SA1

Functional implications of RyR-DHPR relationships in skeletal and cardiac muscles

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Excitation-contraction coupling in muscle cells requires an interaction between L type calcium channels of surface membrane/T tubules (the dihydropyridine receptors, DHPRs) and the calcium release channels or ryanodine receptors (RyRs). of the sarcoplasmic reticulum(SR), Immunolabelling with specific antibodies shows that the two types of calcium channels form part of discrete macromolecular complexes, called calcium release units, that are located at the surface membrane and/or along the T tubules. The complexes include several SR proteins (calsequestrin, triadin, junctin), RyR associat ed proteins (Homer, FKBP12); the DHPRs and a docking protein that connects SR and surface membranes (junctophilin). Skeletal and cardiac muscles contain three types of RyRs: (RyR1 and RyR3 for skeletal; RyR2 for cardiac) and two forms of the a1 subunit of DHPRs (a1 s and a1c). The location of these isoforms and their positioning relative to each other are quite informative in regard to their functional interactions and to the role they play in excitation-contraction coupling. We have explored this question in a variety of native muscles and in cells engineered for null mutations of either channel and induced to express chimeric forms of the cardiac and isoforms. a1sDHPR and RyR1 are essential components of the skeletal muscle e-c coupling machinery and they are located in coextensive and interlocked arrays. Within the arrays, groups of four DHPRs (forming a tetrad) are located at predetermined specific locations relative the four subunits of every other RyR1 in the array. Grouping of DHPRs into tetrads requires the presence of RyR1. This association provides the structural framework for the reciprocal signalling that is known to occur between the two types of channels. RyR3 are present in some but not all skeletal muscles, always in association with RyR1, and in ratios as high as 1:1 ratio with the latter. RyR3 neither induce formation of tetrads by DHPRs, nor sustain e-c coupling. In CRUs of muscle fibres, RyR3 are located in a parajunctional position, in proximity of the RyR1-DHPR complexes. It is thus expected that they may be indirectly activated by calcium liberated via the RyR1 channels. RyR2 channels, the only isoform present in cardiac muscle, have two locations. One is at CRUs formed by the association of SR cisternae with domains of surface membrane and/or T tubules that contain DHPRs. In these cardiac CRUs, RyR2 and a1cDHPR are in proximity of each other, but not closely linked. This is in keeping with the proposed indirect DHPR-RyR interaction during e-c coupling of cardiac muscle. A second location is on SR cisternae that are not directly linked to surface membrane/T tubules. The RyR2 in these cisternae, which are often several microns away from any DHPRs, must necessarily be activated indirectly, Cardiac/skeletal RyR chimerae expressed in RyR null cells and a1s/a1c DHPR chimerae expressed in a1 null skeletal muscle cells show that DHPR tetrads are always induced by the presence of Either DHPR or RyR chimerae that restore skeletal-type e-c coupling. Several regions of RyR and at least one critical region of DHPRs are necessary for tetrad formation, but the molecular domains necessary for tetrad formation are not exactly the same as those required for an inter-molecular interaction. Animal experimentation conformed to local standards.

SA2

Electrophysiological analysis of conformational changes initiating excitation-contraction coupling in skeletal muscle

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Excitation contraction coupling in skeletal muscle begins with propagation of a surface action potential into the transverse (T) tubules to give an electrical signal that gates a steeply voltage-dependent release of intracellularly stored Ca^{2+} through the sarcoplasmic reticular (SR) membrane that increases up to *e*-fold with incremental depolarisations of 2–4 mV. It is thought to take place at localized triadic, T-SR, junctions where the geometrical proximity between nevertheless electrically separate membranes permits their close interaction. This transduction process is likely to involve T-tubular Ca^{2+} channel-dihydropyridine receptors (DHPRs) that act as voltage sensors, acting upon SR ryanodine receptor (RyR)-Ca^{2+} release channels. The conformational changes involved in the voltage-sensing process have been studied electrophysiologically in voltage-clamped intact amphibian muscle fibres. Thus, intramembrane rearrangements in charged functional groups in response to voltage change would displace excess charge from the membrane surface. This would be measurable as a charge movement whose characteristics would offer an electrical signature for the conformational change, provided other membrane ionic currents are minimized.

A specific, q_γ, intramembrane component has been identified with conformational changes in the DHPR. Detubulation experiments specifically localize it to the T-tubular membrane. It is sensitive to the contraction inhibitors tetracaine and dantrolene Na and possesses a steep steady-state voltage-dependence and complex, steeply potential-dependent, 'humped' kinetics thereby matching corresponding features of the Ca^{2+} release process. Both processes share a voltage-dependent inhibition by the DHPR inhibitor nifedipine. Such studies emerged with suggestions for the presence of a q_γ system, valence 4 to 6, at a density in the tubular membrane of 200–400 molecules/micron^2, similar to independently derived estimates of DHPR density. Closer kinetic studies identified the complex and steeply voltage-sensitive kinetics of this intramembrane charge with the direct interactions it may have with RyR-Ca^{2+} channel gating. The kinetics of the q_γ charge movement were separated from remaining components of intramembrane charge through shifts in holding voltage. Isochronic plots of such isolated q_γ charge movement suggested a causally independent intramembrane conformational transition with highly nonlinear kinetics whose embedded rate constants were nevertheless uniquely determined by membrane potential.

Recent pharmacological manoeuvres using DHPR-and RyR-specific reagents have thrown light upon the mechanisms responsible for the complex kinetic features. They suggest the existence of co-operative mechanisms that involve direct interactions between the DHPR-voltage sensor and the RyR-Ca^{2+} release channel. This contrasts with the simple kinetics of corresponding charge movements reflecting Ca^{2+} current gating in cardiac muscle, where intracellularly stored Ca^{2+} is released through indirect, Ca^{2+}-induced Ca^{2+} release, mechanisms that would not require such direct functional contact between membrane molecules. Suggestions for a direct interaction between a tubular membrane DHPR and an anatomically close but electrically uncoupled RyR within SR membrane was consistent with steady state findings. These indicated that DHPR

modification reduces the total steady-state q_γ charge but RyR modification by daunorubicin or ryanodine preserves the separate identities of steady-state q_γ charge, compatible with actions remote from the DHPR-voltage sensor. A hypothesis that RyR gating is allosterically coupled to configurational changes in DHPRs in skeletal muscle would next predict that such interactions are reciprocal and that RyR modification should influence the transitions shown by intramembrane charge. This prediction of a RyR-DHPR cross talk was confirmed by findings that the RyR antagonists ryanodine and daunorubicin remove, but further addition of the RyR agonist perchlorate restores, the nonlinearity in the q_γ charge movement. Other pharmacological manoeuvres using the RyR-specific agents caffeine and μM-tetracaine produced comparable selective effects on the kinetics of q_γ charge. Similarly, q_γ charge, together with its complex charging kinetics in perchlorate-containing solutions, persisted in experimental preparations whose Ca^{2+} stores were depleted using the Ca^{2+}-ATPase inhibitor cyclopiazonic acid (CPA), making it unlikely that such effects result indirectly from Ca^{2+} release rather than such direct allosteric contacts.

A simple analytical model describing the energetics for a possible subunit interaction between DHPRs and RyRs successfully reproduced the major characteristics of this q_γ cooperativity, and could form the basis for further quantitative explorations. This adapted the simplest two-state scheme in which intramembrane charge moves across an energy barrier from a resting energy state to an activated state. It permitted the energy barrier to decline as charge reaches the active state following a depolarising voltage step thereby enhancing subsequent charge transfer, and its return to its previous level with charge recovery at the end of the step.

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SA3

IP_3 receptors mediate coupling between membrane potential changes and transcriptional regulation in skeletal muscle cells

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Recently, we have described an inositol 1, 4, 5-trisphosphate (IP3) signalling system in cultured rodent skeletal muscle, triggered by membrane depolarization and affecting gene transcription (Jaimovich *et al.* 2000, Powell *et al.* 2001). Neonatal rat primary muscle cultures (animals were humanely killed) and muscle cell lines were used to study fluorescence calcium signals after loading with fluo-3. Biochemical measurements, immunocytochemical studies and Western blot analysis were performed on cells before and after depolarising protocols. When myotubes were exposed to tetanic electrical stimulation and the fluorescence calcium signal was monitored, the expected calcium signal sensitive to ryanodine and associated to the E-C coupling was seen during stimulation. A few seconds after the stimulus ended, a long lasting second calcium signal, refractory to ryanodine was evident. The onset kinetics of this slow signal was slightly modified in nominally calcium-free medium, as was by both the frequency and number of pulses during tetanus. The role of the action potential was evidenced since, in the presence of TTX, the signal was abolished. The role of the L-type, voltage dependent calcium channel or dihydropyridine receptor (DHPR) as voltage sensor for this signal (Araya *et al.* 2003) was assessed by treatment with agonist and antagonist dihydropyridines (Bay K 8644 and nifedipine) showing an enhanced and inhibitory response respectively. When the dysgenic GLT cell line was used, the signal was absent. Transfection of these cells with the α1S subunit, restored the slow signal. The IP3 mass increase induced by electrical pulses was previous in time to the slow calcium signal. Both IP3R blocker and PLC inhibitor (xestospongin C and U73122) dramatically inhibited the slow calcium response. We have shown that K^+-induced depolarization of rat myotubes elicits a transient increase in the early genes c-fos, c-jun and egr-1 mRNA levels and we can link such increase to calcium signals (Carrasco *et al.* 2003). Both early genes activation and CREB phosphorylation were inhibited by ERKs phosphorylation blockade.We investigated the possibility that slow calcium signals regulate CREB phosphorylation by a PKC-dependent mechanism. Western blot analysis revealed the presence of seven PKC isoforms (PKC α, -β1, -β2, -δ, -ϵ, ζ, and -θ) in rat myotubes. Pre-treatment of myotubes with either bisindolymaleimide I or Gˆ6976, PKC inhibitors with a preference for calcium-dependent isoforms, blocked CREB phosphorylation. Short-term stimulation with the phorbol ester tetradecanoyl phorbol acetate (TPA) which activates calcium-dependent and −independent isoforms but not atypical PKCs was sufficient to promote CREB phosphorylation and activation. Following chronic exposure to TPA that triggered complete down-regulation of all responsive isoforms except PKCα, CREB was still phosphorylated upon myotube depolarization. Immunocytochemical analysis revealed selective and rapid PKC α translocation to the nucleus following depolarization with kinetics similar to those of IP3 production and calcium release. Such nuclear PKCα translocation, as well as CREB phosphorylation and activation, were blocked by the IP3 receptor inhibitor 2-APB and the phospholipase C inhibitor U73122.Our results agree with a general pattern of intracellular signalling that involves DHPR as voltage sensor, activation of phospholipase C, nuclear calcium increase and translocation of PKCα to the nucleus. Subsequent events include CREB phosphorylation and early gene mRNA expression.

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SA4

The control of calcium release from the cardiac SR

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Most of the calcium that activates cardiac contraction is released from the sarcoplasmic reticulum (SR). This release is triggered by the entry of calcium into the cell via the L-type Ca channel leading to opening of the ryanodine receptor (RyR). This process is known as Ca induced Ca release (CICR). It has been suggested that, in addition to CICR there may also be a voltage dependent mechanism of Ca release (Ferrier & Howlett, 2001). However other work fails to find such a mechanism (Trafford & Eisner, 2003) and has suggested that it may result from incomplete inhibition of calcium entry (Piacentino III *et al.* 2000).The amount of Ca released depends on the Ca content of the SR; an increase of SR content increases the amount released. Stable contraction of the heart therefore depends on control of SR

content (Eisner *et al.* 2000). We will review evidence showing that control of SR content depends on the effects of Ca release on surface membrane Ca fluxes. Specifically an increase of SR content increases SR Ca release and this (1) increases Ca efflux from the cell on Na-Ca exchange and (2) decreases Ca entry into the cell via the L-type Ca current. Both of these effects will decrease cell and thence SR Ca content (Trafford *et al.* 1997). This simple homeostatic mechanism has many consequences. For example it predicts that manoeuvres which only increase the open probability of the RyR will have no maintained effect on the amplitude of the systolic Ca transient. Experimentally we find that such an increase of open probability results in a transient increase of the amplitude of the Ca transient accompanied by a decrease of SR Ca content (Trafford *et al.* 2000). Correspondingly, a decrease of open probability produces a transient decrease of the Ca transient amplitude and an increase of SR content (Overend *et al.* 1998). Under some clinical conditions the cardiac output shows 'alternans'. At a cellular level this is seen as alternate large and small systolic Ca transients (D'az *et al.* 2002). We are investigating the hypothesis that this alternation is due to breakdown of the homeostatic mechanism described above. We find that, under alternating conditions there is an increase in the steepness of the relationship between SR Ca content and Ca efflux from the cell. The factors responsible for this will be described.

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SA5

Role of Ca^{2+} release in regulating electrical rhythmicity in visceral smooth muscle

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SA6

Calcium dynamics during electrically stimulated action potential in *Chara*: experiments and model simulations

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The voltage triggered release of Ca^{2+} from internal stores in the green alga *Chara* reveals the hallmarks of an excitable system. The threshold-like dependence of Ca^{2+} mobilisation on electrical stimulation can be simulated by the combination of two models comprised of :i. the voltage dependent synthesis/breakdown of the second messenger inositol 1, 4, 5-trisphosphate (IP_3) and ii. the concerted action of IP_3 and Ca^{2+} on the gating of the receptor channels, which conduct Ca^{2+} release from internal stores. The combined model predicts a complex behaviour of Ca^{2+} mobilisation under periodic stimulation including higher-order phase locking and irregular responses upon increased stimulation frequency. Comparable dependencies on stimulation frequencies were observed experimentally for the voltage stimulated elevation of Ca^{2+} in the cytoplasm and for activation of action potentials. This shows that the all-or-none type activation of the action potential is in reality only the consequence of the preceding all-or-none type mobilisation of volatge stimulated Ca^{2+} from internal stores.

SA7

Voltage control of Ca^{2+} signalling via G-protein-coupled receptors

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G-protein-coupled receptors (GPCRs) form the largest family of surface membrane receptors and are not normally considered to be voltage-dependent. However, in certain tissues there is significant evidence to suggest that IP_3-dependent Ca^{2+} release during activation of GPCRs can be modified by changes in the cell membrane potential via a mechanism independent of Ca^{2+} influx. This phenomenon was initially described in coronary artery smooth muscle cells during activation of muscarinic cholinergic receptors (Ganitkevich & Isenberg, 1993) but has been most extensively characterised during activation of P2Y receptors in the megakaryocyte (MK) (Mahaut-Smith *et al.* 1999). This non-excitable cell type is the precursor of blood platelets and lacks both voltage-gated Ca^{2+} influx and ryanodine receptors. In unstimulated MKs, changes in membrane potential have little effect on $[Ca^{2+}]_i$, however during stimulation of P2Y receptors with ADP, depolarisations potentiate and hyperpolarisations inhibit the Ca^{2+} response. These voltage-dependent $[Ca^{2+}]_i$ changes are due to modification of IP_3-dependent Ca^{2+} release (along with associated store-dependent Ca^{2+} influx) as they are still observed in Ca^{2+}-free or Na^+-free media and are abolished by inhibitors of IP_3 receptors. The mechanism underlying voltage control of P2Y receptor-evoked Ca^{2+} signals is unknown, and can be explained by one of three theories: 1. The receptor, its G-protein or phospholipase-C is directly voltage-dependent; 2. The binding of polar agonists or substrates (eg. the agonist or PIP_2) is altered by membrane voltage; and 3. A voltage-sensitive protein in the plasma membrane is configurationally coupled to IP_3 receptors on the internal stores. Theories 1 & 2 would suggest that IP_3 production can be voltage-dependent, which is currently the working hypothesis since depolarisation evokes Ca^{2+} waves that are indistinguishable from those observed with ADP (Thomas *et al.* 2001). In addition, a pronounced delay of several hundred milliseconds exists between large amplitude depolarisations and the first detectable Ca^{2+} increase, indicative of an event involving a diffusion-limited step. Although we have shown voltage dependence to several types of GPCR in the MK, the response is particularly robust during stimulation of P2Y receptors. Consequently, physiological voltage waveforms can markedly regulate ADP-evoked Ca^{2+} increases (Mason *et al.* 2000; Martinez-Pinna *et al.* 2003). The reasons for the robust nature of the depolarisation-dependence to P2Y receptor Ca^{2+} signalling in the MK is unknown. One explanation is that the P2Y receptors in this cell type (believed to be both $P2Y_1$ and $P2Y_{12}$ receptors as reported for the platelet) are especially voltage-dependent. Alternatively, the extensive membrane invaginations of the MK (Mahaut-Smith *et al.* 2003) may enhance an innate voltage dependence to GPCR signalling, for example by increasing the ratio of surface membrane to cytoplasmic volume. Thus, the mechanism may have more relevance in cells with high specific membrane capacitances or in structures with high surface area: volume such as dendritic spines. Indeed, a role can be proposed for this phenomenon in synaptic integration by way of co-incidence detection, since membrane depolarisations can markedly potentiate the $[Ca^{2+}]_i$ responses to low concentrations of ADP (Guring & Mahaut-Smith, 2003).

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