Inside the SR

A current focus: regulation of Ca and Ca release inside the Ca store (Sarcoplasmic Reticulum, SR).

Starting from a central hypothesis *—there is regulation of Ca^{2+} release from inside the SR—* we ask: (1) Are there control mechanisms dependent on free intra-SR [Ca^{2+}] ($[Ca^{2+}]_{SR}$) or total SR calcium ($[Ca]_{T,SR}$)? (2) Are these mechanisms dependent on ancillary intra-SR proteins, like calsequestrin (CSQ)? To answer we are now quantifying Ca^{2+} release flux upon measured modifications of the SR Ca^{2+} content, or its endowment of proteins. We also modify SR proteins thought to mediate or condition the SR Ca^{2+} effects and change the cell's endowment of two "proteins of SOCE (Store Operated Calcium Entry)", to test whether store-operated Ca^{2+} entry helps sustain $[Ca^{2+}]_{SR}$ during strenuous activity. Four "studies" are now in progress:

(0) We are enhancing measurement of intra-SR calcium in mouse muscle.

By developing an indo-1 based method to determine $[Ca]_{T,SR}$ by subtracting net released from initial Ca (progress is reported in Royer et al, J. Physiol. 2008).

We use a novel approach (fusion with CSQ) to express at high density inside the mouse muscle SR the cameleon biosensor D4.

We are also targeting to the SR "hybrid" sensors (fusion of a small indicator –indo, mag-indo or fura-- and a modified endogenous protein), prepared for us by K Johnsson (École de Lausanne).

These methods are being applied to:

(1) Modify SR Ca^{2+} content and determine consequences for Ca^{2+} release permeability. *The hypotheses are that Reduction of SR Ca content increases release permeability P, reduces SR buffering power B and, unlike cardiac muscle, it does not close release channels.*

(2) Overexpress, knock down or knock out SR proteins and determine consequences for Ca^{2+} release. Experiments, which change the amount of intra-SR proteins from 30 x normal to 0, test the hypotheses that *Ca^{2+} release is modulated by calsequestrin through Ca^{2+} buffering and RyR gating actions* and *Ca^{2+} release either requires triadin or is modulated by it.*

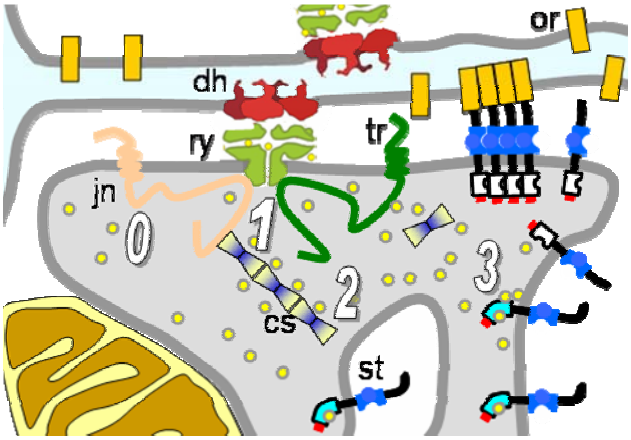

Fig 1. Four specific studies (0-3) examine control of Ca release by SR Ca^{2+} and its proteins. *tr*, triadin; *cs*, calsequestrin; *st,* STIM1; *or*, Orai1. Also shown, *ry*, RyR; *dh*, DHPR; *jn*, junctin. The *releasome* includes *ry*, *tr*, *jn*, *cs* and proteins not shown. We found *or* in t tubule and *st* in SR.. *cs* seems not essential to support Ca release. Do *st* and *or* have a role?

Overexpression and silencing of the genes in adult mice FDB muscles is ascertained by multiple methods and followed by functional, structural and biochemical studies, including measurement of cytosolic [Ca^{2+}] level, $[Ca^{2+}]_{SR}$, total $[Ca^{2+}]_{SR}$, Ca^{2+} release under V clamp or pharmacological stimuli, and at the single fiber and whole muscle level, twitch and tetanic force, force-frequency relationship and fatigability, distribution of proteins at the optical and EM level, structure of membrane systems (by EM) and expression density of *releasome* proteins (RyR, Triadin, Junctin & CSQ), SERCA, and the Ca^{2+} binding proteins (junctate, sarcalumenin, histidine-rich calcium binding protein, and calreticulin). Many collaborating labs are involved in these measurements, including those of R Fitts (Marquette U), D Riley (Med Coll Wisconsin), G Meissner (UNC) and M Fill (Rush).

(3) Determine the role of SOCE molecules in control of $[Ca^{2+}]_{SR}$ and Ca^{2+} release.

We wonder whether mechanisms other than buffering by CSQ are at work to stabilize $[Ca^{2+}]_{SR}$. We combine measurement of SOCE (Launikonis & Ríos, J. Physiol. 2007) and $[Ca^{2+}]_{SR}$ to test *whether SOCE helps stabilize SR Ca content upon sustained activation and fatigue, and whether this function requires STIM1 and Orai1.*

To this end we silence STIM1 and Orai1, and evaluate the effects on fatigability of whole muscles. We measure the same parameters in muscle of mice that are STIM1-null or haplodefficient at the tissue or whole body level (supplied by P Rosenberg, Duke University). We also determine STIM1 movements and putative interactions with Orai1 upon functional depletion, by confocal imaging of voltage-clamped muscle cells expressing YFP-STIM1 and CFP-Orai1.

Background and additional details are given in the following pages.

Background on Ca regulation inside the cellular Ca store.

Ca^{2+} signals share a universal language. 60 years after the original observations of Heilbrunn & Wiercinski (1947) the study of Ca signaling has extended from muscle to all tissues and cells. Ca signaling is often used by a given cell for many different functions. Therefore different signals are coded under different time spans, frequencies, amplitudes, and locations. Various molecules must be dedicated to encoding and decoding these Ca^{2+} messages, risking genetic errors that result in inheritable diseases. They include diseases of SOCE (like severe complex immune disorder or SCID, Partiseti *et al* 1994), diseases of EC coupling affecting cardiac (CPVT, Lahat *et al* 2001) or skeletal muscle (MH, CDs, and more generally myotonias and myodysgeneses, Jurkat-Rott & Lehmann-Horn 2005), plus a large number of alterations in nuclear signaling.

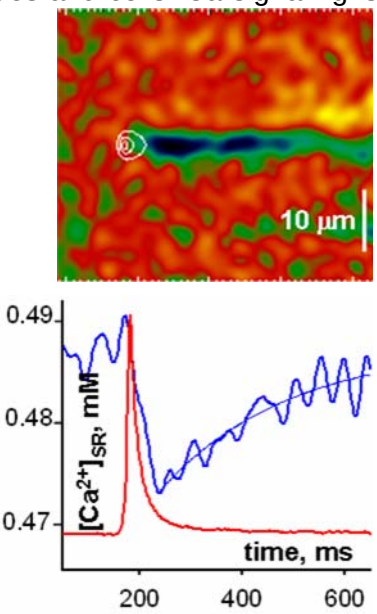

Fig 2. The skrap (blue) reflects a decrease in local $[Ca^{2+}]_{SR}$ of 2-7%. It reaches nadir 80 ms after the spark's peak (red). Launikonis *et al* 2006.

In Ca^{2+} signaling, Ca^{2+} is the message. In electric signaling –when Na^+ enters a cell during action potentials– the message is electricity, Na^+ is just the medium that carries it. In Ca^{2+} signaling instead, Ca^{2+} is the medium *and* the message. Hence, while electric signals move at meters per second, Ca^{2+} signals –if not assisted by electricity– move at the pace of diffusion.

For this reason, Na^+ and K^+ channels can be sparsely distributed and gate independently (*eg* Sigworth, 1994); intracellular Ca^{2+} release channels instead must cluster and rely on mutual interactions to gate open together. Only then they achieve the high local gradients necessary to effectively supply Ca^{2+} to its targets.

The basis of the mutual interaction is the CICR mechanism (Endo *et al* 1970). CICR inherently leads to unstable, all-or-none behavior; however, Ca^{2+} signals stay graded with stimuli. Fabiato first proposed and Stern (1992) then gave formal elegance to the idea, that an equally powerful Ca^{2+}-dependent inactivation (CDI) is needed for signals to remain graded. CICR and CDI are the necessary yin and yang of Ca signaling.

But that is not all: there is evidence (largely from the cardiac muscle field) for a third mechanism, *intra-store Ca^{2+} control* or ISCC. In cardiac muscle ISCC has a unique cybernetic property: it is an *activation* but does not entail positive feedback (because channel opening hastens depletion). Hence it is stabilizing –actually protecting the SR content of heart cells by closing channels when a threshold $[Ca^{2+}]_{SR}$ level is reached on the way to depletion.

Striated muscle must control Ca^{2+} tightly. Cardiac and skeletal muscles constitute prime examples of signaling mechanisms aimed at accomplishing closely related but different goals. Both require a sudden 100-fold elevation of $[Ca^{2+}]_{cyto}$, which must then subside rapidly (Rome *et al* 1996). But the cardiac transient will last 10-50 times longer. The long duty-cycle allows ample opportunity for modulating the transient as demands change. Skeletal muscle instead responds to changes in demand by increasing the frequency of its much briefer transients and differentially controlling motor units. In view of these considerations we hypothesize that *mechanisms of control prevalent in cardiac muscle may apply in skeletal muscle*, but *differences exist, consistent with the divergent functional demands*.

Release termination is "better" in skeletal muscle. The termination of Ca^{2+} release (both at the local level of Ca^{2+} sparks and the global level) occurs earlier and more rapidly in skeletal than cardiac muscle. We therefore launched this project, a few years ago, on skeletal muscle expecting manifestations of control by SR-lumenal Ca^{2+} even stronger than those of cardiac muscle. Strong control was found, but ***not*** in the way we expected.

The SR Ca^{2+} store is robust. In cardiac muscle, "blinks" reveal local depletion ($[Ca^{2+}]_{SR}$ reduction) of ~60% during Ca^{2+} sparks (Brochet *et al* 2005). The "skrap", as we called the skeletal muscle equivalent, is so small that it took averaging thousands to clearly see them (fig 2). We also imaged depletion due to a twitch (fig 3). 200 μM Ca^{2+} is released during a skeletal muscle twitch but the associated reduction in $[Ca^{2+}]_{SR}$ is only ~10%.

This implies existence of an ample intra-SR Ca reservoir, conventionally ascribed to the Ca^{2+}-binding power of calsequestrin, CSQ. Our current research produced intriguing evidence against this widely held idea.

Because the store is robust, depletion cannot play the role in termination of Ca^{2+} release that it has in cardiac cells (Ríos *et al* 2006*). The ISCC mechanism must be very different in the two striated muscle types.

Muscle fatigue involves depletion of Ca^{2+} in the SR. Intra-store Ca^{2+} control mechanisms come to play if there is depletion. Physiologically, $[Ca^{2+}]_{SR}$ declines with sustained exercise and is a factor of "peripheral" muscle fatigue (Allen *et al* 2007). A major goal of our work is to cause fatigue and characterize the changes in Ca^{2+} release that it entails. As we shall show, the prevalent notion that SR Ca^{2+} is well buffered by CSQ is not really supported by our data. Hence we explore an alternative mechanism, SOCE, that may help explain the stability of $[Ca^{2+}]_{SR}$ in the face of sustained activation of Ca^{2+} release.

In striated muscle Ca^{2+} release is the function of a "releasome". This is the array of physically linked and dynamically interacting proteins that execute Ca^{2+} release. The concept stems from the demonstration of quaternary interactions between RyR, triadin, junctin and CSQ (Zhang *et al* 1997). In fig 1 is a minimal view of the releasome, but there are other candidates (Ríos, J. Physiol. 2006). It is thought that control of the RyR by SR Ca^{2+} requires CSQ as primary sensor and triadin as linker. These two proteins are at the focus of our current research.

Cardiac calsequestrin has a dual role. CSQ is thought to be the main buffer of releasable Ca^{2+} (MacLennan & Wong 1971). Work on heart muscle supports this role. A deficit in CSQ2 results in both reduced release duration and premature contractions of heart cells (Terentyev *et al* 2003). These effects are understood as primarily due to a reduction in Ca^{2+} buffering power, which hastens both release termination (as $[Ca^{2+}]_{SR}$ reaches earlier the threshold for channel closing) and recovery of $[Ca^{2+}]_{SR}$ after each beat. Also, CSQ2 affects RyRs in bilayers (Györke *et al* 2004), implying more than just a Ca^{2+} buffering action.

CSQ as main Ca^{2+} "store" of EC coupling? If the releasable Ca^{2+} content in the SR is sufficient to raise myoplasmic concentration to 1- 5 mM (Pape *et al* 1995; Ursu *et al* 2006), and the cell's aqueous volume is > 10 times that of the SR, then total SR [Ca], $[Ca]_{T,SR}$, must be > 10-50 mM. Inside the SR, Ca is largely bound, and CSQ is thought to provide the binding sites (Mac Lennan & Wong 1971). This concept is so seductive that only Volpe & Simon (1991) bothered to test it quantitatively. Their study concluded against the proposed CSQ role, but was generally dismissed because of its other conclusion, that free $[Ca^{2+}]_{SR}$ (calculated dividing putative $[Ca]_{T,SR}$ by buffer power in CSQ) is 3.6 mM. Far from Volpe's fault, this number (much greater than measured $[Ca^{2+}]_{SR}$) constitutes an early warning that the state of Ca^{2+} in the store remains to be established. Please read on…

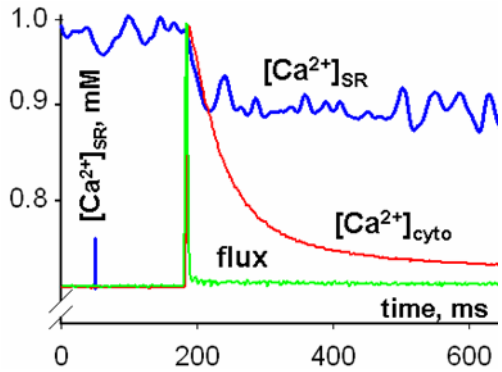

Fig. 3. Cell-wide averages of $[Ca^{2+}]_{SR}$ and $[Ca^{2+}]_{cyto}$ during a twitch. Depletion is ~11%. Launikonis *et al* 2006*.

Knockouts and knockdowns are quite harmless. Paolini *et al* (2007) showed that KO mice devoid of CSQ1 or compensatory CSQ2 live long, with normal twitch peak and tetanic tension and with increased resistance to fatigue. This agrees with results of Wang *et al* (2006) on C_2C_{12} myotubes virtually devoid of CSQ1, and our own studies of a double KO mouse (Section C). While the dearth of effects could reflect compensatory changes (Paolini) or the presence of CSQ2 (Wang), we show below similar results in conditions that exclude compensation. Is CSQ really the main functional reservoir of SR Ca^{2+}?

Three more roles for CSQ. (1) CSQ is proposed to also be a device for smart delivery. As drawn in fig 1, CSQ forms linear chains tethered to the RyR (Franzini-Armstrong *et al* 1987), presumed to efficiently deliver their bound Ca to the channel mouth by a guided diffusion that is fast due to the reduced dimensionality (Adam 1988) of these "calcium wires" (Park, 2004). Our results (Launikonis *et al* PNAS 2006) support the concept of a nonlinear, dynamic SR Ca^{2+} buffer; but is it necessarily CSQ? (2) As we have seen, CSQ has "gating" effects on the RyR. (3) Finally, it is a determinant of shape and size of SR terminal cisternae (TC) during development (*e.g*. Tijskens *et al* 2004) and one of the best targeted SR proteins, almost exclusively to TC, by mechanisms

that remain a puzzle (*e.g.* Gatti, 2001). We exploit effectively this feature in the production of TC-specific probes.

Triadin's roles may be crucial. Trisk95, the main junctional form of triadin, consists of a short cytosolic N terminus, a single transmembrane segment and a large intra-SR moiety with "KEKE" stretches that bind CSQ as well as the RyR (Knudson *et al* 1993) at identified residues (Lee *et al* 2004). Specific manipulations of triadin have not led to a coherent picture. Beginning with triadin additions: in bilayers, *trans*-side triadin increased P_o of RyR1, and was required for effects by CSQ and SR Ca^{2+} (Györke *et al* 2004); in myotubes its overexpression nearly abolished V-induced Ca^{2+} release (Rezgui *et al* 2005); in FDB muscle we are finding no effects of overexpression (fig 24). Turning to triadin reductions: major inhibition of Ca^{2+} release was seen in myotubes with a RyR that did not bind to triadin (Goonasekera *et al* 2007) but silencing caused only a modest reduction in C_2C_{12} myotubes (Wang *et al* 2006) and KO in mice had "no obvious phenotype" (Shen *et al* 2007). We are studying triadin both for its role of mediator of the CSQ-RyR interaction and its potential intrinsic roles. By exploiting the inherent advantages of our approach (claimed in next paragraph) we should be able to decide how crucial triadin is and to what extent its absence can be compensated. ***Junctin*** (Jones *et al* 1995) is a much smaller protein (210 aa *vs* 706 in triadin) that shares triadin's ability to interact with the RyR and bind CSQ but exhibits many functional differences (Kirchhefer *et al* 2006; Yuan *et al* 2007).

The advantage of transient expression. In KO mice the adult phenotype recapitulates all the effects of the molecular deficiency during development, thus rendering any mechanistic interpretation difficult. Our present approach relies instead on our ability to acutely change the endowment of a target protein by enormous factors. Increases in expression require 4 days and silencing 15 days; thus we can change protein levels without affecting cellular or organismal development, or providing time for adaptive reactions.

How does SOCE contribute to lumenal Ca^{2+} regulation? While SOCE (store-operated calcium entry; Putney 1986) helps many types of cells fulfil their basic functions, its physiological role in skeletal muscle has not been defined. Store-operated channels (SOCs) are present, and their activity is increased in Duchenne MD (Vandebrouck *et al* 2002). So are SACs (stretch-activated channels), which have a pharmacology similar to SOCs (Ducret *et al* 2006). SOCs and SACs are involved in muscle development (Formigli *et al* 2007) but their roles in long-term adaptation to mechanical load are not established (*e g* Berchtold *et al* 2000). In non-excitable cells SOCE is known to affect transcription factors and slow responses (Luik & Lewis 2007); in muscle we expect that it will also impact signaling on faster time scales. Can it alter the Ca store at a rate sufficient to affect EC coupling, specifically under patterns of activity that lead to SR depletion and fatigue?

New tools for the study of SOCE. Our work produced a tool to measure SOCE during Ca^{2+} release (Launikonis & Ríos, J. Physiol. 2007*). This method can separate SOCE from other forms of Ca^{2+} entry, including L-type current and excitation-coupled Ca^{2+} entry (ECCE; Cherednichenko *et al* 2004). Combining it with measurements of Ca^{2+} in cytosol and SR, we will evaluate how the store controls Ca^{2+} entry, and how much this flux (which we found to be as high as 1.4 mM/s) contributes to refilling the store.

We also have functional tools to understand the roles of newly discovered *molecules of SOCE*.

STIM and Orai. Four groups, in choreographed accord, recently identified STIM1 (Liou *et al* 2005, 2007; Roos *et al* 2005) and Orai1/CRACM1 (Vig *et al* 2006; Feske *et al* 2006) as, respectively, key ER and plasmalemmal molecules of SOCE. STIM1, the stromal interaction molecule, has features that enable it to coalesce into clusters upon changes in $[Ca^{2+}]_{ER}$ and secondarily bind to the plasma membrane. Orai isoforms 1-3 are named for the Hours, or keepers of the gates of Heaven. Orai1 is a 4-pass plasma membrane molecule whose mutation R91W causes SCID (Liou *et al* 2005), characterized by absence of SOCE in T lymphocytes, plus, and importantly for our project, a congenital myopathy.

In non-excitable cells STIM and Orai come together upon ER depletion to enable SOCE. We are exploring whether this mechanism is at work in skeletal muscle. Both proteins are present (Stiber *et al* 2008; Vig *et al* 2008); STIM is located in junctional and longitudinal SR (figs 26, 27). Myotubes of mice lacking STIM1 and adult fibers of haploinsufficient mice fatigue rapidly (Stiber *et al*, 2008). By direct measurements in these animals, or by knocking STIM and Orai down, we are testing whether these proteins underpin SOCE and through it help maintain skeletal muscle SR load under intense activity. A model of events in non-excitable cells (Liou *et al* 2007) is redrawn in fig 1 for muscle: upon SR depletion the luminal EF hand of STIM1 loses

Ca^{2+}, which leads to clustering and eventual colocalization of clusters with multiple copies of Orai1 at the t membrane; the two together (plus perhaps other molecules) enable SOCE.

Ca^{2+} signaling evolves on three time scales. These are associated with three types of molecules: (1) the millisecond-scale events of EC coupling are largely the function of DHPR and RyR. (2) the seconds-to-minutes scale of exertion that leads to muscle fatigue may cause changes in lumenal Ca^{2+} buffers like CSQ, and (3) the hours-to-weeks scale of activities that cause adaptation and changes in fiber type relies on transcription factors like NFATc1, NF-κB and MAP kinases, activated by Ca^{2+} and other signals (*e g* Tomida *et al* 2003; Shen *et al* 2007, rev. Kramer & Goodyear, 2007).

Function can be compromised in the three time scales of signalling. (1) Failures of the fast signals are found in diseases of EC coupling (including hypo-PP, MH susceptibility and central core and minicore diseases; Jurkat-Rott & Lehmann-Horn, 2005), as well as in aging muscle (Payne *et al* 2004). (2) The mid-scale is affected in the arrhythmogenic CPVT disorder (ter Keurs & Boyden 2007) linked to mutations in CSQ2, and in a skeletal MH-like phenotype developed by CSQ1 KO mice (Dainese *et al* 2008). (3) Diseases of long term Ca^{2+} signaling show striking parallels between muscle and the immune response (Luik & Lewis 2007): NFAT is involved in both, and so are Orai and SOCE. In fact, Orai1 was discovered through its genetic linkage to SCID, which features altered SOCE in T cells and muscle. Orai1 was found to express profusely in all muscles and its deficiency resulted in mice of reduced muscle development (Vig *et al* 2008).

Our work has consequences at all three scales. The impact results not just from the evaluation of roles of specific molecules but also from studying their interactions. Indeed, syndromes and molecular defects are not in simple one-on-one linkage. Thus MH can be caused indistinctly by some mutations in $Ca_V1.1$, RyR, or, as Dainese *et al* show, in CSQ1. Moreover, functional losses of disease, fatigue and aging are interrelated. Duchenne MD courses with reduced resistance to fatigue and probably low $[Ca^{2+}]_{SR}$ (Woods *et al* 2005); one of the triggers of MH episodes is fatiguing exercise; aging muscle is more susceptible to some types of fatigue and some channelopathies are age-sensitive. The importance of context (*i e* interactions that determine the final outcome) is vividly illustrated in the fact that CCD's organismal phenotype of functional loss comes with a mixture of gain and loss of function at the single channel level (Lynch *et al* 1999; Avila *et al* 2001). Therefore, the pathogenesis can be tortuous. Questions on the relationships among deficits of function, the intricate pathophysiology and the rational design of therapeutic corrections will be addressed better as we increasingly understand what SR Ca^{2+} does, and how it is controlled.