(7.6). After deletion of the last five (∈″KESYY), or two (∈″YY), COOH-terminal residues of those pumps, uncoupled current was recorded also in extracellular Na. We used two-microelectrode recording to measure uncoupled, and coupled Na/K transport, currents as those inhibited by 10-30 mM ouabain in *Xenopus* oocytes expressing C113Y or C113Y-∈″YY Na/K pumps. To investigate whether the uncoupled current traverses the same principal pathway followed by transported Na and K ions we used two methods to close that pathway. In one method, we formed stable BeF_X-Na/K-pump complexes, trapped in an E2P-like state with closed cytoplasmic-side gates, by injecting oocytes with 1mM BeF_X. In the other, we closed the extracellular access pathway by modifying a Cys substituted for T806, at the outer end of TM6, with 1 mM extracellular MTSET. Both methods abolished coupled Na/K pump transport current, as well as Na current flow through palytoxin-bound C113Y Na/K pump-channels. But neither method diminished uncoupled current at −180 mV, suggesting either that the responsible ions do not traverse the principal pathway shared by transported Na and K ions or, if they do follow that route, that they do not travel as hydrated cations. [NIH HL36783]

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Intracellular Proton Binding is Voltage-Dependent and Rate-Limiting for the Gastric H,K-ATPase Under in vivo Conditions

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Compared to the extensively studied Na,K-ATPase reaction cycle, much less is known about the voltage-dependent steps in the pump cycle of gastric H,K-ATPase. Due to the overall electroneutral transport stoichiometry of the proton pump, its voltage-dependent ion transport properties cannot readily be determined by standard electrophysiological techniques.

Therefore, we use a combination of two biophysical techniques to investigate H,K-ATPase activity: voltage-clamp-fluorometry on the tetramethylrhodamine-6-maleimide-labeled H,K-ATPase variant αS806C to monitor the voltage-dependent distribution between E_1P/E_2P-states (Ref. 1) and voltage-controlled Rb^+ uptake measurements to assess the steady-state ion transport activity under various pH and ionic conditions in *Xenopus* oocytes.

Both the steady-state E_1P/E_2P-distribution and Rb^+ uptake of the gastric H,K-ATPase are highly sensitive towards changes in intracellular pH (which can be achieved by adding weak organic acids like butyric acid to the extracellular solution), whereas even larger changes in the extracellular pH do neither influence the conformational E_1P/E_2P-equilibrium nor transport activity. An intracellular acidification of approximately 0.5 pH units results in a large negative shift (~100 mV) of the voltage-dependent fluorescence amplitudes and an approximately two-fold acceleration of the reciprocal time constants at positive membrane potentials.

One possible interpretation of these results is that proton binding takes place in a shallow intracellular ion access channel (apparent well depth: 0.3-0.5).

Since maximal rubidium uptake at saturating concentrations is strongly stimulated by intracellular acidification, the voltage-sensitive intracellular proton binding step is apparently rate-limiting for the overall transport activity under physiological conditions. These findings highlight the need for cellullar mechanisms which increase the availability of protons at the cytoplasmic face of the pump, such as CO_2-producing mitochondria, sub-membrane carbonic anhydrase and the basolateral Cl^-/HCO_3^- exchanger that are characteristic for parietal cells.

References
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Conformational Dynamics of a Fluorescent Probe Attached to the Sarcoplasmic Reticulum Ca^{2+}-ATPase (SERCA) Studied by Molecular Simulations

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We have performed molecular dynamics simulations, based on a new X-ray crystal structure of fluorescent-labeled SERCA, in order to establish a more rigorous foundation for analyzing fluorescence data from this system. Site-specific labeling of a protein with a fluorescent probe can provide insight into local structural dynamics, based on fluorescence quenching or anisotropy measurements, or based on fluorescence resonance energy transfer (FRET) to another label. SERCA was labeled at position Cys674 in the P-domain with the fluorescent probe IAEDANS. The crystal structure of IAEDANS-labeled SERCA was determined to 3.4Å resolution, which was sufficient to show the IAEDANS label in close proximity to residues Arg615 and Arg620. This structure was used as a starting point for molecular dynamics simulations and conformational sampling calculations of the fluorescent probe and its protein environment. To be able to perform these simulations, we developed CHARMM force-field parameters for the fluorescence probe IAEDANS. Quantum chemistry calculations have also been performed on the ground state and excited states of IAEDANS, to determine the orientation of the transition dipole moment. The transition dipole autocorrelation functions and reorientation times were calculated from the simulated trajectories and compared with experimental measurements by fluorescence anisotropy. These results validate our computational approach and establish a reliable framework for analysis of fluorescence experiments in this system. This work was supported by NIH (GM27906, AR007612) and the Minnesota Supercomputing Institute.

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Measuring the Dissociation Constants of Ligands from PMCA Complexes by a Photoactivatable Phosphatidylcholine Membrane Domain Probe

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The purpose of this work was to obtain structural information about conformational changes of the plasma membrane Ca^{2+} pump (PMCA) in the membrane region upon interaction with ATP, Ca^{2+}, calmodulin and acidic phospholipids. To this end, we have quantified labeling of PMCA with the photoactivatable phosphatidylcholine analog [^{125}I]TID-PC/16, measuring the shift of conformation E_2 to the auto-inhibited conformation E_1I and to the activated E_1A state, titrating the effect of Ca^{2+} and ATP under different conditions. With this method we were able to measure apparent and equilibrium constants for the dissociation of Ca^{2+}, ATP and calmodulin and other ligands from PMCA complexes through the change of transmembrane conformations of the pump. The results indicate that the PMCA possesses a high-affinity site for Ca^{2+} regardless of the presence or absence of activators. Modulation of pump activity is exerted through the C-terminal domain, which induces an apparent auto-inhibited conformation for Ca^{2+} transport but does not modify the affinity for Ca^{2+} at the transmembrane domain. The C-terminal domain is affected by calmodulin and calmodulin-like treatments driving the auto-inhibited conformation E_1I to the activated E_1A conformation and thus modulating the transport of Ca^{2+}. The data further suggest that the hydrophobic transmembrane domain of the PMCA undergoes major rearrangements resulting in altered lipid accessibility upon Ca^{2+} binding and activation. With grants from ANPCYT, CONICET, UBACYT and NIH.

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A Phospholamban-Cardiac Ca^{2+} Pump Fusion Protein Retains Full Functional Regulation

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To study the molecular mechanism by which phospholamban (PLB) inhibits the cardiac Ca^{2+} pump (SERCA2a), we engineered a fusion protein with PLB tethered to the C-terminus of SERCA2a (SER-20G-PLB). A 20 glycine-residue linker was inserted between the C-terminus of SERCA2a and the N-terminus of PLB to allow the PLB-tether either to bind to SERCA2a at its inhibition site, or to diffuse away from SERCA2a in the membrane, permitting enzyme activation. SER-20G-PLB was expressed in insect cell microsomes and compared to normal WT-SERCA2a co-expressed with normal WT-PLB. SER-20G-PLB ran at 110 KDa and 550 KDa on SDS-PAGE, demonstrating that the fusion protein exists as both monomers and homo-pentamers, like WT-PLB which ran at 5 KDa and 25 KDa. In the Ca^{2+}-uptake assay, SER-20G-PLB transported Ca^{2+}, showing that the pump remained fully active. Importantly, the Ca^{2+}-uptake by SER-20G-PLB was stimulated by the anti-PLB antibody, 2D12, in a similar fashion as occurred with WT-SERCA2a co-expressed with WT-PLB. Further, in the Ca^{2+}-dependent ATPase assay, the Ca^{2+} concentration for half-maximal activation (K_{Ca} value) was 0.26 ± 0.01 μM for SER-20G-PLB, identical to 0.25 ± 0.01 μM for WT-SERCA2a co-expressed with WT-PLB, both larger than 0.16 ± 0.01 μM for WT-SERCA2a expressed alone. Thus, SER-20G-PLB has intrinsically decreased apparent Ca^{2+} affinity, the hallmark of PLB inhibition. Finally, the L31A mutation in the PLB-tether, which disables normal PLB function, also prevented Ca^{2+}-ATPase inhibition by the tether (K_{Ca} = 0.16 ± 0.01 μM). Thus, SER-20G-PLB retains a fully active Ca^{2+} pump, which is intrinsically regulated by its flexibly anchored PLB-tether. The fusion protein, with a built-in 1:1 molar stoichiometry between PLB and SERCA2a, provides a unique system to address dynamic interactions between the two proteins situated in the membrane.