AuPS/ASB Meeting - Adelaide 2010

Symposium: New insights into the molecular architecture of the heart and their implications for heart disease

Monday 29th November 2010 - Hickinbotham Hall - 14:30

Chair: Yue-kun Ju & David Allen

The molecular architecture of the heart's conduction system in health and disease

M.R. Boyett and H. Dobrzynski, Cardiovascular Medicine, University of Manchester, 46 Grafton Street, Manchester, M13 9NT, UK. (Introduced by Yue-kun Ju)

The cardiac conduction system (CCS) acts as the heart's wiring system and is vital for the initiation and coordination of the heart beat (Boyett, 2009). The main tissues comprising the CCS are the sinoatrial node (the pacemaker of the heart), atrioventricular node (responsible for slow action potential conduction from the atria to the ventricles and, thereby, a delay between atrial and ventricular systole) and the His-Purkinje system (responsible for fast action potential conduction throughout the ventricles and, thereby, the synchronised contraction of the ventricles) - they were discovered in the late 19th and early 20th centuries. The electrical activity of the CCS is ultimately dependent on the expression of ion channels and, using quantitative PCR and immunohistochemistry, we are mapping the expression of ~80 ion channel subunits in the CCS (*e.g.* Chandler *et al.*, 2009). Unsurprisingly, the expression pattern is very different from that in the working myocardium, but appropriate to explain the electrical activity of the CCS. For example, pacemaking in the sinoatrial node can be explained by the poor expression of the background K^+ channel, $K_{ir}2.1$, the expression of the pacemaker ion channel, HCN4, and perhaps the high expression of the low voltage-activated Ca^{2+} channels, $Ca_v1.3$ and $Ca_v3.1$; slow conduction through the atrioventricular node can be explained by the poor expression of the major Na^+ channel, $Na_v1.5$, and the major gap junction channel, Cx43; fast conduction through the His-Purkinje system can be explained by the high expression of $Na_v1.5$ and expression of the large conductance gap junction channel, Cx40. The heart rate of the mouse is about 100× faster than that of the human and this can be explained by differences in ion channel expression: analysis suggests that channels carrying outward current are generally more highly expressed in the mouse sinoatrial node and this is expected to result in a shorter action potential (essential in the case of a higher heart rate) and channels carrying inward current are also generally more highly expressed in the mouse sinoatrial node and this may be responsible for the faster heart rate of the mouse.

There is dysfunction of the CCS in the aged heart, in heart failure (HF), atrial fibrillation and possibly diabetes (Boyett, 2009). For example, a substantial proportion (~16%) of HF patients die of bradyarrhythmias and there is dysfunction of all parts of the CCS in HF: there can be sinus bradycardia and prolongation of atrioventricular nodal conduction and heart block, and ~26% of HF patients have left bundle branch block. We and others are showing a widespread remodelling of ion channels in the CCS in heart failure – for example, in a rat model of heart failure (following pulmonary hypertension), 14 out of 21 transcripts for ion channels and related proteins were altered in the sinoatrial node (the CCS appears peculiarly sensitive to HF – for example, in this model only 4/21 transcripts were altered in the atrial muscle. The dysfunction of the CCS in disease is likely to be the result of the remodelling of ion channels - for example, the sinus bradycardia in HF and atrial fibrillation has been attributed to a downregulation of HCN4 in the sinoatrial node (Boyett, 2009). There is even evidence of 'dysfunction' in trained athletes: sinus bradycardia, prolongation of atrioventricular nodal conduction and heart block and incomplete right bundle branch block. The effects of athletic training have been attributed to 'high vagal tone', but there is no evidence for this and instead it is more likely to be the result of a remodelling of ion channels (Boyett, 2009). The CCS is more extensive than originally thought and specialised nodal-like tissue encircles the tricuspid and mitral valves, pulmonary artery and aorta and is potentially responsible for atrial tachycardias and ventricular outflow tract tachycardias (Boyett, 2009).

M.R. Boyett. (2009). 'And the Beat Goes On'. The cardiac conduction system: the wiring system of the heart. *Experimental Physiology* **94,** 1035-1049.

Chandler NJ, Greener ID, Tellez JO, Inada S, Musa H, Molenaar P, Difrancesco D, Baruscotti M, Longhi R, Anderson RH, Billeter R, Sharma V, Sigg DC, Boyett MR, Dobrzynski H. (2009). Molecular architecture of the human sinus node - insights into the function of the cardiac pacemaker. *Circulation* **119,** 1562-1575.

Distribution and functional role of IP_3R receptors in mouse sino-atrial node

Y.K. Ju, Department of Physiology (F13), University of Sydney, NSW 2006, Australia.

Inositol 1,4,5-trisphosphate receptors (IP_3Rs) have been implicated in the generation of cardiac arrhythmias, although the mechanism involved is unclear. In mammalian sinoatrial node (SAN), where the heart beat originates, the expression and functional role of IP_3Rs have not been investigated.

In order to determine whether SAN cells express IP_3Rs and their functional role in cardiac pacemaking, we used a range of techniques to study mRNA and protein expression and distribution. We first examined mRNA expression of *Ip_3rs*, *Hcn4*, *Ryr2* and *Stim1* genes across different regions of the mouse heart, including central SAN, peripheral SAN, AV node, atria and ventricle. We found that all three *Ip_3r* isoforms were expressed in the SAN and other regions of the heart. In contrast, *Hcn4* expression was highest in the central SAN and showed progressive reduction in peripheral SAN, AV node, atria and ventricle.

Whole mount SANs were co-labelled with Cx43 antibody and either IP_3R1 or IP_3R2 antibody. Cx43 antibody was used to distinguish central SAN from peripheral SAN. We found very weak labelling of IP_3R1 in the central SAN, identified by absence of Cx43. IP_3R1 labelling appeared in the peripheral SAN, especially in the interatrial septum, which also showed strong expression of Cx43. In contrast, the entire SAN, including the central and peripheral SAN and the surrounding atrial tissue, was uniformly labelled with IP_3R2 antibody. The results suggested that while the SAN expressed three IP_3Rs isoforms, IP_3R2 was the only protein isoform detected in the central SAN and isolated single pacemaker cells. We also found that Ca^{2+} sparks induced by membrane-permeable IP_3 (IP_3-BM) were predominately located near the sarcolemma (within 1.5 μm). The IP_3R agonist endothelin-1 (ET-1) induced sinoatrial arrhythmias as revealed by SAN electrical mapping. ET-1 and IP_3-BM increased intracellular Ca^{2+} and pacemaker firing rate whereas the IP_3R antagonist, 2-aminoethoxydiphenyl borate (2-APB), decreased Ca^{2+} and firing rate. Both of these effects were absent in SANs from IP_3R2 knockout mice. We estimate that the contribution of Ca^{2+} release from IP_3Rs to normal heart rate could be up to 14 % based on the spontaneous firing rate of isolated SANs from WT v. IP3R2 KO mice. The results provided clear evidence that the heart rate modulated by ET-1 and 2-APB was *via* their interaction with IP_3Rs.

All animal experiments were approved by animal ethics committees of all listed research institutes. IP_3R2 knock out mice are gift from Dr. Ju Chen. The details of gene targeting and generation of IP_3R2-deficient mice were published previously (Li *et al.*, 2005).

Li X, Zima AV, Sheikh F, Blatter LA, Chen J. 2005) Endothelin-1-induced arrhythmogenic Ca^{2+} signaling is abolished in atrial myocytes of inositol-1,4,5-trisphosphate(IP3)-receptor type 2-deficient mice. *Circulation Research* **96(12):** 1274-81.

This study was supported National Health and Medical Research Council of Australia (program grant 354400 and project grant 570926) and the Health Research Council of New Zealand.

Proteins in the lumen of the sarcoplasmic reticulum determine cardiac RyR channel activity, cardiac function and the structure of Ca^{2+} release units

A.F. Dulhunty and N.A. Beard, John Curtin School of Medical Research, Australian National University, PO Box 334, ACT 2601, Australia.

The activity of ryanodine receptor (RyR2) Ca^{2+} release channels in the intracellular sarcoplasmic reticulum (SR) Ca^{2+} store of cardiac myocytes must be low during diastole for efficient Ca^{2+} accumulation by the SR for release during systole and for appropriate lowering of cytoplasmic Ca^{2+}. RyR2 channels become "leaky" in inherited and acquired disorders in which high cytosolic [Ca^{2+}] during diastole leads to delayed after depolarisations (DADs) and arrhythmias (catecholaminergic polymorphic ventricular tachycardia (CPVT)). The "leaky" RyR can also deplete the SR Ca^{2+} store, reducing systolic Ca^{2+} release and cardiac output.

Low RyR2 activity during diastole in healthy myocytes is maintained by the integrated actions of ligands and associated proteins. Cytoplasmic proteins that depress RyR2 activity include members of the glutathione transferase family and the chloride intracellular channel protein, CLIC-2 (Hewawasam *et al.*, 2010). RyR2 channels are exquisitely sensitive to luminal [Ca^{2+}]. However luminal proteins that influence the response of RyR2 to luminal Ca^{2+} and their binding sites on the minute luminal domain of RyR2 are only just being defined. The luminal proteins include the Ca^{2+} binding proteins calsequestrin (CSQ2) and the histidine rich protein (HRP) as well as the membrane spanning triadin and junctin, which bind to RyR2 and to CSQ2. Mutations in CSQ2 as well as RyR2 lead to DADs and CPVT. To understand how luminal factors regulate cardiac output, numerous transgenic models have been developed in which the luminal proteins have been knocked out, under expressed or over expressed (Knollman, 2009; Fan *et al.*, 2008). The general consensus is that none of the proteins is essential, *i.e.* animals survive with altered expression, but are often unable to cope with stress and develop CPVT. A major change with CSQ2 knockout is proliferation of the SR to maintain the Ca^{2+} store. The structure of the SR is also influenced by triadin and junctin expression. However the transgenic studies do not reveal the role of the targeted protein, how it interacts with RyR2 or the mechanisms that allow it to regulate RyR2 and Ca^{2+} release, because of the compensatory changes in SR structure and in expression of other proteins.

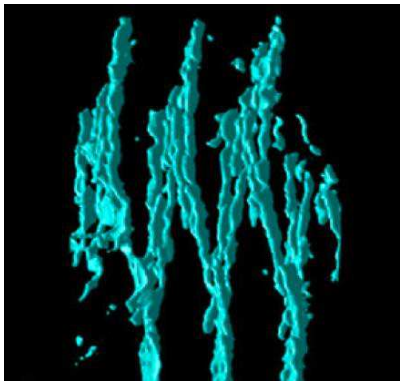
In contrast to the explosion in transgenic models, basic studies of the role of luminal proteins in cardiac SR function are few. This is also in contrast to extensive studies of interactions between the luminal proteins in skeletal muscle, albeit from only a handful of laboratories. A long held assumption, that we now believe is incorrect, is that the luminal proteins interact with RyR2 in the heart in the same way as they interact with RyR1 in fast-twitch skeletal muscle. As a consequence interactions between fast-twitch skeletal proteins have been extrapolated to the cardiac system, despite the fact that there are different isoforms of the RyR, CSQ and triadin in the two tissues and the demands on the Ca^{2+} store are vastly different. This is particularly true for CSQ, where the polymerization of the skeletal isoform dictates both Ca^{2+} binding capacity and its ability to influence RyR1 activity in response to changes in luminal [Ca^{2+}]. Cardiac CSQ2 does not polymerize when exposed to physiological free [Ca^{2+}] of ~1 mM and an ionic strength of ~150 mM, in contrast to CSQ1 which is mostly polymerized under these conditions (Wei *et al.*, 2009). In hindsight it was not surprising that we find that CSQ2 activates RyR2, while CSQ1 inhibits RyR1 (Wei *et al.*, 2009). Our preliminary evidence suggests that the interaction between junctin and RyR2 may also be isoform-specific. Our hypothesis is that CSQ2 acts to ensure strong Ca^{2+} release from cardiac SR with each heart beat, in contrast to its role in conserving the SR Ca^{2+} store in skeletal muscle for occasional maximal exertions.

Knollmann BC. (2009) *Journal of Physiology* **587:** 3081-3087.
Fan GC, Yuan Q, Kranias EG. (2008) *Trends in Cardiovascular Medicine* **18**: 1-5.
Wei L, Hanna AD, Beard NA, Dulhunty AF. (2009) *Cell Calcium* **45:** 474-484.
Hewawasam R, Liu D, Casarotto MG, Dulhunty AF. Board PG. (2010) *Biochemical Pharmacology* **80:** 381-388.

A new twist in cardiac muscle: dislocated and helicoid arrangements of myofibrillar z-disks in mammalian ventricular myocytes

M.B. Cannell, I.D. Jayasinghe, D.J. Crossman, D. Baddeley and C. Soeller, Department of Physiology University of Auckland New Zealand. (Introduced by Yue-Kun Ju)

The general structure of cardiac muscle cells has been established by light and electron microscopy for some time, but detailed information on 3D organization is less common as is the organization of proteins that enable high speed signal transduction to take place. To provide information for detailed modeling of cardiac cell function we have been examining the organization of myofibrils, sarcoplasmic reticulum, t-tubules and associated proteins by confocal fluorescence microscopy. These studies show that the basic 3D organization of the myofilaments is complex, with dislocations and helicoidal structures evident (see figure). Such a complex structure may arise during normal growth but it also has important implications for arrhythmogenesis *via* calcium wave propagation. While the sarcoplasmic reticulum calcium release channel (RyR) is generally close to z-lines and separated laterally by ~0.6 um, the longitudinal separation should be set by the sarcomere length which is considerably longer 1.9-2.2 μm. Therefore one would expect that a Ca wave would be able to propagate more easily in the transverse direction than longitudinally. However, it is well known that calcium waves propagate throughout the cell rather uniformly (*e.g.* Berlin, Cannell & Lederer, 1989). Our observation (Jayasinghe *et al.*, 2010) of dislocated z-lines that have RyRs associated with them helps explain this paradox; the dislocations coupled with the jitter in longitudinal RyR spacing will assist longitudinal calcium wave propagation.



For normal excitation-contraction coupling, calcium influx *via* l-type calcium channels (DHPRs) triggers the release of calcium from RyRs and 2D analysis of the colocalization of DHPRs and RyR in rat suggested that most (~60%) of the DHPRs were grouped opposite the RyRs (Scriven, Dan & Moore, 2000). However, when analysis is performed in 3D, the colocalization is significantly lower (~45%) (Fletcher *et al.*, 2010). This recent finding underscores the importance of studying structure in 3D especially when the underlying resolution of the microscope is insufficient at the spatial scale of interest. In human, we find an even lower level of colocalization which suggests that normal EC coupling may arise from a combination of tightly coupled release triggered by DHPR activtion plus a component due to local calcium diffusion from nearby DHPRs and Ryrs.

At the near molecular scale, we find that RyRs are not packed into junctional regions as a single cluster but rather as smaller groups of RyRs forming super clusters. This again suggests that local diffusion between release sites plays an important role in normal excitation-contraction coupling. It is possible that this partial uncoupling of RyRs from DHPRs serves to protect excitation contraction coupling during the normal trafficking of proteins and sub-cellular remodeling which would be important as the heart never has a chance to rest and rebuild. On the other hand, this looser coupling makes arrhythmogenesis *via* calcium waves more likely and may help explain the propensity of the remodeled, diseased heart to develop arrhythmias.

1. Berlin, J.R., Cannell, M.B. & Lederer, W.J. (1989) ITI in single rat cardiac ventricular cells: Relationship to fluctuations in intracellular calcium. *Circulation Research* **65,** 115-126.
2. Jayasinghe, I.D., Crossman, D.J., Soeller, C., Cannell, M.B. (2010) A new twist in cardiac muscle: dislocated and helicoid arrangements of myofibrillar Z-Disks in mammalian ventricular myocytes. *Journal of Molecular and Cellular Cardiology* **48(5),** 964-971.
3. Scriven, D.L., Dan, P., Moore, E.D.W. (2000) Distribution of proteins implicated in excitation-contraction coupling in rat ventricular myocytes. *Biophysical Journal* **79,** 2682-2691.
4. Fletcher, P.A., Scriven, D.L., Schulson, M.N., Moore, E.D.W. (2010) Multi-image colocalization and its statistical significance. *Biophysical Journal* **99,** 1996-2005.

Mechanisms of contractile dysfunction in lamin A/C-deficient hearts

D. Fatkin, Sr Bernice Research Program in Inherited Heart Diseases, Molecular Cardiology Division, Victor Chang Cardiac Research Institute, Darlinghurst, NSW 2010, Australia.

Mutations in the *LMNA* gene that encodes the nuclear lamina proteins, lamin A and lamin C, have been associated with diverse human disorders and are the most common cause of familial dilated cardiomyopathy (DCM). The mechanisms by which nuclear protein defects result in cardiac contractile dysfunction are incompletely understood. Mice with a targeted deletion of the *Lmna* gene develop DCM and are a useful model to study disease pathogenesis. Homozygous *Lmna* knockout ($Lmna^{-/-}$) have severe DCM and die by 6-8 weeks of age, while heterozygous ($Lmna^{+/-}$) mice develop a milder phenotype in adult life with reduced survival after 40 weeks. Previous studies have established that lamin A/C-deficient nuclei have increased deformability and reduced survival when subjected to biaxial strain. On the basis of these findings, it has been proposed that mechanical stress-induced apoptosis contributes significantly to the development of DCM in patients with *LMNA* mutations. The aim of our study was to test this "structural hypothesis" by determining the effects of interventions to modify mechanical stress in $Lmna^{+/-}$ mice. Serial echocardiography and tissue studies were performed in wildtype (WT) and $Lmna^{+/-}$ mice before and after exercise training, thoracic aortic constriction (TAC), and administration of a β-adrenergic receptor-blocking drug, carvedilol. Echocardiography was performed in anaesthetized mice (avertin 2.5%). Mice were ventilated and anaesthetized for surgical procedures with ketamine (75 mg/kg), xylazine (20 mg/kg) and atropine (0.6 mg/kg). Tissue analyses were performed post-mortem in excised hearts. We first evaluated the biophysical properties of isolated cardiomyocytes from mice aged 12 weeks (prior to DCM) and found changes in nuclear size and shape in $Lmna^{+/-}$ mice, as well as altered distribution of perinuclear desmin and enhanced swelling responses to hypo-osmotic stress. Groups of 12 week-old WT and $Lmna^{+/-}$ mice underwent a 6-week exercise training program. Contrary to our predictions, neither moderate nor severe intensity exercise training induced cardiomyocyte apoptosis (assessed by TUNEL assay and caspase-3 activation) or DCM. Sustained left ventricular pressure overload generated by TAC resulted in apoptosis and contractile dysfunction in WT and in $Lmna^{+/-}$ mice, with no differences in severity between the genotype groups. We found however, that regular moderate exercise training attenuated DCM development in male $Lmna^{+/-}$ mice. Oral administration of carvedilol from 6 weeks to 40 weeks also had a protective effect against DCM in male $Lmna^{+/-}$ mice.

These data indicate that lamin A/C-deficient cardiomyocytes have intrinsic structural defects but that mechanical stress-induced apoptosis is not a major determinant of DCM. We propose that altered cytoskeletal stability due to loss of normal nuclear anchoring may impair force transmission and promote DCM in $Lmna^{+/-}$ hearts. Exercise training and carvedilol administration from an early age are promising strategies for DCM prevention and warrant further clinical evaluation.