Current research plans

While our long term goal is to “understand Ca signaling” in muscle, by which we mean to understand the sequence of cellular events that determine the contractile response to the electrical action potential, we have recently focused on one aspect of this general problem. We would like to understand the generation of the cell-wide (or global) Ca$^{2+}$ release of excitation-contraction (EC) coupling (which allows Ca$^{2+}$ to move from store to cytosol) in terms of contributions from individual channels. Fig 1 shows the time course of voltage-elicited global Ca$^{2+}$ release in mammals and amphibians. Fig 2 shows the local Ca$^{2+}$ events that occur during these pulses. Our goal requires understanding (1) how the global release results from the local events (superposition of events as in fig 2A will NOT reproduce the global transient in fig 1B), and (2) how the local events are generated from individual channels. The structural (i.e. dual release channels, restricted space, etc.) and functional complexity (i.e. RyR-RyR & DHPR-RyR interactions) of the cellular EC coupling environment prescribes a combination of in vitro and in cell strategies. In the next few years we plan to bridge this knowledge gap by applying new approaches to test the 4 hypotheses that follow.

Hypothesis 1 is being tested by quantification of CICR and CDI in adult cells. 1.1: Is there CICR? How much and with what threshold? 1.2: Is there CDI? 1.3: Can 3 classes of sparks (described by Zhou et al. 2005*) be put in correspondence with different RyR channel isoforms or their combinations? 1.4: Are the concerted sparks (described by Zhou et al, 2005*) the result of opening of multiple channels synchronized by allosteric interactions?

Hypothesis 2 is tested by comparing responses of genetically altered adult cells. We will characterize the Ca$^{2+}$ control properties (response to $V_m$, sparks, CICR and CDI) of the cells in the 2nd row of fig 3. Mouse FDB and frog tibialis a. will be studied under patch and Vaseline-gap clamp, respectively. Caged Ca$^{2+}$ will be introduced and [Ca$^{2+}$]$_{cyt}$ monitored to evaluate cage load. Experiments 2.1 will test the role of RyR3 by addition to a cell that lacks it. 2.1: Expression of RyR3 in mice is now routinely induced in our lab by electroporation (a technique of DiFranco et al, 2005). ~50% of acutely dissociated cells exhibit a “frog” phenotype and are being studied biochemically and functionally. EM in a collaborating lab is characterizing the location of RyR3. Rapidly accumulating results of this aim made us reformulate it very ambitiously. The main goal is to define how the exogenous isoform changes the workings of Ca$^{2+}$ release, both globally and locally. Additionally we will use 3 combinations of 2 isoforms afforded by this preparation (RyR3 alone, RyR1 alone, RyR1+3) to test the hypothesis formulated in aim 1.3 on classes of sparks. 2.2: silencing RyR1. cDNA for small RNA interference (siRNA) will be introduced to reduce the density of RyR1 in adult mouse (“silencing”). EM will monitor whether silencing results in disruption of the RyR lattice, reduction in couplon size, or both. Responses (to V or Ca$^{2+}$) that simply scale down with RyR density will indicate little channel-on-channel interaction in the wild type.

Hypothesis 3 will be tested by comparing two regions in genetically altered myotubes represented in fig 3. In each case, Ca$^{2+}$ control will be compared in two regions: one peripheral, where SR is docked with T tubules, and one central, T tubule-free, with undocked SR (Zhou et al, 2006). RyR1s docked with T tubules will face a narrow gap. In undocked regions, RyRs will be facing the wide cytosol. 3.1: in RyR3(-/-)-mice

*All references to our work have been hyperlinked to actual documents in the web page « publications »
myotubes, the differences between these regions cannot be due to isoform differences, and will be due to either cytosol geometry or the presence of the DHPR. 3.2: The double mutant mdg(+/-) RyR3(-/-) will decide between these alternatives. 3.3: Comparing RyR1(-/-) with RyR3(-/-) will establish the isoform-specific properties in undocked regions.

Hypothesis 4 is being tested by overexpression of the intra-SR buffer calsequestrin, and “silencing” experiments, which reduce the concentration of calsequestrin by interfering with its expression using siRNA.

Current Issues in Excitation-Contraction Coupling

Ca²⁺ signals of muscle as paradigm. Ca²⁺ is perhaps the most ubiquitous intracellular signal. Its role was first established in muscle, where Ca²⁺ transients are fastest and most massive. Muscle’s devices continue to be watched as possible models for neurons, nuclear gene-regulatory signals, and linkers of store-operated plasmalemmal channels to intracellular sensors.

Crystalline packing of membrane molecules is a distinctive feature. The characteristic features of muscle EC coupling include (i) mechanical contact between sensors and channels in separate membranes, a feature now demonstrated in control of neuronal syntillas (JV Walsh, personal communication) and (ii) the quasi-crystalline 2-D arrangement of release channels. Other molecules feature tight ordered arrays, including SERCA pumps, gap junction channels, acetylcholine receptors, aquaporins and bacteriorhodopsin. While ordered clustering should induce functional consequences, these have been elusive so far. One of the central goals of our laboratory is to define inter-channel interactions in these clusters, and how they affect signaling.

Muscle Ca²⁺ signals require massive, rapidly gated Ca²⁺ release. In fast twitch muscle, the functional goal of EC coupling is to cause near saturation of troponin C and then make it Ca²⁺-free, all in a few milliseconds. This requires as much as 1 mM Ca²⁺ (referred to fiber volume) moving from stores to cytosol. Because Ca²⁺ is a multi-use messenger, EC coupling is a high-wire act which keeps fast muscle “near death” (Carafoli, 2005). The signals must thus be exquisitely tuned, with multiple and perhaps redundant controls. The fast rate of change of free [Ca²⁺]cyto results from the difference between two large fluxes (release and removal), accomplished by finely interspersed Ca²⁺ sources and sinks with the capability of fluxes of up to 250 mM/s for release and 50 mM/s for removal. The peak release rate is reached in less than 1 ms in the mouse and turned off entirely in ~ 4 ms (Rome et al, 1996), requiring activation of about 80% of the channels in the frog, and perhaps 50% in the mouse. Such degree of activation is achieved by the interplay of a number of mechanisms to amplify release and then abruptly end it. Crucial are interactions of the channels with the signaling ion itself. CICR is the key means of opening in heart, but its actual role in skeletal muscle continues to be debated (e.g. Endo, 2005). One of our goals is to quantify CICR under different circumstances.

But Ca²⁺ release always remains under voltage control. Even if there is CICR, Ca²⁺ release flux, which is physiologically started by change in V_m, is graded with V_m and remains under its control while evolving as shown in fig 1. The decay of release of up to 250 mM/s for release and 50 mM/s for removal. The peak release rate is reached in less than 1 ms in the mouse and turned off entirely in ~ 4 ms (Rome et al, 1996), requiring activation of about 80% of the channels in the frog, and perhaps 50% in the mouse. Such degree of activation is achieved by the interplay of a number of mechanisms to amplify release and then abruptly end it. Crucial are interactions of the channels with the signaling ion itself. CICR is the key means of opening in heart, but its actual role in skeletal muscle continues to be debated (e.g. Endo, 2005). One of our goals is to quantify CICR under different circumstances.

But Ca²⁺ release always remains under voltage control. Even if there is CICR, Ca²⁺ release flux, which is physiologically started by change in V_m, is graded with V_m and remains under its control while evolving as shown in fig 1. The decay of release

*All references to our work have been hyperlinked to actual documents in the web page « publications »
Our current work directly addresses the mechanism of (Stern et al, 1997). How it can do this is a crucial question for stable function, which is being addressed in our current work.

Stoichiometric contact is key to control. A tetrads of DHPRs (V sensors) overlaps one RyR in detailed correspondence with its homomers (Block et al, 1988; Wolf et al, 2003), establishing an interaction near the vertices of the RyR tetramer that is bilaterally specific (i.e. requires the RyR1 and DHPR, Nakai et al, 1997; Protasi et al, 2002, 2000). But the structural determinants for “skipping” every other RyR in the pattern remain unknown, as do the functional implications of this arrangement. An old speculation that attributed different mechanisms of activation (V_m or Ca^{2+}) to alternate members of the RyR array (Ríos & Pizarro, 1988) is indefensible in its original form. Our hypothesis 2 updates that speculation by assigning isoform specificity to the activation method.

Are Ca^{2+} sparks the building blocks of global release? Ca^{2+} sparks were discovered in cardiac muscle (Cheng et al, 1993) and then demonstrated by us in skeletal muscle (Tsugorka et al, 1995*). Impressed by their rapid termination (fig 2), which mimics the inactivation/decay of the global response (fig 1), researchers thought of the cell-wide transient as a superposition of sparks (Klein et al, 1997; Shirokova et al, 1999*). That the situation is more complicated is shown by at least four sets of results: 1) the V sensor activates sparks, but it can also terminate them (therefore, spark kinetics is V_m-dependent; Lacampagne et al, 2000). 2) individual V sensors may cooperate to elicit sparks (no one-to-one link between V sensors and spark generators, Brun et al, 2003). 3) Sparks may not be the same when occurring in isolation vs. when part of a massive response. 4) Ca^{2+} sparks may not even be part of the normal response of mammalian muscle, which may result just from embers (fig 2; Csernoch et al, 2004*; Baylor, 2005). In a nutshell, it is not possible to go from the local signals of fig 2 to the global release of fig 1 by just adding sparks or embers. Our current work directly addresses the mechanism of sparks. Once this is understood, it will be possible to predict interactions within larger responses, thus fulfilling our goal of synthesis of global release.

Amphibians and mammals play by different rules. Three isoforms of the RyR channel are coded by different genes. “Cardiac” isoform 2 and “brain” RyR3 share greater homology than with RyR1. In adult mammals, limb muscles have exclusively RyR1; muscles that “beat”, i.e. engage in repetitive contractions, like masseter and diaphragm, have substantial RyR3. Isoforms 1 and 3 (α and β) are about equally present in most muscles of amphibians, birds and fish (Ogawa et al, 2002), as well as developing mammalian muscle up to D4 (Chun et al, 2003). In the first report of isoform-related functional differences (Shirokova et al, 1996*) we showed that the ratio of Peak/Steady release flux (fig2) had a marked maximum at intermediate V_m in the frog, but was flat in the rat (fig 5). A simple model assuming that RyRs were either V_m- or Ca^{2+}-sensitive (Shirokova et al, 1996*) showed that a V_m-dependent P/S ratio was a hallmark of amplification by CICR. This, and the direct relationship between [Ca^{2+}]_cyto and spark frequency (Klein et al, 1996) are still core arguments for the involvement of CICR in the generation of Ca^{2+} sparks. As arguments, however, they are fairly indirect.

Ancillary proteins continue to be found. The cast of molecules that modulate release channels keeps increasing. We have summarized them and their putative roles in a brief note (Ríos, 2006). With kinases and phosphatases also at work it can be said that Ca release is the function of a supramolecular “signalosome”. The importance of conserving the signalosome intact has been noted by Baylor (2005) who found substantial differences between sparks of intact and cut cells. Our focus remains on RyR-RyR and DHPR-RyR interactions. As for other  

*All references to our work have been hyperlinked to actual documents in the web page « publications »
players, known and unknown, we strive to keep their presence constant (e.g., by adopting conservative permeabilization methods).

The “couplon” simulates sparks from first principles. In 1997 we introduced the concept of couplon, defined as the set of RyRs, their sensors and associated molecules on one side of one junctional segment of T tubule (Stern et al, 1997*). The evolution of this set of channels was derived by a full biophysical simulation which incorporated activation by V sensors and Ca\(^{2+}\), inactivation by Ca\(^{2+}\), and took account of diffusion in the junctional gap (geometry in fig 6). This simulation not only solved the “paradox of control”, i.e. release could be terminated by repolarization at any time, but it simulated sparks. We have extended the couplon simulation to cardiac muscle (Stern et al, 1999).

Dual arrays of dual isoforms. Two newer findings call the couplon model into question. In the mammal, responses to V_m are embers, not sparks (fig 2 and Progress Report). In amphibians and other Ca\(^{2+}\) spark-ing taxa, RyR3 lie in parajunctional arrays, leaving the triadic junction for RyR1 only (fig 7). Thus, the two coexisting sets of channels differ in molecular structure, array geometry and, perhaps most importantly, the geometry of the facing volume—a busy, 15 nm gap for RyR1, the wide cytosol for RyR3. These two sets really constitute two different systems for signaling. A current goal is to clarify how these work; alone (to understand the mammal) or together (in the frog, the chicken, and mammalian newborns). The current approach, which produces and studies single- and dual-isof orm systems, with or without V sensors, in different cytosolic geometries, should be able to separate the key effects of molecular and geometric factors.

Interactions within the SR. An interaction between RyR2 and calsequestrin (CSQ), whereby CSQ “senses” [Ca\(^{2+}\)]_SR, mediates in the heart the promotion of sparks by elevated SR load. Conversely, depletion of intra-SR Ca\(^{2+}\) is believed to exert, again via CSQ, the crucial gating action that terminates sparks (Terentyev et al, 2002, 03, 05). This view was bolstered by the description in heart muscle of a “blink” of local [Ca\(^{2+}\)]_SR depletion (Brochet et al, 2005), large enough to possibly have a role in termination. From ongoing studies we know that a similar picture will not apply to skeletal EC coupling. Skeletal terminal cisternae have a much greater volume, and much more CSQ of a greater binding capacity (Park et al, 2003). Besides, depletion caused by sparks in skeletal muscle is too small to support a robust termination signal (cf. Launikonis et al, PNAS 2006*) and even large imposed depletion is incapable of altering spark duration in skeletal muscle (Launikonis et al, J. Muscle Research 2006*).

EC coupling deficits are found in disease, fatigue and aging. The work of our lab addresses basic physiology and mechanism, and strives to understand how things work when they work well. However, translational implications are multiple. The functions that interest us become altered in three categories of conditions: disease, muscle fatigue and aging. Several diseases of muscle involve alterations in the structure and functions of the main EC coupling molecules. (rev. Jurkat-Rott & Lehmann-Horn, 2005). Most specifically related to our work are hypokalemic periodic paralysis (hypo-PP type 1), susceptibility to malignant hyperthermia (MH) and central core and minicore diseases (CCD) (Loke & MacLennan, 1998). CCD and MH are associated with inherited alterations of two molecules central to our studies. In the voltage sensor CaV1.1 a loss-of-function defect causes hypo-PP type 1 and point mutation R1086H leads to malignant hyperthermia, (Monnier, 1997). The latter relieves a basal inactivation by CaV1.1, also demonstrated in our recent work (Zhou et al, 2006*). Several MH mutations affect the cytosolic part of RYR1 and increase sensitivity to Ca\(^{2+}\)-related activators. To some extent, CCD and MH loci overlap. 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). That MH can be caused indistinctly by some mutations in CaV1.1 and RyR underscores the intimate consort between these molecules; that gain-of-function at the channel level may result in cellular loss-of-function stresses the importance of context (i.e. interactions that determine the final outcome).

Muscle fatigue is a loss of functional ability upon exertion that has complex manifestations and originates at multiple levels. The more strictly defined short-term fatigue is a purely myogenic effect of repeated contractions, due in large measure to a

*All references to our work have been hyperlinked to actual documents in the web page « publications »
reduction in Ca\textsuperscript{2+} release traced to a lower Ca\textsuperscript{2+} availability inside the store (Allen, 2004). Lower [Ca\textsuperscript{2+}]_{SR} reduces the driving force for release, which may change activation and/or inactivation gating (our Hypothesis 1). Thus fatigue is, in good part, a functional alteration of EC coupling.

Ageing muscle also exhibits loss of function. While traditionally attributed to the loss of muscle (i.e. contractile protein) mass, the functional impairment was recently shown to be greater than the loss of protein, which points at alterations in EC coupling (rev. Delbono 2002). In ageing mice a fraction of fibers shift to cardiac-type EC coupling, with no isoform switch (Payne et al, 2004). With the hypotheses of this application in mind one immediately asks: are RyR1 migrating parajunctionally? Are junctions moving to the plasmalemma? The structural studies that we are performing will help understand what happens functionally when RyRs are not docked to the T tubule membrane.

These three broad types of functional loss (in disease, fatigue and ageing) are interrelated. For instance, one of the triggers of MH episodes is fatiguing exercise, ageing muscle is more susceptible to some types of fatigue and some channelopathies are age-sensitive. Furthermore, the pathogenesis is in many cases intricate and puzzling (as the production of MH, an RyR-linked condition, by a CaV1.1 mutation, or the production of a condition usually linked to Na channel changes by a mutation in the same Ca channel). While none of these issues are specifically addressed in current work, questions regarding the relationships among deficits of function, the intricate pathophysiology and the rational design of therapeutic corrections, will be addressed better as we increase our understanding of basic mechanisms.

**Recent advances**

**Comparative EC coupling, a multi-part saga.** Having found that rat muscle does not respond with sparks to depolarizing pulses, we (Shirokova et al, 1998*) and Ward et al (1999, 2000) suggested that sparks require RyR3. This was not quite right; Conklin et al, 1999, Shirokova et al, 1999* and Ward et al, 2001, found that expression of either isoform suffices to produce sparks in 1B5 cells. This set the stage for advances of the current funding period that defined the spark of adult mammalian muscle.

**5. The spark of mammalian muscle.** Under conditions established by our postdoc W.G. Kirsch (2001) we examined thousands of sparks in the rat, which as shown in fig 8 are much wider than the frog’s. Their spatial profile is flatter than a Gaussian (they are platykurtic; Izu, 1998). We named “protoplatykurtic”, or PPK, events that are flat from their beginning, evidence of a spatially extensive source (Zhou et al, 2005*). Indeed, the underlying Ca\textsuperscript{2+} release current calculated by the “backward” algorithm (Ríos et al, 1999*) topped at ~75 pA, implying ~150 simultaneously open channels. Frequent “lone embers” could be simulated by currents of ~0.5 pA, implying that they are the work of single channels. The importance of embers became clear with the next advance.

**6. The elementary events of Ca\textsuperscript{2+} release in mammals are not sparks.** Csernoch et al, 2004*. To isolate the individual events in the mammal’s response to depolarization, we used techniques of Woods et al, 2002, for a reliable “Chemplex-gap” clamp of EDL fibers. The isolation of elementary events was achieved either by low V pulses, or by partial recovery from inactivation. The events (fig 2) were similar to “lone embers” of permeabilized fibers. Their Ca\textsuperscript{2+} current was ~0.5 pA, consistent with an individual open channel. Unlike frogs, in mammals channels appear to be individually operated.

The achievement was incomplete. Whole-cell Ca\textsuperscript{2+} release synthesized by superposition of embers had a qualitatively correct waveform, but stunted kinetics. This discrepancy suggests that the partial inactivation

*All references to our work have been hyperlinked to actual documents in the web page « publications »
imposed in order to isolate events alters the mechanisms that lead to the sharp peak (either weakens CICR or CDI, or reduces SR depletion). Therefore Ca$^{2+}$ sparks, never seen under low $V_m$ stimulation or partial repriming, could have a greater role in fully polarized cells at high $V_m$. This question will be tackled with new approaches in the next period. Either channel density or couplon size will be reduced by RNA interference (Aim 2). If the above hypothesis is correct, then the Ca$^{2+}$ release of the silenced fibers will inactivate less.

7. **Steady changes of cytosolic [Ca$^{2+}$] and [Mg$^{2+}$]. Amphibians.** We compared sparks in amphibian and mammalian muscle while applying various [Ca$^{2+}$] or [Mg$^{2+}$] to the cytosol. In steady state the [Mg$^{2+}$]-dependence of the frequency $f$ of sparks in the frog was well predicted by the “classic” two-site model of RyR modulation (Meissner et al, 1986; Laver et al, 1997). The prediction (eqn.1 of Zhou et al, 2004*) works, provided that an attenuation factor $\kappa$ <<1, a basal inhibition of sparks, is included. Either channels do not open at the frequency expected from their bilayer behavior, or single channel openings trigger sparks only occasionally.

8. **Steady changes of cytosolic [Ca$^{2+}$] and [Mg$^{2+}$]. Mammals.** Zhou et al. 2004*. The results were strikingly different in the rat. The Ca$^{2+}$ activation site was hardly recognizable, with $K_{Ca} =$350 µM. Mg$^{2+}$ only inhibited at greater than 4 mM. The attenuation factor $\kappa$, however, was still necessary. In conclusion, the work showed crucial differences between control of Ca$^{2+}$ release in the two taxa, but also revealed a basal inhibition of sparks, common to amphibians and mammals. These differences will be studied further in the next period, to separate the molecular (i.e. isoform) effects from the geometric (parajunctional vs. junctional).

Advances in the next set regard control by voltage and Ca$^{2+}$.

9. **Direct application of Ca$^{2+}$ by photorelease.** Zhou et al, 2004*. To probe activation and inactivation we used methods of Escobar et al, (1995, 1997) and B.J. Simon (unpublished), to apply spatially homogeneous increases in [Ca$^{2+}$]$_{cyt}$ by photorelease from NP-EGTA. Fig 10 shows an image in xy mode and fig 11 a line scan. The time course of [Ca$^{2+}$]$_{cyt}$ was monitored with fluo-4 and fitted by a model of Ellis-Davies et al, 1996, to calculate the applied [Ca$^{2+}$]. In both frog and rat the application of large spikes (up to 300 µM) increased the frequency of sparks (fig11A) and caused global release, dramatically in many cases. The increase, however, often occurred after a delay, which could be an actual lag in CICR, or an indication that the effect is mediated by increase in SR load. These experiments also evidence a high threshold for CICR and an even higher one for CDI, which matches other evidences that RyRs are inhibited in situ. The mechanism of this inhibition was the topic of a recent paper, Zhou et al, 2006*.

10. **Selectively interfering with voltage sensors.** Brum et al, 2003*. Two quantitative models of control can simulate whole-cell Ca$^{2+}$ release. One is the couplon, which assumes that V-operated channels may add forces to activate other channels. Then there is an attractive procedure of Klein et al, 1997, to construct the whole-cell waveform by summation of Ca$^{2+}$ sparks. Klein’s method assumes that the time course and $V_m$ dependence of release in the partially inactivated system are the same as those of the fully primed situation, only amplitude is scaled down. Such scaling property implies that individual V sensors are masters of a cohort of slave channels, constituting a unit that has in itself all the time- and $V_m$-dependent properties of global release. (Masters can be “killed” one by one, but those that remain standing will still command their cohort and produce sparks of the same time course). In particular, such V sensors do not interact mutually.

We disproved this picture in whole-cell experiments in which V sensors were partially inactivated by depolarized holding potentials. We found that a kinetically normal transient of Ca$^{2+}$ release (as that in fig 1 A) could still be obtained, but by a pulse to a higher $V_m$. This is against predictions of the “slave cohort” model, where kinetics does not change when V sensors are decimated by inactivation because kinetics is a property of the cohort. In contrast, the couplon model reproduced the findings. Thus, the couplon model seems to be correct in its essential feature: V sensors and their associated release channels do not constitute independent units, they can interact. In the next period we will update the model by introducing Ca$^{2+}$-sensitive channels in a parajunctional position.

![Fig 11. Cell-wide Ca$^{2+}$ photorelease](image)

*All references to our work have been hyperlinked to actual documents in the web page « publications »*
11. “Nanoscale” CDI. Pizarro & Ríos, 2004*. We evaluated the effects of SR load on global Ca release by combining in the same cell the EGTA/Phenol Red method for SR Ca content (Pape et al, 1995) with our own method to measure Ca$^{2+}$ release. The results add evidence that Ca$^{2+}$ coming through open channels inactivates them (and their neighbors) to terminate release. The key result is in fig 12. As SR is depleted, the rate of rise of global release permeability $P$ is unchanged, but its inactivation becomes slower (inset). This implies that the initial rate of channel opening does not change with [Ca$^{2+}]_{SR}$. Ergo, the effect occurs after the channels open, it is cytosolic, not lumenal. This is “nanoscale” CDI, as the range of [Ca$^{2+}$] sufficiently elevated near an open channel to inactivate neighbors is only tens of nm (Rengifo et al, 2002*). The CDI concept, first suggested for skeletal muscle by Baylor et al in 1983 and supported by work in our lab, and results of P. Pape and others, should now be accepted as fact. The present result identifies its hallmark: decrease in peak $P$ and rise time as SR load increases, without change in initial rate of channel opening. This “signature” is being used to probe CDI at the local level.

12. Inhibition of RyRs by the DHPR. Zhou et al, 2006*. Studies under # 7-9 above revealed a basal inhibition of the RyRs. We demonstrated that it is due to the DHPR, showing first that areas of myotubes endowed with T tubules have fewer sparks than central areas (fig 13), and then that the inhibition was absent in myotubes from DHPR-null mdg mice. The observations define two regions of the developing cell (fig 13): a periphery, where the SR engages with functional T tubules and plasmalemmal dyads, and the center, which is free of T tubules. There the SR is un-docked, presumably DHPR-free, and facing a large cytosol rather than a narrow junctional gap. The constructs in fig 3 delineate a path to systematically isolate and probe the effects of the narrow geometry (which should radically alter Ca$^{2+}$ diffusion), those of the DHPR, and those depending on the nature of the RyR isoform.

13. Concerted and sequential activation. Zhou et al, 2005*. In special media, local events of frog muscle settle into two discrete categories: concerted and sequential. They are easy to tell apart (fig 14, left). In concerted sparks some 100 channels activate in unison. In this case, the frog behaves like the rat (fig 8). Sequential events obviously involve a “bucket-brigade” of channels, passing activation along. They do not occur in the adult mammalian limb muscle. To explain the functional duality we offered a model (fig 14D) with one idea: that the determinant factor is geometry of the volume of cytosol facing the channels. In both cases activation is by CICR. It is fast if it happens in the triad (where the volume is small and high Ca$^{2+}$ spreads fast), giving rise to concerted events. In the parajunctional regions it is slower (justifying sequential events). This idea is attractively parsimonious, but concerted activation is a prime candidate to also involve allosteric transitions in the 2-D crystal grid of channels. These hypotheses are being tested in current work. Concerted is especially important for the mammal, which exhibits no sequential propagation. Whether RyR1 or geometry is determinant of the activation type will be directly addressed with the study of constructs that change isoforms and geometry.

Fig 12. As [Ca$^{2+}]_{SR}$ is reduced, inactivation becomes slower, (inset) and peak $P$ grows. Rise time increases and initial rate of rise does not change. Pizarro & Ríos, 2004.

*All references to our work have been hyperlinked to actual documents in the web page « publications »
14. SEER, a novel technique for imaging [Ca$^{2+}$]$_{SR}$. We described a new way of using ratiometric dyes that multiplies the dynamic ranges of their shifts in excitation and emission, thus increasing sensitivity to a point where confocal imaging of [Ca$^{2+}$] in small organelles becomes feasible, even beautiful. SEER has sufficient spatial resolution to separate the two TC in a triad (fig 15) or image [Ca$^{2+}$] in individual mitochondria, and sufficient temporal resolution to follow depletion associated with sparks (fig 16). The technique, crucial to all specific aims, has now been used in frog (Launikonis et al, 2005*) and mammalian myotubes (Zhou et al, 2006*). Its application to adult mouse FDB is illustrated in the next section.

15. Skraps. SEER imaging of [Ca$^{2+}$]$_{SR}$ allowed us to define the images of depletion due to sparks (Launikonis et al, 2006a*). We call them “skraps” (the mirror image of the word “sparks”). It can be seen that the depletion amounts to only ~3 % of the SEER ratio, or ~ 5% in [Ca$^{2+}$]$_{SR}$. This is too small to be a signal or factor for termination of release. Hence we will proceed with the assumption that (i) termination of release in sparks is not determined by the reduction in [Ca$^{2+}$]$_{SR}$ and (ii) the driving force for Ca$^{2+}$ release during a sparks is approximately constant, a fact confirmed by the rather constant Ca$^{2+}$ flux during a spark (Zhou et al, 2005*).

16. Sparks at measured [Ca$^{2+}$]$_{SR}$ SEER also allowed us to compare the properties of Ca$^{2+}$ sparks at varied, known, [Ca$^{2+}$]$_{SR}$. The conclusion (Launikonis et al, 2006b*) is that skeletal Ca$^{2+}$ sparks are affected little by a 50% change in [Ca$^{2+}$]$_{SR}$. This is in striking contrast with cardiac muscle. In a summary comparison (Rios et al, 2006) we conclude that depletion during sparks or twitches (or cardiac beats) is 5 to 10-fold lower (relative to initial content) in skeletal than cardiac muscle. The difference is consistent with the striking difference in volume and buffering power of the TC in skeletal vs cardiac muscle. Moreover, even large artificial depletion does not cause major gating changes in skeletal muscle. The result is intellectually clarifying, and allows us to focus almost exclusively on the effects of cytosolic Ca$^{2+}$.

*All references to our work have been hyperlinked to actual documents in the web page « publications »