SA18

Regulation of cardiac myofilaments by phosphorylation.

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Cardiac function is regulated by environmental cues that activate intracellular protein kinases through calcium, cyclic nucleotides and lipid second messengers. The contractile apparatus and associated regulatory proteins represent major targets of protein kinases in the heart. Phosphorylation serves to regulate myofilament properties such as calcium affinity of the regulatory switch, rates of transition through the cross-bridge cycle, power output versus efficiency and rate of relaxation during diastole. One of the major phosphoproteins on cardiac myofilaments is troponin I, the inhibitory subunit of the troponin-tropomyosin complex. Transgenic mouse models in which five previously identified phosphorylation sites on troponin I were mutated to alanine or to aspartate, namely serines$^{23/24/43/45}$ and threonine^{144}, have been especially informative. These 'non-phosphorylatable' or 'pseudophosphorylated' troponin I constructs rescue the lethal phenotype of cardiac troponin I null mice, and are therefore able to support near normal cardiac function. Similarly, a construct with only two of five sites mutated to alanine, namely serines$^{23/24}$, supports normal cardiac function and permits the remaining three sites for protein kinase C (PKC) to be investigated without cross-talk with cyclic AMP dependent protein kinase (PKA) sites. This presentation will describe recent investigations of the function and regulatory properties of these rescued transgenic mice, which in effect harbor troponin I with defined phosphorylation status at several sites and no confounding endogenous troponin I.

Intact hearts and isolated myocytes from rescued cTnI-Ala_2, cTnI-Ala_5 and cTnI-Asp_5 mice responded nearly normally to agonists of G-protein coupled receptors. The positive inotropic response to the β-adrenergic agonist isoproterenol was largely unaltered, but twitch narrowing was blunted if troponin I could not be phosphorylated on PKA sites serines$^{23/24}$. Similarly, rescued myocytes showed normal positive inotropic responses to endothelin-1, but twitch broadening was blunted if troponin I could not be phosphorylated on PKC sites. Studies in skinned myocardial preparations from rescued mouse lines generally reinforced these observations. Desesensitization of myofilaments to calcium was blunted if troponin I could not be phosphorylated by PKA, whereas sensitization to calcium was blunted if troponin I could not be phosphorylated by PKC. These observations suggest dynamic phosphorylation of troponin I by PKA and PKC reciprocally that can alter myofilament calcium sensitivity and in turn impact twitch duration. In related studies, treatment of skinned myocytes with the βII isoform of PKC caused a pronounced increase in calcium sensitivity in cTnI-Ala_2 myocytes, but not in cTnI-Ala_5, cTnI-Asp_5 or WT mouse myocytes. Several lines of evidence indicate that this sensitizing effect of PKC does not involve phosphorylation of myosin regulatory light chains. The sensitizing effect of PKC may have escaped detection in past investigations if PKA sites on troponin I were cross-phosphorylated by the PKC treatment. These observations indicate that one or more of the three PKC sites on cardiac troponin I may be responsible for the enhanced myofilament calcium sensitivity observed in failing hearts, as a consequence of up-regulation of PKC-βII.

SA20

Inositol 1,4,5-trisphosphate has both a positive inotropic and a pro-arrhythmic effect in rat ventricular and atrial myocytes

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The role of inositol 1,4,5-trisphosphate ($InsP_3$) in cardiac myocyte function is unclear and controversial. Although agonists that activate $InsP_3$ generation are positive inotropic agents in the heart and have been implicated in various cardiac pathologies, it is unclear whether $InsP_3$ is responsible for any of their effects. We investigated the expression and function of $InsP_3$ receptors ($InsP_3Rs$) in rat ventricular and atrial cardiac myocytes. Rats were killed in accordance with schedule 1 Home Office regulation, and myocytes were enzymatically isolated using a standard procedure (Mackenzie et al., 2001). Cells were electrically paced at 0.3 Hz using field electrodes and calcium changes were monitored using single cell photometry or confocal imaging.

Stimulation with a membrane-permeant form of $InsP_3$ ($InsP_3$ ester; 2 μM) caused a modest positive inotropic response in both atrial and ventricular myocytes. The most prominent effect of the $InsP_3$ ester was to provoke the occurrence of spontaneous diastolic calcium transients. These events were rare in control recordings. For both cell types, the spontaneous calcium transients evoked by $InsP_3$ ester were prevented by the $InsP_3R$ inhibitor 2-aminoethoxydiphenyl borate (2-APB; 2 μM).

Endothelin-1 (ET-1) acts via multiple signalling pathways to modulate the inotropic status of the heart and is known to cause arryhthmias. ET-1 stimulation of atrial or ventricular myocytes caused a significant positive inotropic effect and also triggered substantial numbers of spontaneous calcium transients. These events were inhibited by 2-APB, suggesting that $InsP_3Rs$ were responsible for the increased automaticity.

The patterns of spontaneous calcium transients were similar in $InsP_3$ ester- or ET-1-treated cells. For both stimuli, the spontaneous diastolic calcium signals largely occurred on the falling phase of the previous action potential-evoked calcium transient. In addition, the spontaneous calcium signals appeared to be due to the triggering of calcium sparks and/or action potentials. Beta-adrenergic stimulation (using isoproterenol; 100 nM) also caused spontaneous calcium transients, but the characteristics of these signals were distinct from those evoked by $InsP_3$ ester or ET-1. The spontaneous calcium transients evoked by isoproterenol typically occurred >1s after the previous action potential when the calcium level had recovered to diastolic levels. Furthermore, the events caused by isoproterenol were largely observed as calcium waves with few action potentials. 2-APB did not affect the characteristics of isoproterenol-induced spontaneous calcium transients.

Our data support a role for $InsP_3$ in mediating a modest inotropic effect in the heart. The most prominent effect of $InsP_3R$ stimulation appears to be the triggering of pro-arrhythmic spontaneous calcium transients. Direct stimulation of $InsP_3Rs$ in atrial

or ventricular myocytes mimics the pattern of response observed with the natural cardioactive hormone ET-1. The properties and mechanism of the spontaneous calcium signals evoked by $InsP_3R$- and ET-1-stimulation appear to be distinct from those triggered by increases in cyclic AMP.

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SA21

Nuclear IP3 receptor isoforms regulate nucleoplasmic calcium transients and modulate transcription factors in muscle cells

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Membrane depolarization of skeletal muscle cells induces slow IP3-mediated calcium signals in which dihidropyridine receptors act as voltage sensors (1) and that regulate the activity of transcription factors such as CREB, jun and fos (2), slow calcium signals with different kinetics can also be evoked by tetanic pulses (3) and several hormones (4). These signals appear unrelated to contraction and with a distinct nuclear component. Using primary cultures from neonatal rats (humanely killed) skeletal muscle, we worked with both whole cells and with a preparation of purified, intact nuclei obtained after hypotonic shock and differential centrifugation. Western blot analysis, binding of 3-HIP3, immuno-gold labelled and fluorescent antibodies against all three types of IP3 receptors (IP3R) were used together with the calcium-sensitive dyes fluo-3, mag-fluo 4 or fluo 4-dextran were used both in confocal microscopy and fluorimetry to asses nuclear [Ca2+] changes. Both immuno-fluorescence and immuno-electron microscopy localized mainly type 1 IP3R in the nuclear envelope, type 3 IP3R in the nucleoplasmic region and type 2 IP3R in the cytoplasm when the number of particles in the different nuclear compartments was quantified, of more than 1500 particles counted, type 1 IP3R gold particles were found mainly in clusters of varying numbers (2 to 30); more than half of the gold particles were found in groups of the three or more particles, 72 % of the total count being in the vicinity of one or more particles. 47 % is associated to the nucleoplasm, 21% to the nucleolus, 25% to the inner nuclear membrane and 7% to the outer nuclear membrane. Type 3 IP3R gold particles were also near the inner nuclear membrane (20 %) whether 54%. Were found in nucleoplasm 26 % of particles were found in the nucleolus. Also in in clusters, reaching 78 % in relation to isolated particles. The outer nuclear membrane was not labeled. Isolated myonuclei responded to IP3 with transient [Ca2+] elevations. The same result was obtained with the 1B5 cell line, not expressing ryanodine receptors. Nuclear Ca2+ increase triggered by IP3 evoked CREB phosphorylation. These results may be correlated to those showing nuclear translocation of protein kinase C isoforms as well as PKC- dependent CREB phosphorylation (5). The ensamble of results support the idea that Ca2+ signals mediated by nuclear IP3R in myotubes are part of a distinct Ca2+ release component that originates in the nucleus and is likely to participate in gene regulation mediated by CREB.

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SA23

IGF-I gene expression and splicing towards MGF in skeletal muscle in response to exercise.

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Muscle hypertrophy and tissue repair involves local as well as systemic growth factors. Using muscles that were stretched and/or electrically stimulated our team cloned an RNA transcript which has been called mechano growth factor (MGF) (Yang *et al.* 1996). This is a splice variant of the IGF-I gene but it has a reading frame shift, making its carboxy peptide sequence different from that of the systemic or liver type of IGF-I (IGF-IEa). In order to determine the role of MGF and particularly its unique C terminal peptide sequence in muscle maintenance we have studied certain disease states in which muscle mass is not maintained. It was found that dystrophic muscle cannot respond to mechanical stimuli by producing MGF in the same way as normal muscle. Also, studies in the muscles of old rats and elderly people have shown that the ability to produce MGF in response to resistance exercise declines markedly with age (Owino *et al.* 2001; Hameed *et al.* 2003). However, in the case of elderly people it is improved by the administration of growth hormone (Hameed *et al.* 2004). The function of MGF is illustrated by experiments using muscle cells in culture which have been transfected with the cDNA of MGF and of IGF-IEa. These have shown that the role of MGF, in particular its carboxy peptide, is to activate the proliferation of muscle satellite (stem) cells (Yang *et al.* 2002). This has now been shown to be true following muscle damage *in vivo* (Hill and Goldspink, 2003). IGF-IEa, which is also produced is the main supplier of "mature IGF-I", but it is clear that the splice variant MGF has a separate and important role in replenishing the stem cell pool, which is important for muscle mass regulation and repair. MGF is much more potent than recombinant IGF-I or the systemic type of IGF-I in inducing hypertrophy as MGF apparently "kick starts" the process by inducing proliferation of the satellite cells to provide the extra nuclei for the growth and/or repair of this post-mitotic tissue.

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