Ion Channel Activity of Muscle Regulatory Proteins Sarcolipin and Phospholamban

Nick DesLauriers

PI: Dr. David D. Thomas, Minnesota Muscle Laboratory

Postdoc Mentor: J. Michael Autry

Context: The proposed research will investigate the role of two key proteins, sarcolipin (SLN) and phospholamban (PLB), in muscle contractility. Both SLN and PLB monomers have a **primary regulatory function** of inhibiting calcium (Ca) transport of the sarcoplasmic reticulum Ca-ATPase (SERCA). SLN inhibits SERCA in skeletal muscle, while PLB inhibits SERCA in cardiac muscle. Adrenaline phosphorylates SLN and PLB, reversing SERCA inhibition and stimulating muscle contraction. This research project will examine a potential **second function** of SLN and PLB: the formation of ion transport channels.

The idea of this **second function** was first proposed in a 1988 paper published by the Jones group (Krannert Institute of Cardiology at Indiana University). The paper describes experiments where isolated canine cardiac PLB was added to lipid bilayers. PLB was reported to form channels through the bilayers, and further, the bilayers were reported to be permeable to cations (Kovacs, J Biol Chem 1988). Thus, the paper suggested that PLB may serve as a Ca leak channel, inhibiting Ca flux in the sarcoplasmic reticulum. In 1989, the Piggott group (Smith Kline and French Research, Ltd.) published a paper providing potential support to this PLB channel hypothesis. The paper reported that expression of PLB was lethal to *E. coli*, presumably due to formation of pentameric Ca leak channels (Cook, Biophys J 1989).

Similar ion channel research has been conducted on SLN. Research papers published in 2007 and 2009 by the Veglia group (University of Minnesota) describe experiments where SLN was expressed in lipid bilayers for electrical conductivity tests (Becucci, Biophys J 2007, 2009b). Data indicated that increased inorganic cation concentrations did not increase conductivity in the bilayers, suggesting the bilayers were impermeable to cations. However, there was an observed increase in conductivity upon increased concentrations of small inorganic anions, such as chloride, phosphate, or sulfate. As a result of the permeability of SLN-incorporated bilayers to small anions but not cations, researchers suggested that SLN forms phosphate uptake channels.

The Thomas group (University of Minnesota) conducted further research that suggests channel formation of SLN and PLB (**Fig. 1**). A recent paper describes how expression of SLN and PLB in *E. coli* cell membranes led to reduced bacterial viability (Autry, J Biol Chem 2011). This result was attributed to the possibility of SLN and PLB forming ion channels in the inner cell membrane, allowing fatal concentrations of ions into the cell to cause death. The group went on to express a mutant of SLN in *E. coli* that hindered higher-order oligomeric formation.

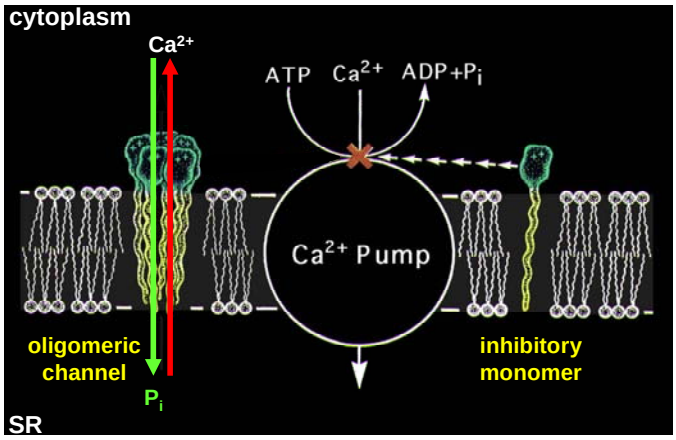

Fig. 1. **Proposed ion channel activity of SLN (P_i) and PLB (Ca).** We propose that SLN channels activate contractility and PLB channels inhibit contractility. Figure by Autry and DesLauriers.

Viability of the cells was consequently restored, which the group attributed to the possibility that these higher-order oligomers were necessary to create channel structure. Due to ion conditions in a cell, it is predicted that a Ca leak channel would inhibit muscle contraction (*red arrow* in **Fig. 1**), while a phosphate uptake channel would activate contraction (*green arrow* in **Fig. 1**) (for further discussion, see Impacts of Research below, plus reviews by Arkin, Annu Rev Biophys Biomol Struct 1997 & Becucci, Biophys J 2009a).

Hypotheses: (1) *That SLN serves as a phosphate (P_i) ion transport channel in muscle cells and* **(2)** *that PLB serves as a Ca ion transport channel in muscle cells.*

From **hypothesis (1)** we proposed that following SLN expression in bacterial cells, an increase in phosphate ion concentration outside the bacterial membrane would result in reduced bacterial cell viability. From **hypothesis (2)** we proposed that following PLB expression in bacterial cells, an increase in calcium ion concentration outside the bacterial membrane would similarly result in reduced viability. These predictions stem from the idea that if ion channels are formed as in **hypotheses (1)** and **(2)**, the increased extracellular ion concentration would result in excessive ion flow into the cells via these channels and cause cell death, as suggested by Cook et al.

Methods: SLN, PLB, and CAT protein were expressed in DH5α strain *E. coli*, as previously described (Autry, J Biol Chem 2011). IPTG, a glucose derivative, was used to induce protein expression, and proteins were expressed with a six-histidine residue tag. Luria-Bertani broth (LB) was used for solution phase rapid bacterial cell growth.

In Experiment 1, overnight cultures (2 ml) were started of each CAT, PLB, and SLN-expressing *E. coli* ± 45 mM phosphate (P_i). LB contains 5 mM phosphate . After 16 hours, expression cultures in fresh LB medium (25 ml) were started by seeding from overnight cultures (1:100 dilution). A spectrophotometer was used to measure absorbance values over time, using Abs 600 nm (scatter from cells) as an indicator of cell density and growth. Values were read every hour, where the first 4 hours were pre-culture to Abs=0.6 in log phase growth. IPTG was

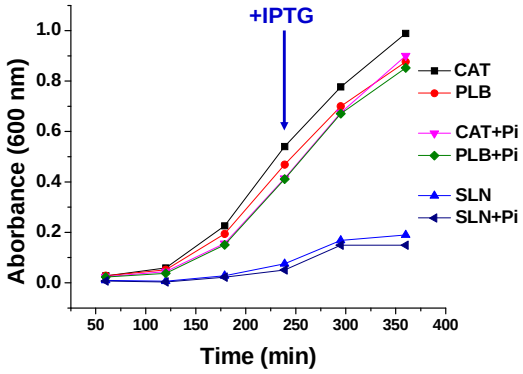

Fig. 2. **Growth curves of CAT, PLB, SLN cultures ± phosphate (t=3 IPTG induction). Expression of** SLN inhibits growth. P_i addition further inhibits.

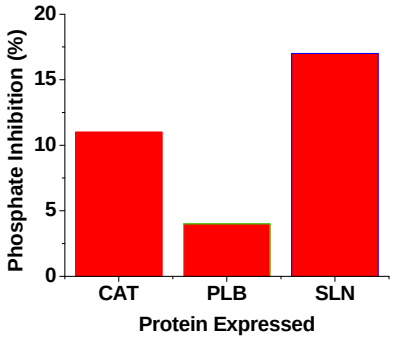

Fig. 3. **Phosphate inhibition of bacteria growth.** Phosphate addition causes greater inhibition in SLN expression..

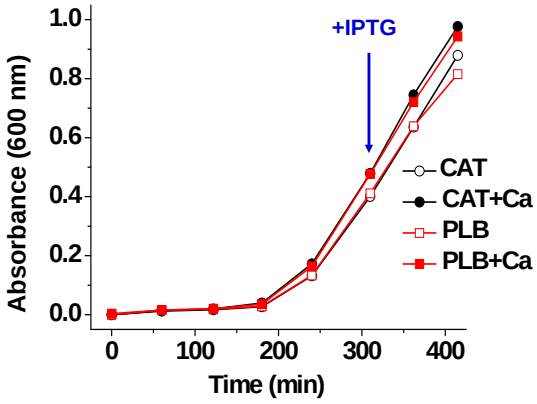

Fig. 4. **Lack of Ca effect on PLB-expressing cells**. Same as CAT control.

added at 3 hrs hours. In Experiment 2, the effect of Ca was tested. LB contains 0.1 mM Ca. The same procedure was followed using overnight cultures ± 5.0 mM added calcium.

Results: In Experiment 1 the expression of SLN substantially hindered cell growth relative to the expression of the control protein, CAT (**Fig. 2**). Before IPTG induction (t= 3 hrs), the absorbance values of SLN cultures in the absence of added phosphate (5 mMP_i) were on average down to 15% of the values of the CAT cultures and after induction, at 17%. The expression of PLB also hindered cell growth relative to CAT, but not as dramatically. Before IPTG induction the absorbance values of PLB cultures relative to CAT were on average down to 87% and after induction, at 85%.

With the addition of extra phosphate (50 mM P_i), inhibition of growth was seen with each CAT, PLB, and SLN cultures relative to phosphate-free cultures. However, P_i inhibited SLN 2-3 more effectively than CAT and PLB (**Error! Reference source not found.**). This indicated SLN-induced phosphate-sensitivity for E. coli growth, as predicted by **hypothesis (1)**.

In Experiment 2, the addition of calcium promoted cell growth of both cultures expressing CAT and PLB (SLN was not tested). Specifically, after IPTG induction, both CAT and PLB cultures (+Ca) averaged an increase in growth of 114% relative to cultures lacking calcium (**Fig. 4**). This was contrary to our hypothesis, which predicted PLB-induced inhibition of growth with calcium addition. Furthermore, the increase in growth in both CAT and PLB cultures was similar, showing that the increase in growth is not PLB-induced.

Conclusions: The dramatic inhibitory effect on *E. coli* growth caused by SLN expression, as noted in other papers, was confirmed with this research. Further, with the addition of phosphate, we saw that growth inhibition in cells expressing SLN was 2-3 times greater than inhibition in cells expressing CAT and PLB. This result is consistent with our hypothesis that SLN can self-assemble into phosphate transport channels. However, both CAT and PLB cultures also displayed inhibition of growth, albeit to a lesser extent. Thus, the question arises of whether or not the observed inhibition is due to another mechanism not unique to SLN. In order to determine whether the inhibition observed is in fact a direct consequence of SLN channel formation, more research needs to be conducted. A first step will be to increase phosphate concentrations to see if inhibition in SLN cultures becomes even more pronounced relative to CAT and PLB. Further, we will conduct FRET assays of oligomerization to correlate with antibacterial assays, as well as measure growth when monomeric mutants are expressed that would be predicted to disrupt channel formation.

As noted in previous papers, PLB expression was also shown to inhibit *E. coli* growth, though to a lesser extent than SLN. However, contrary to our hypothesis, there was no PLB-induced effect on *E. coli* growth with the addition of calcium. Though this is inconsistent with our hypothesis, more experimentation is needed to clarify this result. It is possible that the amount of calcium that was introduced into the media may simply have not been enough to kill the cells if PLB channels are in fact self-assembling. Alternatively, our hypothesis may be incorrect altogether and PLB may not self-assemble into a Ca transport channel. Moncelli et al. have proposedthat PLB forms potassium (K^+) channels (Smeazzetto Phys Chem Chem Phys 2011), and Rousseau et al. have proposed that PLB involvement forms chloride (Cl^-) channels (Decrouy, J Mol Cell Cardiol. 1996). In order to further investigate the PLB channel hypothesis, future experiments will test 20 mM Ca and other ions (K^+, Cl^-, Mg^{2+}).

REFERENCES

Structural perspectives of PLB, a helical transmembrane pentamer.
Arkin IT, Adams PD, Brünger AT, Smith SO, Engelman DM.
Annu Rev Biophys Biomol Struct. 1997;26:157-79. Review.

Oligomeric interactions of SLN and the Ca-ATPase.
Autry JM, Rubin JE, Pietrini SD, Winters DL, Robia SL, Thomas DD.
J Biol Chem. 2011 Sep 9;286(36):31697-706.

An electrochemical investigation of SLN reconstituted into a mercury-supported lipid bilayer.
Becucci L, Guidelli R, Karim CB, Thomas DD, Veglia G.
Biophys J. 2007 Oct 15;93(8):2678-87.

On the function of pentameric PLB: ion channel or storage form?
Becucci L, Cembran A, Karim CB, Thomas DD, Guidelli R, Gao J, Veglia G.
Biophys J. 2009a May 20;96(10):L60-2.

The role of SLN and ATP in the transport of phosphate ion into the sarcoplasmic reticulum.
Becucci L, Guidelli R, Karim CB, Thomas DD, Veglia G.
Biophys J. 2009b Nov 18;97(10):2693-9.

The expression of canine cardiac PLB in heterologous systems.
Cook EA, Huggins JP, Sathe G, England PJ, Piggott JR.
Biochem J. 1989 Dec 1;264(2):533-8.

Biochemical regulation of sarcoplasmic reticulum Cl- channel from human atrial myocytes: involvement of phospholamban.
Decrouy A, Juteau M, Proteau S, Teijiera J, Rousseau E.
J Mol Cell Cardiol. 1996 Apr;28(4):767-80.

PLB forms Ca-selective channels in lipid bilayers.
Kovacs RJ, Nelson MT, Simmerman HK, Jones LR.
J Biol Chem. 1988 Dec 5;263(34):18364-8.

PLB generates cation selective ion channels.
Smeazzetto S, Schröder I, Thiel G, Moncelli MR.
Phys Chem Chem Phys. 2011 Jul 28;13(28):12935-9.