Calcium Dysregulation and Cardiac Pathology

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Cell Biology and Molecular Medicine
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Ca^{2+} is a major intracellular messenger

- The idea that Ca^{2+} could be a carrier of signals originated over 100 years ago with the observation by S. Ringer that isolated hearts could only be made to contract if Ca^{2+} was added to the perfusion medium

- After a long interval of time, work performed in 1947 by Heilbrunn and Wiercinski demonstrated that Ca^{2+} triggers the contraction of frog muscles when injected, whereas no contraction followed the injection of Na+, K+ or Mg^{2+}

- Calcium (Ca^{2+}) plays a vital role in the regulation of muscle contractility, growth and gene expression

Heart diseases associated with abnormal Ca^{2+} cycling

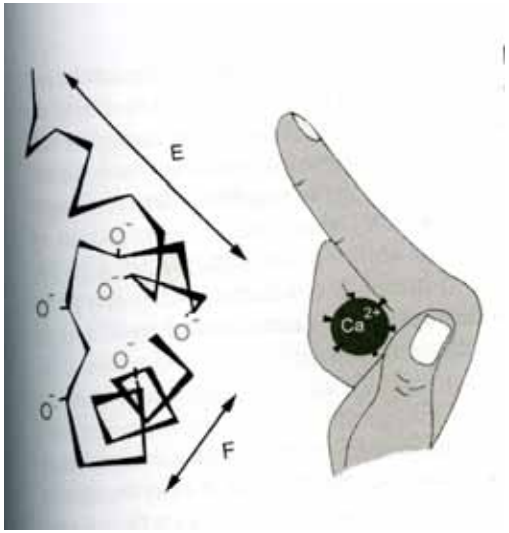
- Heart Failure
- Arrhythmias
  -Atrial fibrillation
  -Ventricular tachycardia

  Mutations in Ca^{2+} handling protein genes-cause cardiac pathology

Ca^{2+} handling proteins

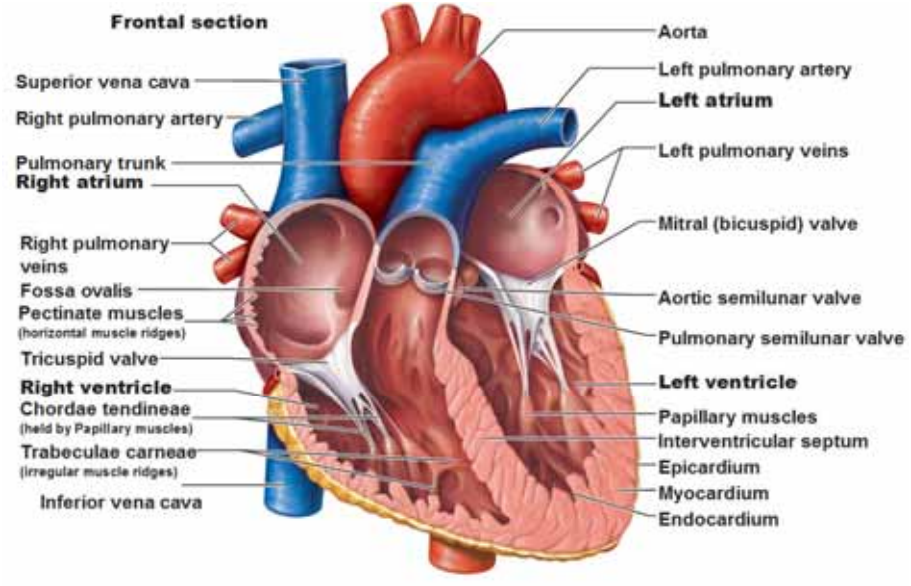
- **Ca^{2+} BINDING PROTEINS**

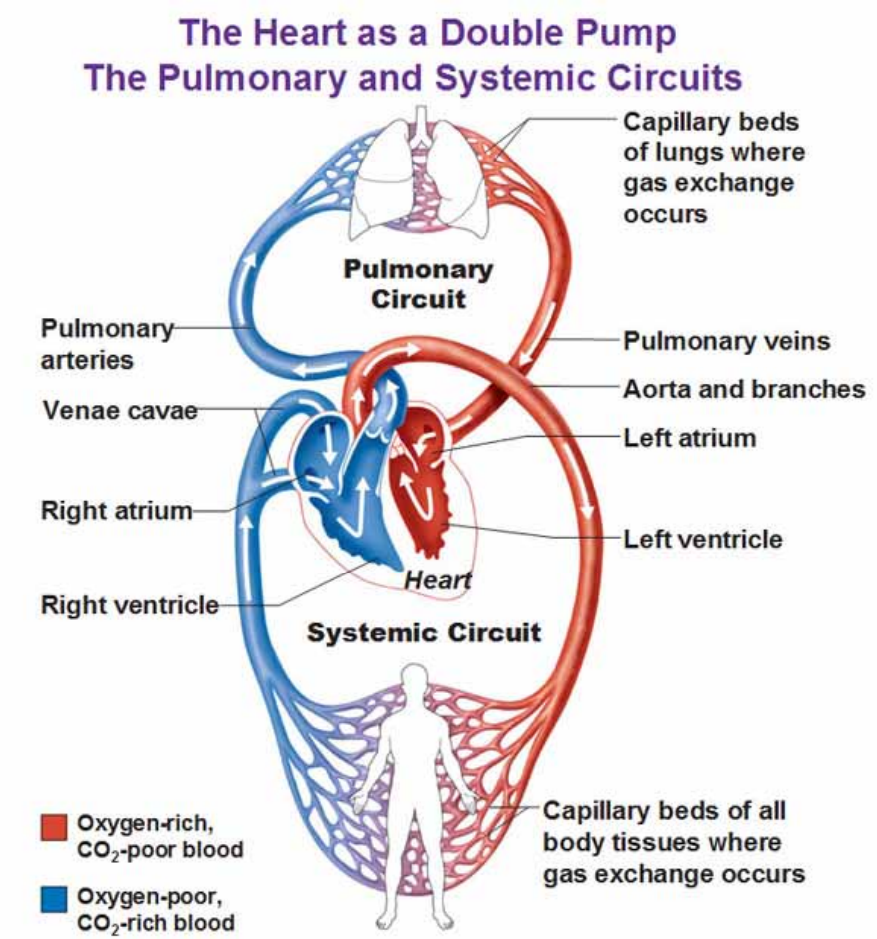
  The most important proteins able to bind Ca^{2+} with the affinity and specificity required for the regulation of its concentration in the intracellular environment belong to the EF-hand family, which now contains hundreds of members; some only regulate only one Ca^{2+} dependent process (enzyme), e.g., troponin C, while others are not target-specific, e.g., calmodulin.



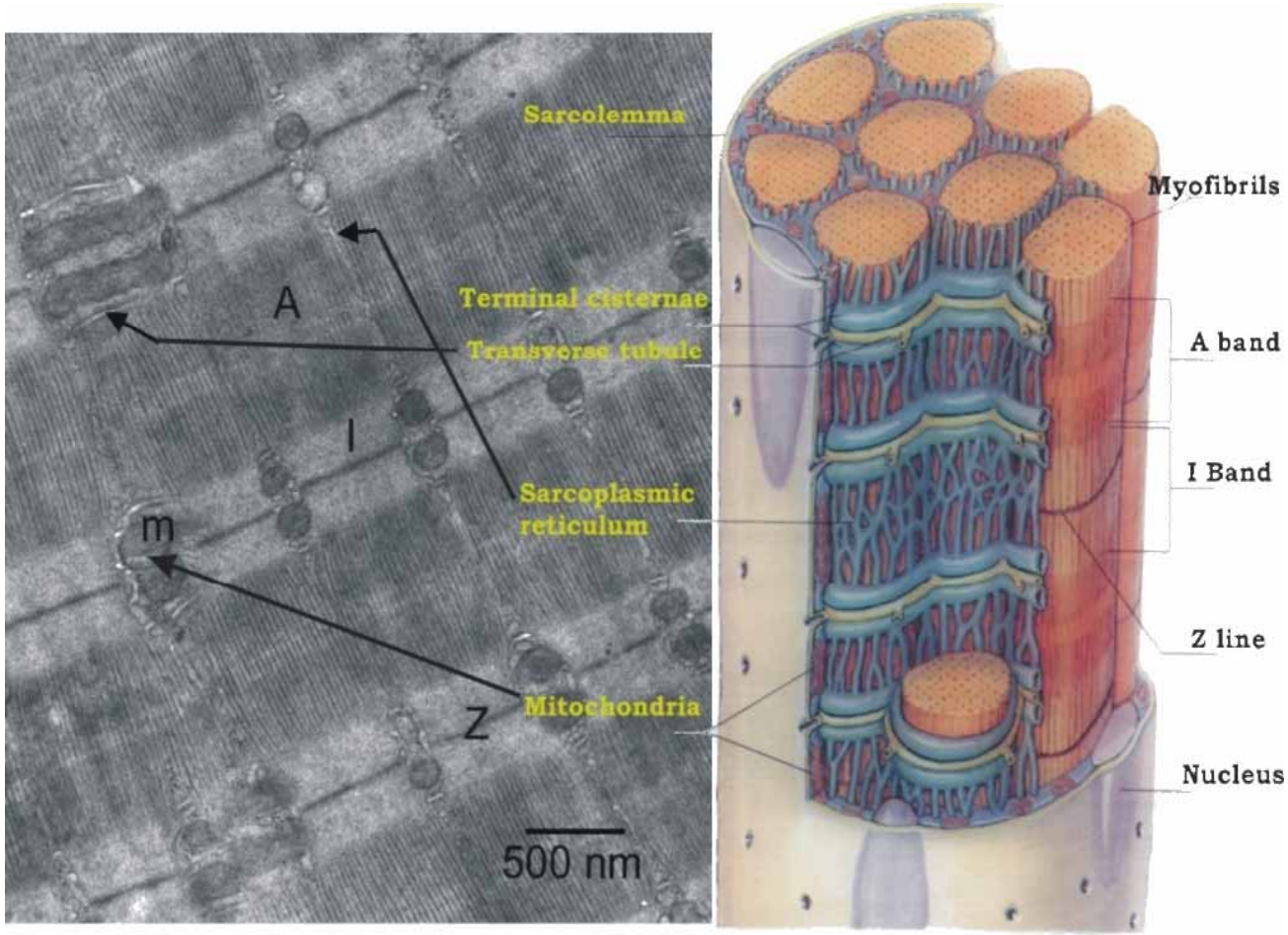
- **Ca^{2+} TRANSPORT PROTEINS**

Function of the heart





Ultra structure of a muscle Cell



Electron microscopic image.

Idealized image of ultrastructure of the muscle cell.

Cardiac muscle-Ultrastructure

TABLE 1-1

Components of a Working Myocardial Cell (Rat Left Ventricle)

Component	Percentage of Cell Volume
Myofibrils	47
Mitochondria	36
Sarcoplasmic reticulum	3.5
Subsarcolemmal cisternae	0.35
Sarcotubular network	3.15
Nuclei	2
Other (mainly cytosol)	11.5

TABLE 1-2

Membrane Surface Areas in a Working Myocardial Cell (Rat Left Ventricle)

Membrane	μm^2 Membrane Area Per μm^3 Cell Volume
Plasma membrane	0.465
Sarcolemma	0.31
t-Tubules	0.15
Nexus	0.005
Total sarcoplasmic reticulum	1.22
Subsarcolemmal cisternae	0.19
Sarcotubular network	1.03
Mitochondria	20

Modified from Page (1978).

Cell polarization

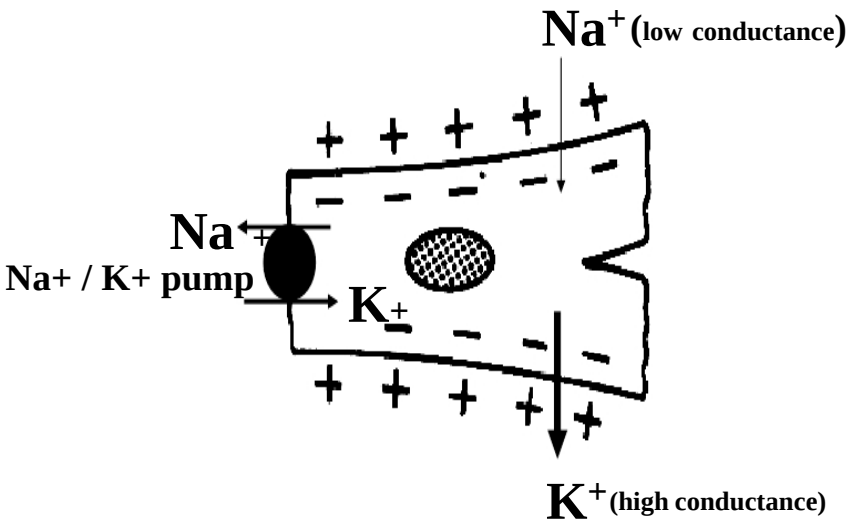
- Electric changes within the myocyte initiate myocyte contraction

- Cardiac cells have an electrical potential across the cell membrane. It can measured by inserting a microelectrode into the cell and measuring the electrical potential in millivolts (mV) inside the cell relative to the outside.

- If measurements are taken with a resting cardiac myocytes, **the resting membrane potential** (Em) will be -85mV. This is determined by the concentrations of positively and negatively charged ions across the cell membrane, the relative permeability of the cell membrane to these ions and ionic pumps that transports ion across the cell membrane.

- The conc. of Na^+, K^+, Cl^- and Ca^{2+} are most important in determining the membrane potential

The resting cardiomyocyte is negatively charged



The membrane potential (Em) is calculated as follows:

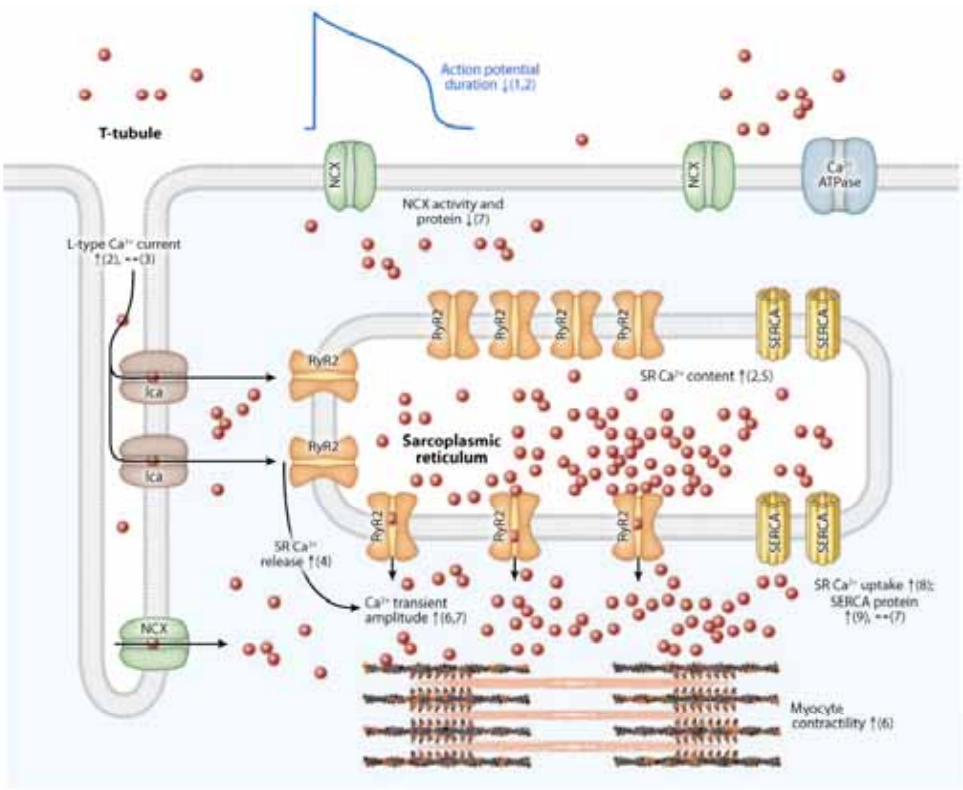
Em = 61.5 ln (PK Ko/Ki + PNa Nao/Nai)

P, conductance; o, extracellular; i, intracellular

- In CMs, the conc. of K^+ is the most important in determining the resting mem. potential.
- The conc. of K^+ is high (150 mM) inside and low outside (4mM)-chemical gradient
- The opposite situation is found for Na^+ (outside-145 mM) .
- Through Na^+ / K^+ pump, the cell extrudes Na^+ and accumulates K^+
- In such a cell, K^+ diffuses out and leaves behind negatively charged proteins and potential difference across the membrane.
- Na^+ tends to spontaneously enter the cell and does it slowly because its permeability is low.
- At rest, the plasma membrane is impermeable to Ca^{2+}

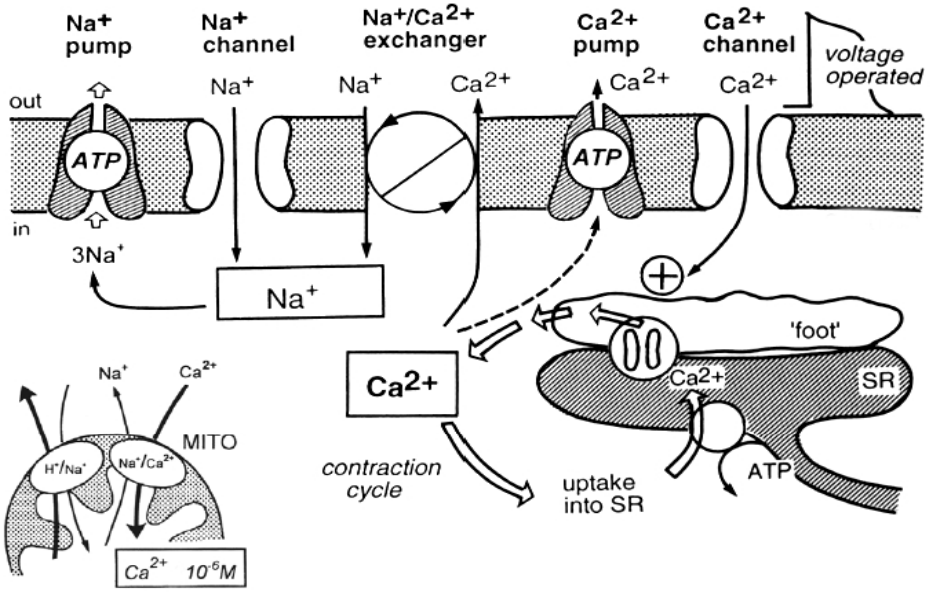
Excitation-Contraction Coupling

- In cardiac E-C coupling, a small amount of Ca^{2+} enters through the L-type Ca^{2+} channel (LTCC) during membrane depolarization
- This Ca^{2+} influx triggers a large scale release of Ca^{2+} from the Sarcoplasmic Reticulum (SR) membrane via Ryanodine Receptor (RyR)
- Released Ca^{2+} binds to myofibirillar proteins, TnC-induce muscle contraction.
- Relaxation is initiated by the reuptake of Ca^{2+}



Terracciano CM, et al. 2010.
Annu. Rev. Med. 61:255–70

Mechanisms of Ca^{2+} reuptake



<u>Ca^{2+} is extruded from the cytosol through pumps and exchangers</u>

➢ **Sarco(endo)plasmic reticulum Ca^{2+} ATPase (SERCA)**. Pumps back Ca^{2+} inside the SR. This represents 90% of reuptake. SERCA function requires ATP to pump Ca^{2+} against the gradient.

➢**The sarcolemmal Na^+/Ca^{2+} exchanger (NCX)**. Pumps Ca^{2+} out of the cell through an exchange with Na^+ (1 Ca to 3 Na). It represent 5% of calcium removal. The NCX does not use ATP because Na^+ follows its spontaneous electrochemical gradient, which provides the energy.

➢**The mitochondrial Na^+/Ca^{2+} exchanger .** Pumps Ca^{2+} into the mitochondria through an exchange with Na^+ , which represents about 2% of reuptake.

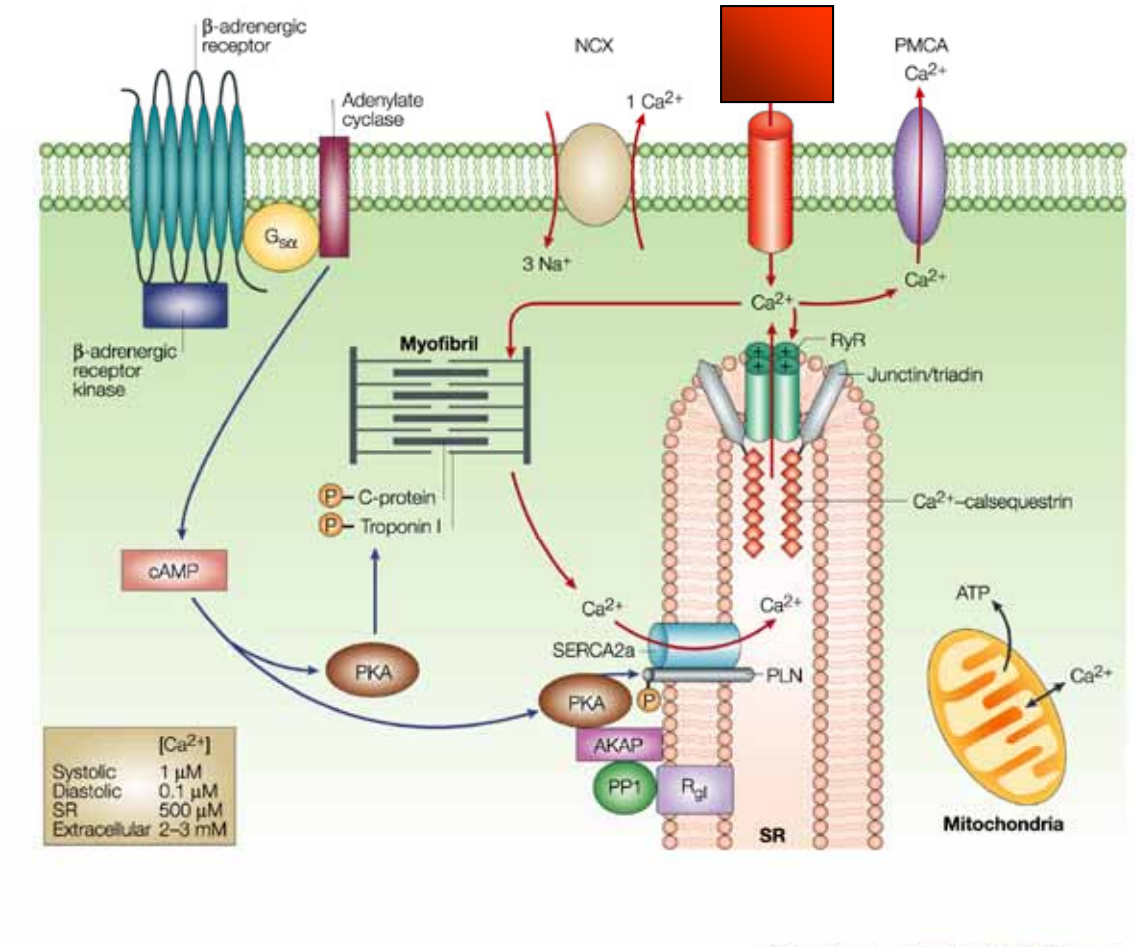
➢**The sarcolemmal Ca^{2+} ATPase**. Pumps back Ca^{2+} in the extracellular milieu, which represents 1% of the reuptake mechanisms. This pump requires ATP to overcome the Ca^{2+} gradient.

Plasma membrane in the maintenance of Ca^{2+} homeostasis

- Calcium influx: Plasma membrane Ca^{2+} entry channels: Voltage-operated Ca^{2+} channels (VOCCs) and Store operated Ca^{2+} channels (SOCCs)

- Calcium efflux: Plasma membrane (sarcolemmal) Ca^{2+} ATPase (PMCA) and Sodium-Calcium Exchanger (NCX)

Calcium cycling in the heart



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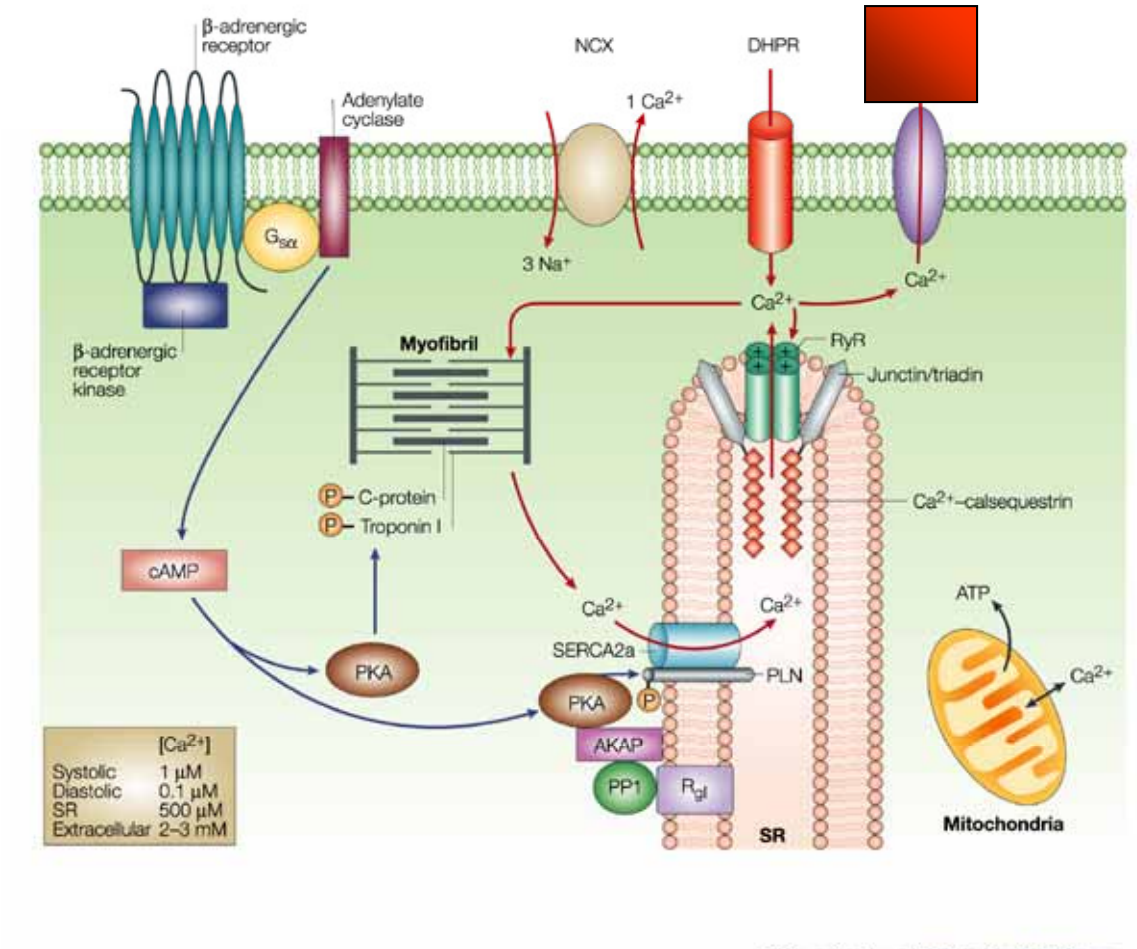
L-type Ca^{2+} Channels

- VOCCs- L-type Ca^{2+} channels controls the depolarization induced Ca^{2+} entry into the heart.
- Two types: Low-voltage activated and high-voltage activated (based on the activation threshold). Combination of auxiliary subunits $\alpha_2\delta$ (2 &3) and γ (1-5)regulates the LVA and HVA channels
- **$Ca_{v1.2}$**- cardiac L-type Ca^{2+} channel-plays important role in E-C coupling
- Prolonged Ca^{2+} current delays cardiac myocyte repolarization and increase the risk of arrhythmia.
- **Heart failure, ischemia**- Downregulation and alterations in the Ca_v channel activity
- Missense mutation G406R (glycine Arginine) results in **Timothy syndrome**, a rare autosomal dominant disorder- Characterized by physical malformations, neurological and developmental defects, **including cardiac arrhythmias and structural heart defects.** Timothy syndrome often ends in early death

Calcium cycling in the heart



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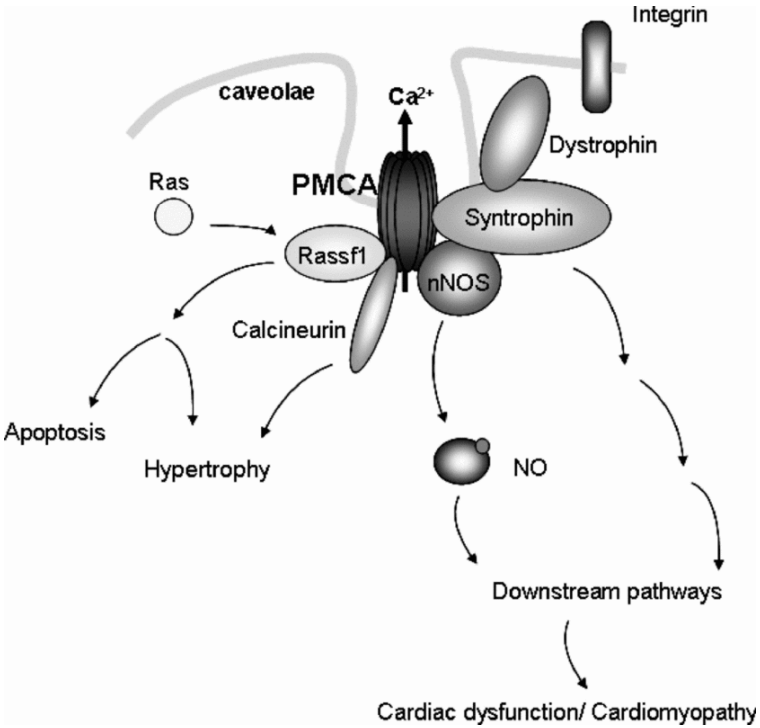
PMCA

- The plasma membrane/sarcolemmal Ca^{2+} ATPase (PMCA) uses energy to pump Ca^{2+} ions out of the cytosol into the extracellular milieu, usually against a strong chemical gradient.
- This energy expenditure is necessary to maintain a relatively low intracellular net Ca^{2+} load.
- Mammals have four genes (ATP2B1-ATP2B4), encoding the proteins PMCA1 through PMCA4. Transcripts from each of these genes are alternatively spliced to generate several variant proteins that are in turn post-translationally modified in a variety of ways.
- PMCA4 is the cardiac isoform

PMCA

- PMCA localized in caveolae (invaginations of PM), which contain signaling molecules-suggest that PMCA may involve in signaling



PMCA

- Transgenic overexpression of PMCA4b

  -reduced inotropic response to β-adrenergic stimulation
- Transgenic overexpression of mutant PMCAct120 (active form of the pump)

  -unable to bind and regulate nNOS

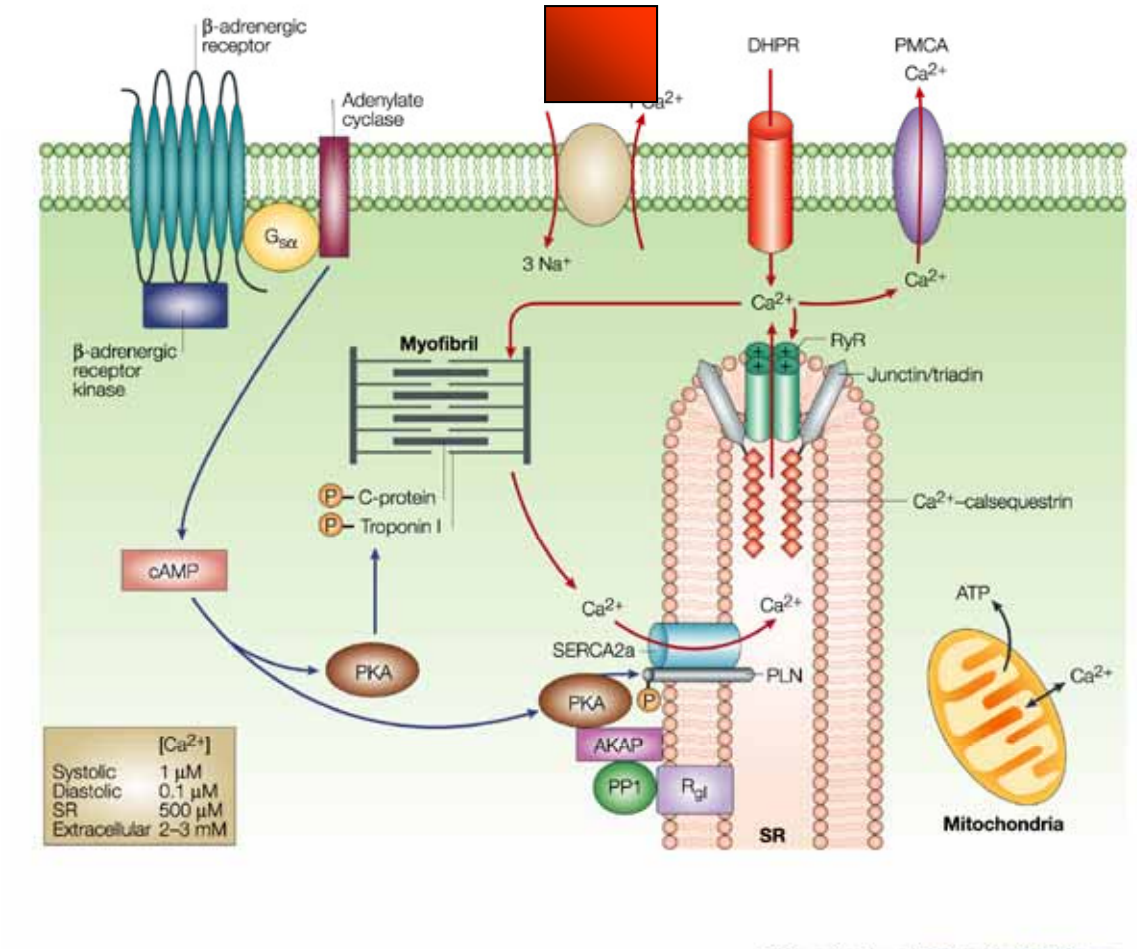
  decreased hypertrophic response under chronic stimulation with β-adrenergic agonist
- PMCA4 gene knockout mouse model

  - Limited cardiovascular phenotype

  -Possibly due to compensation by other PMCA isoforms

  -reduction in bladder smooth muscle contractility
- Tissue-specific and inducible knockout mouse model is necessary to study the function

Calcium cycling in the heart



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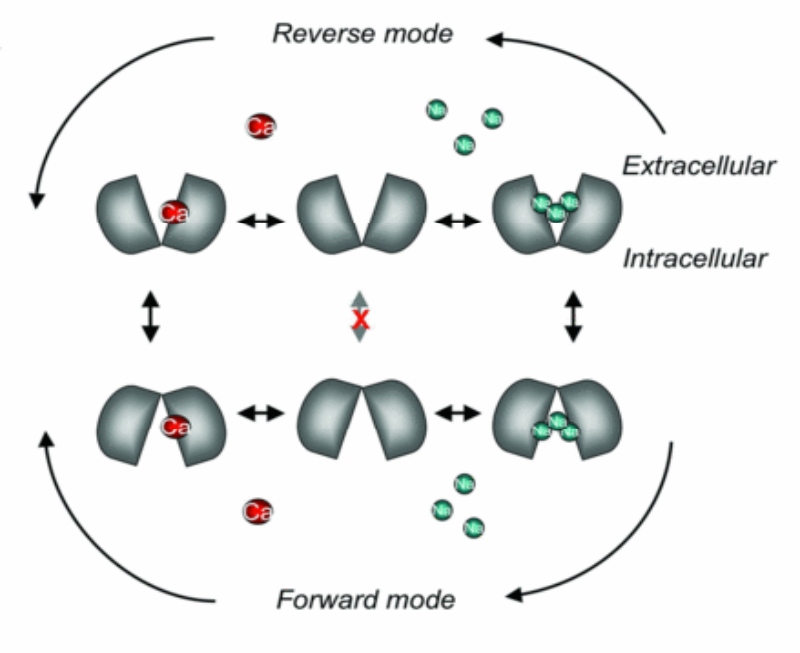
NCX

- $Na+/Ca^{2+}$ exchange play a crucial role in Ca^{2+} extrusion and operated with a stoichiometry of 3 Na+ ions to one Ca^{2+} ion
- 3 different isoforms from a single gene by alternate splicing.
- Splicing at the 5' UTR produces three independent start site for the same coding sequences-driving expression in different tissues.
- Alternative splicing within the coding region –various isoforms
- NCX1-originally identified in heart, but also expressed at high levels in brain and kidney and at lower levels in other tissues
- NCX2- abundant in neurons and all parts of brain
- NCX3-expressed selectively in skeletal muscle and low levels in brain

NCX-FUNCTION

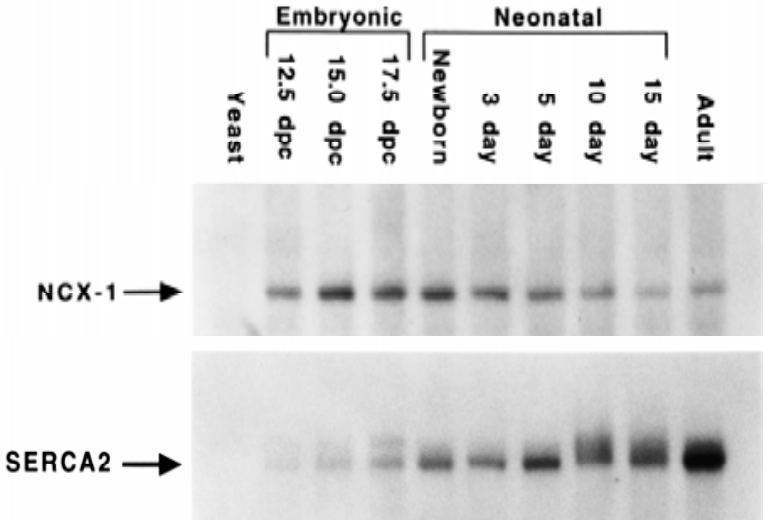
- Apparent affinity for Ca^{2+} is in micromolar range and so it is not maximally activated until Ca^{2+} level rises
- Apparent affinity for Na+ is 10-20 mM (resting level)
- 3 Na^{+} ions/1 Ca^{2+} ions
- During **diastole** Ca^{2+} will be extruded out of PM
- Operation of NCX is fully reversible
- During **systole** with memb.depol. Na^{2+} level rise adjacent to the PM through VOSC, NCX1 reverses operation and allows a small amount of Ca^{2+} entry.



This Ca^{2+} did not trigger SR Ca^{2+} release, but may play an important regulatory role in the E-C coupling process by altering the VOCC

NCX-REGULATION

- Developmentally regulated-higher in neonatal heart/declining with age
- Antithetically regulated in expression with SERCA2
- Up-regulated during cardiac overload and in end-stage heart failure
- Increased in cultured myocytes upon α1-adrenrgic stimulation.



NCX-REGULATION

- NCX1 interacts with **ankryin**, a cytoskeletal protein in the T-tubular region. KO for ankryin shows decreased stability, reduced expression and mislocalization of NCX1

- NCX1 has been shown to co-localizes with caveolin-3

- **Phospholemman (PLM),** modulator of Na, K, ATPase activity, also shown to regulate NCX1 activity.

- When phosphorylated at Ser68 by PKA, the C-terminus of PLM binds to NCX at two regions in the cytosolic loop and inhibits its function. *This would promote the Ca^{2+} entry during β-adrenergic stimulation.*

- PLM KO exhibit enhanced NCX1 function

- NCX1 function can be modulated by adrenergic stimulation via PKA and PKC pathway. However, phosphorylation of NCX1 remains controversial

NCX-PHYSIOLOGY

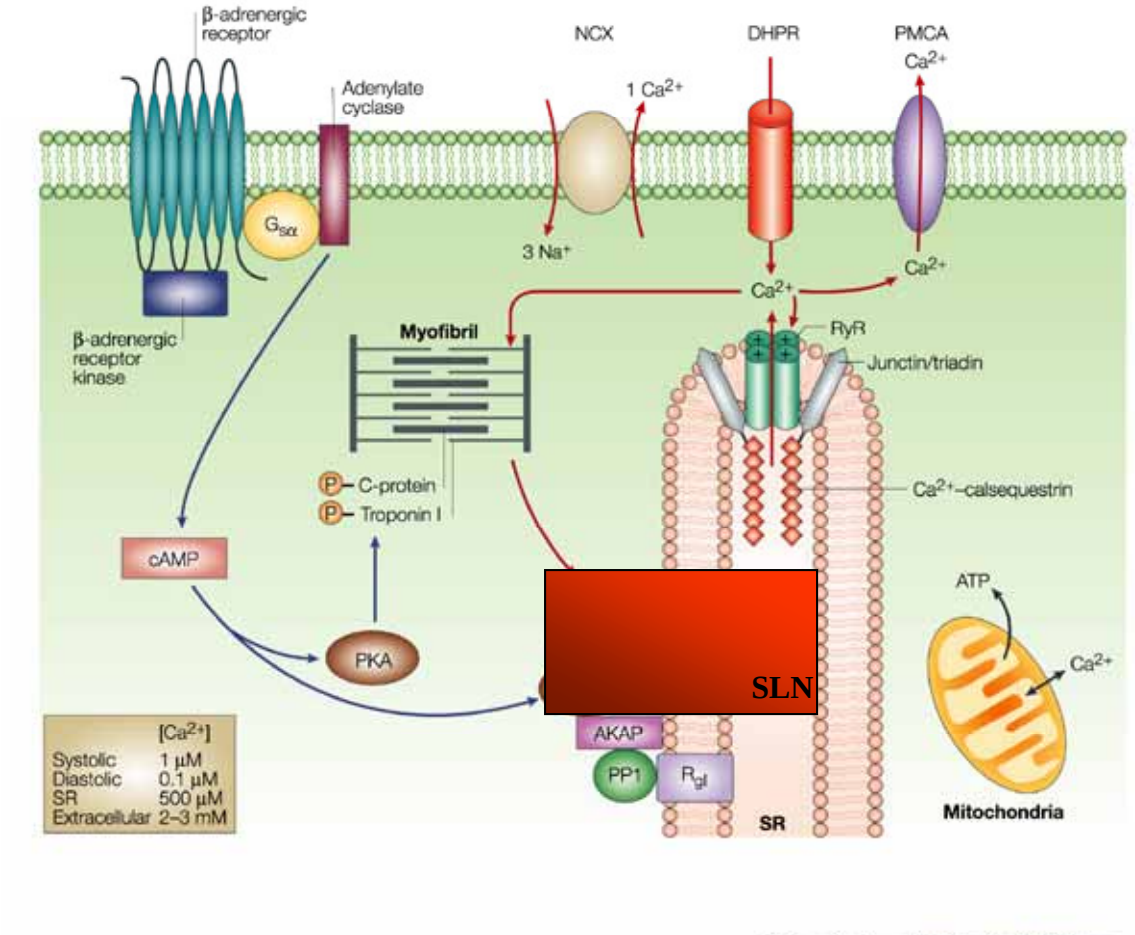
- NCX1-predominant Ca^{2+} efflux pathway-essential to cardiac Ca^{2+} homeostasis.

- NCX also generates depolarizing current (carried by Na+ ions) which may contribute to the shape and length of the action potential

- Global knockout-embryonically lethal (8.5 dpc) attributed to a cardiac defect-indicates that NCX function can't be compensated

- Cardiac specific KO was not lethal-modest decrease in cardiac function-progress to hypertrophy and HF. Reduction in L-type Ca^{2+} channel activity

Calcium cycling in the heart



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Sarco(endo)plasmic Reticulum Ca^{2+} ATPase (SERCA)

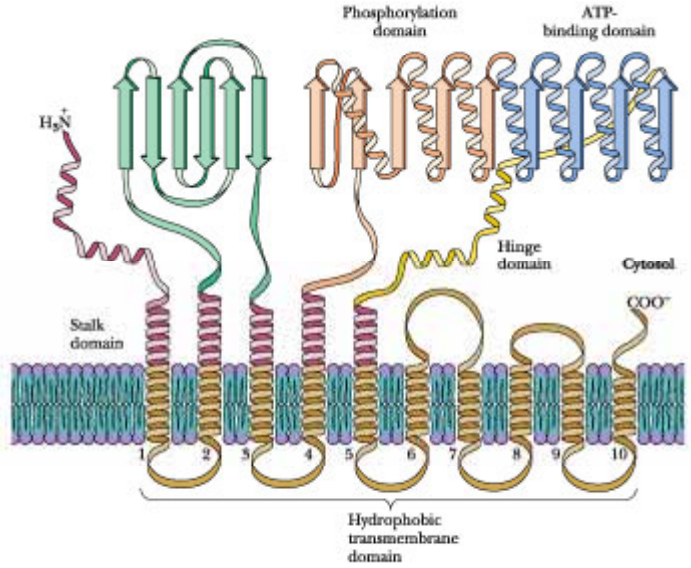
The SERCA pump serves a dual function:

(1) to cause muscle relaxation by lowering the cytosolic calcium

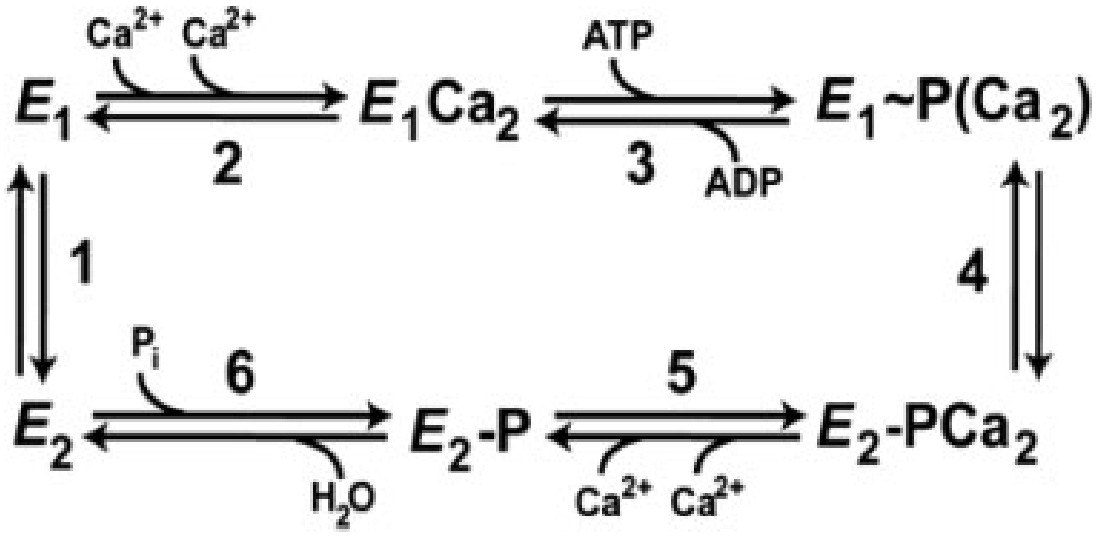
(2) at the same time to restore SR calcium store necessary for muscle contraction

SERCA Isoforms

- SERCA pump is a single polypeptide of Mol.wt 110 kDa and is localized both in the ER and SR membrane.
- The SERCA pump utilizes the energy derived from ATP hydrolysis to transport Ca^{2+} across the membrane
- In vertebrates, there are three distinct genes encoding SERCA 1(2 isoforms), 2 (2 isoforms), and 3 (6 isoforms) isoforms
- SERCA1a and 1b-skeletal muscle
- **SERCA2a-Cardiac**
- SERCA2b- Ubiquitous, predominant in ER
- SERCA3-endothelial cells.

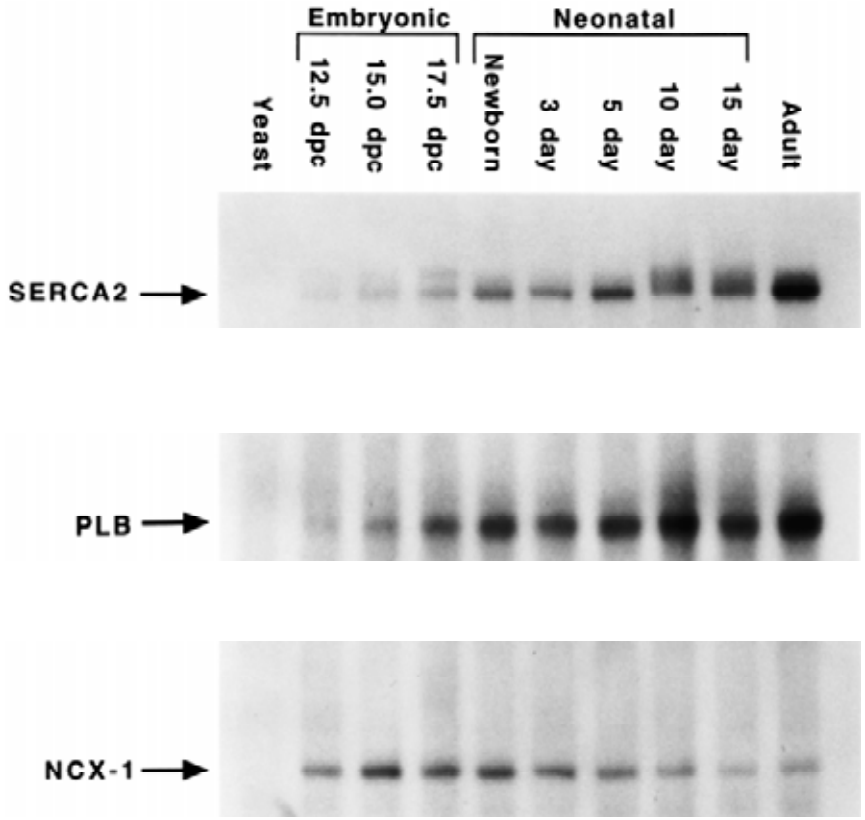


The kinetic model of SERCA pump



SERCA2a

- SERCA2a remains the predominant isoform during development and in adult stages
- Aging-related changes in SERCA levels have been observed both in animal models of aging and in human senescent myocardium.

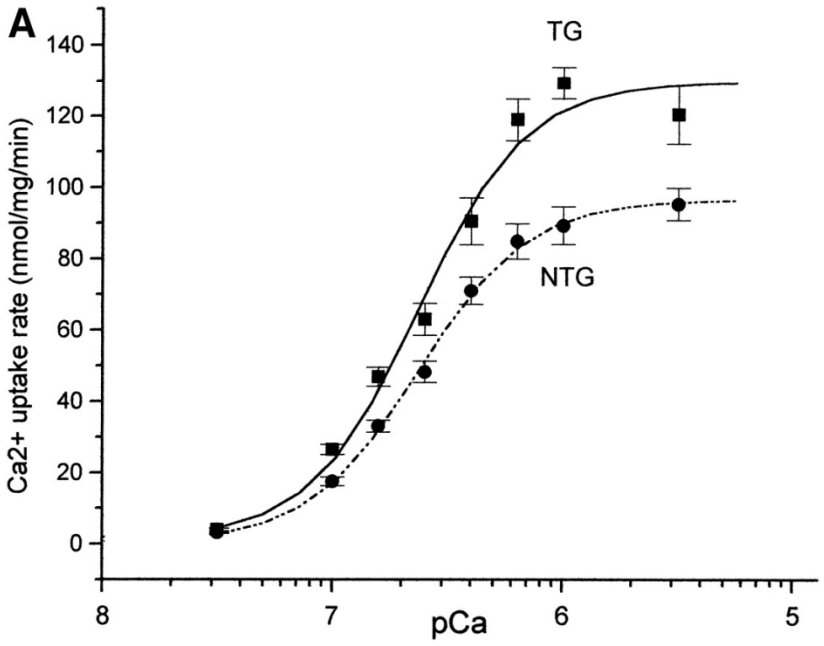


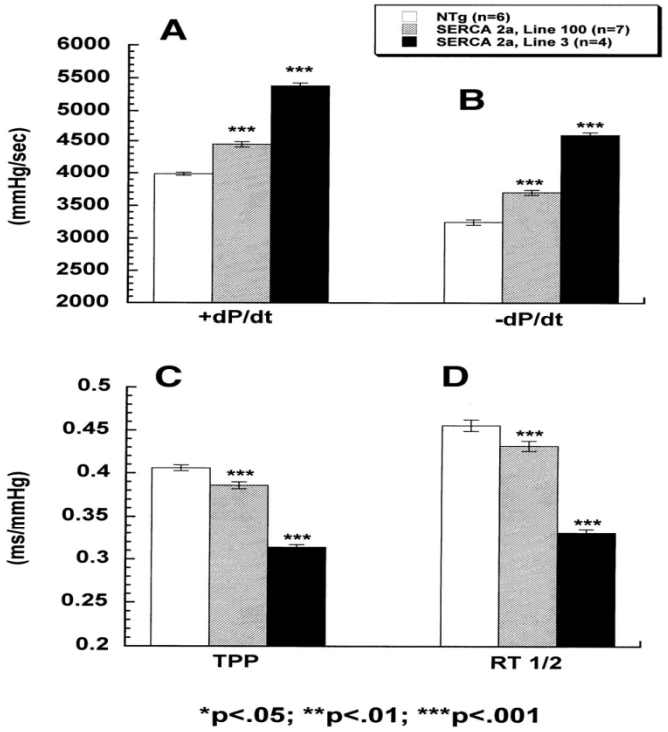
SERCA2 levels are altered during cardiac patho-physiology

- Studies from animal models and heart failure patients: SR Ca^{2+} transport is decreased in heart failure.
- SERCA2a mRNA and protein levels were found to be decreased in failing human hearts.
- In some studies, the expression levels of SERCA2a was found to be unaltered despite a decrease in SR Ca^{2+} transport function.

Transgenic approaches

Cardiac Specific Overexpression of SERCA2a





Increased maximal velocity of SR Ca^{2+} uptake and rates of contraction and relaxation were increased

Baker et al Circ Res. 1998

Transgenic approaches

SERCA2-Gene knockout mice

- Disruption of both copies of the SERCA2 gene is lethal, whereas heterozygous mice with one functional allele are alive and reproduce well.

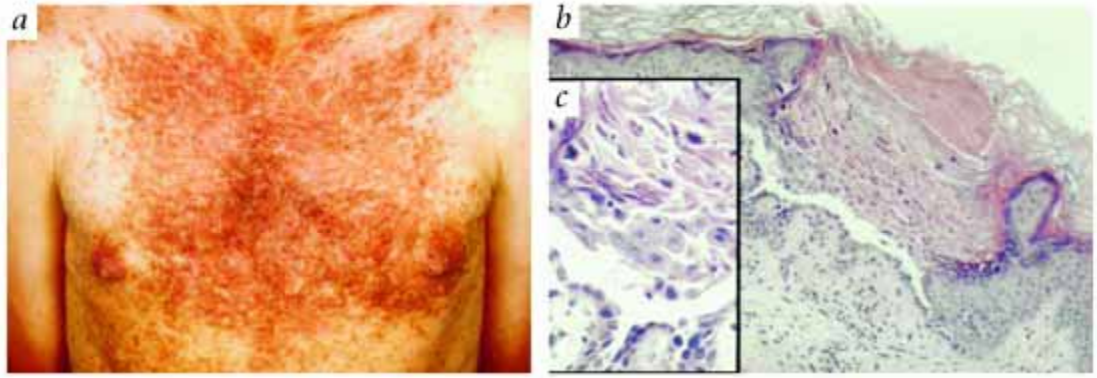
- SERCA2a protein levels and the maximal velocity of SR Ca^{2+} uptake were reduced by approx 35%.

- Measurements of in vivo cardiac function revealed reductions in heart rate, mean arterial pressure, systolic ventricular pressure, and maximal rates of both contraction and relaxation.

- SERCA2 heterozygous mice did not develop cardiac pathology, contrary to expectations. However, these mice are more susceptible to cardiac stress (pressure-overload).

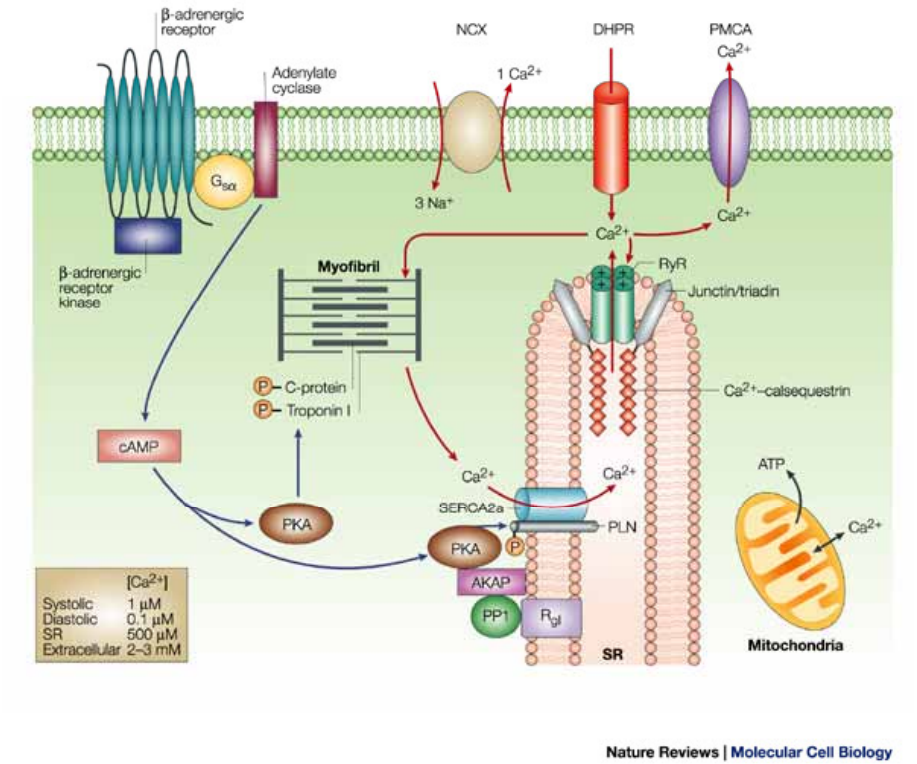
- The heterozygous mutant (+/-) mice develop squamous cell tumors of the forestomach, esophagus, oral mucosa, tongue, and skin.

Mutations in SERCA gene cause Darier's disease



- Mutations in the *SERCA2* gene are extremely rare.
- Missense mutations affecting one copy of the *SERCA2* gene causes an autosomal- dominant skin disease "Darier's disease" in humans.
- Darier's disease is characterized by keratinized squamous epithelial cells.
- These patients do not manifest any heart disease, showing that a single *SERCA2* allele is sufficient to maintain cardiac muscle function.

Regulation of SERCA pump



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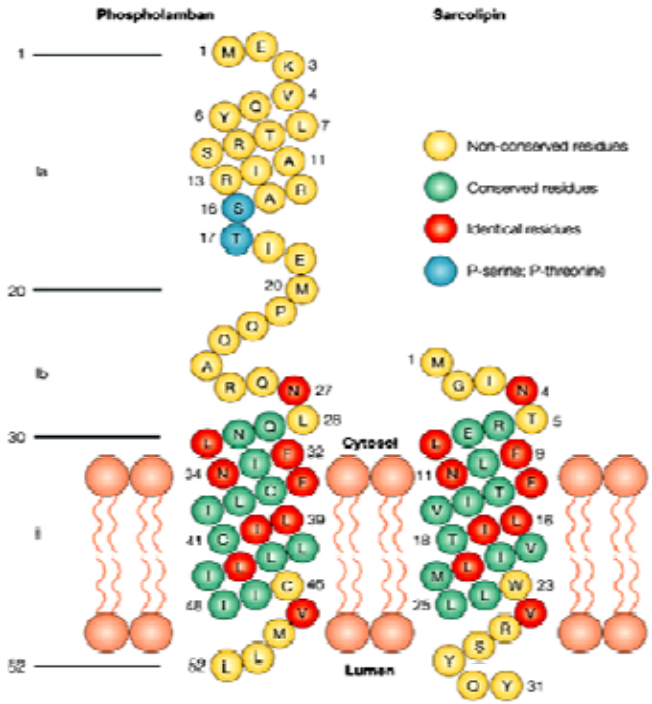
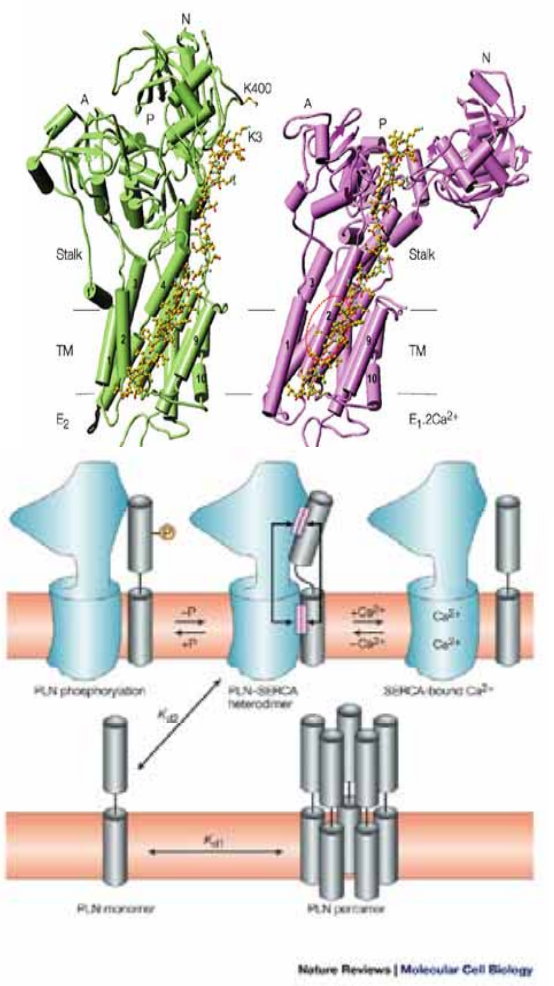
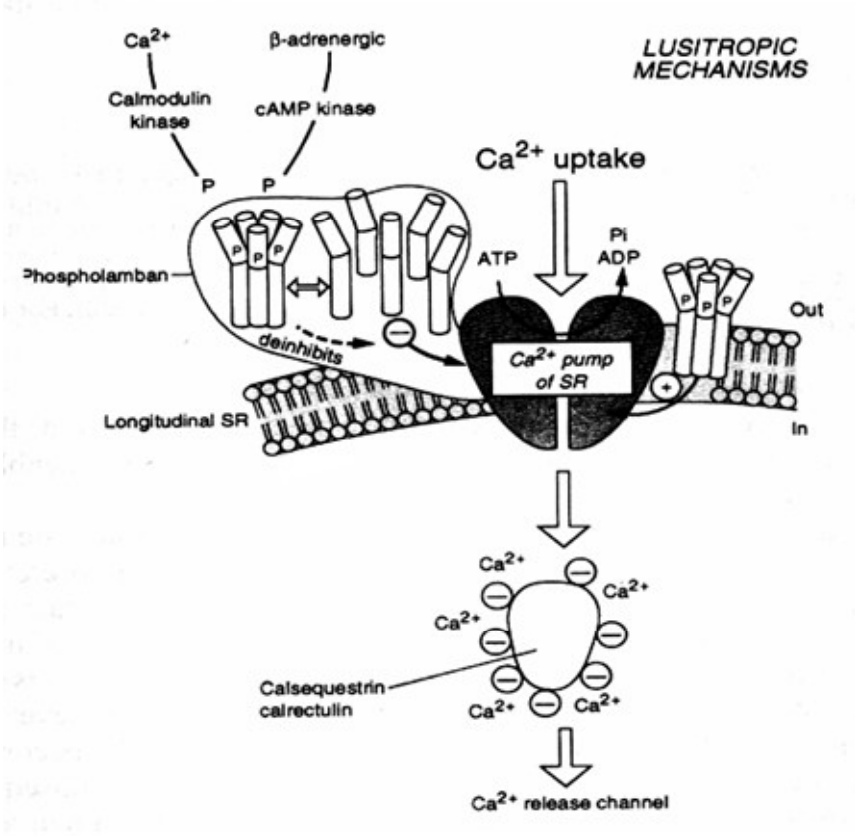


PLN/SLN interacts with SERCA2

- PLN interacts with SERCA through the TM domain.
- The mutation of Leu321 in M4 and Val795, Leu802, Thr805 and Phe809 in M6 of SERCA diminishes the SERCA-PLN interaction.
- These domains also involved in Ca^{2+} binding
- PLN fits into the groove of M2, M4, M6 and M9 domains at E2 stage, when Ca^{2+} binds and forms E1.2 Ca^{2+} the groove becomes narrower because of the large movement of helix M2, forcing PLN out of this inhibitory effect.
- Phosphorylation of PLN at Ser16 by PKA and/or at Thr17 by CaMKII disrupts the interaction.



Regulation of phospholamban



Phospholamban is phosphorylated on:

- **Serine 16 by Protein kinase A**
- **Threonine 17 by Calcium-calmodulin protein kinase**

Both phosphorylation have cumulative effect and are most often combined in vivo

Phospholamban and Cardiac Pathology

No change in PLN levels during HF; however, SERCA levels decreased. Thus, a decrease in SERCA relative to PLN results in inhibition of SR Ca^{2+} uptake

In human, PLN mutation at Arg9Cys-linked to dilated cardiomyopathy

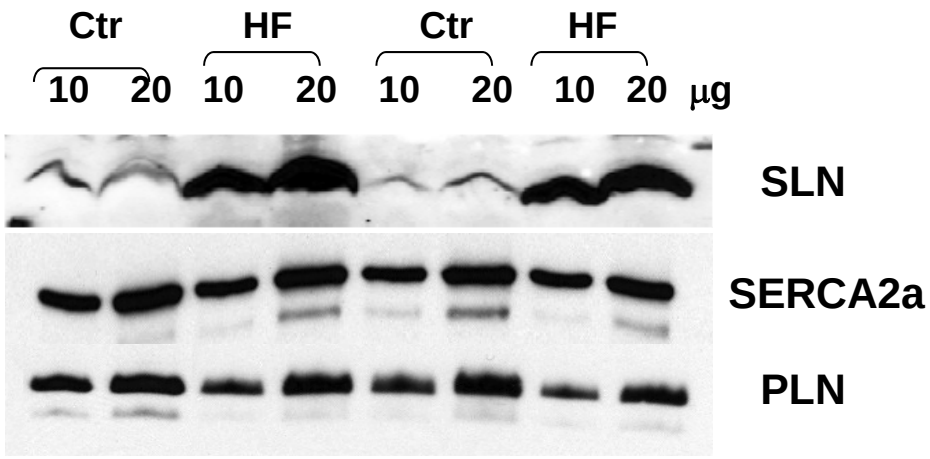
Another mutation at Leu39stop in two large Greek families-Heterozygous inheritance results in LV hypertrophy; homozygous inheritance results in dilated cardiomyopathy

Sarcolipin and Cardiac Pathology

1. Expression of SLN increased in the atria of heart failure patients and in the atria animal models of hypertrophy

2. Expression decreased in atrial fibrillation

3. Expression of SLN increased in the LV of MR patients and DMD mouse models

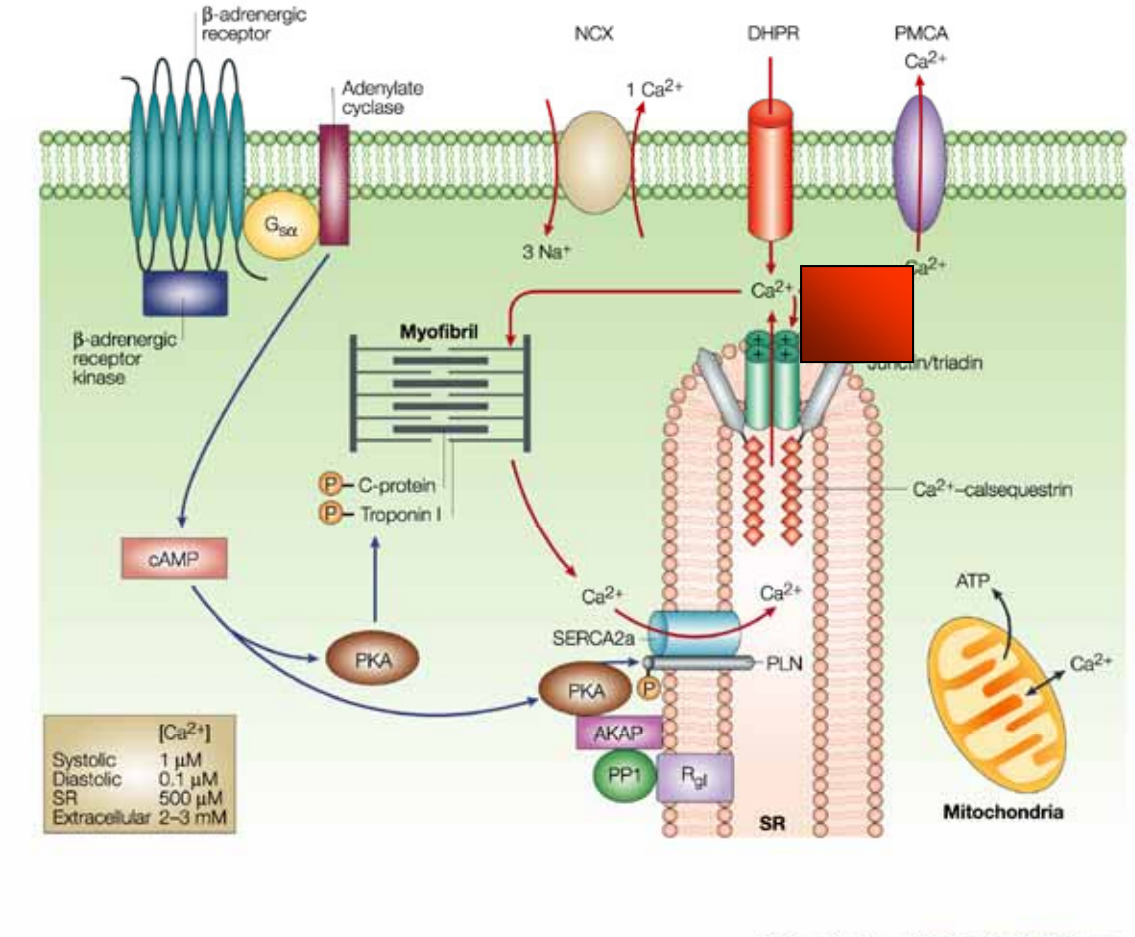


SERCA function is decreased in cardiac pathology

- SERCA2a mRNA and protein levels were found to be decreased in failing human hearts. PLN and SLN levels/function are altered. This could contribute for the decreased SR Ca^{2+} transport in failing myocardium and cardiac dysfunction.

- SERCA2a level is not altered in atria of AF patients, but SLN level decreased –increased SR Ca^{2+} transport function and arrhythmias

Calcium cycling in the heart



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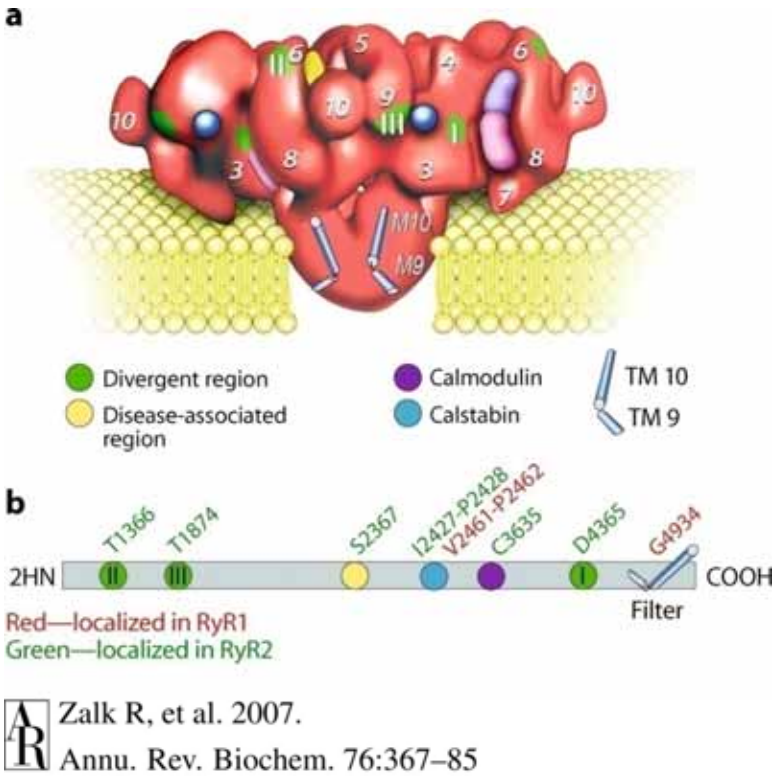
Ryanodine Receptor (RyR)

- Ca^{2+} release channel on the ER/SR (Mol.wt. ~565 kDa)
- Forms a homotetrameric complex (~2200 kDa)
- N-terminal forms a large cytosolic scaffold, which interacts with regulatory proteins creating a molecular signaling complex.
- Three different isoforms: RyR1, RyR2, RyR3
- RyR1-predominatly expressed in skeletal muscles
- RyR2-abundant in heart
- RyR3-found in heart, brain, spleen and testis

RyR Macromolecular complex



Zalk R, et al. 2007.
Annu. Rev. Biochem. 76:367–85

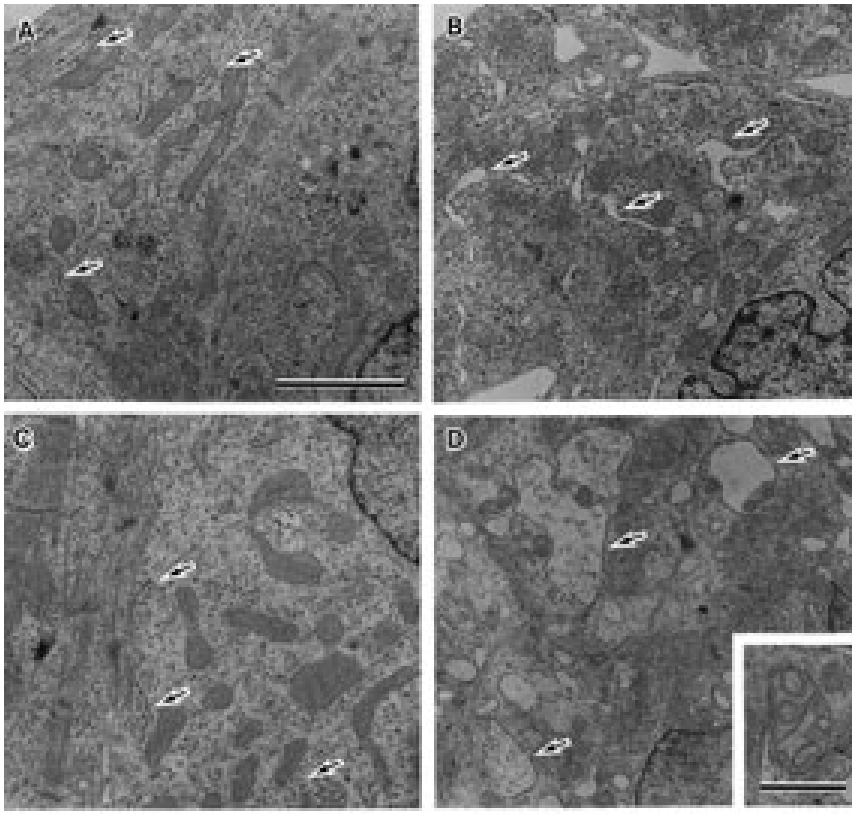
Calmodulin (CaM)

- CaM binds to RyR1 & 2 at a 1:1 stoichiometry
- CaM inhibits RyR2 at all Ca^{2+} conc.
- Binding of CaM to Cav1.1 channel suggest RyR and Cav channel are functionally interact

Calstabin (Cal)

- Ca^{2+} channel stabilizing proteins: Cal1 (FKBP12)
- Cal2 (FKBP12.6) with peptidyl-prolyl-cis-trans isomerase activity
- Localized in the cytoplasmic portion of RyR
- Depletion-increases RyR channel open probability

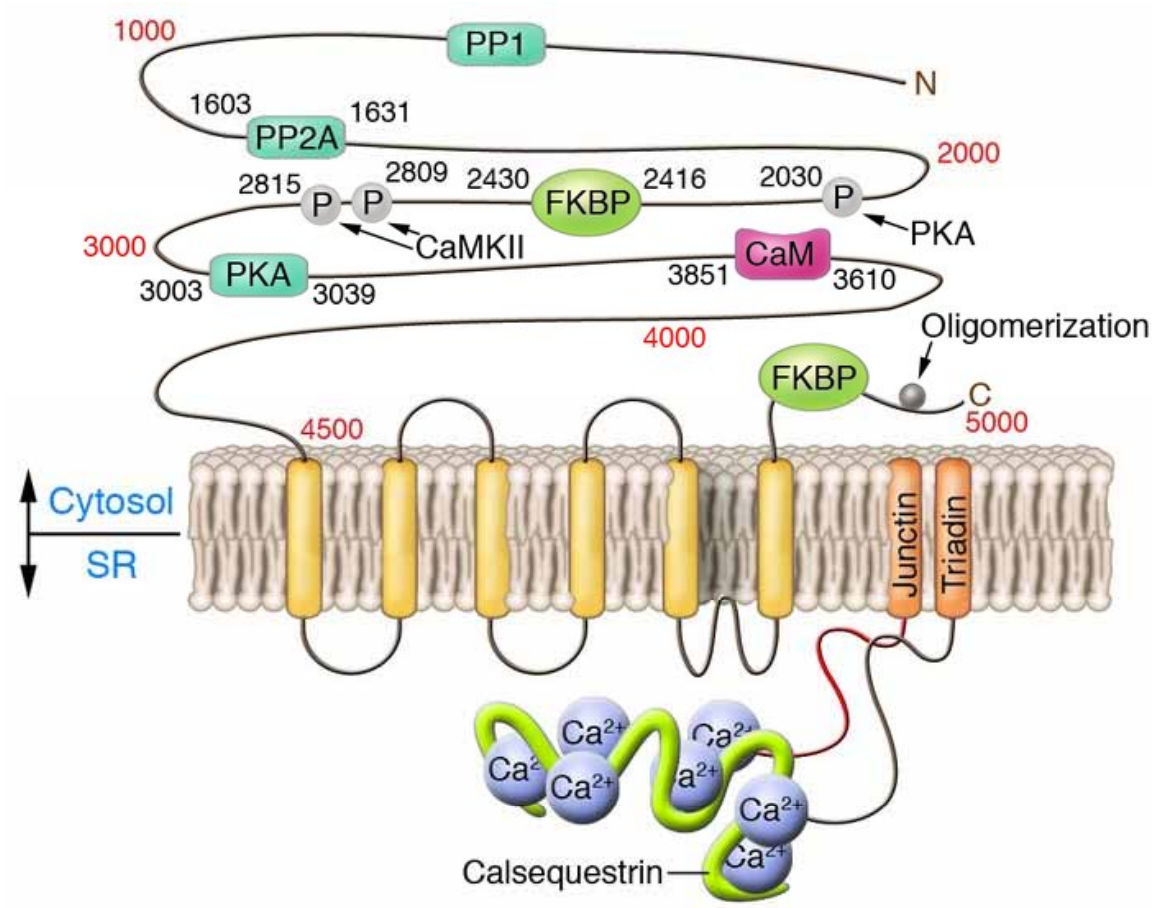
Ultrastructural abnormalities in cardiac myocytes from RyR2-deficient embryos.



(**A**) E8.5 wild-type, (**B**) E8.5 mutant, (**C**) E9.5 wild-type, (**D**) E9.5 mutant embryos. Abnormally large vacuoles were found in mutant myocytes, and the growth of the vacuoles in size were observed during the embryonic development in the mutant mice. The developing SR in control myocytes and the SR carrying swelling parts and abnormal vacuoles in mutant myocytes are indicated by arrows.

Takeshima H, et al., EMBO J. 1998 Jun 15;17(12):3309-16

Regulation of RyR



N-terminal forms a large cytosolic scaffold, which interacts with regulatory proteins creating a molecular signaling complex.

Regulation of RyR by PKA

- RyR2 function as a macromolecular complex in SR Ca^{2+} release.

- RyR2 is associated with PKA and PP1 & PP2A (phosphatases). This interaction is mediated by muscle A-kinase-anchoring protein (mAKAP)

  β-adrenergic stimulation phosphorylates RyR2 at S2808- a transient decrease in binding affinity to FKB12.6 results in an increase in Ca^{2+} release.

- However, chronic PKA phosphorylation of RyR2 results in incomplete channel closing and Ca^{2+} leak during diastole

Regulation of RyR CaMKII

- RyR2 complex also associates with CaMKII by an unidentified targeting mechanisms.

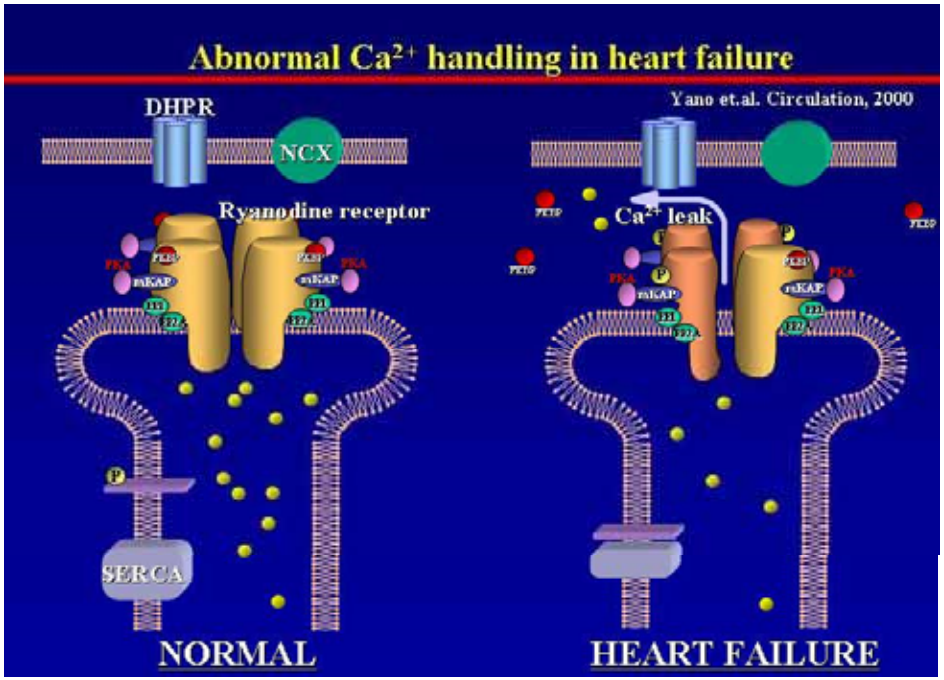
- S2814 was identified as CaMKII phosphorylation site.

- β-adrenergic stimulation- phosphorylates RyR2 at S2814- a transient decrease in binding affinity to FKB12.6 - increase in Ca^{2+} release.

- CaMKII phosphorylation increase single channel open probability but to a small extent than PKA phosphorylation

RyR Dysfunction & Cardiac Pathology

- In HF, RyR2 is PKA hyperphosphorylated contributing to intracellular SR Ca^{2+} leak.
- RyR2 dysfunction has been linked to heart failure and arrhythmias
- Missense mutations in the cardiac RyR2 [N-terminus (176-420), central region (2246-2504) and C-terminus (3778-4950)]has been associated with Catecholaminergic polymorphic ventricular tachycardia (CPVT) and possibly with a form of arrhythmogenic right ventricular dysplasia.

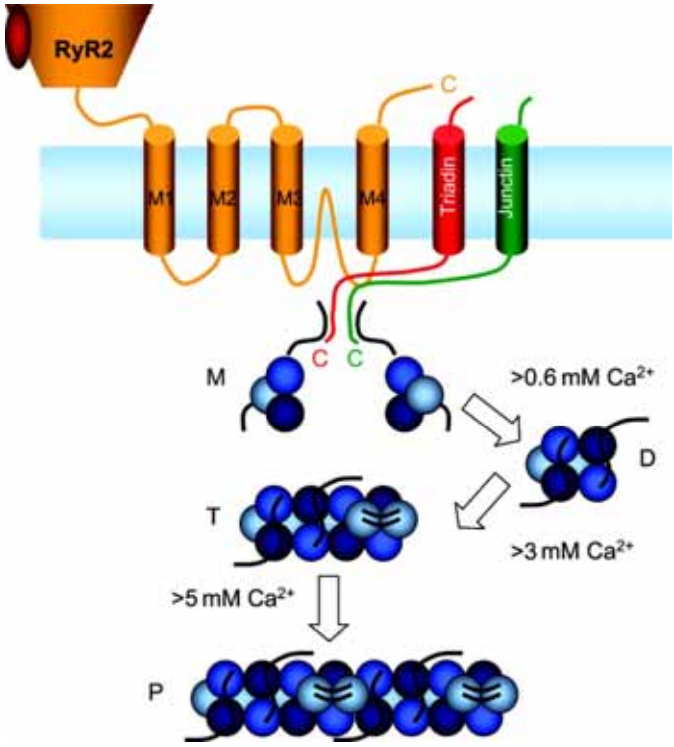


CPVT has a high risk of stress-induced juvenile sudden death and no specific treatment exist

Other SR proteins

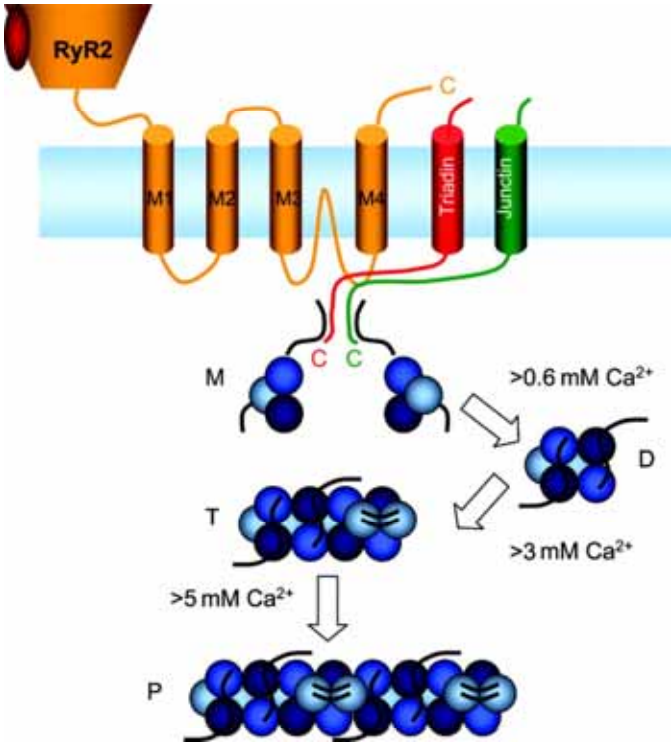
Proteins involved in luminal calcium regulation

- Calsequestrin (CASQ)
- Triadin (TRD)
- Junctin (JCN)
- Histidine-rich Ca-binding protein
- Calreticulin
- Sarcolumenin



Calsequestrin (CASQ)

- CASQ1 –Skeletal muscle;
- CASQ2-Cardiac isoform
- 44 kDa and highly acidic with more than 37% of the AA are represented by either Asp or Glu at the C-terminus.
- It can bind up to 800 nM of Ca^{2+} per mg protein (cardiac isoform binds less)
- Form polymers when Ca^{2+} conc. is high and controls RyR2 open probability probably via triadin (TRD) and junctin (JCN)



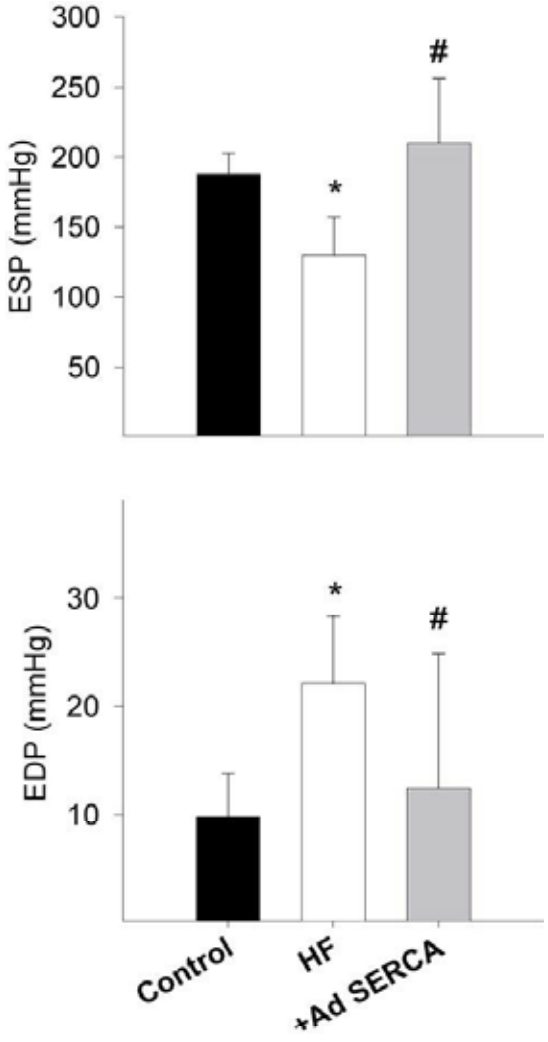
CASQ: Transgenic Approach

- Overexpression cause hypertrophy and HF
- Increased SR Ca^{2+} content.
- Less Ca^{2+} transients and Ca^{2+} sparks
- Increased expression of triadin and junctin.
- CASQ KO-Normal but develops Polymorphic ventricular tachycardia upon Catecholamines such as epinephrine (adrenaline), norepinephrine (noradrenaline), and dopamine
- CASQ2 mutation R33Q (Arginine to Glutamine) and D307H (Aspartic acid to Histidine)-cause CPVT

Therapeutic aspects

- Adenoviral gene transfer of SERCA2a into the failing heart improves the cardiac function

- Inhibition of PLN function by anti-sense or by inhibitors improve the cardiac function in the animal models of HF



Kaye DM, et al. 2008.
Annu. Rev. Med. 59:13–28

Therapeutic aspects

- The calcium channel blocker flunarizine has been documented to inhibit L-, T- and N-type Ca^{2+} channels. Prevents early-afterdepolarization (EAD) and Long QT syndromes-due to arrhythmias. Also effective on Delayed AD (DAD) dependent ventricular tachycardias.-Mechanism is still unknown.

- JTV519 (K201)-prevents Ca^{2+} leak from SR by stabilizing calstabin2 (FKBP12.6) to RyR and improves cardiac function

- CaMKII inhibitors such as W7 and KN93-used as anti-arrhythmic agents

- Ranolazine is a novel anti-anginal agent- acts as a multichannel ion blockers. At low conc.- blocks selectively Na^{+} current. At high conc. Inhibits K^{+} and Ca^{2+} current.