Physical principles of mechanosensitive channel gating by bilayer deformation forces

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Mechanosensitive (MS) ion channels have been documented in cells belonging to a wide variety of prokaryotic and eukaryotic organisms (Hamill & Martinac, 2001). The channels show great diversity in their conductance, selectivity and voltage dependence, while sharing the property of being gated by mechanical force exerted on cell membranes. They act as membrane-embedded mechanoelectrical switches, which open in response to lipid bilayer deformations. This process is critical to the response of living organisms to direct physical stimulation, as in touch, hearing, osmoregulation and other physiological responses. In prokaryotes MS channels were first documented in *Bacteria* and later in *Archaea*. Among prokaryotic MS channels studied to date, the best characterized are the MS channels of *E. coli*, which has three types of MS channels based on their conductance and sensitivity to applied pressure: MscM (M for mini), MscS (S for small) and MscL (L for large). Bacterial MS channels were the first shown to sense directly membrane tension in the lipid bilayer caused by external mechanical force applied to the cell membrane. The validity of the bilayer model has been well documented for MS channels in bacteria as well as in archaea (Hamill & Martinac, 2001).

MscL, the bacterial MS channel of large conductance, has become a prototype MS channel to study the structure–function relationship in this class of ion channels. Within a few years of the cloning of the *mscL* gene the 3D structure of the MscL protein was determined by X-ray crystallography (Chang *et al.* 1998), showing that the MscL channel is a homopentamer. A site-directed spin labelling (SDSL) study of MscL using electronparamagnetic resonance (EPR) spectroscopy provided complementary structural information about the conformation of the closed MscL channel (Perozo *et al.* 2001). To probe the molecular mechanism of how mechanical force gates MscL we have recently evaluated two physical mechanisms as triggers of MscL gating by bilayer deformation forces: (i) the energetic cost of protein-bilayer hydrophobic mismatches and (ii) the geometric consequences of bilayer intrinsic curvature.

Structural changes in MscL from *E. coli* were studied under zero transbilayer pressures using both the patch clamp and EPR spectroscopic approaches (Perozo *et al.* 2002*a, b*). To examine the role of hydrophobic mismatch in MscL gating we recorded the activity of MscL in phosphatidylcholine bilayers of different thickness ranging from 16 (PC16) to 20 (PC20) hydrocarbons per acyl chain. We found that decreasing bilayer thickness lowered MscL activation energy (Fig. 1A). Furthermore, the EPR spectroscopic analysis indicated that a structurally distinct closed channel intermediate was stabilized in PC14 bilayers (Perozo *et al.* 2002*b*). Although hydrophobic mismatch alone was unable to open the channel, the importance of hydrophobic mismatch for MscL mechanosensitivity results from stretch-induced bilayer thinning, which stabilizes the open conformation of MscL due to a better hydrophobic match with the open compared with the closed conformation of the channel (Perozo *et al.* 2002*a*).

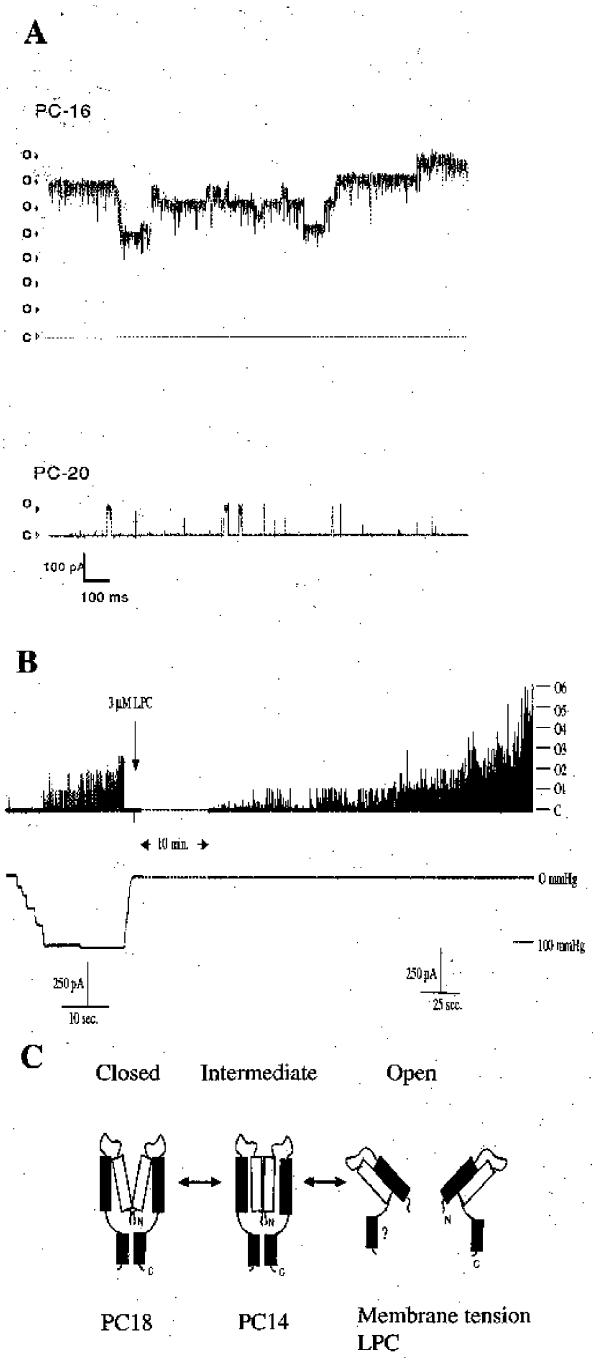

Figure 1. A, hydrophobic surface matching. Representative single-channel current traces of MscL reconstituted in phosphatidylcholines of different acyl chain lengths. Traces show 16:1 dipalmitoleoyl-phosphatidylcholine (PC16) and 20:1 eicosenoyl-phosphatidylcholine (PC20). Data were obtained at room temperature holding the membrane at +30 mV and negative pressures of 10 mmHg (PC16) and 60 mmHg (PC20) applied to the patch pipette. B, stabilization of open MscL with mixtures of phosphatidylcholine/lysophosphatidylcholine. Activation of MscL in the presence of lysophosphatidylcholine (LPC). LPC (3 μM) was applied to the external solution in the absence of applied pressure. Pipette voltage was +30 mV. C, model of structurally distinct conformations during MscL gating. Manipulating the lipid environment surrounding the channel can trap at least three distinct conformations: (i) the closed state is stable in PC18, (ii) PC14 stabilizes a closed

conformation further along the kinetic path, and (iii) the fully open state can be stabilized by addition of LPC in one leaflet of the lipid bilayer. (Adapted from Perozo *et al.* 2002*a*.)

Changes in membrane intrinsic curvature induced by the external addition of lysophosphatidylcholine (LPC) produced a dramatic increase in MscL single-channel activity in the absence of applied pressure (Fig. 1B). In EPR experiments addition of LPC to the external leaflet of the liposome bilayer generated massive spectroscopic changes in the narrow constriction that forms the channel 'gate', trapping the channel in the fully open state (Perozo *et al.* 2002*b*) (Fig. 1C), which suggested that it was the *asymmetry* in the lateral pressure profile between the two monolayers that caused the channel to open. Using EPR spectroscopy and site-directed spin labelling we have determined the structural rearrangements that underlie MscL closed-to-open transitions (Perozo *et al.* 2002*b*). Transitions to the open state were accompanied by massive rearrangements in both TM1 and TM2 helices, as shown by large increases in probe dynamics, solvent accessibility and the elimination of all inter-subunit spin-spin interactions. The open state is highly dynamic, supporting a water-filled pore of at least 25 Å, lined mostly by the TM1 helix (Fig. 2). This pore size is in agreement with the single-channel permeation studies, which by using large organic cations indicated a MscL pore of 30–40 Å in diameter (Hamill & Martinac, 2001). Our studies suggest a plausible molecular mechanism of gating in mechanosensitive channels.

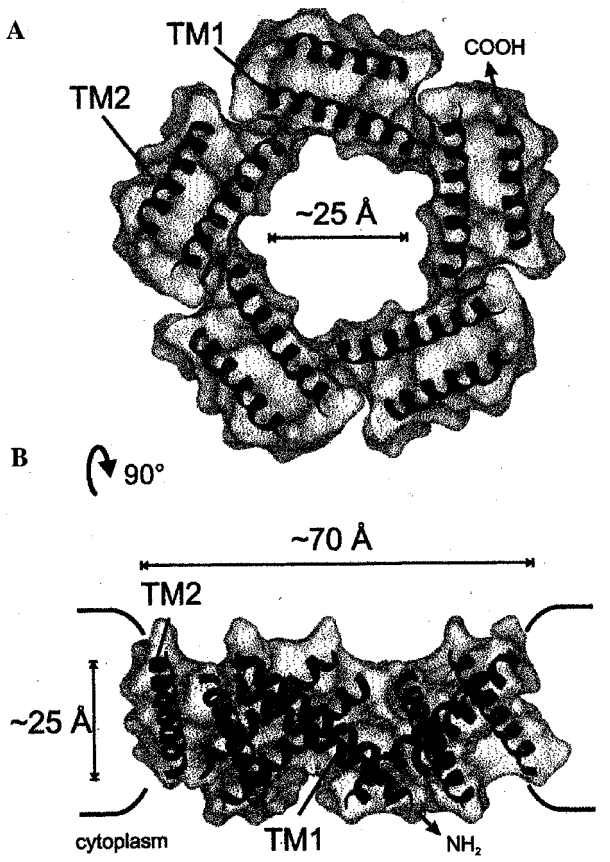

Figure 2. Structure of MscL in the open state. Extracellular (A) and side (B) views of TM1 and TM2 in the open state. Structures are shown within a translucent molecular surface representation of the whole molecule (without extracellular loop or the C-terminal end). The side view is shown in relation to a hypothetically distorted bilayer. (Adapted from Perozo et al. 2002*b*.)

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Stretch activated channels – an overvalued mechanism of mechanoelectric feedback in whole heart

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Since their description in skeletal muscle (Guharay & Sachs, 1984) stretch-activated channels (SACS) also appear in heart (Craelius *et al.* 1988). Many of their electrophysiological characteristics have an equivalence in intact heart, where monophasic action potentials (MAPs) are used for qualitative insights into electrophysiology. The preparations include isolated perfused heart – frog (Lab, 1978), mammalian ventricle (Franz *et al.* 1985; Hansen *et al.* 1990; Dick & Lab, 1998). Notably, there is a type of reversal potential (Lab, 1978; Zabel *et al.* 1996) where a stretch early in the action potential produces a repolarising tendency, whereas if late it produces depolarisation. At the 'reversal potential' the voltage time course remains unchanged. The equivalence extends not only to intact heart i*n situ*, in mammalian experimental preparations (Dean & Lab, 1987), but it also applies in man (Taggart *et al.* 1988). Importantly, the mechanical perturbation can give rise to clinically relevant premature beats. The likelihood that SACs are operative is further strengthened: gadolinium, a proposed SAC blocker, reduces the effects of stretch (Hansen *et al.* 1990). The same applies to streptomycin (Gannier *et al.* 1994; Eckardt *et al.* 2000). We have found, however, that streptomycin's effects can be heterogeneous in intact atrium (Babuty & Lab, 2001).

In the face of the foregoing persuasive studies, how can one contemplate the possibility that SACS are overrated in producing the electrophysiological changes in intact heart? This can be facilitated – correlation does not mean causality – a source of many a pitfall in interpretation. What are the alternatives?

Essentially one has to reflect on alternative mechanoelectric transducers in heart. Any membrane channel associated with the cytoskeleton could be a candidate (see Fig. 1, SACs/channels/receptors), for stress–strain could be transmitted to the channel in this way. There is evidence that the ATP-activated potassium (K_{ATP}) channel is not only attached to the cytoskeleton, but it has been suggested to be mechanosensitive (Van Wagoner, 1993): similarly the L-type Ca channel (Matsuda *et al.* 1996). We have shown (Gu *et al.* 2002) that several ion channels are fixed (probably via cytoskeleton) in Z grooves and around T-tubule openings. This means that they could be subject to transmitted forces. Although this makes them candidates for mechanoelectric transduction, we have yet to investigate this aspect. However, several other channels may be mechanosensitive, including sodium channels (Tabarean *et al.* 1999) and potassium channels such as TREK-1 (Maingret *et al.* 2002) (which appears not to involve the cytoskeleton), and IKAA (Kim, 1992), ion exchangers (Perez *et al.* 2001). However, invoking all the foregoing to substitute the SAC mechanism for explaining the observations in

intact heart violates 'Occam's razor' which needs the fewest hypotheses to explain an observation. If force transmission via the cytoskeleton has multi-channel effects, it should be operating on a beat-to-beat basis, continuously modulating the action potential. If so, perturbing the cytoskeleton should have electrophysiological effects. Pilot experiments in our laboratory show that depolymerising cytoskeletal f-actin modulates the MAP.

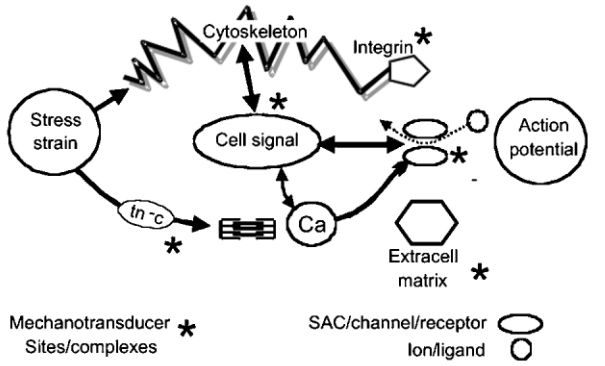

Figure 1. Cell stress/strain is distributed to several potential mechanotransducers to affect electrophysiology.

Another mechanoelectric transducer in the actively beating heart is via the binding constant of troponin c (tn-c) for calcium (see Fig. 1). Papillary muscle shortening reduces the binding constant for Ca and this increases intracellular Ca to influence transmembrane charge movement (Lab *et al.* 1984). Isometric contraction reduces the action potential duration (APD), whereas shortening prolongs it – sometimes taking the form of an afterdepolarisation which can, as in stretch of intact heart described above, produce premature beats (Kaufmann *et al.* 1971). Superficially, it appears difficult to supplant the SAC mechanism with this one, for intraventricular distension should produce a sustained stretch and isovolumic (isometric) contraction. However, contraction at the segment level is counter-intuitive. The segment often stretches early in systole (accompanied by MAP reduction) and shortens much later (accompanied by MAP prolongation, or afterdepolarisation). That is, the tn-c mechanism mimics the SAC mechanism.

Alternative mechanotransducers also reside in cell 'signalsomes' (see Fig. 1, cell signal, SACs/channels/receptors). One notable example is the β receptor. We have shown that β agonism or manipulation can significantly modulate mechanically induced electrophysiological changes (Horner *et al.* 1996). Raised intraventricular pressure results in catecholamine release from the ventricle (La Farge *et al.* 1970) and conceivably ventricular mechanical changes affect the cell signal chain involving β agonist/receptor, ATP, cAMP and Ca channels

Finally, other cell 'signalsomes' have also been implicated as being mechanosensitive and, mostly via intracellular Ca, affect membrane electrophysiology. These include AT2 and ET1 receptors (see brief overview: Lab, 1999) which would affect PLC via G_q. This can alter intracellular Ca, firstly, via IP_3 and SR Ca release, and secondly through PLC to facilitate membrane translocation (DAG/RACK/PKC) to influence Na–H exchange, and so intracellular Na via Na–Ca exchange. An under-investigated but potentially important mechanism invokes mechanically induced changes in ATP (Kawakubo *et al.* 1999)

In conclusion, it is conceivable that the plethora of channels, exchangers, and cell 'signalsomes' that exist in heart to transduce mechanical to electrical energy render SACs to just being one of many contributors to cardiac mechanoelectric feedback or transduction, and not the main one.

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Stretch-induced increments in $[Na^+]_i$ (mouse ventricular myocytes)

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Recently, we have published (Kamkin *et al.* 2000) that local axial stretch can activate whole-cell currents (I_{SAC}) through non-selective cation channels (SACs; for review, see Bett & Sachs, 1999). Here, we measured changes in intracellular sodium concentration ($[Na^+]_i$) as they were expected from the simultaneously recorded I_{SAC}.

Ventricular myocytes were superfused by a solution composed of (mM): 150 NaCl, 5.4 KCl, 1.8 $CaCl_2$, 1.2 $MgCl_2$, 10 glucose and 5 Hepes/NaOH (pH 7.4, 37 °C). Patch pipettes were filled with 140 KCl, 5 Na_2ATP, 5.5 $MgCl_2$, 10 BAPTA and 10 Hepes/KOH (pH 7.2). Local mechanical stimuli (m.s.) were applied via a glass stylus whose distance to the patch electrode was increased by approximately 20 %. Voltage dependence of mechanosensitive currents was evaluated from *I–V* curves obtained by a series of 20 voltage clamp pulses (1 Hz) that started from −45 mV (holding potential) and went to potentials between −100 and +50 mV (170 ms), current components were separated by comparing *I–V* curves before and after m.s. Stretch-induced changes in intracellular sodium concentration $[Na^+]_i$ were analysed by 3-D imaging of ventricular myocytes that had been loaded with 20 μM cell-permeant sodium green or by electronprobe microanalysis of cryosections (Wendt-Gallitelli *et al.* 1993).

Axial stretch of ventricular myocytes prolonged the action potential, depolarised the resting membrane and caused extra systoles. The most prominent stretch-sensitive current component was a stretch-activated inward current (I_{SAC}) that

followed modest outward rectification, reversed close to 0 mV and was blocked by 8 μM $GdCl_3$ or 30 μM streptomycin, respectively. Long periods of stretch (< 5 min) induced a nearly voltage-independent outward current (Cs^+ substitution for extra- and intracellular K^+ ions); the sensitivity of this current to removal of e.c. Cs^+ or to strophanthidin let us attribute this current to intracellular Na^+ accumulation followed by electrogenic Na^+ extrusion.

Pseudo-ratiometric imaging of sodium green fluorescence indicated that a 2 min axial stretch can double $[Na^+]_i$, which is an increment in $[Na^+]_i$ twice as big as the one induced by a 1 Hz stimulation. Stretch increased $[Na^+]_i$ with spatial heterogeneities, in 'hot spots' $[Na^+]_i$ could be as high as 50 mM. Co-imaging with ANEPPS localized most of the $[Na^+]_i$ hot spots close to the surface membrane. Co-imaging with tetramethyl rhodamine indicated that $[Na^+]_i$ hot spots were outside the mitochondria. After the stretch, the $[Na^+]_i$ hot spots dissipated with a time constant of approximately 1 min, suggesting that Na^+ accumulated in a space of restricted diffusion.

Electron probe microanalysis (EPMA, lateral resolution of 20 nm × 20 nm). Myocytes were shock-frozen during axial stretch. EPMA of cryosections (Wendt-Gallitelli *et al.* 1993) indicated that a 2 min stretch increased the total concentration [Na] (sum of free $[Na^+]_i$ plus bound $[Na]_i$) by a factor of approximately 2. In the cytosol close (100 nm) to the sarcolemma (100 nm) [Na] increased from 26 to 58 mmol l^{-1} and in the central cytosol from 19 to 36 mM. In mitochondria underneath the sarcolemma [Na] increased from 21 to 44 mM and in central mitochondria from 10 to 20 mM. [Na] increased from 25 to 44 mM in the nuclear envelope and from 25 to 37 mM in the nuclear matrix.

Local axial stretch can increase the intracellular sodium content; this is indicated by the increase in the free and total sodium concentration. The increments in [Na] can be attributed to Na^+ influx through SACs (charge −400 pA × 240 s) on the assumption that only a third of the influxing Na^+ is extruded by the Na^+,K^+-ATPase. The result that [Na] and $[Na^+]$ were higher underneath the sarcolemma than in the centre of the cell is discussed as a result of restricted diffusion of Na^+ ions in a 'fuzzy space' delimited by membranes of sarcolemma and subsarcolemmal SR or sarcolemma and subsarcolemmal mitochondria, respectively.

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SAC blockade inhibits stretch-induced atrial fibrillation

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Atrial fibrillation (AF) is the most common sustained arrhythmia, yet the mechanisms that lead to AF are incompletely understood. The occurrence of atrial fibrillation is often associated with haemodynamic or mechanical disorders of the heart, which lead to intra-atrial pressure or volume overload. Therefore myocardial stretch might play an important role in the development of AF. This is also emphasized by the common clinical finding that atrial dilatation correlates with the susceptibility to atrial fibrillation. The electrophysiological changes that occur in response to mechanical perturbations are referred to as mechanoelectrical feedback, a phenomenon that has been recognized in both experimental settings and humans. Atrial stretch caused by ventricular contraction modulates the atrial flutter cycle length in man, while in isolated hearts, acute atrial dilatation facilitates the induction and maintenance of atrial fibrillation.

We hypothesized that stretch-activated channels (SACs) might be involved in the arrhythmogenic effect of acute atrial stretch. SACs are found in many cardiac tissues and species including humans. Non-selective cationic SACs pass Ca^{2+} as well as Na^+ and K^+, whereas others selectively carry K^+ and possibly Cl^-. Block of SACs has previously shown to decrease arrhythmic responses to acute stretch in ventricular myocardium.

We studied the effect of SAC on the inducibility of atrial fibrillation in the isolated Langendorff perfused rabbit heart. In the unstretched atrium at a pressure of 0 cmH_2O, burst pacing did not produce AF. With an increase in atrial pressure, AF could be induced in all preparations. At low pressures, the sinus rhythm recovered spontaneously. At higher pressures, AF was sustained. AF inducibility increased and average duration of AF episodes lengthened as a function of pressure. Sustained AF terminated promptly upon lowering the intra-atrial pressure. We demonstrated that Gadolinium (Gd^{3+}), a generic blocker of stretch-activated channels, reduced the ability of stretch to potentiate atrial fibrillation in a dose-dependent manner. After application of Gd^{3+} (50 μM) in 16 hearts, intra-atrial pressure needed to be raised to 19.0 ± 0.5 cmH_2O to allow perpetuation of AF (*vs.* 8.8 ± 0.2 cmH_2O at baseline, $P < 0.001$).

SACs have been inhibited by a variety of non-specific agents like Gd^{3+}, streptomycin and amiloride in patch-clamp experiments. Their lack of specificity prevents clinical applications as blockers of SACs. It was recently reported that spider venom from the tarantula *Grammostola spatulata* could block SACs in single myocardial cells and very recently the active agent from the venom, GsMtx-4, was purified. It is a 4 kD peptide of the cysteine knot family that specifically blocks SACs as well as volume-activated cation currents in rat primary astrocytes and in hypertrophic dog ventricular cells.

After application of 170 nM GsMtx-4 in the isolated rabbit heart ($n = 10$), induction of AF required a significantly higher atrial pressure: 18.5 ± 0.5 *vs.* 8.8 ± 0.4 cmH_2O at baseline ($P < 0.001$). Sustained AF was obtained at 24.8 ± 0.6 cmH_2O after GsMtx-4 *vs.* 11.6 ± 0.3 cmH_2O at baseline ($P < 0.001$). The average duration of AF decreased at intra-atrial pressures between 10 and 27.5 cmH_2O ($P < 0.05$). These GsMtx-4-related effects were readily reversible upon wash-out.

To study the effect of SAC blockade on atrial excitability, the right atrial effective refractory period (ERP) was determined ($n = 7$). ERP progressively shortened with an increase in atrial pressure. On average, the ERP shifted from 71 ± 7 ms at 0.5 cmH_2O to 39 ± 2 ms at 20 cmH_2O ($P < 0.05$). GsMtx-4 did not significantly alter the ERP at any pressure level. This was in accordance with previous findings during Gd^{3+} application.

Our results demonstrate that Gd^{3+} and GsMtx-4, the only known specific blocker of SACs, reduce the vulnerability to atrial fibrillation during acute atrial dilatation. They confirm the predicted effects of SAC blockade on stretch-induced arrhythmias. In our model, electrical stimulation could only elicit atrial fibrillation when the atrium was preconditioned by stretch, while increased dilatation also increased the duration of AF. SAC blockade decreased the probability of initiation and increased the probability of spontaneous recovery of AF. This result suggests the involvement of SACs in the process.

The present data reaffirm that myocardial stretch shortens refractoriness in the atria. According to the multiple wavelet hypothesis, AF represents multiple wavefronts encountering excitable tissue. Shortening of refractoriness shortens the excitation wavelength and thereby favours re-entrant activity. Yet, after SAC blockade we found that local atrial refractoriness was not significantly altered while vulnerability to AF was markedly reduced. This implies that GsMtx-4 is acting at specific sites that initiate AF rather than by simply lengthening the ERP through generic changes in excitability. Importantly for potential clinical applications, GsMtx-4 had no significant side-effects on the preparation, thereby opening the field for a new class of anti-arrhythmic agents. The availability of a specific reagent to modify SACs will permit study of the coupling between mechanical strain and electrical excitability in further detail.

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Mechanically induced potentials in atrial fibroblasts from rat hearts are sensitive to hypoxia/reoxygenation

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Cardiac fibroblasts are electrically non-excitable cells with a resting membrane potential that is sensitive to mechanical stretch (Kiseleva *et al.* 1998). Increased sensitivity of the membrane potential of atrial fibroblasts to mechanical stretch has been implicated in the pathophysiology of cardiac arrhythmia after myocardial infarction (Kamkin *et al.* 2002). The cellular mechanisms that underlie the membrane potential changes of cardiac fibroblasts in response to tissue ischaemia are unknown. In this study we analysed the effect of hypoxia/reoxygenation, which are major components of tissue ischaemia/reperfusion, on the electrical function of atrial fibroblasts. Intracellular microelectrode recordings were performed simultaneously with isometric force measurements on spontaneously contracting right atrial tissue preparations from adult rat hearts, which were excised under deep ether anaesthesia. The standard perfusate contained (mmol l^{-1}): 118 NaCl, 2.7 KCl, 1.2 $CaCl_2$, 1.2 $MgSO_4$, 2.2 NaH_2PO_4, 25 $NaHCO_3$ and 5 glucose. The osmolarity of the solution was 290 ± 3 mosmol; pH was adjusted to 7.4. The perfusate was bubbled either with carbogen (5 % CO_2 and 95 % O_2) to adjust P_{O_2} to ~80 kPa, or with a 5 % CO_2 and 95 % N_2 mixture to lower the oxygen tension to 3 kPa. Lowering the oxygen tension in the extracellular perfusate increased resting force (RF), dramatically reduced active force (AF) development (Table 1), and decreased the resting membrane potential (E_m) of the cardiac fibroblasts from −23 ± 5 to −16 ± 4 mV (Table 2, n = 35).

Table 1. Time course of P_{O_2}, resting force (RF) and active contractile force (AF) during hypoxia/reoxygenation (1 mN of RF is a preload)

t (min)	P_{O_2} (kPa)	RF (mN)	AF (mN)
0	80 ± 3	1 ± 0	1.1 ± 0.15
0.5	38.3 ± 1	1 ± 0	1.1 ± 0.1
2	3.5 ± 0	1.5 ± 0.07	0.33 ± 0.07
4	60 ± 2	2.4 ± 0.1	0.15 ± 0.05
5	72.2 ± 3	1.91 ± 0.06	0.37 ± 0.06
7	80 ± 3	1.33 ± 0.04	0.95 ± 0.2
11	80 ± 2	1.16 ± 0.02	1.1 ± 0.2
20	80 ± 3	1 ± 0	1.1 ± 0.15

Values are means ± S.E.M. Student's paired t test was used for statistical comparisons. $P < 0.05$ was considered significant.

The following reoxygenation led to the following decrease in E_m to −5 ± 2 mV. These electrical changes were associated with increased frequency of spontaneous contractions of the right atrial preparations during hypoxia (Table 2). The further tissue reoxygenation increased the resting membrane potential to maximally −60 ± 8 mV and significantly lowered rhythmic contractile activity. The nature of depolarization during hypoxia connected with the compression of fibroblasts that results in opening of mechanosensitive channels. This possibility is shown in isolated cardiac fibroblasts during the compression and stretch. The double increase E_m cannot be explained by the decrease in RF to initial values. These mechanisms may include a rise of cytoplasmic Ca^{2+}, which is thought to contribute to impaired contractile function of the myocardium during acute ischaemia/reperfusion injury (Jennings *et al.* 1985). Increased intracellular Ca^{2+} may activate Ca^{2+}-operated K^+ channels and thereby hyperpolarize the membrane potential of the fibroblasts (Brooks *et al.* 1995; Kiseleva *et al.* 1996). Our findings indicate that the membrane potential of cardiac fibroblasts and the contractile function of the atrial myocardium are sensitive to hypoxia. It is suggested that alterations in the electrical function of atrial fibroblasts may contribute to cardiac arrhythmia during ischaemia/reperfusion injury of the heart.

Table 2. Time course of resting membrane potential (E_m) and amplitudes of mechanically induced potentials (VMIP) of atrial fibroblasts in response to hypoxia/reoxygenation

t (min)	E_m (mV)	VMIP (mV)	Frequency of spontaneous contractions
0	−23 ± 5	20 ± 3	100 %
0.5	−23 ± 5	20 ± 3	100 %
2	−16 ± 4	10 ± 4	107 ± 2 %
4	−5.0 ± 2	0 ± 2	114 ± 2 %
5	−15 ± 6	9 ± 5	108 ± 1 %
7	−52 ± 7	44 ± 6	94 ± 1 %
11	−60 ± 8	54 ± 6	85 ± 1 %
20	−24 ± 4	21 ± 4	100 %

Values are means ± S.E.M. Student's paired t test was used for statistical comparisons. $P < 0.05$ was considered significant. Strikingly, the changes in rhythmic activity of spontaneous contractions correlated closely with alterations in force development and electrical function of the atrial fibroblasts during hypoxia/reoxygenation.

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Myocyte/non-myocyte interactions in the heart: clues to an alternative mechanosensor for cardiac MEF

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Cardiac myocytes are believed to form an electrically conducting network of cells that is completely isolated from non-myocytes in the heart. Non-myocytes, like fibroblasts, form one of the largest cell populations in the heart. In particular, the healthy mammalian sino-atrial node (SAN) is very rich in fibroblasts (Shiraishi *et al.* 1992), which are mechanosensitive (Stockbridge *et al.* 1988) and, if electrically coupled to each other and/or adjacent cardiac myocytes, they would affect origin and spread of cardiac excitation (Kohl *et al.* 1999). The interaction of cardiac myocytes with other mechanosensitive cells (like fibroblasts), may participate in the cardiac mechanoelectric response that occurs during physiological and pathophysiological mechanical changes (Kohl *et al.* 1999). Preliminary findings of structural and functional studies suggest the presence of a direct electrical communication between myocytes and fibroblasts in the heart (De Maziere *et al.* 1992; Kohl *et al.* 1994).

Our aim was to solve the question of whether there is regular myocyte-fibroblast and/or fibroblast-fibroblast coupling in rabbit SAN.

We studied the distribution of the three main cardiac connexins (Cx43, Cx40 and Cx45) and myocyte-fibroblast coupling in the native SAN tissue, using a combination of immunohistochemical techniques and confocal microscopy. We used a triple labelling approach that provides, in addition to connexin localisation, identification of the coupled cell types (using anti-vimentin antibodies to mark fibroblasts and anti-myomesin antibodies for myocytes).

Our results show that there is gap junctional coupling, not only between myocyte and myocyte, but also between myocyte and fibroblast, and fibroblast and fibroblast. We found that Cx40 is mainly associated with fibroblasts and not myocytes, and that it is involved in fibroblast-fibroblast coupling in the rabbit SAN. Cx45 was expressed by both myocytes and fibroblasts of the SAN and formed junctions coupling either myocytes or fibroblasts, as well as fibroblasts and myocytes. Cx43 was found at the interface between SAN and *crista terminalis*, where it is longitudinally organized along the atrial myocytes arranged in bundles of atrial muscle coming into the node. Cx43 was absent in the nodal cells and fibroblasts.

We furthermore observed, in the SAN, a correlation between myocyte and fibroblast cells organisation and cell-type connexin expression. We identified two distinct fibroblast populations in the centre of the SAN: fibroblasts expressing Cx40 in fibroblast rich areas devoid of myocytes, and fibroblasts expressing Cx45 in regions of the node where both myocyte and fibroblast cells are intermingled.

In addition, dye transfer studies support the existence of functional coupling between myocytes and fibroblasts.

Our results show that the general assumption that connexins exclusively link cardiomyocytes to each other has to be modified. Here we demonstrated that, in the rabbit SAN: (i) fibroblasts and fibroblasts, as well as fibroblasts and myocytes, are structurally coupled by gap junctional channels, (ii) fibroblasts are coupled by Cx40 or Cx45, (iii) fibroblast-myocyte are coupled by Cx45 and (iv) cardiac fibroblasts can express multiple connexin isoforms in spatially distinct patterns.

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Mechanoelectric feedback and arrhythmias: the role of refractoriness in the induction and termination of atrial fibrillation

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Atrial fibrillation (AF) is often seen in heart failure or mitral valve disease with left atrial enlargement, and it is not uncommon that sinus rhythm is restored when, after surgical repair of the mitral valve, the size of the atria diminishes. This clinical observation suggests that mechanoelectric feedback may play an important role in the occurrence of atrial fibrillation. Theoretical formulations and high-density mapping have shown that the electrophysiological mechanism responsible for the maintenance of AF is the irregular propagation through the atria of several wavefronts. Inhomogeneity in electrophysiological properties, excitation wavelength (refractory period × conduction velocity) and tissue mass are determinant factors for the initiation and persistence of re-entrant arrhythmias. According to this, atrial stretch may contribute to atrial fibrillation development by increasing the atrial size, by decreasing the refractory period and/or the conduction velocity and by increasing their spatial dispersion. Modulation of the atrial electrophysiological parameters by stretch has been documented in both experimental studies and humans. In humans, we have shown that atrial stretch caused by ventricular contraction modulates the cycle length of atrial flutter by affecting the refractory period and the conduction properties of the underlying re-entrant circuit (Ravelli *et al.* 1994). Numerous studies have demonstrated stretch-induced changes in atrial refractoriness, although in the intact atrium conflicting results exist.

We have studied the effects of stretch on atrial refractoriness and vulnerability to atrial fibrillation in the isolated rabbit heart (Ravelli & Allessie, 1997). Atrial dilatation was obtained by raising the level of an outflow cannula in the pulmonary artery after occlusion of the caval and pulmonary veins. Increasing atrial pressure resulted in a progressive shortening of the atrial refractory period and monophasic action potential duration. All these changes were completely reversible after release of the atrial stretch. Dilatation of the atria was a major determinant for the vulnerability to atrial fibrillation. The inducibility of AF increased with progressively higher pressure levels and was strictly dependent on the refractory period. Atrial fibrillation was not induced at refractory periods > 70 ms. For refractory periods shorter than 50 ms, the inducibility of atrial fibrillation increased to 80 %. Similar results have been recently obtained in humans (Tse *et al.* 2001).

Whereas atrial stretch favoured the induction of AF by a premature beat, lowering of the atrial pressure invariably terminated AF. We have been investigating the mechanical modulation of atrial fibrillation by stretch release. Lowering of the atrial pressure causes a lengthening of AF cycle length and an increase in the degree of organization of atrial fibrillation, which leads to the arrhythmia termination. A gradual prolongation of

the refractory period together with the diminishment of atrial size causes a progressive decrease in the average number of wavelets, which increases the chance of AF interruption.

These results provide evidence for mechanoelectrical feedback as a potential mechanism for atrial re-entrant arrhythmias by critical shortening of atrial refractoriness. An increase of spatial heterogeneity in refractoriness and/or conduction by stretch may also facilitate wavefront fragmentations by creating regions of functional block. In addition to changes in the substrate, stretch-induced atrial triggers may play a role in atrial fibrillation genesis. Recently ithas been proposed that clinical atrial fibrillation may start by stretch-activated atrial premature beats originating from pulmonary veins. The experimental observation of stretch-induced afterdepolarizations producing triggered atrial arrhythmias may support this hypothesis (Nazir & Lab, 1996). In the development of chronic atrial fibrillation, structural changes caused by chronic stretch should also be considered in addition to stretch-induced electrophysiological changes.

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Mathematical modelling of mechanical effects on action potential duration in heterogeneous myocardium

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Myocardial heterogeneity affects cardiac mechano-electrical function, but how exactly remains unclear. This is partially due to the lack of simplified models for the study of this subject. We developed a fundamental model of cardiac heterogeneity (duplex), consisting of two separate muscle segments that are mechanically coupled (in series or parallel). Muscle segments may either be (1) biological samples (trabeculae), (2) mathematical models, or (3) a combination thereof (Solovyova *et al.* 2002).

Here, we studied mechano-electrical interactions in heterogeneous 'in series' duplexes, consisting of two virtual muscles (VMs). Each VM constitutes a mathematical model of guinea-pig ventricular electromechanical activity (Garny *et al.* 2001), developed by the Ekaterinburg and Oxford teams. Mechanical parameters for individual VMs were set to simulate a 'fast' and a 'slow' VM (Fig. 1A). In isolation, action potential duration (APD) was found to be longer for the slow muscle than for the fast one (in spite of identical parameters for the electrical blocks of both muscles). This difference in APD was produced by the difference in cross-bridge kinetics influencing Ca^{2+} dissociation from Troponin C, which affected Ca^{2+} transient and, consequently, Na^{+}–Ca^{2+} exchange current.

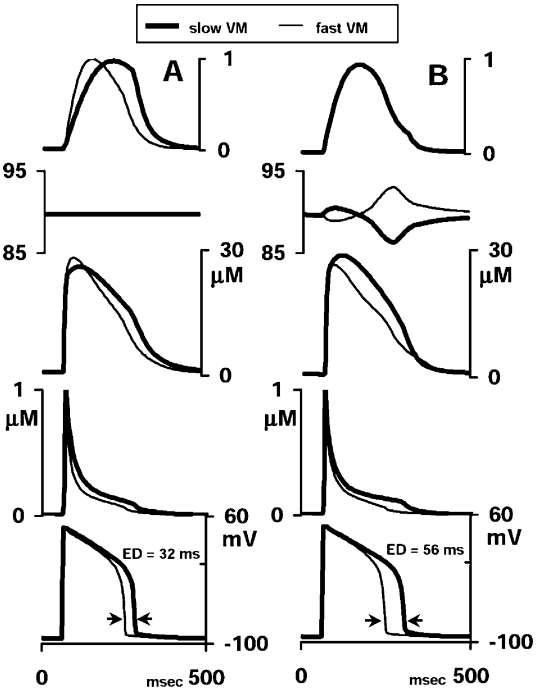

Figure 1. Electromechanical characteristics of individual VMs. A, in isolated conditions; B, within 'in series' duplex. From top to bottom: isometric force (arbitrary units); muscle length normalized by L_{max}; [Ca-TnC]; $[Ca^{2+}]_i$; AP.

In the duplex setting, we investigated isometric contractions of in series heterogeneous duplexes. External length of the duplex was fixed, whereas length of individual elements varied dynamically, due to the asynchronous mechanical activity of components. This *internal* mechanical interaction produced alterations in both mechanical and electrical activity of each muscle (see Fig. 1B for comparison with control behaviour in isolated conditions shown in Fig. 1A). Namely, APD of the fast muscle decreased further, while APD of the slow one increased, causing an increase in electrical dispersion (ED), defined as the time difference between 90 % repolarization. This ED may be modulated by introduction of a time lag (ΔT_{stim}) in electrical activation of duplex elements, to simulate the differences in the speed of electrical (up to 3.5 m s^{-1}) and mechanical (up to 350 m s^{-1}) 'conduction' in cardiac tissue. Not unexpectedly, ED decreased when stimulation of the fast muscle was delayed and *vice versa*. However, there was a significant difference between the ΔT_{stim} value and the effect on ED (Fig. 2A and B). Furthermore, applying ΔT_{stim} for individual muscles in *homogeneous* duplexes (pairs of either fast or slow muscles) caused ED that significantly exceeded the delay value (Fig. 2C). Model analysis showed that these alterations in APD of mechanically interacting muscles were caused by non-linear mechanodependent changes in their Ca^{2+} kinetics, affecting ED mainly via the Na^{+}–Ca^{2+} exchange current during the late plateau phase of the AP.

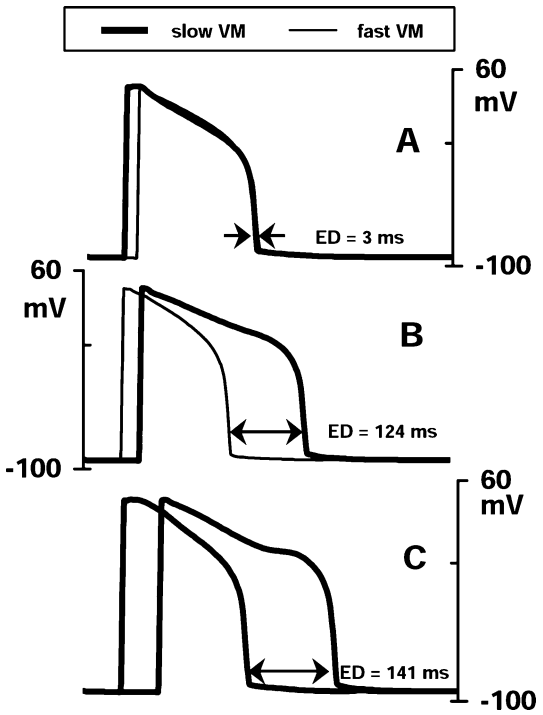

Figure 2. A, 20 ms stimulation delay of the fast VM in the heterogeneous duplex; B, 30 ms stimulation delay of the slow VM; C, 60 ms stimulation delay of one muscle in the *homogeneous* duplex.

We conclude that (i) myocardial electromechanical heterogeneity is an important determinant of ED; (ii) that this may be studied in reduced models of heterogeneity, consisting of only two elements; and (iii) that our virtual duplex model may provide a framework for developing experimentally testable hypotheses on this subject.

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Signalling pathways that underlie the slow inotropic response to myocardial stretch

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Myocardial stretch determines cardiac performance. Upon stretch, there is an immediate increase in contractility (the well-known Frank-Starling mechanism) and a secondary slow increase in contractility (the Anrep effect). The Anrep effect is due to an increase in Ca^{2+} supply to the myofilaments. This phenomenon is observed in the intact heart, in isolated muscle and in the single myocyte, yet the signalling pathway(s) and ultimate mechanism(s) that underlie this slow increase in contractility are a subject of continuing debate.

Several kinases have been identified as playing a role in the slow response to stretch. The protein kinase A (PKA) pathway was the first to be highlighted. We have observed a functionally significant increase in myocardial cAMP during the slow response in ferret papillary muscle (Calaghan *et al.* 1999). Endothelin 1 (ET1) and angiotensin II (Ang II)-stimulation of protein kinase C (PKC) has also been implicated. In ferret papillary muscle, ET_A and ET_B receptor antagonism attenuated the slow response, although blockade of Ang II receptors was without effect (Calaghan & White, 2001). Other studies using cardiac muscle from the cat have implicated Ang II upstream from ET1 (Pérez *et al.* 2001). Most recently, Vila Petroff *et al.* (2001) proposed that activation of the PtdIns-3-OH kinase-Akt-endothelial NO synthase axis mediates the slow increase in $[Ca^{2+}]_i$ seen following stretch of rat cardiac myocytes.

In terms of effectors, the target protein phosphorylated by PKA has yet to be identified although, given that the slow response in muscle is not attenuated by pharmacological blockade of the L-type Ca^{2+} channel or the sarcoplasmic reticulum (SR) (e.g. Chuck & Parmley, 1980; Kentish & Wrzosek, 1998), we speculate that increased cAMP and consequent stimulation of PKA occurs in a compartment inaccessible to these sites. Evidence suggests that ET1-dependent activation of PKC during the slow response stimulates Na^+–H^+ exchange and thereby Ca^{2+} influx, via reverse mode Na^+–Ca^{2+} exchange (Pérez *et al.* 2001). The work by Vila-Petroff *et al.* (2001) is consistent with S-nitrosylation of the ryanodine receptor enhancing the capacity of the SR to release Ca^{2+}.

There is evidence that the mechanism that underlies the slow response in the single cell is different in some respects to that seen in muscle. For example, ET1 antagonism does not attenuate the slow response in the single rat myocyte (Calaghan & White, 2002); neither is an increase in intracellular $[Na^+]$ seen upon stretch in the single cell, as it is in muscle (Hongo *et al.* 1996). Furthermore, when endocardial endothelium is removed from papillary muscle using Triton X-100, the slow response is absent (Calaghan *et al.* 2001). One interpretation of these data is that in intact muscle the slow response is predominantly a paracrine phenomenon, and there is no autocrine release of ET1 from the myocyte.

Although these pathways and effectors have been identified to date there is still a great deal that is not understood. One anomaly is the finding of enhanced capacity of the SR to release Ca^{2+} in the single myocyte (Vila-Petroff *et al.* 2001), coupled with evidence from muscle that a functional SR is not required for the slow response (e.g. Kentish & Wrzosek, 1998). The difference between the mechanisms that underlie the response of intact muscle and single cells to stretch could be ascribed to paracrine function of endothelial cells (or fibroblasts) present in muscle; however, it is also possible that mechanotransduction pathways are different in cells that are physically isolated from each other.

Finally, what of the components of the cell that act as mechanotransducers? The stretch-activated channel (SAC) has been identified as one such mechanotransducer. We have found recently that the SAC blocker streptomycin (40 μM) reduces the magnitude of the slow response by 80 % in the rat cardiac myocyte. Cationic SACs could allow entry of Ca^{2+}, and Ca^{2+}-dependent changes in action potential may also be important in this scheme. The role of other potential mechanotransducers (such as the cytoskeleton) in the slow response has yet to be established.

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The role of a volume-sensitive cation current in the genesis of spontaneous depolarizations and triggered activity in the failing heart: a potential cause of lethal arrhythmias

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In congestive heart failure (CHF), ventricular arrhythmias are often caused by triggered activity (Tomaselli & Marban, 1999). Nuss *et al.* (1999) described spontaneous depolarizations (SD) that occur during electrical diastole in single ventricular myocytes isolated from a canine CHF model and hypothesized that a chronically activated depolarizing current may cause them. A volume-sensitive, inwardly rectifying, poorly selective cation current ($I_{Cir,swell}$) was identified in the same canine model (Clemo *et al.* 1998). $I_{Cir,swell}$ is a candidate for the current underlying SD because it is chronically activated under isosmotic conditions in the setting of CHF.

To test this hypothesis, the correlation between $I_{Cir,swell}$ activity and frequency of SD was examined in single ventricular myocytes obtained from a rabbit aortic regurgitation model of CHF and from normal rabbits. The amphotericin perforated-patch clamp technique was used to avoid unpredictable cell swelling and changes in membrane currents that often slowly occur with ruptured patches. Ramp voltage clamps (28 V s^{-1}) were applied to determine current–voltage (*I–V*) curves. Current clamp (2–5 nA, 2 ms pulse) was used to elicit a train of action potentials (AP) at 0.2 Hz for 50 s, and then the stimulus was turned off to allow detection of SD (> 5 mV depolarization). The activity of $I_{Cir,swell}$ was decreased by osmotic manipulation of cell volume or by addition of the $I_{Cir,swell}$ blockers Gd^{3+} (Clemo & Baumgarten, 1997) or GsMTx-4, a *G. spatulata* peptide toxin (Suchyna *et al.* 2000).

Under isosmotic conditions (1.0 T), an inwardly rectifying current that was attenuated by hyperosmotic (1.5 T) cell shrinkage was seen in 25 of 30 CHF cells. The difference current at −80 mV was -2.9 ± 0.10 pA pF^{-1}. Application of GsMTx-4 (0.4 μM, graciously provided by Dr Fred Sachs, SUNY Buffalo, USA) significantly reduced this inward rectifying current, as shown in Fig. 1. The GsMTx-4-sensitive current at −80 mV was -1.8 ± 0.09 pA pF^{-1} ($n = 10$). Similar results were found for Gd^{3+} (10 μM). Hyperosmotic shrinkage and application of $I_{Cir,swell}$ blockers had no effect on whole-cell currents of normal cells.

Following a train of stimulated AP, SD were seen in 24 and spontaneous AP in 15 of the 30 CHF cells, as demonstrated in Fig. 2. In contrast, a train of AP elicited SD in only 2 and spontaneous AP in 0 of 30 CHF cells after $I_{Cir,swell}$ was blocked by shrinkage in 1.5 T. Moreover, in cells with SD, the $I_{Cir,swell}$ blockers Gd^{3+} and GsMTx-4 reduced the occurrence of SD by 85 and 90 %, respectively. In normal myocytes under isosmotic conditions, neither SD nor spontaneous AP were observed.

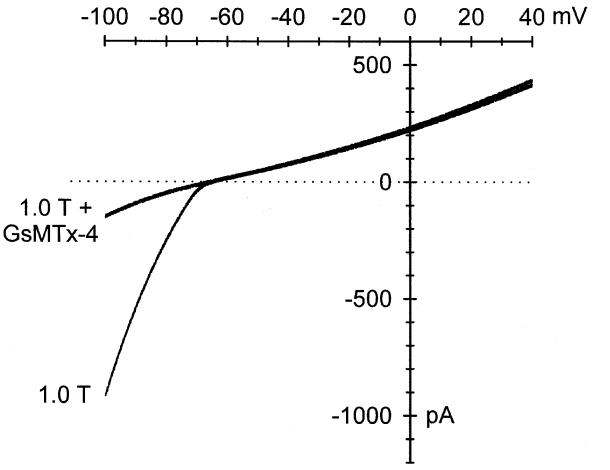

Figure 1. Whole-cell currents in the absence and presence of GsMTx-4 under isosmotic (1 T) conditions in a CHF cell.

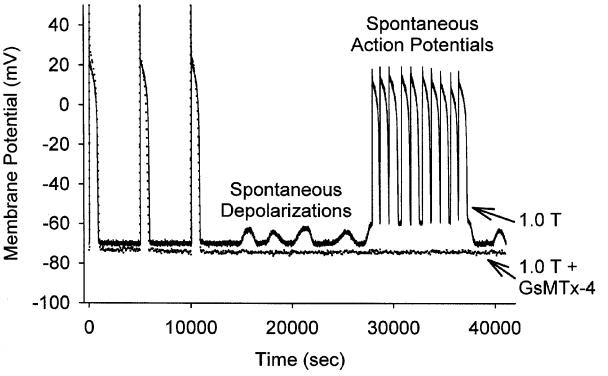

Figure 2. SD and spontaneous AP in the absence and presence of GsMTx-4 in a CHF cell under isosmotic (1 T) conditions.

This study showed that: (1) $I_{Cir,swell}$ is chronically active under isosmotic conditions in ventricular myocytes taken from a rabbit aortic regurgitation model of CHF; (2) under the same conditions, SD and spontaneous AP are present; and (3) attenuation of $I_{Cir,swell}$ reduces the frequency of SD and spontaneous AP. These results suggest that chronic activation of $I_{Cir,swell}$ may underlie the genesis of SD in failing ventricular myocytes and may potentiate triggered cardiac arrhythmias in the setting of heart failure. Novel blockers of $I_{Cir,swell}$ such as GsMTx-4, which inhibits atrial fibrillation in an isolated, perfused rabbit heart model (Bode *et al.* 2001), also may be efficacious in preventing the triggered arrhythmias seen in CHF.

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Differential gene expression and current density of TREK-1 in rat ventricle: implications for MEF

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In the heart, the process by which mechanical forces on the myocardium can induce electrical disturbances (mechano-electric feedback or MEF) has recently excited much interest, because it seems likely that it is responsible for many clinically important arrhythmias. The cellular mechanisms underlying mechanoelectric feedback are uncertain, but it seems likely that they involve mechanosensitive ion channels in the myocardial cell membrane (Sakin, 1995; Hu & Sachs, 1997). The electrical response of the myocardium to stretch can be either depolarising or hyperpolarising (Zabel *et al.* 1996), suggesting that two different types of channels may be involved. Indeed, stretch-sensitive non-specific cation channels (SACs) and potassium channels have been described (Kim, 1992).

We have used patch-clamp techniques to investigate the properties of a mechanosensitive potassium channel recorded in isolated ventricular myocytes from adult rats. The channel is highly selective for potassium ions, has a conductance of *ca* 100 pS, and responds strongly to membrane stretch applied as hydrostatic pressure across the membrane patch. Until recently the molecular identity of this channel was unclear. However, in 1998, a new family of potassium channels was cloned (the 4T2P channels: Lesage & Lazdunski, 2000). Members of this gene family are widely expressed in a variety of tissues such as brain, kidney and heart. The channels fall into three phenotypic groups: (1) the acid-sensitive channels TASK-1, -2 and -3, (2) the mechanosensitive channels TREK-1 and -2 and TRAAK and (3) the weakly inwardly rectifying channels TWIK-1 and -2. In the mechanosensitive subfamily only TREK-1 has been found to be expressed in cardiac tissues. TREK-1, when expressed in heterologous systems, has been shown to have the combination of properties that matches our functional measurements in cardiac cells (Maingret *et al.* 1999), and RT-PCR has shown a gene similar to TREK-1 to be expressed in the heart (Aimond *et al.* 2000).

The function of TREK in the heart is still a matter of speculation. However, it has been known for some time that the cardiac action potential has a very different morphology in different parts of the heart, a crucial determinant of this action potential heterogeneity being the variation in the expression level of the voltage-dependent potassium channel Kv4.3. If TREK showed a similar heterogeneous expression pattern, it would provide clues as to its function. We have now shown that TREK is indeed differentially expressed in epicardial and endocardial cells from rat hearts, and that the level of expression correlates with the size of an anaesthetic-induced current in these cells.

This differential expression of a mechanosensitive channel may be related to the distribution of stress across the ventricular wall. In this case, the factors that govern the gene expression of TREK, and similar channels, and the way that these change in pathological states such as heart failure and hypertrophy may provide fruitful avenues for new investigations.

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