a pSD5 vector was transcribed in vitro. Xenopus oocytes were injected with a 50-nl mixture of 5 ng of Xenopus β<sub>3</sub> and 15 ng of mutated *Xenopus* α<sub>1</sub> complementary RNAs, and incubated at 18 °C for 1–3 days. Current recordings and analysis. Currents were recorded in outside-out excised patches at 22–24 °C as described previously<sup>5,18</sup>. The internal (pipette) solution contained 125 mM NaOH, 100 mM sulphamic acid, 20 mM HCl, 10 mM HEPES, 1 mM EGTA, 1 mM MgCl<sub>2</sub> and 5 mM MgATP (pH 7.4). The external solution contained 125 mM NaOH or TMA-OH, 125 mM sulphamic acid, 10 mM HEPES, 5 mM BaCl<sub>2</sub>, 0.5 mM CaCl<sub>2</sub>, 1 mM MgCl<sub>2</sub> (pH 7.6) and 100 μM ouabain. Palytoxin (Wako) was added (from 100 μM aqueous stock solution) at 50 nM, with 0.001% bovine serum albumin and 1 mM Na-borate. MTS reagents (Toronto Research Chemicals) were added from ice-cold (~0 °C) 100 mM aqueous stock solutions immediately before use, and were refreshed at 1.5-min intervals to maintain reactivity during prolonged (≥2 min) applications4. Alteration of palytoxin-induced current by MTS reagents was calculated as follows: percentage inhibition of  $I_{\rm palytoxin}$  equals  $100 \times (1 - I_{\rm after}/I_{\rm before})$ . Here  $I_{\text{after}}$  represents steady palytoxin-induced current at  $-50 \,\text{mV}$  after MTS reagent application, and Ibefore represents the same current just before MTS reagent application. Data are given as mean  $\pm$  s.e.m.

**Model building.** The *Xenopus* Na $^+$ ,K $^+$ -ATPase  $\alpha_1$  subunit homology model was built from the Ca $^{2+}$ -ATPase E2 $^+$ BeF3 $^-$  structure (ref. 12; Protein Data Bank code 3B9B) using SWISS-MODEL (http://swissmodel.expasy.org) as described previously $^5$ . Structural figures were prepared with PyMOL version 0.97 (http://www.pymol.org).

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**Supplementary Information** is linked to the online version of the paper at www.nature.com/nature.

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