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Mechano-Regulation of the Beating Heart at the Cellular Level
– Mechansensitive Channels in Normal and Diseased Heart –

O. Friedrich¹, S. Wagner¹, A.R. Battle²,³, S. Schürmann¹, B. Martinac³,*

¹ Institute of Medical Biotechnology, Friedrich-Alexander-University Erlangen-Nuremberg, Paul-Gordan-Str.3, 91052 Erlangen, Germany
² School of Pharmacy, Griffith University Gold Coast Campus, Gold Coast, QLD 4222, Australia
³ Molecular Cardiology and Biophysics Division, Victor Chang Cardiac Research Institute, Lowy Packer Building, 405 Liverpool St, Darlinghurst, Sydney, NSW 2010, Australia

oliver.friedrich@mbt.uni-erlangen.de, soeren.wagner@kfa.uni-erlangen.de, sebastian.schuermann@mbt.uni-erlangen.de, a.battle@griffith.edu.au

*:corresponding author: e-mail: B.Martinac@victorchang.edu.au

phone: +61-292958743
FAX: +61-292958770
Abstract:

The heart as a contractile hollow organ finely tunes mechanical parameters such as stroke volume, stroke pressure and cardiac output according to filling volumes, filling pressures via intrinsic and neuronal routes. At the cellular level, cardiomyocytes in beating hearts are exposed to large mechanical stress during successive heart beats. Although the mechanisms of excitation-contraction coupling are well established in mammalian heart cells, the putative contribution of mechanosensitive channels to $\text{Ca}^{2+}$ homeostasis, $\text{Ca}^{2+}$ signaling and force generation has been primarily investigated in relation to heart disease states. For instance, transient receptor potential channels (TRPs) are up-regulated in animal models of congestive heart failure or hypertension models and seem to play a vital role in pathological $\text{Ca}^{2+}$ overload to cardiomyocytes, thus aggravating the pathology of disease at the cellular level. Apart from that, the contribution of mechanosensitive channels (MsC) in the normal beating heart to the downstream force activation cascade has not been addressed. We present an overview of the current literature and concepts of mechanosensitive channel involvement in failing hearts and cardiomyopathies and novel data showing a likely contribution of $\text{Ca}^{2+}$ influx via mechanosensitive channels in beating normal cardiomyocytes during systolic shortening.

Keywords: cardiomyocytes, heartbeat, mechanosensitive channel, transient receptor potential channel, calcium homeostasis
Contents

1. Abstract: ......................................................................................................................2
2. Introduction: ..........................................................................................................4
3. Excitation-contraction coupling in the beating heart .............................................7
4. Mechanosensitive channels in cardiac molecular biology and heart disease ......8
   3.1. Transient receptor potential channel (TRP) expression in the heart ...............8
   3.2. Mechanosensitivity of TRPC channels ............................................................12
   3.3. Blockers of mechanosensitive channels .........................................................14
5. Mechanosensitive channels and heart disease: a TRP to pathophysiological
   mechanisms ..............................................................................................................17
   4.1. Mechano-electrical coupling (MEF) and arrhythmogenesis ..........................17
   4.2. MsC and cardiac hypertrophy ........................................................................19
   4.3. MsC and dystrophic cardiomyopathy ............................................................21
5. Role of MsC in normal cardiac homeostasis ......................................................22
   5.1. Contribution of MsC to Ca\(^{2+}\) signals in the beating heart .......................23
   5.2. Methods ........................................................................................................25
   5.2.1. Isolation of adult rat cardiomyocytes ..........................................................25
   5.2.2. Confocal microscopy recordings of global Ca\(^{2+}\) transients during field
          stimulation .........................................................................................................26
   5.3. Results and Discussion ...............................................................................27
6. Concluding Remarks ..........................................................................................29
7. Acknowledgements: ..........................................................................................29
8. References: .........................................................................................................30
9. Figure Legends: ....................................................................................................43
1. Introduction:

The human beating heart is a high-endurance bio-engine that is adapted to vast biomechanical stress. It continuously performs about 100,000 beats per day, thereby pumping about 7,000 litres of blood through the circulatory system. As a discontinuous pump, the heart chambers cycle between filling and ejection, i.e. diastole and systole for the ventricles, and experience intrinsic hydrostatic pressures that range between passive filling pressure and the active systolic ejection pressure. For the different chambers, pressures vary widely (Fig. 1), the left ventricle being exposed to the highest pressure amplitude (~120 mmHg; ~16 kPa) due to its valvular connection to the high pressure system by the aortic valve (Pasipoularides, 1992; Sacks et al., 2009). During systole, peak pressure rate can exceed +2,000 mmHg/s and turns largely negative around -1,000 mmHg/s during diastolic relaxation in the left ventricle (Cohn et al., 1972). Cardiac catheterization protocols in patients with heart failure show markedly compromised dP/dt rates with vastly increased endsystolic, but more importantly, enddiastolic left ventricular pressures (LVESP: left ventricular endsystolic pressure, LVEDP: left ventricular end diastolic pressure) and less than 50 % of normal ejection fractions (Mak et al., 2012). Thus, the beating heart as a whole is prone to large pressure-induced mechanical strains that result from (i) volume overload in case of reduced ejection fraction and increased ventricular enddiastolic filling volume in most heart failure models or (ii) pressure overload in case of increased afterloads (e.g. arterial hypertension). However, even under physiological conditions, these pressure gradients impose substantial strain on cardiomyocytes. According to Laplace’s law, an increase in ventricular pressure is associated with an increase in wall tension that is conferred to longitudinal stretch of the ventricular wall elements, i.e. fibroblasts, collagen, but moreover, single
cardiomyocytes in case of passive filling. On the other hand, during systolic force generation, cardiomyocytes themselves actively produce wall tension through their contractile activity (Zhong et al., 2011; Gordon, 1976). Although pressure profiles in healthy and diseased beating heart are very well known, the quantitative role of wall stress to cardiac pumping patho(-physiology) is a relatively unexplored phenomenon (Zhang and Karemaker, 2012; James et al., 2000). Moreover, estimates of wall tension in clinical studies, e.g. in a recent study in pulmonary arterial hypertension, have remained qualitative, at best (Marcus et al., 2008). From a biophysical point of view, it would be important to know the exact profiles of membrane stretch experienced by individual cardiomyocytes during diastolic and systolic ventricular pressure fluctuations in the beating heart under normal and pathological conditions. In mechanical terms the cell membrane can be seen as the lipid bilayer fortified with cytoskeleton and extracellular matrix, whose function (in simplistic terms), is to attenuate the effects of mechanical force on the cell membrane and thus help to increase a dynamic range of membrane responsiveness to mechanical force fluctuations. However, surprisingly, such studies are still not at hand. It can be expected that membrane stress and strain are actively involved in the cardiac regulation of cardiomyocyte fluid and ion homeostasis as well as fine tuning of contractility (McNary et al., 2011). Indeed, this aspect of cardiac mechanosensation and mechanotransduction has been widely recognized to play a vital role in the development and maintenance of several cardiac pathologies (Fanchaouy et al., 2009; Komuro, 2000) and even cardiomyocyte death in reperfusion injury (Otani et al., 2006).

Mechanosensitive channels have been identified as important players in the development of cardiac hypertrophy and are associated with several forms of heart failure (Seth et al., 2009; Ohba et al., 2007; Stiber et al., 2009). In addition,
expression of a number of genes in the heart has been modulated by stress (Asakura & Kitakaze, 2009), The canonical transient receptor potential channels (TRPC) have in particular become a major focus of interest, as mechanical deformation of ventricular cardiomyocytes is associated with modulated expression of some of these TRPCs and this, in turn, may alter Ca\textsuperscript{2+} handling of ventricular myocytes (Dyachenko et al., 2009). Thus, the molecular basis of mechanosensation conveyed by mechanosensitive channels in the heart may have important implications not only for the diseased but also the normal beating heart. Therefore, within the current special issue on the ‘Biophysics and Molecular Biology of the Beating Heart’, we address this important issue and review the current literature on mechanosensation and mechanosensitive channels in normal and diseased heart. After giving a brief overview about current concepts of excitation-contraction coupling in the heart and the ion channels involved, we present a classification of mechanosensitive channels inherent to cardiac molecular biology before moving on to heart failure models involved with mechanical stress and mechanosensation-triggered hypertrophy response. The relationship of mechanosensitive channels in the heart and their involvement in ion and contractile regulation is discussed. Lastly, the issue of mechanosensitive channels contributing to normal homeostasis and function of the normal beating heart is highlighted by our own results on global Ca\textsuperscript{2+} transients in normal field-stimulated cardiomyocytes in the presence and absence of specific blockers of mechanosensitive channels (MsC). We show that during normal contraction, there is Ca\textsuperscript{2+} influx contribution through putative MsC that may provide an additional positive-inotropic component during systolic ejection in normal beating hearts but may also drive cellular damage from a certain ‘tipping point’ in heart disease.
In conjunction with this review we wish to bring attention of our readers to an excellent and extensive recent text on mechano-electric coupling in the heart (Kohl 2011). This text consists of a series of 65 chapters written by prominent scientists and clinicians currently active in the area of cardiac electrophysiology. The chapters encompass a wide range of topics on stretch effects on heart rhythm from basic to translational science and their clinical relevance. This text will be a valuable addition to everyone's library working in this exciting field of basic and clinical research.

2. Excitation-contraction coupling in the beating heart

The cycling of Ca\(^{2+}\) ions from the external bulk solution and the internal sarcoplasmic reticulum Ca\(^{2+}\) store into the cardiomyocyte myoplasm and back, in response to membrane depolarization during an action potential with activation and relaxation of the contractile apparatus (excitation-contraction or ‘ec’-coupling), belongs to the most extensively clarified and reviewed mechanisms of beating heart. A very good source of literature is given by outstanding work from Donald M. Bers (Bers, 2001; Maier & Bers, 2002; Bers & Gua, 2005; Bers, 2008). A simple model that does not yet involve likely contributions from mechanosensitive channels is shown in Fig.2 describing the cellular mechanisms involved during action potential spread through the cardiac syncytium. Fast upstroke depolarization during the action potential activates sarcolemmal and tubular DHPR L-type Ca\(^{2+}\) channels that allow transsarcolemmal Ca\(^{2+}\) influx. This, in turn, activates sarcoplasmic RyR2 channels to release SR Ca\(^{2+}\) in a process called ‘Ca\(^{2+}\)-induced Ca\(^{2+}\) release’ (CICR). The increase in myoplasmic Ca\(^{2+}\) in the ~µM range then activates the crossbridge cycle via Ca\(^{2+}\) binding to troponin C (TnC). Dissipation of Ca\(^{2+}\) from TnC terminates contraction and initiates relaxation through four major subsequently competing myoplasmic Ca\(^{2+}\) removal
processes: Ca$^{2+}$ uptake by mitochondria (not shown in Fig. 2; blocked e.g. by Ru360), SR Ca$^{2+}$ uptake by SERCA2 (can be blocked by cyclopiazonic acid), extrusion by Na$^+$/Ca$^{2+}$-exchanger NCX (blocker e.g. KB-R7943) and the plasmalemmal Ca$^{2+}$ ATPase (PMCA; blocker e.g. carboxyeosin). Under resting conditions, some small Ca$^{2+}$ leak from the SR must occur to prevent store-overload and for various other reasons (e.g. Kong et al., 2007). Such spontaneous SR Ca$^{2+}$ release events can be visualized as Ca$^{2+}$ sparks that are much more common in a leaky RyR2 environment in cardiomyocytes than in skeletal muscle (RyR1) and also play a role in heart failure (Kerfant et al., 2011).

3. Mechanosensitive channels in cardiac molecular biology and heart disease

3.1. Transient receptor potential channel (TRP) expression in the heart

So far, only few eukaryotic channels have met the criteria for mechanotransduction, i.e. conveying mechanical stimuli into electrical or chemical cellular signals. Amongst those fall the transient receptor potential channels (TRP), K(2P) channels, ENaC, NMDA receptor channels, Piezo proteins etc., that are most relevant for mammalian tissues (Arnadottir & Chalfie, 2010; Fabian et al., 2008; Kloda et al., 2007; Coste et al., 2012; Kim et al., 2012), and there is still some debate as to whether some of their activation through mechanical stimuli is direct or indirect (Sharif-Naeini et al., 2008; Gottlieb et al., 2008). Specific mechanisms may include changes in tension, shear stress or curvature of membrane bilayers or cytoskeleton, hydrophobic mismatch, lipid metabolism, kinases or phosphatases (Pedersen & Nilius, 2007; Maroto et al., 2005; Spassova et al., 2006; Lansman & Franco-Obregon, 2005; Putney, 2005). In
the case of TRP channels both mammalian TRPC6 (Spassova et al. 2006) and yeast
TRPY1 (Palmer et al., 2001; Zhou et al., 2005) channel are thought to be
mechanically sensitive because they can be activated by pipette pressure or by
osmotic swelling. Given that unlike bacterial MscL and MscS channels (Martinac,
2011), neither of them has been reconstituted and activated in liposomes it remains
unclear whether they are directly activated by membrane tension. Furthermore,
similar to TRPC1, the functional expression of TRPC6 in African green monkey
kidney (COS) or Chinese hamster ovary (CHO) cells raised additional doubts about a
specific role of this channel in mechanosensitive channel activity (Gottlieb et al.,
2008). In the cardiovascular system, more than ten different members of the TRP
family are expressed, most of them in vascular smooth muscle and endothelial cells
(Inoue et al. 2006). Mammalian TRP isoforms (Fig. 3A) consist of six transmembrane
domains with a pore loop probably placed between the fifth and sixth domain. Most
mammalian TRP channels act as nonselective cation channels with a moderate to
high Ca^{2+} permeability except for TRPM4 and TRPM5 (Petersen et al., 1995; Launay
et al., 2002; Inoue, 2005). TRP channels are grouped within their superfamily
according to either receptor-relationships (e.g. TRPV – vanilloid receptor-related;
TRPM – melastatin-related), the classical membrane lipid-related protein G or
tyrosine kinase phospholipase C (PLC) activation (TRPC – canonical transient
receptor channels, TRPC1-7) or other relationships to inherited diseases (e.g. TRPP
– polycystine-related; TRPML – mucolipidin-related) (Montell, 2005). The TRP
members usually show a complex activation and regulatory pattern that involves a
multitude of several possible stimuli even within the same isoforms, as for example
most canonical TRP isoforms have been implicated in store-operated and/or
receptor-operated Ca^{2+} entry (Pani et al., 2012; Mohl et al., 2011, Olah et al., 2011),
as well as to act as stretch-sensors (Maroto et al., 2005; Spassova et al., 2006)
while TRPVs not only respond to cell stretch but also to vanilloids, heat, acid and several small signaling molecules (for review see Inoue et al., 2006). The expression profiles of members of the TRP family have been quite extensively characterized in the vascular system, e.g. vascular smooth muscle cells and endothelial cells however, information for the heart is less abundantly available. mRNA analysis of human TRPC isoforms in whole human heart homogenates revealed an expression ranking of TRPC1 > TRPC4 ≥ TRPC6 > TRPC5 >> TRPC7 while little data is available for other isoforms (Inoue et al., 2006; Riccio et al., 2002). Using comparative RT-PCR analysis, Fonfria et al. (2006) found a high expression of TRPM7 in whole human heart tissue. However, this isoform seems to be also ubiquitously expressed in many other tissues. As those aforementioned studies could not provide more detailed information on the cell-type specific distribution of those TRP members, it was necessary to perform further immunochemistry and immunofluorescence microscopy approaches. It should be noted that primers as well as antibodies are available for most TRP members (Albert et al., 2009; Flockerzi et al., 2005), although their crystal structure is still largely unknown (Li et al., 2011). Using commercial TRPC antibodies, recent studies revealed novel information about the cellular expression of some TRPC isoforms. In normal rat hearts, TRPC1 was visualized in myocytes of the left ventricle and atrium in immunohistochemistry preparations. TRPC1 signals were detected at the cell membrane and also colocalized with phalloidin stains in confocal images in single isolated ventricular myocytes, suggesting a t-tubular localization of TRPC1 in ventricular myocytes that was not present in atrial myocytes (Huang et al., 2009). However, a TRPC1−/− knockout mouse shows no apparent phenotype leading to a conclusion that TRPC1 is not a necessary component of mechanosensitive and store-operated ion channel complexes in vascular smooth muscle (Dietrich et al., 2007). Another study aiming to
clarify the subcellular distribution of TRPC3 channels in normal rat ventricle using polyclonal anti-TRPC3 antibodies (αA-, αB-TRPC3; Goel et al., 2002) found strong colocalization of TRPC3 with the Na⁺/K⁺-ATPase (NKA pump) and the Na⁺/Ca²⁺ exchanger (NCX) in ventricular confocal immunofluorescence sections (Goel et al., 2006). Longitudinal and transverse sections revealed that this colocalization was confined to tubular membranes and intercalated disk regions only and not to the sarcolemma. Furthermore, the authors also found TRPC3 to co-immunoprecipitate with NCX and NKA in rat ventricles and suggested interaction between those binding partners, although this still awaits confirmation with e.g. FRET technology.

Interestingly, Goel et al. (2006) also aimed to elucidate the relationship of TRPC3 with the sarcoplasmic reticulum. There was no overlap between TRPC3 and SERCA protein in confocal images and only a close proximity of TRPC3 with RyR2 showed a few overlapping regions within the lateral resolution of the confocal microscope. In HEK cells expressing heterologous TRPC3, co-immunoprecipitation also suggested interaction of TRPC3 and NCX which was substantiated by glutathione S-transferase pull-down showing interaction with the TRPC3 cytosolic C-terminus (Rosker et al., 2004). Intracellular Ca²⁺ fluorescence recordings in TRPC3 overexpressing cells showed cytoplasmic Ca²⁺ increase through TRPC3 which seemed not to be Ca²⁺ entry itself but Na⁺ entry that, in turn, stimulated the reverse-mode of NCX, because this increase was almost abolished when NCX was blocked with KB-R9743 (Rosker et al., 2004). However, it still remains to be elucidated whether this mechanism also applies to heart muscle tissue. Interestingly, a recent comparative study on TRPC confirmed a strong expression of TRPC3 in rat ventricle but none in mouse or guinea pig ventricle (Dyachenko et al., 2009). As for TRPC6, the same study was able to detect strong TRPC6 expression in homogenates from mouse ventricular cardiomyocytes detected with a commercial antibody. Furthermore, the authors
managed to localize TRPC6 to the transverse tubules but not the sarcolemma in three-dimensional immunofluorescence recordings (Dyachenko et al., 2009). As another species difference, rat ventricular cardiomyocytes did not show any tubular TRPC6 but only TRPC3. A similar cellular distribution pattern of TRPC6 in single murine ventricular cardiomyocytes, i.e. main localization to the tubules, was observed by Ward et al. (2008), while Mohl et al. (2011) recently obtained single cell immunofluorescence data with a pore-blocking anti-TRPC6-antibody that were compatible with a distribution of TRPC6 also at the sarcolemma in normal mouse cardiomyocytes. This plasmalemmal distribution even increased within minutes following treatment with the $\alpha_{1A}$-agonist methoxamine by TRPC6 redistribution from the tubules to the plasma membrane. The TRPC6 translocation was successfully prevented by pre-treatment with the $\alpha_{1A}$-antagonist prazosin (Mohl et al., 2011). These functional data provide first evidence for a plasticity of TRPC distribution related to sympathetic activity and adrenergic channel regulation.

3.2. Mechanosensitivity of TRPC channels

The recent literature mostly indicates only the isoforms TRPC1, -3, and -6 to be involved in cardiac regulation although TRPC1$^{-/-}$ knockout mice show no apparent phenotype (Dietrich et al., 2007), as previously mentioned. To span the link to MsC in the heart, their mechanosensitivity has thus to be established. Even though the patch clamp method may not be the best assay for channel density because the whole cell amplitudes do not necessarily agree with prediction of whole cell currents, previous studies had shown that heterologous expression of human TRPC1 in *Xenopus* oocytes substantially increased the density of mechanosensitive channels and cation currents across the membrane in patch clamp recordings after applying different step pressures to the membrane (Maroto et al., 2005). Moreover, these currents were
abolished by co-treatment with TRPC1-specific antisense RNA and single channel
currents were practically identical when comparing human TRPC1 with *Xenopus*
TRPC1, which share over 80% of homology (Maroto et al., 2005). Single channel
conductances were given as ~50 pS at -50 mV and ~10 pS at 50 mV for both
species. This result is opposite to the results of the heterologous expression of
TRPC1 in COS and CHO cells (Gottlieb et al., 2008), showing that although both
TRPC1 and TRPC6 can be highly expressed in these cells, the mechanosensitive
currents are not significantly altered by overexpression of these proteins. As
commented by Wu et al. (2010) ‘It is possible that TRPC1 homomer channels are
functional, but the activating stimulus has not yet been found. Alternatively, TRPC1
may function as a homomer in the endoplasmic reticulum and reach the plasma
membrane only when co-assembled with other TRP (or TRPC??) subunits. A more
detailed examination of previously proposed homomeric TRPC1 channels is required
before TRPC1 can be assumed to form a plasma membrane channel by itself.’ This
is in agreement with our view that further studies are needed to understand the
trafficking, maturation and/or functioning of the TRPC channels in different cell types.
Mechanosensitivity of TRPC3 is less well documented. Studies in heterologous
expression systems showed functional NCX1/TRPC3 coupling (Rosker et al., 2004)
and TRPC3 could be activated by angiotensin II, PLC and DAG to mediate Ca\(^{2+}\)
influx (Onohara et al., 2006). Recently, mechanical stretch induced a similar ROS
production as direct TRPC3 activation with 1-oleoyl-2-acetyl-sn-glycerol (OAG) in
neonatal cardiomyocytes of a dilative cardiomyopathy mouse model (MLP\(^{-/-}\)). There,
the authors also found a large increase in TRPC3 expression (Kitajima et al., 2011)
in contrast to the abovementioned studies in adult animals (Dyachenko et al., 2009).
However, OAG is an unspecific activator of TRPC3/6/7 (Zhang et al., 2011). Although
the study of Kitajima et al. (2011) may point to potential mechanosensitivity, a more
direct approach is required to establish TRPC3 mechanosensitivity. Unlike TRPC3, there is a paucity of evidence for TRPC6 being mechanosensitive. In
TRPC6-overexpressing HEK293 cells, Spassova et al. (2006) recorded non-selective
cation currents with a typical TRPC6-like outward rectification upon hypo-osmolar cell
swelling. In uninduced HEK293 cells, the osmotically induced currents were only
marginal (Spassova et al., 2006). Although this activation of TRPC6-mediated
currents was independent of PLC activation the osmotically induced activation cannot
be unambiguously interpreted as being stretch-related activation given that changes
in membrane stress with osmotic stimulation were only shown on red blood cells and
cells with disrupted cytoskeleton (Johnson, 1994; (Morris and Horn 1991); Zhang and
Hamill, 2000). In murine ventricular cardiomyocytes, whole-cell patch clamp
recordings revealed immediate activation of non-selective ion currents when the
myocyte membrane was deformed via a glass stylus. In addition, a stretch-related
deactivation of \( I_K \) currents was observed (Dyachenko et al., 2009). Application of
OAG induced similar currents as in response to membrane deformation. Both
mechano-induced, as well as OAG-induced currents were blocked when a pore-
blocking anti-TRPC6 antibody was present in the pipette (Dyachenko et al. 2009).
Thus, TRPC6 in cardiomyocytes act as a mechanosensitive channel.

3.3. Blockers of mechanosensitive channels

The identification of mechanosensitive channels in the heart has to rely on (i) the
observation of direct activation of a non-selective ion conductance upon mechanical
membrane stress or \( \text{Ca}^{2+} \) influx in case of a given \( \text{Ca}^{2+} \)-selectivity as well as (ii) a
response to known modulators or blockers of such channels. For a long time, \( \text{Gd}^{3+} \)
and streptomycin were the only available blockers for MsC. \( \text{Gd}^{3+} \), a member of the
lanthanide series, has been known to inhibit both stretch-activated and -inactivated
channels in skeletal muscle (Lansman, 1990; Franco-Obregon & Lansman, 2002). However, its use is limited by its unspecificity and binding to physiological anions and proteins (Caldwell et al., 1998). Aminoglycoside antibiotics have also been found to block MsC, as well as other channels (Hamill and McBride, 1997). Streptomycin has been widely used but requires relatively large concentrations (~200 µM in most studies) and interferes with ribosomal protein translation (Yeung et al., 2005). It is also potentially ototoxic which limits its use as a blocker of MsC in vivo. It is supposed to block the open channel pore.

Specific MsC blockers have not been available for a while. ‘In a desperate attempt’ to find such drugs, the group around Frederick Sachs turned towards ‘the most antithetical approach of funding agencies: a blind search’ (Bowman et al., 2007). When screening invertebrate venoms for their potential to block mechanosensitive currents in patch-clamp recordings in astrocytes and rabbit ventricular cardiomyocytes during osmotic shock or patch pipette suction, they were able to obtain a potent hit from the venom of the spider Grammostola spatulata (Suchyna et al., 2000). The identified 35 amino acid peptide was called GsMTx-4, the only drug known to specifically affect cationic mechanosensitive channels (Bowman et al., 2007). Although it has been shown to block stretch-sensitive channels, e.g. the eukaryotic cation selective Piezo1 (Bae 2011), GsMTx-4 has not been tested on other channels, thus restricting statements about its exclusive selectivity for MsCs. GsMTx-4 has been chemically synthesized (Ostrow et al., 2003), its structure clarified (Oswald et al., 2002) and it is commercially available today (e.g. http://www.peptanova.de/products/Biologically-Active-Peptides/Ion-Channel-Blocker/GsMTx-4.html). GsMTx-4 acts as a so-called amphipath, that is, it interacts with lipid bilayers to penetrate them with its hydrophobic surface while the charged
regions probably determine its penetration depth into the bilayer (Bowman et al., 2007). Since its discovery, GsMTx-4 has been more and more widely used to study specific signaling effects on MsC and their potential contribution to pathophysiology of disease in the nervous system (e.g. Alessandri-Haber et al., 2009; Park et al., 2008), skeletal muscle (e.g. Teichmann et al., 2008; Whitehead et al., 2006), smooth muscle (e.g. Fanchaouy et al., 2007; Ducret et al., 2010) and the heart (e.g. Iribe et al., 2009; Dyachenko et al., 2009; Ward et al., 2008). GsMTx-4 has also been directly linked to specificity for some MsC, in particular members of the TRPC family, i.e. TRPC1 and TRPC6, in heterologous expression systems (Fig.3) (Maroto et al., 2005, Spassova et al., 2006) as well as ‘in situ’. In HEK cells, Bae and colleagues (2011) directly observed a potency of GsMTx-4 in the micromolar range to block Piezo-1 currents in outside-out patches. However, GsMTx-4 peptide did not block currents of the mechanically gated channel TREK-1 in outside-out patches (Bae 2011). For the heart, Williams & Allen (2007), for instance, observed that GsMTx-4 reduced chronically elevated resting Ca$^{2+}$ levels in ventricular cardiomyocytes from dystrophic mdx mice pointing towards an MsC-mediated component. They also found increased levels of TRPC1 in those myocytes (see also Ward et al., 2008). Although this suggests a direct relationship of the investigated TRPC channel being the only source of aberrant Ca$^{2+}$ signaling in diseased heart, caution has to be taken, as in contrast to the controlled heterologous expression experiments, such conclusion is only supported by indirect proof ‘in situ’. This becomes more evident from the fact that several other ion channels have also been shown to be mechanosensitive in the heart (e.g. Kir2.2, TREK-1; Dyachenko et al., 2009; Tan et al., 2004). Since pore-specific antibodies of TRPC are available now, it is therefore, a convincing strategy to compare GsMTx-4’s effects with those of anti-TRPCs (Dyachenko et al., 2009; Mohl
et al., 2011). This will especially help to further elucidate the role of TRPC channels in cardiac disorders more specifically.

4. Mechanosensitive channels and heart disease: a TR\text{IP} to pathophysiological mechanisms

The number of studies addressing the role of mechanosensitive channels to mechanisms of heart dysfunction in disease models associated with increased mechanical stress by far outweighs those studies elucidating their role in normal heart function, especially for the beating heart. The following sections thus, summarize current concepts for the involvement in cardiac disease before addressing evidence for MsC involvement in normal heart function.

4.1. Mechano-electrical coupling (MEF) and arrhythmogenesis

\textit{Commotio cordis} refers to a condition of mechanically induced arrhythmias without structural damage to the heart. This relationship between mechanical impact on electrical activity of the heart has long been recognized and termed `\textit{mechano-electrical feedback}' (MEF). MEF may also account for conditions of acute cardiac death in ischemia (Kohl et al., 2006, 2001). MEF forms an almost instantaneous electrical response to mechanical stress that can also be observed as sudden temporary asystole and/or subsequent fibrillation of isolated hearts upon manual manipulation in a Langendorff-preparation. In particular, atrial fibrillation occurs more readily upon acute pressure-overload, and this mechanism unequivocally involves MsC, as the threshold for atrial arrhythmias is significantly shifted towards larger chamber pressures by GsMTx-4 (Bode et al., 2001). The non-specific blocker streptomycin, on the other hand, did not abolish stretch-induced changes in
pacemaker activity in the SA node of mice (Cooper & Kohl, 2005). The exact prediction of hypo- or hyperexcitability in cardiomyocytes is not straightforward, because different MsC entities may be involved. Some K\(^+\) channels have been found to be mechanosensitive, e.g. ventricular Kir2.3 channels being deactivated (Dyachenko et al., 2009), atrial K\(_{\text{ATP}}\) channels being activated by mechanical (e.g. hypotonic swelling) stimuli (van Wagoner, 1993). In conditions of myocardial ischemia, mechanosensitivity of K\(_{\text{ATP}}\) was even increased. This, in conjunction with falling ATP levels in ischemic heart regions, would substantially relieve K\(_{\text{ATP}}\) inhibition thus, favoring AP duration shortening. In a very elegant study, White et al. (1993) were able to show a reduction of AP duration and amplitude upon a ~10 % stretch in field-stimulated ventricular cardiomyocytes but could not give the identity of the involved channel at that time. In general, the gating of many voltage-gated channels is affected by mechanical forces in the lipid bilayer (Morris et al., 2006; Beyder et al., 2010). This prompted Catherine Morris to propose that ‘in the intensely mechanical environment of the myocardium and its vasculature, kinetics of voltage-gated channels might be routinely modulated by reversible and irreversible changes in bilayer structure’ (Morris, 2011). Consequently, voltage-gated channels may also be collectively contributing to mechanoelectric feedback in the heart. On the other hand, most studies regarding mechanical stress during ischemia now favor tachycardiac arrhythmias reflected as atrial or ventricular fibrillation that is due to depolarization associated with extra-beats and action potential prolongations (Kamkin et al., 2005). Microelectrode and patch clamp recordings on stretched ventricular myocytes revealed activation of non-selective stretch-activated currents (Kamkin et al., 2005; Zeng et al., 2000). Spontaneous arrhythmias in acute regional ischemia were also recently confirmed in a modeling approach to be initiated by stretch-induced membrane depolarization (Jie et al., 2010). There is no concluding evidence yet as to
whether the sustained depolarization through MsC during stretch and mechanical
overload in ischemic output failure is due to a major Ca\(^{2+}\) component or non-specific
cation component. TRPC1 has been proposed to contribute to cardiac MEF as a
mixed cationic Ca\(^{2+}\) permeable channel (Huang et al., 2009; Patrick et al., 2010)
while other authors conclude that no TRPC subtype has yet been related to MsC
activity in arrhythmia (Vassort & Alvarez, 2009). Very recently, however, Sabourin et
al. (2011) provided the first evidence that TRPC channels regulated
electromechanical activity in the developing heart via interaction with the Ca\(_{v}1.2\)
channel. TRPC inhibition by SKF-96365 resulted in negative chrono-, dromo- and
inotropic effects and AV-block (Sabourin et al., 2011). However, for ischemia-induced
arrhythmias, the involvement of specific TRPC isoforms and their mode of action still
await experimental clarification. One suitable experiment could be induction of
diastolic depolarizations in stretched ventricular myocytes monitored by patch clamp
and Ca\(^{2+}\) fluorescence recordings in the absence and presence of pore-blocking
specific anti-TRPC antibodies. This would significantly help to improve our
understanding of TRP family involvement in this important MEF related disorder seen
in ischemic arrhythmias.

4.2. MsC and cardiac hypertrophy

It has long been recognized that mechanical loading of cardiac muscle in vivo and in
vitro induces immediate-early gene activation and hypertrophy signaling. Such genes
include, e.g., c-fos, c-jun, c-myc, and unspecific MsC blockade with Gd\(^{3+}\) neither
changed RNA levels nor the rate of protein synthesis in stretched rat ventricular
myocytes (Sadoshima et al., 1992). A wide variety of stretch-activated signaling
pathways had been identified (e.g. MAPK, JAK/STAT, PKC, NFAT) that are linked to
cardiomyocyte hypertrophy and cellular growth and those are seen as the milestone
for pathologic cardiac hypertrophy and fibrosis in conditions of chronic volume or pressure-overload in the heart (Lammerding et al., 2004). Interestingly, upregulation of members of the TRP family has been found in cardiomyocytes in response to prolonged pressure overload, e.g. TRPC1/3/6 following aortic banding (Ohba et al., 2007) or TRPM4 in hypertension models (Guinamard et al., 2006; see Inoue et al., 2009 for review). Aberrant expression of TRPC3/6 isoforms in the vascular system may substantially increase long-term blood pressure due to activation of the vasoconstrictive DAG and PLC pathway (see Inoue et al., 2009). Activation of the rennin-angiotensin system with subsequent systemic increase in angiotensin II is known to mediate cardiac hypertrophic remodeling. AT1 receptor mediated cardiomyocyte hypertrophy has been shown to be related to activation of TRPC3/6 and nuclear NFAT translocation (Onohara et al., 2006) which, in itself, may again upregulate TRPC6 expression in a positive feedback loop once a pathological hypertrophy response has been initiated (Kuwahara et al., 2006). Upregulation of mechanosensitive TRPC1 has further been linked to calcineurin/NFAT signaling in pressure-overload cardiomyopathy (Ohba et al., 2007). In rat neonatal cardiac myocytes, Onohara et al. (2006) found that angiotensin II activated DAG-sensitive TRPC-like currents that seemed to provide a pre-depolarization of the membrane to activate L-type Ca\(^{2+}\) channels for subsequent Ca\(^{2+}\) influx to trigger the NFAT activation and hypertrophic response. OAG increased nuclear NFAT translocation but was completely abrogated when voltage-dependent Ca\(^{2+}\) channels were blocked with nitrendipine (Fig.4) (Onohara et al., 2006). Thus, TRPC channels in the hypertrophic response seem to be inevitably coupled to ec-coupling in the hypertrophic response pathway. Whether this pathway is also valid for pure mechanical stress response remains to be confirmed. In another study of a murine model of dilative cardiomyopathy (muscle LIM protein deficiency, MLP\(^{-/-}\)), increased
TRPC3 activity following OAG-application or mechanical stress was linked to CAMKII and NOX-mediated reactive oxygen species (ROS) production that was inhibited by pyrazole-3 treatment (Kitajima et al., 2011). ROS, in turn, is known to activate the NFAT pathway in cardiomyocytes and thus, initiates/sustains hypertrophy (Kalivendi et al., 2005). Finally, the cardiac TRPC family is completed by recent studies showing that TRPC currents induced in adult cardiomyocytes subjected to pressure overload (transverse aortic constriction, TAC) were not present in TRPC1−/− mice. These mice were protected from a hypertrophic response (Seth et al., 2009). Those animals had much higher levels of inactive phosphor-NFATC3 in heart lysates suggesting that the calcineurin/NFAT pathway was silenced and thus, clearly TRPC1 dependent (Seth et al., 2009). The study also showed significantly reduced levels of the pro-hypertrophic factors ANF (atrial natriuretic factor) and BNP (brain natriuretic peptide) and preserved expression of SERCA2A as compared to almost diminished levels in wt-TAC mice in agreement with expression profiles in heart failure (Seth et al., 2009). Thus, a paucity of recent evidence shows that TRPC channels are indeed crucially involved in the development of cardiac hypertrophy. For further reading, there are more detailed reviews available (Kurdi & Booz, 2011; Eder & Molkentin, 2011; Watanabe et al., 2008).

4.3. MsC and dystrophic cardiomyopathy

Duchenne muscular dystrophy is a very-well studied inherited muscle disease where the lack of the subsarcolemmal scaffold protein dystrophin renders muscle membrane weak and more permeable to external Ca^{2+} entry in response to stretch (Yeung et al., 2005) or aberrant intracellular microdomain Ca^{2+} signaling in response to tubular osmotic swelling (Teichmann et al., 2008). Patients also develop a progressive dilative cardiomyopathy. In isolated cardiomyocytes from dystrophic mdx
mice, abnormal Ca\textsuperscript{2+} entry through stretch-activated membrane channels, short-lived microruptures and the NCX pathway have been determined using osmotic membrane challenges (Fanchaouy et al., 2009). Cardiomyocytes from old mdx mice had substantially elevated myoplasmic resting Ca\textsuperscript{2+} concentrations that approached the wt levels within 20 min following application of 10 \mu M GsMTx-4 (Williams & Allen, 2007). Alongside of this, those dystrophic cardiomyocytes also had about threefold increased expression levels of TRPC1 thus, TRPC1 being suggested to significantly contribute to dystrophic dilative cardiomyopathy (Ward et al., 2008). Apart from these clues, not much has been determined for the dystrophic cardiomyopathy in conjunction with specific involvement of selected TRPC isoforms members, although for skeletal muscle, much more detail is available (Allen et al., 2010; Millay et al., 2009; Vandebrouck et al., 2002).

5. Role of MsC in normal cardiac homeostasis

As mentioned before, the role of mechanosensitive channels, i.e. TRPC members, in the normal heart has been less well highlighted during the last couple of years. In the developing heart of chicks, pacemaking activity was found to be regulated by a functional macromolecular complex between TRPC and L-type Ca\textsuperscript{2+} channels where the latter served its obvious role while the TRPC channels were suggested to refill the relatively limited sarcoplasmic Ca\textsuperscript{2+} stores and insure sustained embryonic cardiac activity despite a grossly still undeveloped SR (Sabourin et al., 2011). However, how the situation changes through maturation is not yet known. It may be that TRPC channels play a pivotal role as physiological growth stimulus in maturing beating cardiomyocytes for ‘balanced’ or ‘adapted’ hypertrophy and that studies on their pathophysiological involvement in cardiac diseases may indicate a ‘tipping point’
for mechanical strain to initiate subsequent pathological signaling in hypertension or heart failure (Kurdi & Booz, 2011; Fujiwara et al., 2010; Wu et al., 2010). In vascular smooth muscle, TRPC6 has been shown to play a functional role in the physiological, arterial myogenic response (Patel et al., 2010). In the normal heart, MsC have been linked to ‘Starling’s law of the heart’. For the slow force response (SFR) that develops over several minutes following a stretch they may reflect a contributing mechanism additionally to the largely known increase in Ca\(^{2+}\) sensitivity of the myofilaments (Allen et al., 1974). The SFR ‘per se’ is independent of ec-coupling because it can develop in the resting period in response to stretch (Kockskamper et al., 2008). Recently, Iribe et al. (2009) found an immediate and transient increase in Ca\(^{2+}\) spark frequency in response to cardiomyocyte stretch that was not related to MsC because application of GsMTx-4 did not have any effect. This seems to be in contrast to the situation in skeletal muscle where a clear MsC-involvement had been shown in some disease states (Teichmann et al., 2008; Shkryl et al., 2009).

5.1. Putative contribution of MsC to Ca\(^{2+}\) signals in the beating heart

Corresponding experiments on osmotic sparks in isolated cardiomyocytes seem sparse, if available at all. Also, resting spark activity under osmotic challenge would not reveal a possible contribution of MsC in the beating cardiomyocyte, as Ca\(^{2+}\) sparks will be masked in the large Ca\(^{2+}\) transient signal. Likewise, passive stretch probably does also not reveal all potential contributions of MsC in the actively contracting cardiomyocyte. To date, most experiments focusing on MsC and Ca\(^{2+}\) homeostasis in cardiomyocytes use passive stretch (e.g. Gannier et al., 1994; Iribe et al., 2009) or isometric stretched-contractions (Ward et al., 2008). However, surprisingly, the mechanosensitive component to Ca\(^{2+}\) signaling in the actively contracting cardiomyocyte during systole has never been addressed. This is
important since axial stretch most likely activates plasmalemmal MsC components while tubular membrane becomes slack. On the other hand, during active shortening, plasma membrane is supposed to become slack while tubular stress occurs, if one assumes a largely preserved cell volume during systolic-diastolic cycles (see Fig. 1 C). Evidence for the first mechanism comes from a recent confocal imaging study that quantified the morphology of t-tubules under axial strain in single cardiomyocytes. Already 5% stretch decreased the mean tubular length from ~2.12 µm to ~2.07 µm which became significant at 15% stretch at 1.90 µm (McNary et al., 2011). The authors already implicated relevance for mechanosensitive channels in the tubules (McNary et al., 2011). However, direct evidence is still missing. To address this important question of whether MsC contribute to global \(^{2+}\text{Ca}\) transients via an additional \(^{2+}\text{Ca}\) influx during systolic/diastolic cycling in beating cardiomyocytes, we performed confocal \(^{2+}\text{Ca}\) fluorescence microscopy recordings in field-stimulated single rat cardiomyocytes paced at different frequencies in the absence and presence of GsMTx-4, a likely specific inhibitor of stretch-activated channels in myocardium (Fanchaouy et al. 2009; Suchyna et al. 2000). Our hypotheses to be tested were: (i) during contraction, there is an additional high-GsMTx-4 sensitive \(^{2+}\text{Ca}\) influx that results in a larger \(^{2+}\text{Ca}\) transient amplitude, and (ii) \(^{2+}\text{Ca}\) transient decay reflecting the cellular \(^{2+}\text{Ca}\) extrusion mechanisms is accelerated by application of high-GsMTx-4 due to a somewhat smaller amount of myoplasmic \(^{2+}\text{Ca}\) available for faster extrusion. Although this may not reflect a proof for the mechanosensitivity underlying the \(^{2+}\text{Ca}\) transient properties on a beat-to-beat basis, this approach, which is currently being extended also for other drug combinations (e.g. aminoglycosides) is a promising first presentation of a GsMTx-4 sensitive component of global \(^{2+}\text{Ca}\) transients. Future studies will also have to carefully address ways to determine the state of the membrane (e.g. concomitant
capacitance recordings) to rule out the possibility that other gatekeeping \( \text{Ca}^{2+} \) influx regulators, e.g. DHPR or NCX were directly modified by larger concentrations of GsMTx-4 as used in our and many other studies.

5.2. Methods

5.2.1. Isolation of adult rat cardiomyocytes

Rat left ventricular myocytes were enzymatically isolated from male Wistar rats (250 – 300 g). Rats were deeply anesthetized with sodium pentobarbital (400 – 800 mg/kg i.p.), the chest opened and the heart quickly excised. All procedures complied to the guidelines of the local Animal Welfare regulations and the experiments were approved by local ethics authorities. The hearts were cannulated and retrograde perfusion through the ascending aorta fist applied with \( \text{Ca}^{2+} \)-free, modified Krebs-Henseleit (KHS) solution (in mM: KCl 5, MgSO\(_4\) 2, Na-pyruvate 5, NaHCO\(_3\) 20, glucose 10, Hepes 20, Na-glutamate 25, Na-acetate 3, creatinine 6, taurine, 30, N-(2-mercaptoproprionyl)-glycine 4, insulin 5 I.U.) at 10 ml/min for at least 3 min at 37 °C. The solution was then exchanged to \( \text{Ca}^{2+} \)-free KHS containing 1 mg/ml collagenase (type 2, Worthington, Australia) for 20 min and then flushed with fresh solution for 2 min. The left ventricle was cut off at the septum, diced into several pieces and gently minced. The dispersion was filtered through a 200 µm mesh and the filtrate of dissociated cells stored at 37 °C in \( \text{Ca}^{2+} \)-free KHS. Before recordings, cells were \( \text{Ca}^{2+} \) adapted through increasing (~0.25 mM) \( \text{Ca}^{2+} \) steps to a final \( \text{Ca}^{2+} \) concentration of 1.8 mM.
5.2.2. Confocal microscopy recordings of global Ca\textsuperscript{2+} transients during field stimulation

Before recordings, cardiomyocytes were stained with 5 µM fluo-4 AM for 20 min at 37 °C and then transferred to a slim Perspex recording chamber equipped with two platinum wires at the side walls that were connected to an external pulse generator (STIM, Scientific Instr., Heidelberg, Germany) for external field stimulation. Cells were flushed with fresh solution to wash out excess dye and the chamber mounted on the stage of a confocal laser scanning microscope (Olympus FV300, Olympus, Hamburg, Germany). Fluo-4 was excited with the 488 nm line of an Ar\textsuperscript{+} laser and fluorescence collected >510 nm. Images were obtained in XT line-scan mode scanning along the long axis of an individual cardiomyocyte at 2 ms/line. Line-scan recording was performed over five seconds while simultaneously applying field-stimulation pulses at either 0.5 Hz, 1 Hz or 2 Hz. Each cardiomyocyte was aimed for each stimulation frequency for a 5 s period only then allowed to rest for 1 min before applying the next frequency train. The tarantula (\textit{Grammostola spatulata}) peptide GsMTx-4 (PeptaNova, Leimen, Germany) was added from a stock solution to a final bath concentration of 5 µM and the stimulation and Ca\textsuperscript{2+} transient recording protocol repeated. XT images were processed using a custom-written image processing algorithm in the MatLab environment. For the measurement of relative systolic/diastolic intracellular Fluo-4 Ca\textsuperscript{2+} amplitudes and decay rates of transients, XT data were averaged in the spatial domain. Transients were detected automatically in low-pass filtered differentiated datasets. For each detected transient, (i) an exponential decay function was fitted to the original data using a nonlinear least squares algorithm that yielded the rate of decay and (ii) the normalized peak size was calculated as \((F_{\text{max}} - F_{\text{min}})/(F_{\text{min}} - F_{\text{bgrd}})\). The fluorescence background level was measured in the original XT image in an area outside the cell. The results for all
detected transients were collected in treatment groups (control vs. GsMTx-4) and stimulation frequency (0.5 Hz, 1 Hz, 2 Hz) to calculate mean values and standard error of the mean (s.e.m.). To test whether the differences between control and GsMTx-4 data were significant for each stimulation frequency, an analysis of variances (ANOVA) was performed for a confidence level of p<0.05. For the visual presentation, data pairs were selected for Fig. 5.

5.3. Results and Discussion

Fig.5 A shows a representative example of XT line scan recordings in one single rat cardiomyocyte under control conditions and 5 min following application of 5 µM GsMTx-4. The line-averages of the first transient are shown for each case next to the images. For better comparison of kinetics between the two cases, their amplitudes have been matched. By eye, there is hardly any noticeable difference. Fig. 5B shows results from a screening assay using up to several hundred transients that were automatically analyzed by the MatLab algorithm to extract decay rates and fluorescence amplitudes. It is interesting to note that for all frequencies tested, decay rates were always significantly higher after blockade of MsC by GsMTx-4 while amplitudes were smaller. The difference in decay rate, although significant, is a subtle one and accounts for approx. 5 – 10 % of the control values. As an example, two exponential decays are superposed in the right most panel in Fig.5 A that correspond to the mean values at 1 Hz. The difference is hard to detect by the naked eye and becomes only apparent after analysis of many transients. This may indicate why such an effect might have remained unnoticed so far.

The reduction of transient amplitudes and acceleration of transient decay after block of mechanosensitive channels is compatible with a model where under normal
beating activity with cell shortening and tubular stretch, activation of tubular MsC results in an additional Ca\(^{2+}\) influx supporting the regular L-type Ca\(^{2+}\) influx already in the normal heart. A higher resulting myoplasmic Ca\(^{2+}\) concentration will then be subject to a prolonged extrusion time compared to the situation with blocked MsC, given that this surplus Ca\(^{2+}\) has to be extruded by the main removal systems (PMCA, SERCA, NCX, mitochondria) and MsC themselves do not participate in removal. The question arises which Ca\(^{2+}\) extrusion mechanism might be mostly affected by this additional MsC-mediated Ca\(^{2+}\) influx in the beating heart to slow down overall extrusion. Because additional Ca\(^{2+}\) influx prolongs and eventually increases membrane depolarization at the plateau of the myocyte action potential, transporters that are likely to depend on voltage regarding their activity are strong candidates. As such, the Na\(^+\)-Ca\(^{2+}\) exchanger is known to be electrogenic and to depend on membrane potential to some extent. At more positive potentials, the charge moved by Na\(^+\)/Ca\(^{2+}\) exchange is declining and hyperpolarization increases Ca\(^{2+}\) extrusion rates (Vedovato & Rispoli, 2007; Niggli & Lipp, 1994). Whether the relative contribution of MsC in the normal beating cardiomyocyte derived from our data also reflects the situation in the beating heart, needs further research. For example, one shortcoming of our approach is given by the pure isotonic contraction cycle as compared to auxotonic contractions in the beating heart. Future experiments involving single cardiomyocyte force transducer settings in combination with fluorescence microscopy will provide adequate measures to apply an afterload to single cardiomyocytes and to adapt the axial and vertical strain more to the ‘in vivo’ condition.
6. Concluding Remarks

Although mechanotransduction has long been recognized as an integral part in various cardiomyopathies, research on the role and identity of mechanosensitive channels in the normal heart are still an emerging field of cardiac research. After a paucity of studies that addressed resting stretch and passive reactions to axial stretch in single cardiac cells, the time has now come to address cardiac cells and their potential to sense and react to mechanical stimuli during the whole contractile cycle of normal cardiac activity. Alongside with this, the ongoing research on structure of MsC and specific drugs has provided us with valuable tools for cardiovascular research. One of the most specific and potent blockers, GsMTx-4, has fueled research on mechanotransduction in the last decade and is now being accompanied by novel pore-blocking antibodies to dissect molecular identities of MsC in question. For the heart, the TRPC family may be a very important player in health and disease and it will be very interesting to see whether our findings provided here for the involvement of MsC to Ca\(^{2+}\) handling in the normal beating cardiomyocyte are waiting to be completed by the name for the channel in question.

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**Figure Legends:**

**Figure 1: Mechanical stresses in the beating heart.**

A, The beating heart cycles through systolic and diastolic pressure changes that direct the flow between adjacent chamber and outlets. Indicated are typical pressure ranges in some heart chambers and central vessels. In the left ventricle, left ventricular pressure (LVP) experiences the largest pressure gradients and kinetics. The pressure differential dP/dt as a measure for contractility reaches large peak values of ~2,000 mmHg/s during systole and shortly drops below zero during ventricular filling (according to Cohn et al., 1972) in the normal heart. B, ventricular pressure changes translate to the ventricle wall according to *Laplace’s law*. Cardiomyocytes are expected to experience a longitudinal increase in tension during systole due to passive membrane strains via ventricular filling and also during systole due to active tension development of the contractile apparatus. C, suggested distribution of local membrane tension in single cardiomyocytes during systole and diastole. Various different mechanosensitive channels (MsC) have been localized to the sarcolemma and the transverse tubules (see text). During diastole or experimental myocyte stretch, the sarcolemma is expected to experience longitudinal stretch while the tubules may experience a slack. Thus, sarcolemmal MsC may contribute extensively to Ca$^{2+}$ influx in diastole and
myoplasmic Ca\textsuperscript{2+} overload in dilative cardiomyopathies. During a heart beat (systole), sarcolemmal slack does not contribute to Ca\textsuperscript{2+} influx through MsC apart to the Ca\textsuperscript{2+} influx through voltage-gated DHPR channels but the stretched tubules may contribute to an additional Ca\textsuperscript{2+} influx through MsCs. DHPR: dihydropyridine-receptor. RyR2: ryanodine-receptor. Vol.: cell volume.

**Figure 2: Excitation-contraction coupling in beating cardiomyocytes.** The sketch shows two adjacent cardiomyocytes connected by gap junctions that allow electrical coupling between cells for the rapid spread of action potentials (AP). The drawing shows the membrane charge distributions at a given time snapshot with the right cell just becoming depolarized at the upstroke of the AP while the left myocyte is fully depolarized. The major Ca\textsuperscript{2+} players are represented by the DHPR L-type Ca\textsuperscript{2+} channel, the NCX and the plasmalemmal Ca\textsuperscript{2+} ATPase (PMCA) at the outer membrane (sarcolemma and tubules) and the internal release channel RyR2 and the SR Ca\textsuperscript{2+} pump (SERCA). EC-coupling in cardiomyocytes is governed by the mechanism of Ca\textsuperscript{2+}-induced Ca\textsuperscript{2+} release (CICR) and the local Ca\textsuperscript{2+} concentrations are also given. Gap junctional currents are carried by cations from the depolarized to the polarized cell initiating local membrane depolarization and a new action potential. Note that the NCX runs in reverse mode early in systole where local Na\textsuperscript{+} concentration is high underneath the membrane.

**Figure 3: TRP channels as prime candidates for mechanosensitivity in the mammalian heart.** A, Dendrogram of TRP channel families and their genetic relation (scale bar indicates genetic distance). Black bold isoforms indicate major distribution in the cardiovascular system (from Inoue et al., 2006). B, Mechanosensitivity of human TRPC1 transcripts expressed in *Xenopus* oocytes. Mechanosensitive
currents during patch clamp recordings in response to increasing steps of pipette suction are shown at patch potentials of 50 mV and -50 mV. Expanded trace shows single channel currents of about 2 pA amplitude (from Maroto et al., 2005). C, Mechosensitivity of TRPC6 channels expressed in HEK293 cells. Patch clamp recordings show a current of ~200 pA at 80 mV in response to hypoosmolar membrane stretch which was not further increased by addition of the TRPC activator OAG. In control HEK cells, such currents were markedly reduced. The IV-curves for the TRPC6 non-selective cation currents showed outward rectification (from Spassova et al., 2006). D, Model of stretch and/or diacylglycerol (DAG) activation of TRPC6 in the membrane and its inhibition by the specific blocker GsMTx-4. Mechanical TRPC6 activation involves thinning of the membrane due to longitudinal shear stress and channel opening which is prevented by GsMTx-4 inserting into the outer leaflet and modifying lipid tension in the vicinity of the channel. PLC activation cleaves charged IP₃ from PIP₂ to produce a more lipophilic head group on DAG which in turn, changes local curvature of the membrane to open TRPC6. GsMTx-4 is believed to relieve this curvature-induced stress (from Spassova et al., 2006).

**Figure 4: Involvement of TRPC channels in cardiac hypertrophy signaling.**

Angiotensin II as one major hypertrophy stimulus induces PLC second messenger cascades with DAG production. The latter increases local membrane curvature and induces membrane stress to activate TRPC family members (see Fig. 3). Non-selective cation influx through TRPC is coupled to L-type Ca²⁺ channels via sustained pre-depolarization and facilitated DHPR activation. Sustained Ca²⁺ influx triggers the calcineurin-NFAT pathway that results in cardiomyocyte hypertrophy (from Onohara et al., 2006).
Figure 5: Putative contribution of mechanosensitive channels to Ca\textsuperscript{2+} transients in field-stimulated beating cardiomyocytes. A, paired line-scan recordings from one cardiomyocyte field-stimulated at 1 Hz under control conditions and after application of 5 µM GsMTx-4. Scale bar: 400 ms. The corresponding line-averaged transients are shown for the first transient, respectively. The two transients shown were normalized to the same peak amplitude for better comparison of kinetics. From the raw transients, normalized peak amplitudes (see Methods) and exponential fit decay rates were obtained. B, shows the mean decay rates and normalized amplitudes of all transients recorded for control and GsMTx-4 conditions. There is a significant putative contribution of MsC Ca\textsuperscript{2+} influx to decay rate and Fluo-4 amplitudes. However, one has to bear in mind that although the decay rates suggest a significantly faster decline of Fluo-4 transients when MsC are blocked such differences appear only subtle when directly comparing transients by eye in their exponential phase. As an example, two exponential decays have been plotted in (A) that exactly reflect the mean decay rates for 1 Hz in (B).
Fig. 1
Fig. 2
Fig. 3
Figure 8: Schema of Ang II-induced NFAT activation in cardiac myocytes. In cardiac myocytes, stimulation of AT1R induces NFAT activation through Gqα, PLC signaling pathway. DAG, generated by PLC activation, directly activates TRPC3 and TRPC6 (TRPC3/C6). Activation of TRPC3/C6 causes slow increases in the membrane potential to a positive direction (∆VC) and concomitantly increases the frequency of spontaneous firing due to activation of L-type Ca2+ channel. The Ca2+ influx through L-type Ca2+ channel activates calcineurin-NFAT pathway, which leads to hypertrophic responses in cardiomyocytes. From Orlov et al., 2008

Fig. 4
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