performed in HeLa cells expressing MCU-V5 and Mfrn2-GFP. Results: In RLM, Ca²⁺ and Fe²⁺ (250 μM) each stimulated respiration to a nearly identical degree, and both were completely blocked by Ru360. In UMSCC22A cells, mRNA and protein expression of Mfrn2 was 2-3-times that observed in UMSCC1 cells. High Mfrn2-expressing UMSCC22 cells also had 3-fold greater rates of mitochondrial Ca²⁺ and Fe²⁺ uptake. After Mfrn2 knockdown (55% decrease), rates of mitochondrial uptake of both Ca²⁺ and Fe²⁺ decreased by ~75%. All uptakes were blocked by Ru360. In HeLa cells co-transfected with MCU-V5 and Mfrn2-GFP, anti-GFP beads pulled down MCU-V5, whereas anti-V5 beads pulled down Mfrn2-GFP. COX-IV was not pulled down by beads, indicating that the interaction between MCU and Mfrn2 was specific.

The mitochondrial calcium uniporter should more appropriately be called the mitochondrial Ca²⁺/Fe²⁺ uniporter.

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K⁺ Translocation by the Giant Axon of the Humboldt Squid Na⁺/K⁺ ATPase
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The Na⁺/K⁺ pump is a membrane protein which plays a fundamental role in maintaining the Na⁺ and K⁺ electrochemical gradients in animal cells. When internal and external Na⁺ is absent the pump can only undergo K⁺ translocation reactions. At equilibrium, the distribution of the different protein conformations depends on the rate constants of each step leading to K⁺ binding and unbinding. If some of these rate constants are voltage-dependent, sudden changes in membrane electric potential will shift the binding-unbinding equilibrium. In those translocation reactions K⁺ has to travel a fraction of the membrane electric field generating a transient current signal. Here, K⁺ pump currents were measured under voltage clamp conditions using the giant axon of the Humboldt squid, which due to its large diameter (1 -1.5 mm) allows the detection of these charge movements. By using H2DTG, a reversible inhibitor of the squid NAD+, K⁺ pump, we were able to obtain H2DTG-sensitive transient currents in response to voltage jumps in K⁺/K⁻ conditions. Kinetics of these transient currents shows two main components, that in contrast to their Na⁺ counterpart, appeared to be uncoupled. The origin of the fast component appears to be the movement of ions along an access channel that it is always open, suggesting that the gate that occlude K⁺ ions is deep in the permeation pathway. On the other hand, charge displacement distribution and rate constants of the slow component show a clear dependence on the K⁺ external concentration revealing that the entrance of the K⁺ to the Na⁺/K⁺ pump from the external side is a voltage-dependent step. Supported by FIRCA grant R03 TW008351 and U54GM087519, GM030376, NS64259, HL36783 and the Intramural Program of the NINDS/NIMH and FONDECYT 1110430.

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Conformational Rearrangements of the Na⁺/K⁺ ATPase During Na⁺/K⁺ Occlusion/Deocclusion Transitions Assessed by Site-Directed Fluorescence
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The Na⁺/K⁺ ATPase is a E₁-E₂-type pump formed mainly by two subunits: α and β, and is responsible for Na⁺ and K⁺ homeostasis, thereby fundamental for cellular life. Most of the voltage dependence of the pump cycle originates from the steps associated with extracellular Na⁺ binding and release, namely occlusion and deocclusion (E₁P and PE₂). To gain further insights into the conformational rearrangements of the squid Na⁺/K⁺ pump during the E₁P and PE₂ transitions, we have used site-directed fluorometry under voltage-clamp. We engineered single cysteine mutants facing the outside of the α and β subunits of the squid Na⁺/K⁺ pump, as targets of a cysteine-reactive fluorescent reporter (6-TMR). These constructs were found functional when expressed in Xenopus oocytes under voltage-clamp conditions and we obtained simultaneous electrical and fluorescence recordings. We have identified two positions on the α-subunit: N894C (linker M7-8) and D740C (linker M5-M6), and two on the β-subunit: D74C (top of the TM helix) and D116C (external cap-helix), that produce voltage dependence fluorescence changes during the E₁P/PE₂ transitions. Fluorescence intensities produced by N894C, D74C and D116C constructs were correlated with its respective voltage dependent Na⁺/K⁺ translocation currents following a Boltzmann distribution. Interestingly, kinetics of the fluorescence and electrical signals from N894C showed remarkably similar voltage dependence, suggesting functional and conformational correlation during E₁P/PE₂ transitions. Surprisingly, fluorescence signal from D116C produced a biphasic kinetics behavior, indicating a complex movement of the β and/or the α with respect to the β subunit. These findings begin to delineate a region in the α-subunit that is moving during the E₁P/PE₂ transitions. Supported by U54GM087519 and GM030376.

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State-Dependent Movement between the First and Last External Loops of the Na⁺/K⁺ Pump α Subunit
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Previously we reported that conserved Na⁺/K⁺ pump α subunit residues D128 (loop L1-2, shank-α numbering) and R979 (L9-10) reach atomic proximity in E₂ (Artigas, 2009, Biophys J 96(3):145a). L1-2 is expected to move throughout the pump cycle. We used double cysteine substitutions to address the relative displacement of L1-2 with respect to R979. We mutated R979C and concomitantly introduced a cysteine at each of the residues within L1-2, from Q118 (external end of TM1) to L132 (within TM2), and analyzed the functional effect of reducing and oxidizing reagents on these double cysteine mutants heterologously expressed in Xenopus oocytes. The pump current (Ip) induced by 3 mM K⁺ under two-electrode voltage clamp in the absence of Na⁺ was measured; first without redox treatment, then after 15 min in 10 mM TCEP (a reducing agent) and subsequently, following oxidation with copper phenanthroline (100 μM Cu²⁺, applied in N-Methyl-D-glucamine). TCEP increased the Ip of E122C/R979C (20%), E124C/R979C (20%), P125C/R979C (36%), Q126C/R979C (52%) and D128C/R979C (150%), whilst Cu²⁺:Phe induced similar effects, in the opposite direction than TCEP, with a maximum reduction of Ip (~80%) in D128C/R981C. The effect of Cu²⁺:Phe on the charge movement without K⁺ in the presence of Na⁺ was also studied. Cu²⁺:Phe abolished charge movement of D128C/R981C and modified the characteristics of the transients of the other double cysteine mutants that showed effects of crosslinking in Ip. Our results indicate that residue 128 (at the end of a rigid TM2-helix) must separate from R979 in order for TM2 to perform its required motions, while crosslinking residues further in the flexible loop does not block Ip, but modifies the E1-E2 conformational equilibrium in the presence of Na⁺. Supported by R15NS081570-01A1.

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Single-Molecule Measurements to Investigate the Negative Cooperativity in Na⁺/K⁺ -ATPase
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The Na⁺/K⁺ ATPase, a cell membrane ion motive ATPase, uses energy from the hydrolysis of ATP to move Na⁺ out of and K⁺ into cells, thus maintaining the relative sodium potential and cellular volume. To investigate how this pump functions, we isolated ATPase from duck suprarnaortal salt glands and labeled it with Cy3-maleimide (Cy3-ATPase). In bulk experiments, we found that the fluorescence of Cy₃-ATPase decreases in the presence of ATP (Biochim Biophys Acta 2009; 1794:1549-1557). The kinetics of this ATP-induced fluorescence decrease exhibited negative cooperativity and could be explained in terms of protein aggregation. To further explore the phenomenon of negative cooperativity on the level of individual monomers, we used single-molecule total internal reflection fluorescence (SM-TIRF) microscopy. Protein monomers were solubilized and reconstituted into lipid vesicles to investigate the effect of varying ATP concentration on the fluorescence. Data from SM-TIRF experiments, analyzed using a hidden Markov model (HMM), suggest that the Cy₃-ATPase exists in dynamic equilibrium between a high fluorescence state (unquenched) and a low fluorescence state (partially quenched). These kinetics are characterized by either rapid or slow transitions between these states. Two subpopulations are observed, one where the transitions between the states occur rapidly and the other where the kinetics are slower. Preliminary analysis of the data suggests that ATP shifts the population distribution from those exhibiting rapid transitions to those exhibiting slow transitions. Here, we report on the analysis of these effects and the implications of the above observations on the working of the pump.

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The Molecular Mechanism of Na⁺, K⁺ -ATPase Mutation in Fused Renal Adenoma
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Mutations within ion transporting proteins may severely affect their ability to properly traffic ions and thus perturb the delicate balance of ion gradients.