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Structural Dynamics of Calcium Transport Proteins Detected by Saturation Transfer Electron Paramagnetic Resonance
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We have used conventional and saturation transfer electron paramagnetic relaxation (EPR) to probe the structural dynamics of the integral membrane protein phospholamban (PLB), as a function of phosphorylation and the presence of its regulatory target, the sarcoplasmic reticulum calcium ATPase (SERCA).
Our goal is to elucidate the mechanism of SERCA inhibition by PLB, which is needed for the rational design of therapies to improve calcium transport in muscle cells.
We used the monomeric mutant AFA-PLB with the rigid electron spin label TOAC incorporated at position 36 in the transmembrane domain, and reconstituted the protein in lipid vesicles.
EPR experiments were performed to determine the nanosecond (conventional EPR) and microsecond (saturation transfer EPR) rotational motions of PLB, as modulated by phosphorylation of PLB at serine 16 (pPLB), and/or addition of excess SERCA.
Neither SERCA binding nor PLB phosphorylation caused significant changes in conventional EPR spectra of PLB, indicating no effect on the nanosecond flexibility of PLB's transmembrane domain.
However, saturation transfer EPR found that addition of SERCA caused a large decrease in microsecond rotational motion for both PLB and pPLB.
Phosphorylation of PLB caused no effect in the absence of SERCA and a slight mobilization in the presence of SERCA.
These results indicate that both PLB and pPLB bind to SERCA, and PLB phosphorylation only slightly increases the flexibility and/or the dissociation constant of the SERCA-PLB complex.
Therefore, PLB phosphorylation relieves SERCA inhibition primarily by changing the structural dynamics of the SERCA-PLB complex, not by dissociation of the complex.
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Time-Resolved Fluorescence and Molecular Modeling of FITC-Labeled Ca-ATPase
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We have detected structural dynamics in the nucleotide-binding domain of the Ca-ATPase (SERCA) using fluorescence spectroscopy and molecular modeling.
SERCA in sarcoplasmic reticulum (SR) vesicles was selectively labeled with fluorescein isothiocyanate (FITC), an affinity probe that specifically react with Lys-515 in the nucleotide-binding site.
FITC-labeled SERCA enters the phosphorylated “E1P-like” conformation with low probe fluorescence (~50% intensity) when SR vesicles are loaded with calcium using acetylphosphorylated “E1P-like” conformation with low probe fluorescence.
We have detected structural dynamics in the nucleotide-binding domain of the Ca-ATPase (SERCA) using fluorescence spectroscopy and molecular modeling.
Ca-ATPase transports Ca2+ into the SR, allowing muscles to relax, and is inhibited by phosphorylamban (PLB) at submicromolar [Ca2+]i.
PLB inhibitor can be relieved by adrenergic stimulation, leading to PLB phosphorylation.
Heart Failure (HF), which contributes to 12% of US deaths, can be caused by a variety of genetic or environmental factors, but a common symptom is decreased SERCA activity.
Several potential therapies for HF aim to increase SERCA activity, including overexpression of the enzyme via rAAV gene therapy.
We are investigating the possibility of using LOF-PLB mutants (PLBm) as alternative gene therapy vectors to increase Ca-ATPase activity.
We have used FRET and FLIM to measure quantitatively measure the ability of several PLBm to compete with WT-PLB to bind to SERCA and activate it.
Experiments in living HEK cells examine the ability of PLBm to compete with WT-PLB, thus rescuing SERCA activity.
Active human SERCA2a and WT-PLB, tagged with fluorescent proteins (e.g., CFP and YFP), are co-expressed in stable cell lines followed by transient co-expression with PLBm.
The effects of PLBm on Ca-ATPase activity and FRET are measured to determine the mutant’s ability to compete with PLBm, both physically and functionally.
Optimal mutants are being tested in a rat model of heart failure.

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Quantification of Calcium Pump Structure Changes in Live Cells
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The sarcoendoplasmic reticulum ATPase (SERCA) is a calcium pump that undergoes conformational changes during catalytic cycling.
To investigate SERCA structure changes in living cells, we fused Cerulean to the A-domain and YFP to the N-, P- or TM-domain of SERCA2a and expressed these “2-color” SERCA constructs in AAV-293 cells.
2-color SERCA constructs were catalytically active as shown by APase activity in vitro and Ca uptake in live cells.
Specifically, the transport activity of 2-color SERCA blunted IP3-mediated Ca transients and increased the size of the Tg-releasable Ca stores.
Both of these effects were reversed by coexpression of exogenous phospholamban (PLB), suggesting that 2-color SERCA function and regulation were intact.
All of the constructs exhibited dynamic FRET changes in response to the pump ligands Ca and thapsigargin (Tg).
The Tg-dependent conformational change was not decreased by coexpression of PLB, nor did PLB slow the kinetics of Tg-binding.
Notably, FRET in ionophore-permeabilized cells was higher with saturating calcium than with EGTA in the extracellular solution.
This suggests a decrease in domain separation distance with the structure transition from E2 (Ca-free) to E1 (Ca-bound).
The data are consistent with closure of the cytoplasmic headpiece with Ca-binding.
These 2-color SERCA constructs provide insight into the structural dynamics of Ca transport and may also be useful for evaluating candidate small molecule regulators of Ca uptake activity.

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Dynamic Conformational Transitions of Sarcoendoplasmic Reticulum Ca-ATPase (SERCA) Quantified by Single Molecule FRET
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We present here the measurement of dynamic transition of SERCA among its various conformational states during its Ca pumping cycle.
This is achieved by monitoring single-molecule FRET (smFRET) between fluorescent proteins fused to the N and A domains of SERCA.
We observe fluctuations in the FRET efficiency by measuring the changes in the fluorescence lifetime of the donor fluorescent protein as a function of time as detergent solubilized SERCA single molecules transit through the laser excitation spot.
Making histograms of these FRET efficiency fluctuations, we detected multiple discrete FRET levels indicating different conformational states.
The mean FRET efficiency values determined from the histograms are used to assign conformational states of SERCA to the FRET efficiency time trajectories.
The fluctuations in these time trajectories then provide us the timescales of the transition of SERCA between its different conformational states.
We detect fast transitions (ms) between the long lived (ns) conformational states of SERCA.
We also measure decrease in these transition timescales in the presence of Ca.
This information provides new insight to the structural dynamics of SERCA during the Ca transport cycle.

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Fluorescence and Functional Measurements of Phospholamban Mutant Competition
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Ca2+ cycling through the SR in muscle cells is largely controlled by the Ca-pump (SERCA), SERCA transports Ca2+ into the SR, allowing muscles to relax, and is inhibited by phospholamban (PLB) at submicromolar [Ca2+]i.
PLB inhibition can be relieved by adrenergic stimulation, leading to PLB phosphorylation.
Heart Failure (HF), which contributes to 12% of US deaths, can be caused by a variety of genetic or environmental factors, but a common symptom is decreased SERCA activity.
Several potential therapies for HF aim to increase SERCA activity, including overexpression of the enzyme via rAAV gene therapy.
We are investigating the possibility of using LOF-PLB mutants (PLBm) as alternative gene therapy vectors to increase Ca-ATPase activity.
We have used FRET and FLIM to measure quantitatively measure the ability of several PLBm to compete with WT-PLB to bind to SERCA and activate it.
Experiments in living HEK cells examine the ability of PLBm to compete with WT-PLB, thus rescuing SERCA activity.
Active human SERCA2a and WT-PLB, tagged with fluorescent proteins (e.g., CFP and YFP), are co-expressed in stable cell lines followed by transient co-expression with PLBm.
The effects of PLBm on Ca-ATPase activity and FRET are measured to determine the mutant’s ability to compete with PLBm, both physically and functionally.
Optimal mutants are being tested in a rat model of heart failure.

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Physical and Functional Interaction of Membrane Proteins Modulated by Electrostatic Interactions with Membrane Surface Charge
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We control the physical interaction and thus functional regulation between integral membrane protein phospholamban (PLB) and sarcoplasmic reticulum