

PS C14

Importance of the threonine residue T148 in the pore region for extracellular block and permeation of the cardiac muscarinic K⁺ channel, Kir3.1–Kir3.4

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The cardiac muscarinic K⁺ channel, Kir3.4–Kir3.4, is a heterotetramer and its pore is asymmetric (Silverman *et al.* 1998). A threonine residue is present in the Kir3.4 subunit (Kir3.4-T148), but not in the Kir3.1 subunit; in Kir3.1 an alanine residue is present instead (Kir3.1-A142). In the Kir2.1 channel, which is homotetramer, the equivalent threonine residue (T141) has been shown to be important in Cs⁺ block (Thompson *et al.* 2000). In Kir2.1, the mutation T141A increases the K_D for Cs⁺ block at \sim 100 mV 50-fold. To test whether Kir3.4-T148 is important for block and permeation of Kir3.1–Kir3.4, we have investigated Cs⁺ block and Rb⁺ and polyamine permeation of the mutant Kir3.1–Kir3.4-T148A channel.

Kir3.1, Kir3.4 and hD2 (human dopamine D2 receptor) were expressed in *Xenopus* oocytes and ionic current was measured with the two-microelectrode voltage clamp technique in the presence of 10 mM dopamine and 90 mM extracellular K⁺.

Extracellular Cs⁺ blocked the wild-type channel with a K_D at -130 mV of $177.5 \pm 4.2 \mu\text{M}$ (mean \pm S.E.M.; $n = 6$), while the K_D of the mutant Kir3.1–Kir3.4-T148A channel was $444 \pm 28 \mu\text{M}$ ($n = 5$); the block exhibited voltage dependence ($\delta = 0.8$ and 0.6 for the wild-type and mutant channels, respectively). This mutation resulted only in 2.5-fold decrease in the channel sensitivity to Cs⁺ block. In the mutant Kir3.1-A142T–Kir3.4 channel, another two threonine residues have been added to the channel pore. Extracellular Cs⁺ blocked the mutant channel with a K_D at -130 mV of 443.6 ± 64 ($n = 7$); the block exhibited a pronounced voltage dependence ($\delta = 2.5$). To test whether the wild-type and mutant channels are permeable to Rb⁺ and polyamine, the 90 mM K⁺ in the bathing solution was replaced by 90 mM RbCl or spermine chloride. The wild-type channel was slightly permeable to Rb⁺ and spermine: with Rb⁺ and spermine, current at -130 mV was, respectively, 0.30 ± 0.02 times ($n = 5$) and 0.36 ± 0.04 times ($n = 5$) that carried by K⁺. The mutant Kir3.1/Kir3.4-T148A channel was markedly more permeable to both Rb⁺ and polyamine: with Rb⁺ and spermine, current at -130 mV was, respectively, 0.58 ± 0.03 times ($n = 6$) and 1.27 ± 0.20 times ($n = 6$) that carried by K⁺.

These results support the hypothesis that the threonine residue in the pore region of the muscarinic K⁺ channel is important for channel block and permeation.

Silverman SK *et al.* (1998). *Biophys J* **75**, 1330–1339.Thompson GA *et al.* (2000). *J Physiol* **536**, 231–240.*All procedures accord with current UK legislation*

PS C15

Agonist-activation of the cardiac muscarinic K⁺ channel, Kir3.1–Kir3.4, may occur at the selectivity filter

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The Kir3.1–Kir3.4 channel is activated by G $\beta\gamma$ subunits released on binding of acetylcholine to the m2 muscarinic receptor. A

mechanism of channel opening has been suggested that involves translocation of pore lining transmembrane helices and the opening of an intracellular gate (Jin *et al.* 2002). Here we show that an extracellular gate at the selectivity filter is critical for agonist activation of the Kir3.1–Kir3.4 channel.

Kir3.1, Kir3.4 and the dopamine D2 receptor (another G protein-coupled receptor) were injected into *Xenopus* oocytes and currents were recorded 24–96 h later using the two-electrode voltage clamp technique; 750 ms voltage pulses were applied to -130 to $+60$ mV from 0 mV in the presence of 90 mM K⁺.

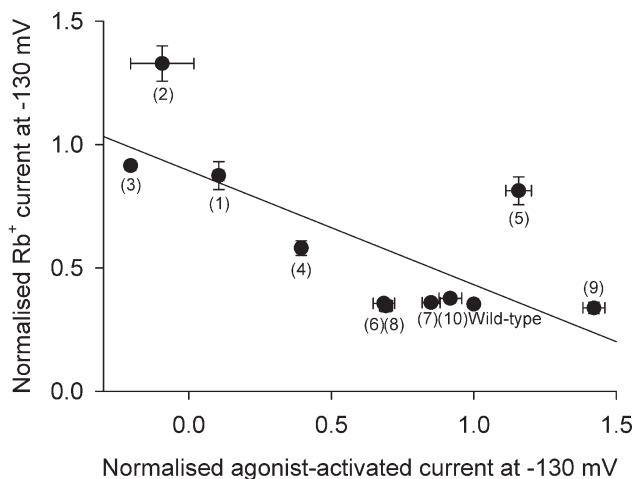


Figure 1. Correlation between agonist activation and selectivity. Data are means \pm S.E.M. ($n = 5$ –23).

Current was seen in the absence of agonist, but on application of $10 \mu\text{M}$ dopamine wild-type Kir3.1–Kir3.4 current was increased by $60.7 \pm 6.4\%$ (mean \pm S.E.M., $n = 23$). Figure 1 shows evidence that the selectivity filter may be involved in agonist activation: the extent of agonist activation (agonist-activated current at -130 mV normalised to that in the wild-type channel) is plotted against a measure of channel selectivity (current at -130 mV with Rb⁺ as the charge carrier normalised to that with K⁺ as the charge carrier). There is a significant correlation ($r^2 = 0.52$; $P < 0.02$) between agonist activation and selectivity. Mutations that abolished selectivity, such as those that disrupted a salt bridge that maintains the selectivity filter structure (Kir3.4-E145Q (1), Kir3.4-R155E (2) or Kir3.4-E145R, R155E (3)), also abolished sensitivity to agonist. Another mutation Kir3.4-T148A (4) that is important for extracellular block and permeation (Makary *et al.* 2003) halved the effect of agonist and doubled the size of the Rb⁺ current. Other mutations within the selectivity filter, such as Kir3.1-E139Q (5) (equivalent to Kir3.4-E145Q), Kir3.1-A142T (6) (equivalent to Kir3.4-T148A) or the double mutation Kir3.1-A142T/Kir3.4-T148A (7), that had little or no effect on selectivity also had little or no effect on agonist-activation. Finally, mutations at the intracellular gate (Kir3.1-F181M (8), Kir3.4-F187M (9) and Kir3.1-F181M–Kir3.4-F187M (10)) had no effect on selectivity and only a small effect on agonist activation.

These results demonstrate a significant correlation between agonist activation and selectivity, which is determined by the selectivity filter, and suggests, therefore, that agonist activation of the Kir3.1–Kir3.4 channel may involve the selectivity filter.

Jin T *et al.* (2002). *Mol Cell* **10**, 469–481.Makary SY *et al.* (2003). *J Physiol* **552**, P, C14 (this meeting).*All procedures accord with current UK legislation*

PS C16

Preferential closed channel blockade of HERG potassium currents by chemically synthesized BeKm-1 scorpion toxin

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The scorpion toxin peptide BeKm-1, originally isolated from *Buthus eupeus*, was synthesized by Fmoc solid phase chemistry and folded by air oxidation. The peptide's effects on heterologous human *ether-a-go-go* related gene (HERG) potassium current (I_{HERG}) and mutant HERG channels expressed in a mammalian cell line (HEK293 cells) were assessed using 'whole-cell' patch-clamp and compared with the commercially available recombinant BeKm-1 (rBeKm-1).

Blockade of I_{HERG} by BeKm-1 was concentration dependent, temperature dependent, and rapid in onset and reversibility. Two protocols, differing only in the duration at depolarized voltage, were adopted to investigate steady-state potency of I_{HERG} blockade by a range of BeKm-1 concentrations. Fractional block of I_{HERG} tails elicited at -40 mV following a depolarizing step to $+20$ mV for either 2000 ms or 400 ms from a holding potential of -80 mV was determined. Data are presented as means \pm S.E.M., or mean plus 95% confidence intervals (CI).

At 37°C , IC_{50} values were 63.3 nM, CI 52.7 – 76.1 nM (2000 ms step) and 15.3 nM, CI 11.2 – 20.1 nM (400 ms step), Student's unpaired t test, $P < 0.05$). These values were not significantly different from IC_{50} values for rBeKm-1 under identical conditions (unpaired t test, $P > 0.05$), indicating the synthesis of an active peptide. At room temperature identical experiments yielded significantly lower IC_{50} values of 9.7 nM, CI 7.2 to 13.0 nM (2000 ms) and 7.6 nM, CI 7.2 to 8.1 nM (400 ms) respectively (unpaired t test, $P < 0.05$). Blockade also exhibited inverse voltage dependence and reverse use and frequency dependence. During a three-step protocol, cells were held at -80 mV and stepped to zero for 2 s; further depolarization to $+80$ mV for 4 s resulted in a dramatic attenuation of I_{HERG} blockade by 25 nM BeKm-1 from $30.0 \pm 3.2\%$ to $2.2 \pm 5.5\%$ ($n = 5$). On stepping back from $+80$ mV to 0 mV for 4 s, BeKm-1 blockade re-developed from $2.2 \pm 6\%$ to $24 \pm 2\%$ (ANOVA, $P < 0.0001$). The attenuated inactivation mutation S631A abolished initial time-dependent relief of BeKm-1 blockade. BeKm-1 blockade of the HERG S6 mutant Y652A was identical in potency to that of wild-type HERG (250 nM BeKm-1; $71 \pm 1\%$ vs. $73 \pm 2\%$ respectively, unpaired t test, $P = 0.4$).

We conclude that synthetic BeKm-1 toxin blocks HERG with identical potency to rBeKm-1, preferentially through a closed (resting) state channel blockade mechanism, although some open channel blockade also occurs. The site of action of BeKm-1 differs from the high affinity site described within the vestibule (Mitcheson *et al.* 2000).

Mitcheson JS *et al.* (2000). *Proc Natl Acad Sci U S A* **97**, 12329–12333.

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PS C17

Hyperphosphorylation of slow delayed rectifier potassium channels in mouse hearts expressing KCNQ1–KCNE1 and over-expressing β_2 -adrenergic receptors

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We studied the effects of β_2 -adrenergic receptors (β_2 -AR) on the slow delayed rectifier current (I_{Ks}) in single ventricular myocytes isolated from hearts of transgenic (TG+) mice expressing an hKCNQ1–hKCNE1 fusion protein and overexpressing the h β_2 -AR. Both transgene constructs were under the control of the α -myosin heavy chain promoter (α -MHC). Strains of TG+ mice overexpressing β_2 -AR ($\beta\text{TG+}$) and hKCNQ1–hKCNE1 (KTG+) were crossed to produce double TG+ (DTG+) mice overexpressing both β_2 -AR and expressing hKCNQ1–hKCNE1.

Animals were humanely killed by intraperitoneal injection of pentobarbital sodium (50 mg kg^{-1}). KCNQ1 and β_2 -AR were found to be in similar subcellular localisations of isolated ventricular myocytes by immunohistochemical techniques combined with laser scanning confocal microscopy. KCNQ1 was localised to the surface sarcolemmal membrane, the transverse tubules and intercalated disc regions (ICD). In contrast, β_2 -AR were located in the T-tubules and ICD, but were absent from the surface sarcolemmal membrane. I_{Ks} density was increased (peak tail current after 60 mV pulses: KTG+ 4.0 ± 0.7 pA pF^{-1} (mean \pm S.E.M.), $n = 8$; DTG+ 9.2 ± 1.7 pA pF^{-1} , $n = 6$, $P < 0.05$, Student's unpaired t test), and activation was shifted in the hyperpolarizing direction (24 mV) in myocytes from DTG+ mice compared to TG+ mice expressing the KCNQ1–KCNE1 transgene alone (KTG+), indicating overexpression of β_2 -AR results in β -AR dependent stimulation of I_{Ks} , even in the absence of exogenous β -AR agonist. In fact, we found I_{Ks} current density in myocytes from DTG+ mice to have similar characteristics to that in myocytes from KTG+ mice plus β -AR stimulation by isoproterenol (ISO, 1 μM) (peak tail current after 60 mV pulses: KTG+ control 5.0 ± 1.7 pA pF^{-1} ; KTG+ ISO 7.3 ± 2.1 pA pF^{-1} , $n = 5$, $P < 0.05$, Student's paired t test, or $67 \pm 7\%$ increase). We found no further increase in I_{Ks} density or change in activation voltage dependence with β -AR stimulation in DTG+ mice indicating β_2 -AR overexpression results in maximal activation of I_{Ks} . Back phosphorylation experiments revealed hyperphosphorylation of KCNQ1 in DTG+ mice overexpressing β_2 -AR. Hyperphosphorylation of KCNQ1 was also detected in lysates from failing human hearts. Thus, overexpression of β_2 -AR in the heart results in hyperphosphorylation of KCNQ1 and maximal activation of I_{Ks} . Hyperphosphorylation of KCNQ1 is also observed in myocytes from failing human hearts. As such, myocytes overexpressing β_2 -AR may provide a good system to examine the effects on ion channel function of chronic activation of β -AR as seen in human heart failure.

All procedures accord with current national and local guidelines

PS C18

Phenylephrine inhibits an acid-sensitive background K⁺ current in rat ventricular myocytes

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Four components to the outward potassium currents of rat ventricular myocytes have been identified on the basis of their voltage- and time-dependent kinetics and pharmacology (Himmel *et al.* 1999; James *et al.* 2002). We have previously shown that the steady-state background K⁺ current, I_{ss} , is inhibited by the G-protein-coupled receptor agonists endothelin-1 and phenylephrine (PE; James *et al.* 2001a,b). The molecular basis to I_{ss} is unclear but may involve the acid-sensitive background K⁺ channel, TASK-1 (James *et al.* 2001a). The aim of the present study was to investigate in detail the properties of the PE-sensitive current.

Male Wistar rats were humanely killed and ventricular myocytes isolated by enzymatic and mechanical dispersion. Whole-cell patch-clamp recordings of membrane currents were made at 35°C (pH 7.35) using a K⁺-rich pipette solution and standard external Tyrode solution containing nifedipine (3 µM) and atenolol (1 µM). Outward currents were elicited by depolarisation to +40 mV (holding potential = -70 mV) in the absence and presence of 10 µM PE. The time course of inactivation of the currents was fitted by a double exponential equation and I_{ss} defined as the time-independent component. PE-sensitive difference currents were obtained by subtraction of I_{ss} recorded in the absence and presence of PE. Data are expressed as means ± S.E.M. and compared using Student's unpaired *t* test. *P* < 0.05 was accepted as significant.

Under control conditions, the PE-sensitive current was 301 ± 36 pA (*n* = 40). Replacement of pipette K⁺ with either caesium or lithium largely abolished the PE-sensitive current (12.4 ± 8.5 pA; *n* = 6 and -20.2 ± 4.2 pA; *n* = 3, respectively; *P* < 0.001), demonstrating the K⁺-selective nature of the PE-sensitive current. The PE-sensitive current was also greatly attenuated by reduction of external pH to 6.1 (38.5 ± 25.4 pA; *n* = 7, *P* < 0.001). Moreover, the PE-sensitive current was inhibited by the TASK-1 K⁺ channel blocker, anandamide (10 µM, 175 ± 29 pA; *n* = 7, *P* < 0.02). On the other hand, the TASK-3 K⁺ channel blocker, ruthenium red (20 µM), did not significantly inhibit the PE-sensitive current (237 ± 38 pA; *n* = 5). Neither was the PE-sensitive current (370 ± 72 pA, *n* = 11) affected by incorporation of the voltage-gated K⁺ channel blocker, tetraethylammonium (TEA, 20 mM) in the pipette solution.

Taken together, these data suggest that PE inhibits an acid-sensitive K⁺ channel with the properties of TASK-1 in rat ventricular myocytes.

Himmel HM *et al.* (1999). *Am J Physiol* **277**, H107–118.

James AF *et al.* (2001a). *Biochem Biophys Res Commun* **284**, 1048–1055.

James AF *et al.* (2001b). *J Physiol* **536**, P, 68P.

James AF *et al.* (2002). *J Physiol* **544**, P, 62P.

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All procedures accord with current UK legislation

PS C19

Effects of diazoxide pretreatment on sarcolemmal K_{ATP} channel activation and functional protection during metabolic inhibition in rat isolated cardiac myocytes

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Diazoxide (DZX) exerts a powerful pharmacological preconditioning effect, protecting cardiac muscle from the effects of ischaemia and reperfusion, and has been proposed to act through a mitochondrial ATP-sensitive K⁺ (K_{ATP}) channel (Garlid *et al.* 1997). However, Kir6.2 knockout mice, which lack sarcolemmal K_{ATP} channel activity, do not show protection by DZX (Suzuki *et al.* 2003). Here, we have investigated DZX protection and its effects on sarcolemmal K_{ATP} channel activity in rat ventricular myocytes.

Myocytes were isolated enzymatically from humanely killed adult rats (Rodrigo *et al.* 2002). Contractions were measured with a video system, action potentials and single channel activity with patch clamp, and [Ca²⁺]_i with fura-2. Metabolic inhibition (MI) was induced by superfusion with substrate-free Tyrode solution containing 2 mM NaCN and 1 mM iodoacetate for 7 min, and all experiments were done at 34°C.

Cells were stimulated at 1 Hz, treated with DZX (100 µM) for 5 min 2 min before MI, and contractile function was assessed 10 min after reperfusion. DZX pretreatment increased the proportion of cells that recovered contractile function from 16.8 ± 2.4 % (mean ± S.E.M., *n* = 21 expts) to 65.0 ± 2.2 % (*n* = 20, *P* < 0.001, Student's *t* test), and also increased the proportion of cells in which diastolic [Ca²⁺]_i recovered to below 250 nM. DZX also accelerated action potential and contractile failure during MI. In both control and DZX-pretreated cells, loss of contraction accompanied failure of the action potential. Contraction failed after 192 ± 6 s (*n* = 57) in control cells and 138 ± 4 s (*n* = 23) in DZX-pretreated cells (*P* < 0.001). In cell-attached patches on unstimulated myocytes, MI led to sarcolemmal K_{ATP} channel activity, but this occurred earlier in DZX-pretreated cells than in control cells (145 ± 24 s, *n* = 8 versus 225 ± 15 s, *n* = 10 respectively, *P* < 0.01). However, local application of DZX by inclusion in the patch pipette did not accelerate channel opening. The blocker of sarcolemmal K_{ATP} channels HMR 1883 (10 µM) delayed action potential failure and also reduced the protective effect of DZX pre-treatment.

Our results show that pre-treatment with DZX increases recovery of function and Ca²⁺ homeostasis after MI and reperfusion in isolated myocytes. Early activation of sarcolemmal K_{ATP} channels leads to failure of the action potential and contraction and it is likely that this contributes to protection by DZX.

Garlid KD *et al.* (1997). *Circ Res* **81**, 1072–1092.

Rodrigo GC *et al.* (2002). *J Mol Cell Cardiol* **34**, 555–569.

Suzuki M *et al.* (2003). *Circulation* **107**, 682–685.

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All procedures accord with current UK legislation

PS C20

Expression of C-terminal fragments of the sulphonylurea receptor SUR2A in rat isolated cardiac myocytes modifies sarcolemmal K_{ATP} currents and contractile responses on metabolic inhibition

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Cardiac sarcolemmal ATP-sensitive K^+ (K_{ATP}) channels form as octamers of four Kir6.2 channel subunits and four SUR2A subunits (Aguilar-Bryan *et al.* 1998). We have shown previously that certain C-terminal fragments of SUR2A co-immunoprecipitate with Kir6.2, and act as dominant negative polypeptides in HEK 293 cells stably expressing Kir6.2 and SUR2A, reducing the number of functional K_{ATP} channels (Rainbow *et al.* 2002). Here, we describe the effects of SUR2A fragments in rat ventricular myocytes.

Ventricular myocytes were isolated enzymatically from humanely killed adult rats, transfected using Lipofectamine 2000 with SUR2A fragments in the pIRES2-EGFP-F vector, and cultured at 37°C in minimal Eagle's medium. After 48 h myocytes were transferred to Tyrode solution. Transfected cells were identified by green fluorescence, and studied using patch clamp or video microscopy. The channel opener pinacidil was used to activate K_{ATP} current.

In cells transfected with vector alone, the current density at 0 mV in 200 μ M pinacidil was 7.04 ± 1.22 pA pF⁻¹ (mean \pm S.E.M., $n = 6$). Transfection with the entire C-terminus of SUR2A (residues 1254–1545) reduced this to 0.39 ± 0.02 pA pF⁻¹ ($n = 6$, $P < 0.001$, ANOVA plus Student-Neumann-Keuls). The 65 amino-acid fragment 1294–1358 was also effective at reducing current density (0.94 ± 0.07 pA pF⁻¹, $n = 6$, $P < 0.001$ *versus* vector alone). In contrast, no reduction in whole-cell K_{ATP} current was observed when the distal C-terminus (residues 1358–1545) was expressed (current density 6.30 ± 0.85 pA pF⁻¹, $n = 6$), and we have used this fragment as a control. We also investigated K_{ATP} channel opening in cell-attached patches on myocytes exposed to metabolic inhibition (MI, substrate-free Tyrode, 2 mM NaCN, 1 mM iodoacetate). SUR2A1294–1358 did not affect the time to opening, but reduced the maximum number of channels seen in the patch to about 20% of that seen in control cells. Transfection with SUR2A1294–1358 delayed action potential failure in cells exposed to MI, and increased the time to contractile failure, but decreased the time to rigor contraction from a control value 4.91 ± 0.14 min ($n = 30$) to 3.07 ± 0.15 ($n = 46$, $P < 0.001$).

Our results suggest that C-terminal fragments of SUR2A containing residues 1294–1358 reduce functional K_{ATP} channel density in cardiac myocytes, and that this can delay action potential failure and contractile failure but accelerate rigor during MI.

Aguilar-Bryan L *et al.* (1998). *Physiol Rev* **78**, 227–245.

Rainbow RD *et al.* (2002). *J Physiol* **544**, P, 21P.

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All procedures accord with current UK legislation

PS C21

Role of brain type sodium current in murine sino-atrial node pacemaking

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Voltage-gated Na^+ channels are the primary molecules responsible for the rising phase of the action potential in electronically excitable cells. The majority of Na^+ channels in the heart are cardiac-type Na^+ channels, but brain-type Na^+ channels have also been recently identified in murine heart including sinoatrial node (SAN) (Maier *et al.* 2002), but the role of brain type Na^+ channels in SAN has not been clarified. In this study, we have isolated and characterized a TTX-sensitive Na^+ current from murine SAN pacemaker cells and examined its role in murine SAN pacemaking.

Hearts were excised from 20–25 g adult C57BL mice humanely killed by cervical dislocation. Single SAN cells were isolated as we described previously (Lei *et al.* 2002a). TTX (100 nM) was used to dissect out sodium channel subtypes from total sodium current. Nifedipine (300 nM) was applied to block $I_{Ca,L}$. External sodium concentration was decreased to 70 mM (replaced by CsCl).

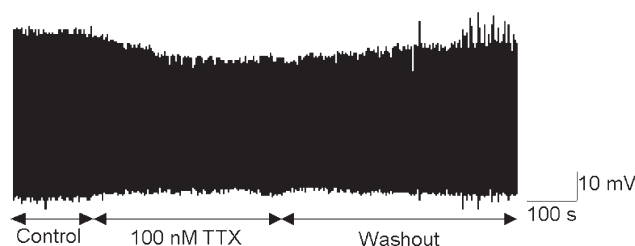


Figure 1 Continuous extracellular potentials (ECPs) recorded from a mouse SAN in absence and presence of 100 nM TTX.

Extracellular potentials (ECPs) were recorded as we described previously (Lei *et al.* 2002b). A TTX-sensitive inward current was recorded by 20 ms depolarising step pulses from a holding potential of -120 mV to various test potentials between -90 and $+40$ mV. At body temperature, the current starts to activate at -60 mV and peaks at -10 mV with a current density of -21.8 ± 2 pA pF⁻¹ ($n = 6$). Block of brain-type Na^+ channels by 100 nM TTX decreases spontaneous beating rate in both intact SAN and isolated pacemaker cells. Figure 1 shows an example of continuous recording of the effect of 100 nM TTX on the leading pacemaker site.

The results suggest that brain-type Na^+ current contributes to murine cardiac pacemaking.

Lei M *et al.* (2002a). *Biophysical Journal* **82**, 605a.

Lei M *et al.* (2002b). *J Physiol* **554**, P, 39P.

Maier SK *et al.* (2002). *Proc Natl Acad Sci U S A* **99**, 3507–3512.

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All procedures accord with current UK legislation

PS C22

 β -Adrenergic regulation of cardiac CaV1.2 channels requires anchoring of cAMP-dependent protein kinase via AKAP15 in rat heart

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In the heart, activation of β -adrenergic receptors increases the activity of L-type CaV1.2 channels through cAMP-dependent protein kinase (PKA)-mediated phosphorylation. We have recently demonstrated that an A-kinase anchoring protein (AKAP15) targets PKA to the carboxy (C-) terminus of skeletal muscle L-type CaV1.1 channels through modified leucine zipper (LZ) interactions (Hulme *et al.* 2002). Sequence alignment of the LZ-like region of CaV1.1 with other members of the CaV1 family revealed a striking conservation of LZ-like motifs, suggesting that AKAP15 may also target PKA to CaV1.2 channels in the heart.

Animals were humanely killed and procedures were approved by the Institutional Animal Care and Use Committee at the University of Washington. Immunoprecipitation experiments using rat heart extracts revealed that AKAP15 co-immunoprecipitates with CaV1.2 channels. Immunohistochemical staining of isolated rat ventricular myocytes showed CaV1.2 channels and AKAP15 to be localised in the transverse tubules. Site-directed mutagenesis studies in transfected HEK293 cells confirmed that AKAP15 directly interacts with the distal C-terminus of the cardiac CaV1.2 channel (residues 2057–2115) via a LZ interaction similar to that identified in the skeletal muscle CaV1.1 channel (Hulme *et al.* 2002). The functional importance of AKAP15 binding to the C-terminus of CaV1.2 channels with regards to β -adrenergic regulation of L-type Ca²⁺ currents in rat ventricular myocytes was examined using the whole cell patch clamp technique. In control cells, isoprenaline (1 μ M) caused a marked increase in the amplitude of the Ca²⁺ current and a hyperpolarising shift in the current-voltage (*I*–*V*) relationship. In contrast, in cells dialysed with a synthetic peptide corresponding to the LZ motif of AKAP15 (AKAP15LZ(38–54)), the isoprenaline-induced increase in Ca²⁺ current was greatly attenuated, and the hyperpolarising shift in the voltage dependence of activation was significantly blocked. Moreover, dialysis of a mutant form of the same peptide had no significant effect on the isoprenaline-induced increase in the amplitude of the Ca²⁺ current.

These data indicate that targeting of PKA to Ca²⁺ channels via a LZ-like interaction between AKAP15 and the CaV1.2 channel plays an essential role in the β -adrenergic-dependent regulation of Ca²⁺ channels in rat ventricular myocytes.

Hulme JT *et al.* (2002). *J Biol Chem* 277, 4079–4087.

All procedures accord with current national and local guidelines

PS C33

Absence of strain-softening in isolated, superfused, passive right-ventricular trabeculae from rat

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The mechanical behaviour of the diastolic heart reflects the passive properties of cardiac tissue, which have been reported to demonstrate 'strain-softening' (Emery *et al.* 1997). This phenomenon is characterised by a stiff stress–strain relationship on the first loading cycle; subsequent cycles at the same extension show a 'softening' of stress. If the tissue is then stretched to some greater extent, the first stress–strain curve of the new loading cycle follows the previous 'softened' locus. Subsequent cycles at the new extension again show 'softening' (Fig. 1B). In contrast to 'stress relaxation', strain softening is irreversible. It has been demonstrated in the pressure–volume behaviour of the 2,3-butanedione monoxime (BDM)-arrested rat heart (Emery *et al.* 1997) and by the shear behaviour of BDM-treated tissue blocks cut from the left-ventricular wall of the pig heart (Dokos *et al.* 2002). In both of these studies, the tissues were unperfused.

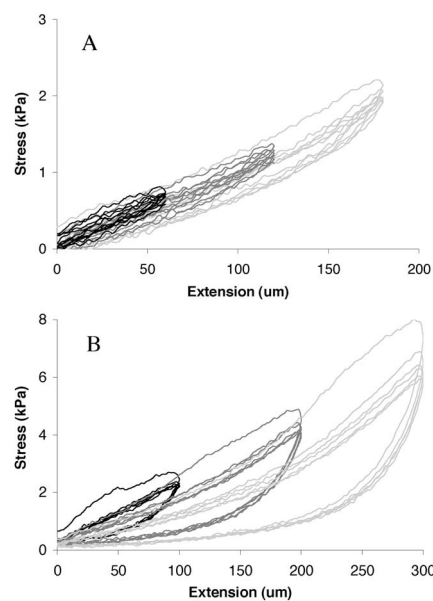


Figure 1. Stress–strain loops for 6 cycles at each of 5%, 10% and 15% extensions of muscle length for a viable (A) and a non-viable (B) trabecula.

To determine whether strain softening occurs in well-oxygenated tissue, we subjected 10 superfused trabeculae, from the right ventricles of humanely killed rats, to repeated strain cycles of 5%, 10% and 15% extension of muscle length. To ascertain the contribution of BDM, we tested half of the preparations first in the absence (–BDM) and then in the presence (+BDM) of 50 mM BDM. The other half experienced the converse order. Stress–strain data were subjected to repeated-measures, split-plot ANOVA ($\alpha = 0.05$). As shown by the typical result in Fig. 1A (–BDM), we found no evidence of strain-softening – in either the presence or absence of BDM. In fact, strain softening was observed (Fig. 1B; note difference of vertical scales) only if trabeculae became non-viable (i.e. had stiffened and failed to respond to electrical stimulation). If strain-softening does not reflect deterioration of the health of the tissue, then it must

reflect the different mode of deformation that we applied (linear elongation *versus* shear or volume expansion), or the different microstructure of trabeculae (axially aligned myocytes and perimysial collagen fibres (Hanley *et al.* 1999) vis-à-vis the complex 3-D geometry of whole-hearts (Le Grice *et al.* 1995) or ventricular tissue blocks). We rule out a contribution from BDM.

Dokos S *et al.* (2002). *Am J Physiol* **283**, H2650–2659.

Emery JL *et al.* (1997). *J Biomech Eng* **119**, 6–12.

Le Grice IJ *et al.* (1995). *Am J Physiol* **269**, H571–582.

Hanley PJ *et al.* (1999). *J Physiol* **517**, 831–837.

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All procedures accord with current national and local guidelines

PS C34

Heart rate variability instrumentation: agreement and reliability

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Short-term heart rate variability (HRV) is used to monitor autonomic function. HRV is a generic term which describes a number of distinct measures. The reliability (test–retest repeatability) of these measures is rarely reported (Sinnreich *et al.* 1998; Marks & Lightfoot, 1999). Comparisons are difficult because of the variation in the number of test conditions, sampling epochs, test–retest durations and statistical analyses. A range of instruments is now available to record HRV and the purpose of this study is to assess the agreement between such systems and their individual reliability.

Subjects were 30 (20 male and 10 female) volunteers (median age 27.5, range 19–59 years). They gave informed consent. The three HRV instruments were: VariaCardio TF5 (Advanced Medical Diagnostics Group Ltd, UK), CardioTens (Meditech Ltd, UK) and a specialised HRV module within a CardioPerfect ECG analysis system (Carido Control Ltd, MN, USA.). Simultaneous, 5 min ECG recordings were made under three conditions: subject supine, subject standing, subject supine with paced (0.2 Hz) breathing. This recording protocol was repeated 1 week later at the same time of day with identical pre-test conditions. Agreement between instruments was assessed using repeated measures ANOVA and intraclass correlation coefficient 3.1 (R). Reliability was assessed using coefficient of variation (CV).

The consistent HRV measurement across each machine were mean normal-to-normal beat interval (NN), the route mean square of the standard deviation of NN intervals (RMSSD), the standard deviation of the NN intervals (SDNN), high and low frequency spectral power in raw units (HF and LF) and in normalised units (HFnu and LFnu). A high level of agreement existed in all HRV measurements under all conditions between the three instruments (median $R = 0.95$, range 0.79–0.99). A small but statistically significant (5.6 ms, $P < 0.05$) difference was found between the NN intervals recorded by the instruments. This were translated into significantly ($P < 0.05$) higher values for SDNN intervals calculated by the CardioTens. The reliability (CV %) was found to be poor for the TF5 (median 26.5, range 1.1–189.4 %) the CardioTens (median 29.6, range 6.2–238.5) and the CardioPerfect (median 35.5, range 7.2–90.4). Similar CVs were commonly produced by all three instruments, for given

measurements, made under given conditions, although this was not consistent.

The results demonstrate that data manipulation is similar in these three instruments. The variability in R–R interval length and SDNN was found to be due to slight variation in the number of R–R intervals included in each instrument's sampling epoch, a function of their data collection protocols. Similarities between all remaining measures demonstrate data interpretation to be so similar between instruments they may be used interchangeably. This will enable data collected between laboratories to be compared.

The results also demonstrate low levels and wide ranges of reliability for HRV measures due mainly to biological variation. This demonstrates the importance of assessing and reporting reliability of HRV parameters under specific experimental conditions. This will provide essential information on which to base any intervention.

Marks BL & Lightfoot JT (1999). *Can J Appl Physiol* **24**, 337–348.

Sinnreich R *et al.* (1998). *Heart* **80**, 156–162.

All procedures accord with current local guidelines and the Declaration of Helsinki

PS C35

Decreased heart rate variability in a ferret model of heart failure

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The presence of pathological left ventricular hypertrophy is associated with the future development of heart failure or sudden cardiac death due to arrhythmias. It has been suggested that assessment of heart rate variability (HRV) may be a useful prognostic index of the likelihood of sudden cardiac death, but this remains controversial. The aim of this study is to investigate, in an animal model of heart failure, whether changes in this electrocardiographic parameter occur and thus are likely to be of any prognostic value.

Animals were anaesthetised with 70 mg kg⁻¹ medetomidine–7 mg kg⁻¹ ketamine. Carprofen analgesic (5 mg kg⁻¹) was administered pre-operatively. Animals were humanely killed with pentobarbitone sodium (200 mg kg⁻¹) administered intraperitoneally at the onset of symptoms of heart failure. Heart failure was induced in adult male ferrets by placing an ameroid occluder around the ascending aorta. The electrocardiogram was monitored continuously in freely moving animals using telemetric devices implanted intra-peritoneally. Heart rate variability was assessed either as the standard deviation of the mean heart rate calculated over a 24 h period or as the change in the inter-beat interval (period between successive normal R waves) in the same 60 min period each day.

Mean heart rate appeared to increase with progression to heart failure from 185 beats min⁻¹ prior to aortic coarctation to 220 beats min⁻¹ with the onset of clinical symptoms ($n = 2$). However, the standard deviation expressed as a percentage of the mean remained constant at (8.5 and 8.4 % respectively). Measurements of inter-beat interval were able to detect differences in the short-term regulation of heart rate during the progression to heart failure. When analysed in this manner, the inter-beat interval decreased with the increase in mean heart rate (0.315 to 0.287 s), but the standard deviation expressed as a

percentage of the mean inter-beat interval decreased (23.5 to 10.9%). Power spectral analysis of inter-beat interval (Fig. 1) revealed that there are a number of frequency components before aortic coarctation (continuous line) that are lost with the progression to heart failure (dotted line).

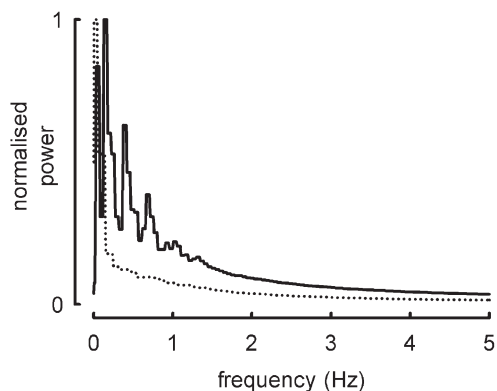


Figure 1. Power spectral analysis of inter-beat interval. Data were obtained from the same animal before aortic coarctation (continuous line) and when clinical heart failure was present (dotted line).

In summary, measurements of mean heart rate over extended periods of time do not appear to be of prognostic value. However, assessment of short-term changes in heart rate through inter-beat interval analysis does appear to reveal several components that alter with the development of heart failure. Further work is required to determine the role of autonomic control of heart rate in this effect.

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All procedures accord with current UK legislation

PS C36

Selective down-regulation of subendocardial ryanodine receptors in a rabbit model of left ventricular dysfunction

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Cardiac ryanodine receptors (RyR2s) play a critical role in excitation-contraction coupling, mediating the efflux of Ca^{2+} from the sarcoplasmic reticulum. In left ventricular dysfunction (LVD) and in heart failure, where Ca^{2+} handling is disrupted, there is considerable controversy over whether RyR2 protein expression is selectively altered. Using a rabbit coronary artery ligation model of LVD, alterations in RyR2 mRNA and protein levels were studied.

Prior to experiments, rabbits were humanely killed with a lethal injection of sodium pentobarbital (1 ml kg^{-1}) and hearts rapidly removed. Subendocardial and subepicardial regions of the left ventricle were analysed 8 weeks after coronary artery ligation and these were directly compared with the same regions in hearts from sham-operated animals. Real-time quantitative Taqman PCR revealed a subendocardial/subepicardial gradient for RyR2 mRNA in both experimental groups, with a decrease in the gradient in samples from animals with LVD.

The mean fold-difference ($\pm \text{S.E.M.}$) in mRNA for RyR2

normalised to GAPDH was 0.35 ± 0.02 (endo, $n = 6$) and 0.55 ± 0.04 (epi, $n = 6$) in the sham-operated group, compared with 0.26 ± 0.02 (endo, $n = 10$) and 0.56 ± 0.02 (epi, $n = 10$) in the LVD group (ANOVA, $P = 0.013$, where $P < 0.05$ is significant). The decrease in gradient observed is due to a down-regulation of RyR2 message in the subendocardial region. Quantitative analysis of RyR2 protein levels revealed similar findings. Two independent methods were used to assess regional RyR2 protein levels. Method 1 used quantitative densitometric analysis from Western blots probed with a specific monoclonal antibody against RyR2. Normalised data revealed down-regulation in the subendocardium in LVD preparations (2.2 ± 0.36 (sham, $n = 7$) vs. 1.2 ± 0.34 (LVD, $n = 7$)) ($P = 0.009$). As with mRNA data, there was no significant change in RyR2 protein levels in subepicardium (3.56 ± 0.53 (sham, $n = 7$) vs. 3.66 ± 0.51 (LVD, $n = 7$)). Method 2 used [^3H]ryanodine binding to assess the amount of RyR protein in subepicardial and subendocardial LV homogenates. Again the same observation was noted. Specific ryanodine binding ($\text{pmol (mg protein)}^{-1}$) was reduced in subendocardial preparations from the LVD group (0.18 ± 0.03 (LVD, $n = 7$) and 0.3 ± 0.04 (sham, $n = 4$), $P = 0.018$) with a small but non-significant decrease in subepicardium (0.39 ± 0.03 (LVD, $n = 7$) and 0.45 ± 0.05 (sham, $n = 4$)). This gave results similar to those obtained with the quantitative immunoblotting.

Taken together, these results reveal the following in this rabbit model of LVD. (i) Subendocardial RyR2 mRNA expression is reduced by $\sim 25\%$ whereas subepicardial message is unchanged. (ii) Subendocardial RyR2 protein is reduced by $\sim 40\%$ with no significant change in subepicardium. Similar data was obtained using two independent methods. (iii) A subendocardial/subepicardial gradient exists in both sham and LVD groups. This is decreased to a similar extent in LVD for both RyR2 message and protein expression ($\sim 20\%$ for mRNA and $20\text{--}30\%$ for protein).

This study highlights the importance of regional analysis in assessing cardiac protein dysfunction and could reconcile some of the controversy surrounding this area.

This work was sponsored by the British Heart Foundation.

All procedures accord with current UK legislation

PS C37

Sarcoplasmic reticulum calcium fluctuation: the key to systolic calcium transient alternans?

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We have recently shown that decreasing the ryanodine receptor (RyR) open probability (P_o) with tetracaine results in alternation of the amplitude of the systolic Ca^{2+} transient (Díaz *et al* 2002). Since RyR opening is triggered by Ca^{2+} entry through the L-type Ca^{2+} channels, this work investigates whether such non-uniform Ca^{2+} release can also result from a reduced L-type Ca^{2+} current (I_{Ca}) trigger. We also wish to know whether changes in sarcoplasmic reticulum (SR) Ca^{2+} content are involved in the development of alternans of systolic release of Ca^{2+} .

Ventricular myocytes were obtained from rats that were humanely killed. The experiments were performed on fluo-4-loaded voltage-clamped single rat ventricular myocytes. Cytosolic Ca^{2+} was monitored using line scan confocal microscopy.

A reduced I_{Ca} trigger, (i.e. depolarisation for 100 ms from -40 to -20 mV) caused non-uniform release of Ca^{2+} and alternation of global Ca^{2+} transient amplitude, as we have previously reported with the RyR inhibitor tetracaine. A typical example of the large variations of Ca^{2+} transient amplitude is shown in Fig. 1. Application of caffeine (10 mM) to empty SR Ca^{2+} abolished alternans, which resumed as the SR Ca^{2+} load recovered (not shown). Quantification of SR Ca^{2+} load following a big release shows that content is $86.4 \pm 9.8 \mu\text{mol l}^{-1}$ and rises to $112.4 \pm 6.7 \mu\text{mol l}^{-1}$ after a small release (means \pm S.E.M., $n = 5$, $P < 0.02$, Student's paired t test). This suggests that the reason for alternation of systolic release is alternation of SR Ca^{2+} content. The large releases we measure are larger, in fact, due to propagation of 'mini-waves' (Díaz *et al.* 2002). As the propagation of waves of Ca^{2+} release requires a threshold amount of Ca^{2+} in the SR these relatively small changes in SR content may be responsible for the non-propagation leading to small releases. A small depolarization can allow alternans to take place by activating only a relatively few release sites as L-type current is much reduced. This leaves a sufficient number of release sites available to take part in any subsequent propagating wave.

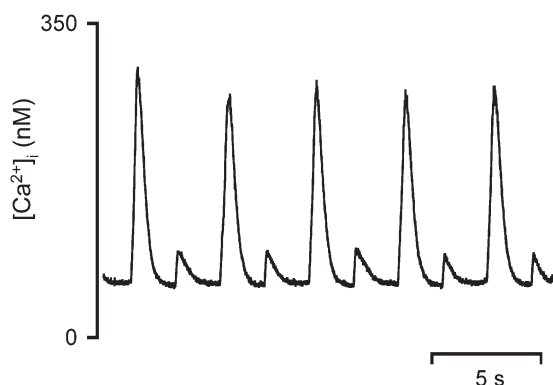


Figure 1. Development of alternans during small pulse stimulation. Changes in Ca^{2+} in response to consecutive (20 mV; 100 ms duration) pulses applied every 2 s from a holding potential of -40 mV.

Díaz ME *et al.* (2002). *Circ Res* **91**, 585–593.

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All procedures accord with current UK legislation

PS C38

Force–frequency relation in frog ventricle

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The myocardial force–frequency relation (FFR) is positive at steady-state in mammals except the rat where it is negative (Layland & Kentish, 1999). In frogs, FFR is positive up to a certain optimum frequency. We propose a mechanism involving a single protein to explain the FFR in the frog-ventricle.

Circular ventricular strips, made from isolated hearts of frogs anaesthetised with ether and then pithed, were used. In one set of experiments, the strips were paced at 0.2 Hz and the effect of rest on post-rest beat amplitude was studied. Under control conditions, there was rest-induced decay (RID) of amplitude.

This is due to a steady diastolic leak of calcium from sarcoplasmic reticulum (SR) to the outside (Subramani *et al.* 2002). We report here that RID was not affected by the following interventions: adrenaline (10 μM ; augments L-type calcium currents and SR calcium pump) ryanodine (10 μM ; ryanodine receptor blocker), neomycin (10 μM ; inhibitor of IP_3 formation) lanthanum (10 μM ; sarcolemmal calcium-pump blocker), and nickel (40 μM ; T-type calcium channel blocker). The proteins whose functions are modified by these agents are therefore not involved in the RID phenomenon. In another protocol, the frequency was stepped up from 0.03 Hz to about 0.5 Hz and then stepped down. The force at a particular frequency was averaged from the two cycles. There was a positive FFR under control conditions. The interventions mentioned above which did not change the RID phenomenon did not affect the positive FFR either. When the direction of the sodium–calcium exchanger (NCX) which is normally calcium-extrusive in diastole (Bogdanov *et al.* 2001) was reversed with (a) low sodium concentration (40 mM, osmolarity corrected with sucrose), (b) high calcium concentration (5 mM) and (c) ouabain (10 μM), the post-rest beat was larger than the pre-rest (rest-induced potentiation, RIP). In addition, the FFR turned negative. With reversal, NCX becomes calcium-acquisitive in diastole. The rest periods now help in accumulating calcium into SR and hence the RIPs.

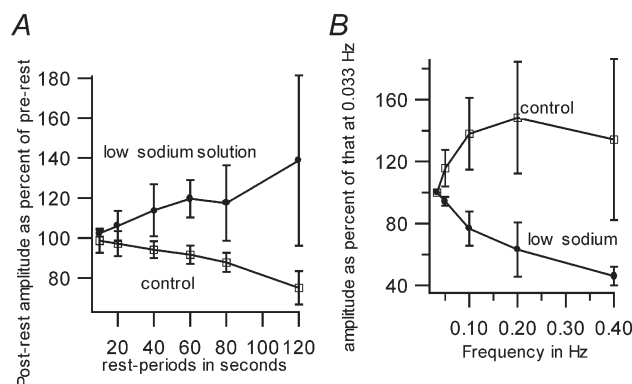


Figure 1. A, rest-induced decay in control solution and rest-induced potentiation in low sodium solution (40 mM, osmolarity corrected with sucrose, other components as in control) in circular frog-ventricular strips. Values are means \pm S.D., $n = 4$, $P < 0.05$ with repeated measures ANOVA. B, positive FFR in control solution turns negative in low sodium solution. Values are means \pm S.D., $n = 4$, $P < 0.05$ with repeated measures ANOVA.

To summarize, when there is RID, FFR is positive, and whenever there is RIP, FFR turns negative (Fig. 1). We hypothesize that when the conditions favour a net leak of calcium from SR during diastole (precisely when the conditions favour the calcium-extrusive mode of NCX), FFR is positive. An increase in frequency shortens diastole and therefore the diastolic calcium leak, thereby augmenting force. On the other hand, interventions which lock the NCX in reverse mode, causing a net uptake of calcium in diastole, were able to change the pattern of RID to RIP and converted the FFR from positive to negative. With net diastolic calcium uptake, lower frequencies can help increase force. In conclusion, the force–frequency relation is dependent on the direction of the sodium–calcium exchanger.

Bogdanov KY *et al.* (2001). *Circ Res* **88**, 1254–1258.

Layland J & Kentish JC (1999). *Am J Physiol* **276**, H9–18.

Subramani S *et al.* (2002). *Br J Pharmacol* **137**, 756–760.

All procedures accord with current national and local guidelines

PS C39

Frequency-dependent changes in sarcoplasmic reticulum Ca^{2+} content and Ca^{2+} transients in rat ventricular myocytes under physiological conditions

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The Ca^{2+} content of the sarcoplasmic reticulum (SR) is central to excitation–contraction coupling. To date, there is little information concerning SR Ca^{2+} content obtained at physiological temperatures and rates of stimulation. In this study we have used the perforated patch technique to elicit action potentials at physiological rates (4–8 Hz) in rat ventricular myocytes.

Adult male rats were humanely killed by cervical dislocation and ventricular myocytes were isolated by collagenase digestion. Changes in $[\text{Ca}^{2+}]_i$ were measured using Fluo-3. Following steady-state stimulation, the SR Ca^{2+} content was measured by switching to voltage clamp mode and rapidly applying 10 mM caffeine (Varro *et al.* 1993). All experiments were performed at 37°C. Data are presented as means \pm S.E.M. from n experiments. Statistical analysis was carried out using repeated measures ANOVA.

Action potential duration, measured at 90% repolarisation, was little altered with stimulation frequency (53 ± 4 , 60 ± 4 and 63 ± 4 ms at 4, 6 and 8 Hz; $n = 6$, not significant (n.s.)). The amplitude of the systolic Ca^{2+} transient was greater at 6 Hz ($P < 0.05$) than at 4 and 8 Hz (411 ± 98 , 315 ± 62 and 325 ± 100 nM, respectively; $n = 7$ –10). Despite this biphasic effect on the amplitude of the systolic Ca^{2+} transient, the SR Ca^{2+} content increased with stimulation frequency (72 ± 6.4 , 101 ± 8.1 and 121 ± 7.9 μM at 4, 6 and 8 Hz, respectively; $P < 0.05$ between all frequencies, $n = 8$ –9).

The decrease in the amplitude of the systolic Ca^{2+} transient on increasing frequency from 6 to 8 Hz arose through the combined effects of a fall in peak systolic $[\text{Ca}^{2+}]_i$ (655 ± 121 and 622 ± 135 nM; $P < 0.05$) and increase in diastolic $[\text{Ca}^{2+}]_i$ (245 ± 49 and 297 ± 70 nM; n.s.). Thus, under the conditions used in these experiments, which have been designed to be as physiological as possible, Ca^{2+} transient amplitude and SR Ca^{2+} content appear to dissociate at high stimulation frequencies. This may arise through frequency-dependent effects on recovery from inactivation of the L-type Ca^{2+} channel and/or SR Ca^{2+} release through the ryanodine receptor. Furthermore, endogenous chronotropic effectors, e.g. adrenergic stimulation, may additionally act on intracellular Ca^{2+} handling such that this relationship may be restored.

Varro A *et al.* (1993). *Pflugers Arch* **423**, 158–160.

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All procedures accord with current UK legislation

PS C40

Fundamental differences in male and female cardiomyocyte function can be related to sarcoplasmic reticulum calcium content and sodium–calcium exchanger activity

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It has long been recognised from whole heart preparations that intrinsic differences in cardiac function exist between males and females (Schaible & Scheuer, 1984). However, the precise subcellular mechanisms responsible remain poorly understood. Recent evidence suggests that sex hormones can alter the expression and function of key proteins involved in intracellular Ca^{2+} handling (e.g. Ren *et al.* 2003). We therefore tested the hypothesis that sex differences in cardiomyocyte function can be related to differences at the level of the sarcoplasmic reticulum (SR) and Na^{+} – Ca^{2+} exchanger (NCX).

Weight-matched male and female guinea-pigs were humanely killed and left ventricular myocytes isolated by enzymatic dissociation. Ca^{2+} transients were recorded during field stimulation in a superfusion chamber (1 Hz, 37°C) by loading cells with indo-1 AM. Electrophysiological recordings were performed using single electrode voltage-clamp.

There was no significant difference in Ca^{2+} transient amplitude between male and female myocytes (indo-1 ratios of 0.19 ± 0.01 and 0.18 ± 0.01 ; means \pm S.E.M., $n = 34$ for both sexes). However, the peak L-type Ca^{2+} current ($I_{\text{Ca,L}}$) was greater in males (-6.3 ± 0.4 pA pF $^{-1}$ ($n = 24$) cf. -5.3 ± 0.3 pA pF $^{-1}$ ($n = 27$), Student's unpaired t test, $P < 0.05$). In order to explain this apparent disparity, we measured SR Ca^{2+} contents by integrating the NCX current induced by rapid caffeine application. Female myocytes had a greater SR Ca^{2+} load (0.33 ± 0.02 pC pF $^{-1}$, $n = 41$) compared with males (0.27 ± 0.01 pC pF $^{-1}$, $n = 42$, $P < 0.01$). This could be related to differences in NCX function, as assessed by measuring the monoexponential time constant of the tail current (τ) following membrane depolarisation (-60 mV to 0 mV). Female myocytes exhibited longer tail τ compared with males (111 ± 31 ms ($n = 22$) cf. 37 ± 3 ms ($n = 18$), respectively, $P < 0.05$), implying a slower rate of Ca^{2+} extrusion via the NCX in females. In support of this, we found a longer time-to-50% decay of the Ca^{2+} transient in females compared with males (256 ± 10 ms ($n = 25$) cf. 227 ± 7 ms ($n = 29$), $P < 0.05$), which became more pronounced following thapsigargin treatment (564 ± 69 ms ($n = 14$) cf. 252 ± 11 ms ($n = 12$), $P < 0.001$).

These data demonstrate important, intrinsic differences in intracellular Ca^{2+} handling between male and female myocytes. Although $I_{\text{Ca,L}}$ is larger in males, SR Ca^{2+} load is greater in females, attributable to slower NCX activity. The end result is that of similar Ca^{2+} transient amplitudes in both sexes.

Ren J *et al.* (2003). *Am J Physiol Heart Circ Physiol* **284**, H1800–1807.

Schaible T & Scheuer J (1984). *Basic Res Cardiol* **79**, 402–412.

Financial support from the British Heart Foundation is gratefully acknowledged.

All procedures accord with current UK legislation

PS C41

Inhibition of SR Ca^{2+} uptake increases Ca^{2+} efflux generated by propagating waves of Ca^{2+} release in isolated rat ventricular myocytes

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During metabolic inhibition in Ca^{2+} -overloaded cardiac myocytes, the frequency of waves of Ca^{2+} -induced Ca^{2+} release (CICR) is decreased and their duration prolonged (Overend *et al.* 2001). These effects are due to decreased opening of the Ca^{2+} release channel (ryanodine receptor) and decreased activity of the SR Ca^{2+} -ATPase (SERCA), respectively. We have previously characterized the effects of inhibition of CICR alone (with tetracaine) on the properties of waves of CICR; waves are less frequent but larger and so generate more efflux of Ca^{2+} from the cell (Overend *et al.* 1997). In the present work we have investigated the effects of inhibition of SERCA alone in rat ventricular myocytes.

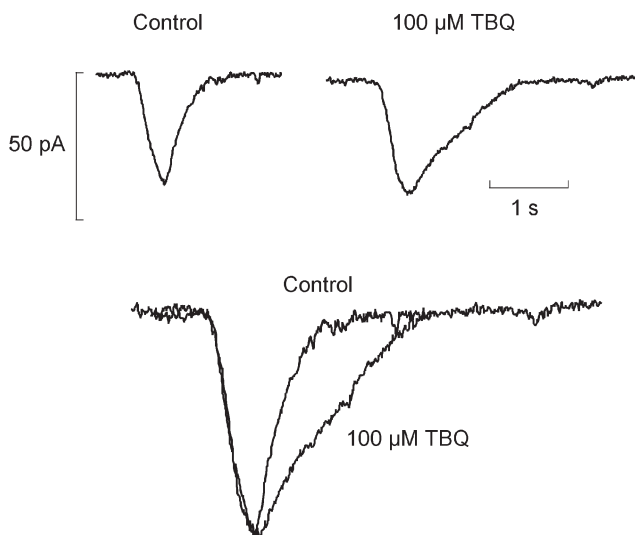


Figure 1. TBQ prolongs the transient inward current accompanying spontaneous waves of calcium release in rat ventricular myocytes.

Ventricular myocytes were isolated from the hearts of rats that were humanely killed. We used 2,5-di-tert-butyl hydroquinone (TBQ, 100 μM) to inhibit SERCA to study the effects on wave propagation. The current records in Fig. 1 are from a Ca^{2+} -overloaded rat ventricular myocyte. Each trace shows Na^{+} - Ca^{2+} exchange current activated by a wave of Ca^{2+} release propagating along the cell. The amplitude of the wave-associated current is unaffected by inhibition of SERCA but wave duration is increased. In TBQ mean current amplitude was $107.6 \pm 6.0\%$ of control (mean \pm S.E.M.; $P > 0.25$, Student's paired *t* test). In these experiments inhibition of SERCA is not complete and waves persist at a low frequency in TBQ. However, as is obvious from Fig. 1, each wave generates more efflux presumably because intracellular Ca^{2+} remains elevated for longer due to slower SR uptake. The mean current integral in TBQ increased to $230 \pm 14\%$ of control (mean \pm S.E.M.; $P < 0.001$, $n = 5$). The greater efflux of Ca^{2+} depletes the SR more, requiring more time for refilling. Therefore, Ca^{2+} efflux generated by waves is

increased by inhibition of CICR or SERCA. Moreover, the increase in wave period seems to depend only on the increase of efflux however it has been brought about. Wave period increases proportionately with Ca^{2+} efflux generated by the waves whether CICR or SERCA is inhibited.

We conclude, therefore, that even with SERCA partly inhibited, the time for refilling of the SR between waves is limited by influx of Ca^{2+} from outside the cell rather than uptake of Ca^{2+} by SERCA.

Overend CL *et al.* (1997). *J Physiol* **502**, 471–479.

Overend CL *et al.* (2001). *Circ Res* **88**, 181–187.

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All procedures accord with current UK legislation

PS C42

Effects of calsequestrin over-expression on excitation-contraction coupling in isolated rabbit cardiomyocytes

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Calsequestrin (CSQ) is a SR resident Ca^{2+} -binding protein that may also modulate other proteins involved in excitation-contraction (E-C) coupling. To evaluate the effect of CSQ over-expression on cardiac excitation-contraction (E-C) coupling, a recombinant adenovirus coding for human calsequestrin (Ad-CSQ) was used to transfect rabbit ventricular myocytes.

Rabbits were humanely killed with an overdose of Euthatal (1 mg kg^{-1}) and their hearts removed. The ventricular myocytes were isolated and placed in culture and infected with Ad-CSQ or an adenovirus coding for β -galactosidase (Ad-LacZ) as a control. Data are expressed as means \pm S.E.M. Comparisons were performed using Students' unpaired *t* test and differences were considered significant when $P < 0.05$.

Twenty-four hours after infection with Ad-CSQ (100MOI), CSQ expression was increased 1.58 ± 0.18 times (mean \pm S.E.M., $n = 10$, $P < 0.05$) as assessed by Western blots. Fura-2 loaded myocytes were voltage clamped from a holding potential of -80 mV to -40 mV (50 ms) and then to 0 mV (150 ms) every 2 s at room temperature in the presence of 5 μM TTX to inhibit inward Na^{+} current. An increased peak-systolic $[\text{Ca}^{2+}]$ was observed after Ad-CSQ transfection (329 ± 32 nM, $n = 23$ vs. 192 ± 25 nM, $n = 16$, $P < 0.05$). L-type Ca^{2+} current amplitude was also increased (3.22 ± 0.33 pA pF^{-1} , $n = 23$ vs. 1.85 ± 0.27 pA pF^{-1} , $n = 17$, $P < 0.05$). Measurements of intracellular Ca^{2+} and associated Na^{+} - Ca^{2+} exchanger current (INCX) upon rapid application of 10 mM caffeine were used to assess SR Ca^{2+} content. The integral of the INCX revealed a significantly higher SR content in the Ad-CSQ group (4.13 ± 0.42 C/F, $n = 8$) compared to the Ad-LacZ group (2.57 ± 0.39 C/F, $n = 6$). When caffeine was applied in the presence of Ni^{2+} the rate of extrusion of Ca^{2+} from the cell was significantly reduced in the Ad-CSQ transfected cells. This latter result suggests that Ca^{2+} extrusion via the sarcolemmal Ca^{2+} ATPase is reduced in the Ad-CSQ group.

In summary, CSQ over-expression has complex effects on E-C coupling including modulation of Ca^{2+} influx and efflux processes.

All procedures accord with current UK legislation

PS C43

Detection of low amplitude Ca^{2+} sparks from confocal line-scan images using permeabilised ventricular cardiomyocytes

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Ca^{2+} sparks within cardiomyocytes are a result of local sarcoplasmic reticulum (SR) Ca^{2+} release due to the transient opening of a cluster of ryanodine receptors (RyRs). Typically the characteristics of Ca^{2+} sparks within these cells are obtained by analysis of line-scan images generated by laser scanning confocal microscopy (LSCM). This is performed using epi-fluorescence excitation of the Ca^{2+} sensitive dye Fluo-3. The detection algorithm (Cheng *et al.* 1999) depends on identifying Ca^{2+} spark events as transient fluorescent signals that are greater than the mean fluorescence value plus an arbitrary amplitude value (CRI) times the standard deviation of the signal. One method, to measure the optimum CRI value is to record the signal from cells in the absence of RyR activity (using SR inhibitors). The CRI value is then set to minimise the number of false events (Lukyanenko *et al.* 2000). However, this approach will exclude small amplitude Ca^{2+} spark events from the analysis.

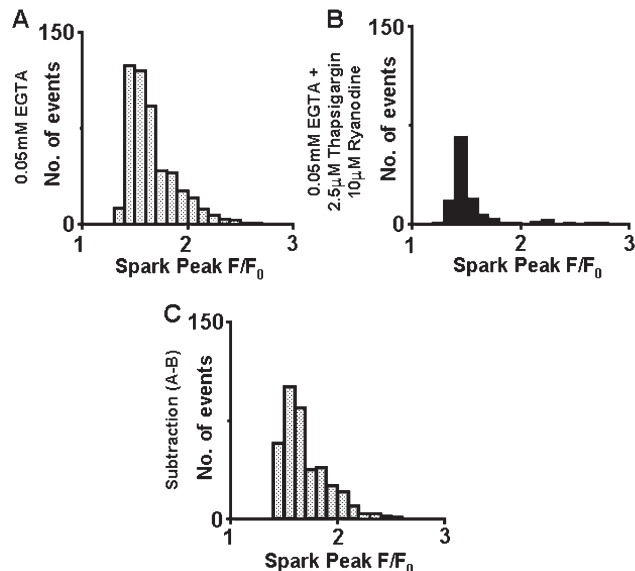


Figure 1. New Zealand White rabbits (2–2.5 kg) were humanely killed, their hearts subsequently removed and cardiomyocytes isolated by enzymatic digestion. Cells were permeabilised using 0.1 mg ml^{-1} β -escin (0.5–1 min). Amplitude distribution histograms of Ca^{2+} spark events within permeabilised rabbit cardiomyocytes (CRI 3.5). A, cells are perfused with 150 nM $[\text{Ca}^{2+}]$, 50 μM EGTA and 10 μM Fluo-3. B, the same cells ($n = 4$) are perfused with the above after the addition of 4 min of 2.5 μM thapsigargin and 10 μM ryanodine. C, subtracted of the events in panel A from panel B to give an indication of the distribution of amplitudes.

We have developed a new method that allows for detection of actual events and estimation of false positives in the same cell. This approach involves performing line-scan recordings before and immediately after the inhibition of RyR activity. Application of the detection algorithm after inhibition of RyR yields an amplitude distribution histogram that reflects purely false event detection. This profile can be subtracted from the initial recording to yield a histogram that represents the contribution of events due to RyR activity (Fig. 1). The permeabilised cardiomyocyte lends itself to this approach since intracellular $[\text{Ca}^{2+}]$ can be maintained before and after the application of SR inhibitors.

Cheng H *et al.* (1999). *Biophys J* **76**, 606–617.

Lukyanenko V *et al.* (2000). *Biophys J* **79**, 1273–1284.

All procedures accord with current UK legislation

PS C44

Modelling of Ca^{2+} wave characteristics in cardiac muscle over a range of cytoplasmic $[\text{Ca}^{2+}]$

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Ca^{2+} wave characteristics in cardiac muscle have been studied over a range of cellular Ca^{2+} values by a number of groups. In one study, the results suggest that Ca^{2+} wave properties are diverse and modulated by the Ca^{2+} loading state (Kaneko *et al.* 2000). In another, only Ca^{2+} wave frequency increases as cellular Ca^{2+} load is increased (Diaz *et al.* 1997).

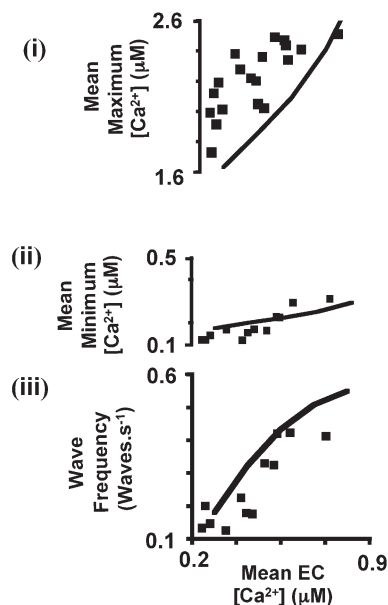


Figure 1. Relationship between mean intracellular $[\text{Ca}^{2+}]$ (IC) and various wave characteristics: (i) maximum $[\text{Ca}^{2+}]$, (ii) minimum $[\text{Ca}^{2+}]$ and (iii) wave frequency. Continuous line represents a typical relationship predicted from initial parameters measured at a mean intracellular $[\text{Ca}^{2+}]$ of 300 nM.

To study the characteristics of Ca^{2+} waves in detail, rabbit ventricular myocytes were isolated from hearts removed after

humanely killing the animal with an overdose of sodium pentobarbitone (100 mg kg⁻¹ i.v.). Spontaneous SR Ca²⁺ release was evoked in isolated myocytes permeabilised with β -escin (0.01 mg) and perfused with a mock intracellular solution containing [Ca²⁺] at 300 nM. Ca²⁺ concentration within myocytes was measured using laser-scanning confocal epifluorescence microscopy to excite the Ca²⁺-sensitive dye Fluo-5F pentapotassium salt (10 μ M). Using previous estimates of cytoplasmic Ca²⁺ buffering (Hove-Madsen *et al.* 1993) the Ca²⁺ fluxes underlying Ca²⁺ waves could be calculated from the time course of the Ca²⁺ wave. Total SR content was assessed by rapid application of caffeine (10 mM).

These calculations suggest the SR Ca²⁺ content during spontaneous release (151 ± 11 μ moles l⁻¹ cell volume, $n = 5$) is not significantly different from the total Ca²⁺ release during each wave (162 ± 7 μ moles l⁻¹ cell volume, s.e.m., $n = 5$). As shown in Fig. 1, model calculations based on initial parameters measured at 300 nM, predicted a frequency and amplitude dependence over a range of cytosolic [Ca²⁺] (300–1000 nM) that was similar to that measured experimentally (filled squares). This indicates that a common threshold mechanism explains the dependency of Ca²⁺ wave frequency and amplitude on the mean intracellular Ca²⁺ levels (up to 1 μ M).

Diaz ME *et al.* (1997). *J Physiol* **501**, 3–16.

Hove-Madsen L *et al.* (1993). *Am J Physiol* **264**, C677–686.

Kaneko T *et al.* (2000). *Circ Res* **86**, 1093–1099.

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All procedures accord with current UK legislation

PS P22

Characterization of cystine uptake in rat cardiac sarcolemmal vesicles

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Little is known about the characteristics of cystine transport and expression of cystine transporters in heart. Such knowledge is likely to be important because the cystine–glutamate exchanger (xCT) has been suggested to be a rate-limiting factor in glutathione synthesis (Sato *et al.* 1998), and glutathione plays a major role in protecting heart cells against oxidative stress (Dhalla *et al.* 2000). The aims of this study were to investigate cystine uptake in cardiac sarcolemmal vesicles under normal conditions and during ischaemia.

Male Wistar rats were humanely killed by cervical dislocation. The hearts were dissected and were either used directly for sarcolemmal vesicle preparation or perfused as Langendorff preparations before sarcolemmal vesicle preparation. Langendorff hearts were perfused at 10 ml min⁻¹ with aerated Krebs solution at 37°C (Javadov *et al.* 2000) for 45 min with (ischaemic) or without (control) 30 min global normothermic ischaemia. Homogenisation and differential centrifugation was used to prepare the vesicles as described previously with the purity of the vesicle preparation assayed by marker enzyme activity (King *et al.* 2001). L-[¹⁴C]Cystine uptake into sarcolemmal vesicles was measured by rapid filtration at room temperature (King *et al.* 2001).

There was no significant difference in the enrichment of Na⁺,K⁺-ATPase activity in vesicles from control compared to ischaemic hearts (6.67 ± 1.15 -fold control *vs.* 8.53 ± 1.01 -fold ischaemic, $n = 3$, means \pm S.E.M.). L-[¹⁴C]Cystine uptake into sarcolemmal vesicles was not sodium dependent but was affected by ischaemia and by pH. The initial rate of 0.03 mM L-[¹⁴C]cystine uptake in vesicles from ischaemic hearts was 66.18 ± 2.91 pmol (mg protein)⁻¹ s⁻¹, which was significantly greater than the 29.86 ± 10.98 pmol (mg protein)⁻¹ s⁻¹ measured in vesicles from control hearts ($P < 0.05$, Student's unpaired *t* test, $n = 3$, means \pm S.E.M.). When the pH was decreased, the initial rate of 0.1 mM L-[¹⁴C]cystine uptake increased from 10.58 ± 6.86 pmol (mg protein)⁻¹ s⁻¹ at pH 7.4 to 104.44 ± 39.73 pmol (mg protein)⁻¹ s⁻¹ at pH 6.2 ($P < 0.05$, *t* test, $n = 3 \pm$ S.D.) in vesicles from control hearts.

This work suggests that cystine uptake into rat cardiac sarcolemmal vesicles is stimulated by low pH and ischaemia.

Dhalla NS *et al.* (2000). *Cardiovasc Res* **47**, 446–456.

Javadov SA *et al.* (2000). *Cardiovasc Res* **45**, 360–369.

King N *et al.* (2001). *Cardiovasc Res* **52**, 84–94.

Sato H *et al.* (1998). *Biochim Biophys Acta*, 85–94.

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All procedures accord with current UK legislation

PS P23

Age-dependent decrease of L-type calcium channel protein expression within the sinoatrial node

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Previously, we have demonstrated the sinoatrial (SA) node contained an area lacking the gap junctional protein, connexin43 (Cx43), and that the area increased proportional to the animal's age (Jones *et al.* 2001). Ca_v1.2 provides a depolarizing current during pacemaker activity in the SA node and reduced expression would produce a decline in SA node function. Now we have investigated changes in protein expression of the L-type calcium channel (Ca_v1.2) within the SA node during ageing.

Guinea-pigs were humanely killed by anaesthetic overdose and the heart removed. The SA node was dissected from the right atrium and 14 μ m sections were dual labelled with anti-Cx43 (Chemicon, USA) and anti-Ca_v1.2 (Alomone, Israel) plus IgG conjugated to FITC/rhodamine and imaged by confocal laser-scanning microscopy. Data are shown as means \pm S.E.M.

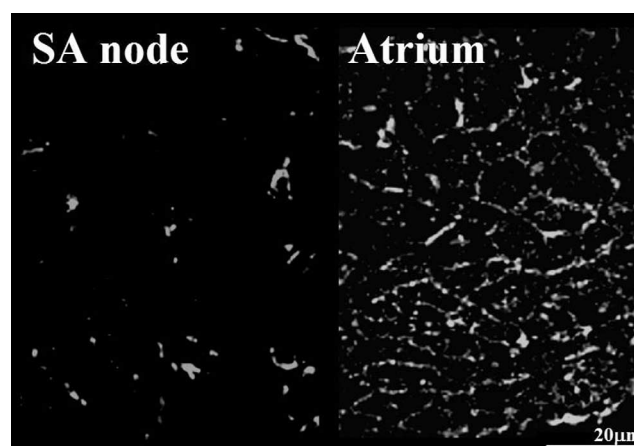


Figure 1. Expression of Cav1.2 protein. Punctate labelling was observed around the outer membrane of atrial cells, but was absent from the centre of the SA node.

Expression of Ca_v1.2 protein was observed as punctate labelling around the outer membrane of atrial cells, but was absent from the centre of the SA node (Fig. 1). The area lacking Ca_v1.2 protein significantly increased from 2.06 ± 0.1 mm² at 1 month ($n = 4$), to 6.23 ± 0.4 mm² at 18 months ($n = 3$), to 7.45 ± 0.9 mm² at 26 months ($n = 4$) and 18.72 ± 2.2 mm² at 38 months ($n = 3$) (ANOVA, $P < 0.0005$). When the SA node preparation was maintained in oxygenated Tyrode solution at 37°C, an extracellular electrode was used to record the intrinsic heart rate, which decreased with age from 249 ± 13 beats min⁻¹ at 1 day to 177 ± 4.5 beats min⁻¹ at 1 month and 152 ± 5 beats min⁻¹ at 38 months ($n = 5$; ANOVA, $P < 0.02$). However, beating of the heart was stopped by 100 μ M nifedipine at 1 day compared with 30 μ M at 1 month and 10 μ M at 38 months ($n = 5$; ANOVA, $P < 0.005$).

We conclude that Ca_v1.2 protein expression decreased in the SA node with age and this may affect the heart rate and sensitivity to nifedipine.

Jones SA *et al.* (2001). *Circulation* **104**, 643, Suppl. S.

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All procedures accord with current UK legislation

PS P24

Glutamate incubation protects isolated rat cardiomyocytes against oxidative stress

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Glutamate loading of cardiomyocytes has been shown to decrease production of reactive oxygen species (ROS) and improve recovery during simulated ischaemia (Williams *et al.* 2001; King *et al.* 2003). However in these studies glutamate loading was achieved during isolation in conditions involving reduced calcium and enzyme perfusion. The aim of this study was twofold, firstly to investigate glutamate incubation as a new method of loading isolated cardiomyocytes, and secondly to characterize the response of these cells to a novel model of oxidative stress involving exogenous H_2O_2 .

Male Wistar rats were humanely killed by cervical dislocation and the hearts dissected. Ventricular myocytes were isolated as described previously (Williams *et al.* 2001) and incubated with/without 5.12 mM potassium glutamate for 1, 2, 3 and 4 h at 20°C. The cells were placed in suspension on the stage of a fluorescence microscope at 37°C and stimulated at 0.2 Hz. Cells were superfused with a Tyrode solution containing 100 μM H_2O_2 with/without 5.12 mM potassium glutamate. Time taken for a field of myocytes to undergo arrhythmia was observed visually and confirmed using an edge-tracking device. Identical superfusion conditions were used to measure ROS. For this myocytes were loaded with 10 μM 5-(and-6)-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate (King *et al.* 2003) prior to glutamate incubation. The intracellular glutamate concentration of myocytes was measured using HPLC. Results are expressed as means \pm S.E.M.

Incubating cardiomyocytes with glutamate for 4 h significantly increased their intracellular glutamate concentration compared with controls (920.9 ± 182.0 vs. 238.5 ± 37.7 nmol (mg protein) $^{-1}$, $n = 5$, $P < 0.05$), whilst 1, 2 or 3 h incubations showed no significant increase (ANOVA). A 4 h glutamate incubation also significantly increased the time to arrhythmia in response to H_2O_2 (25.7 ± 3.4 vs. 14.6 ± 1.1 min, $P < 0.05$, ANOVA, $n = 9-12$). Fluorescence traces for a representative isolation with linear regression lines superimposed indicated a decreased rate of ROS production as the duration of incubation increased.

This work suggests that it is possible to load cardiomyocytes with glutamate by incubating them in glutamate-containing solutions for relatively long periods and that this appears to protect against oxidative stress.

King N *et al.* (2003). *J Mol Cell Cardiol* (in press).

Williams H *et al.* (2001). *J Mol Cell Cardiol* **33**, 2109–2119.

This work was supported by the BHF.

All procedures accord with current UK legislation

PS P25

ER retention of mutant KCNQ1 K⁺ channels expressed in CHO-K1 cells

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Hereditary mutations of KCNQ1 K⁺ channels can lead to the cardiac long QT syndromes, Jervell and Lange-Nielson (JLNS) or Romano-Ward (RWS) syndromes. KCNQ1 is known to mediate cardiac slowly activating delayed rectifier current (I_{Ks}) although it is not clear how mutations in the channel decrease channel function. The aim of this study was to investigate whether the intracellular retention of mutant KCNQ1 channels in the endoplasmic reticulum (ER) is a feasible mechanism to explain the decreased KCNQ1 channel function observed in JLNS/RWS.

Enhanced green fluorescent protein (EGFP) was cloned onto the C-terminus of the long isoform of KCNQ1 and transfected into CHO-K1 cells with or without the accessory subunit, IsK. ER localisation was determined by co-transfection of pDsRed2-ER (an ER-retained red fluorescent protein, Clontech, UK). Images were acquired with a Bio-Rad Radiance 2000 scanning laser confocal microscope. Co-localisation of green and red fluorescence was quantified using LaserPix software (Bio-Rad, UK). Due to variability of transfection efficiency, data are compared to controls recorded in parallel. Data are expressed as means \pm S.E.M. Statistical analysis was performed using Student's unpaired *t* test or one-way ANOVA, as appropriate.

Expression of wild-type KCNQ1 resulted in $37.3 \pm 2\%$ ($n = 44$) of the protein being retained in the ER. Co-expression of IsK significantly reduced this to $27.0 \pm 2\%$ ($n = 47$, $P < 0.05$). When mutant channels were expressed with IsK, of six mutants screened, R243H, E261D, R518X, Q530X, 1008delC and R594Q, all showed significantly increased ER retention compared to KCNQ1+IsK (range 46–53%, $n = 15-26$, $P < 0.001$). A seventh mutant, L273F, also showed increased ER retention, although this was not significantly different from the KCNQ1+IsK control ($36.2 \pm 4\%$, $P < 0.05$, $n = 28$). Incubation of cells at 27°C for 24 h which has previously been shown to recover trafficking of some HERG channel mutants (Zhou *et al.* 1999), did not significantly reduce ER colocalisation of any of the mutants tested ($n = 6-14$). In other experiments, 24 h incubation of channel-modulating drugs had no significant effect on retention of R243H channels being $38.0 \pm 4\%$, $43.3 \pm 3\%$ and $37.2 \pm 5\%$ ER retained in the presence of HMR1556 (100 nM), chromanol 293B (10 μM) and mefenamic acid (100 μM), respectively (control, $38.1 \pm 1.0\%$, $n = 6-15$).

ER retention of KCNQ1 channels is a potential mechanism by which mutations could cause JLNS/RWS. It is not known if this reflects a defect in trafficking ability *per se*, or whether mutations abrogate the ability of the channel protein to interact with IsK. Unlike some mutant HERG channels, KCNQ1 trafficking ability does not appear to be sensitive to either temperature or the presence of pharmacological channel modulators.

Zhou Z *et al.* (1999). *J Biol Chem* **274**, 31123–31126.

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PS P26

Glutamate loading protects isolated cardiomyocytes against oxidative stress by stimulating glutathione peroxidase

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Glutamate loading has been shown to protect single isolated perfused cardiomyocytes against metabolic inhibition and wash-off (Williams *et al.* 2001). The mechanism underlying this protection is unknown. The aim of this study therefore was to test the hypothesis that glutamate loading protects isolated cardiomyocytes during oxidative stress by stimulating the glutathione redox system.

Male Wistar rats were humanely killed by cervical dislocation and the hearts dissected. Ventricular myocytes were isolated with/without 6.4 mM potassium glutamate to stimulate glutamate loading and then loaded with 10 μ M 5-(and-6)-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate (CM-H₂DCFda) as described previously (King *et al.* 2001; Williams *et al.* 2001). Cellular production of reactive oxygen species (ROS) during oxidative stress caused by exposure to 30 μ M H₂O₂ was measured as increases in CM-H₂DCF fluorescence. Reduced glutathione (GSH) levels and the activity of glutathione reductase were measured in control and glutamate-loaded cells with/without exposure to 30 μ M H₂O₂. Finally, the effect of glutamate on glutathione peroxidase activity was measured *in vitro*. All glutathione-related measurements were performed using Calbiochem kits.

Following 15 min exposure to 30 μ M H₂O₂, the control cells were arrhythmic and their fluorescence had increased from 100% to $225.1 \pm 23\%$ ($n = 3$, mean \pm S.E.M., $P < 0.05$, Student's unpaired *t* test). This contrasted with the glutamate-loaded cells which after the same insult were still responding regularly to stimulation and showing no significant change in fluorescence (100% vs. $91.9 \pm 10.2\%$, $n = 3$, mean \pm S.E.M.) suggesting that in these cells the H₂O₂ was removed as rapidly as it entered. GSH levels in glutamate-loaded cells were reduced by 2.0 ± 0.2 nmol (mg protein)⁻¹ ($n = 3$, mean \pm S.E.M.) during H₂O₂ exposure, which was significantly greater than the 1.3 ± 0.2 nmol (mg protein)⁻¹ ($P < 0.05$, unpaired *t* test, $n = 3$, mean \pm S.E.M.) decrease in control cells. This was accompanied by a concentration-dependent stimulation of glutathione peroxidase activity by glutamate *in vitro*. However, there was no difference in glutathione reductase activity between control and glutamate loaded cells before or after H₂O₂ exposure.

These results provide new evidence for a novel cardioprotective mechanism against oxidative stress in which increased intracellular glutamate directly stimulates the activity of glutathione peroxidase. This would lead to an increased capacity to eliminate ROS in the cell.

King N *et al.* (2001). *J Physiol* **533P**, 23P.Williams H *et al.* (2001). *J Mol Cell Cardiol* **33**, 2109–2119.

This work was supported by the British Heart Foundation.

All procedures accord with current UK legislation

PS P27

Inhibition by procainamide and N-acetyl procainamide of HERG K⁺ channels expressed in a mammalian cell line

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Procainamide (Proc) is a Class Ia antiarrhythmic agent used in the treatment of both ventricular and atrial arrhythmias. Its use has been linked with the production of *torsades de pointes* arrhythmias (Lazzara, 1993). Although Proc has been suggested to inhibit HERG (human *ether-à-go-go*-related gene) K⁺ channels (Sesti *et al.* 2000; Yang *et al.* 2001) there has been little detailed study of its blocking characteristics to date.

Measurements of whole cell HERG current (I_{HERG}) were made at $37 \pm 1^\circ\text{C}$ from a mammalian cell line (HEK-293) stably expressing HERG (Zhou *et al.* 1998). I_{HERG} 'tail' amplitude was monitored at -40 mV, following activating pulses to $+20$ mV.

Procainamide was found to exert a concentration-dependent inhibitory effect on I_{HERG} , with a mean half-maximal inhibitory concentration (IC₅₀) of 138.7 μ M ($n =$ minimum of 5 cells for each of six Proc concentrations between 1 μ M and 100 mM; 95% confidence intervals 115.5–166.7 μ M). Inhibition of I_{HERG} by 150 μ M Proc was found to be voltage dependent and also depended on duration of the applied voltage command. Data from a three-step protocol from -80 mV (2 s at $+20$ mV, 4 s at $+80$ mV, 4 s at $+20$ mV) suggested that the level of inhibition was attenuated at a highly positive voltage favouring I_{HERG} inactivation. Collectively, the data obtained suggest that Proc inhibits I_{HERG} in a time- and voltage-dependent fashion favouring the activated over inactivated channel state. In our hands, the major metabolite of Proc, N-acetyl procainamide (NAPA), inhibited I_{HERG} less potently than the parent compound (150 μ M NAPA inhibited I_{HERG} by $23.3 \pm 3.8\%$ (mean \pm S.E.M.) $n = 5$ cf. $61 \pm 3.0\%$ with 150 μ M Proc, $n = 6$; $P < 0.0001$; Student's unpaired *t* test; cf. Yang *et al.* 2001). The observed IC₅₀ for I_{HERG} inhibition by Proc exceeds the clinical concentration range (17–35 μ M, Yang *et al.* 2001), suggesting that neither HERG blockade by Proc itself nor HERG blockade by Proc's major metabolite can readily account for the observed pro-arrhythmia with the drug.

Lazzara R (1993). *Eur Heart J* **14**, Suppl. H, 88–92.Sesti F *et al.* (2000). *Proc Natl Acad Sci U S A* **97**, 10613–10618.Yang T *et al.* (2001). *J Cardiovasc Pharmacol* **38**, 737–744.Zhou Z *et al.* (1998). *Biophys J* **74**, 230–241.

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PS P28

Differential responses at 37°C to insulin of L-type calcium current and cell shortening from guinea-pig isolated ventricular myocytes

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Insulin is an important metabolic hormone that influences ion transport mechanisms and the inotropic state of the heart (Hiraoka, 2003). At room temperature insulin has been reported to increase L-type calcium current ($I_{Ca,L}$) in human atrial and rat ventricular isolated myocytes (Aulbach *et al.* 1999; Maier *et al.* 1999) and this effect could contribute to its influence on cardiac contractility. However, we have recently shown that the effect of insulin on $I_{Ca,L}$ may in fact vary with temperature: a stimulatory effect is observed at room temperature and an inhibitory one at 37°C (Pabbathi *et al.* 2002). The present study was designed to see whether the inotropic effect of insulin is observed in myocytes under experimental conditions expected to result in an inhibitory effect on $I_{Ca,L}$.

Adult male guinea-pigs were killed by cervical dislocation and ventricular myocytes were isolated by a combination of mechanical and enzymatic dispersion. Ionic current measurements were made using the whole-cell patch clamp technique and cell contraction was assessed videometrically by measuring unloaded cell-shortening. Experiments were performed at physiological temperature (37°C).

Under conditions selective for $I_{Ca,L}$ (Cs⁺-based pipette dialysate), peak $I_{Ca,L}$ at a test potential of +10 mV was observed to be smaller in cells pre-incubated in 1 μ M insulin than in insulin-free controls. Dual measurements of membrane current and unloaded shortening were then made (using a K⁺-based pipette dialysate). Lidocaine at 200 μ M was present in the external solution to block residual Na current, and peak $I_{Ca,L}$ and cell shortening were measured during a voltage step from –40 mV to +10 mV. In control cells, $I_{Ca,L}$ amplitude was -2.52 ± 0.4 nA ($n = 6$; mean \pm S.E.M.) and cells contracted by $6.37 \pm 0.87\%$ of resting cell length (mean \pm S.E.M.). In cells pre-incubated in 1 μ M insulin and continuously superfused with insulin-containing solution during recording, $I_{Ca,L}$ amplitude was -1.30 ± 0.26 nA ($n = 6$; $P < 0.05$ compared to control, Student's unpaired *t* test), whilst cells contracted to $10.25 \pm 1.05\%$ of resting cell length ($P < 0.05$ compared to control).

This work suggests that under conditions in which insulin decreases $I_{Ca,L}$ it can nevertheless increase cell shortening.

Aulbach F *et al.* (1999). *Cardiovasc Res* **42**, 113–20.Hiraoka M (2003). *Circ Res* **92**, 707–709.Maier S *et al.* (1999). *Cardiovasc Res* **44**, 390–397.Pabbathi VK *et al.* (2002). *Pflugers Archiv* **443S**, S350, 484.

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All procedures accord with current UK legislation

PS P29

Sex-related differences in ionic currents of guinea-pig ventricular myocytes

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It is well known that women have a higher resting heart rate than men, but the rate-corrected QT interval of the electrocardiogram is significantly longer in women than in men (Bazett, 1920). The basis for this sex difference is unclear, but is likely to reflect the influence of the sex hormones on ventricular repolarisation. Previous reports using the rabbit suggest a key role for testosterone in somehow shortening ventricular repolarisation (Pham & Rosen, 2002). On the other hand, the influence of oestrogen and progesterone remains unclear, partly due to the abbreviated nature of the oestrus cycle in female rabbits. We have therefore recently suggested that the guinea-pig, which has an oestrus cycle similar to the human, may be useful for the investigation of the influence of the sex hormones on ventricular repolarisation (James & Hancox, 2003). However, at present there is no information available concerning the existence of sex differences in ventricular repolarisation in the guinea-pig. The objective of this study was to investigate sex-related differences in ion currents and action potentials recorded from isolated guinea-pig ventricular myocytes.

Male and female guinea-pigs were humanely killed and ventricular myocytes isolated by enzymatic and mechanical dispersion. Oestrus cycles of females were monitored by examination of the vulva and the vaginal membrane and ventricular myocytes isolated on the day of oestrus (day 0) and 4 days post-oestrus (day 4). Myocytes were superfused with a standard Tyrode solution (pH 7.35, 35°C) and ion currents and action potentials recorded by whole-cell patch-clamp using a K⁺-rich pipette solution containing EGTA. Currents were normalised for cell size. Data are expressed as means \pm S.E.M. and statistical comparisons made by one-way ANOVA with Student-Newman-Keuls *post-hoc* test. $P < 0.05$ was accepted as significant.

The L-type Ca²⁺ current (I_{Ca}) measured as the peak inward current on depolarisation to +10 mV (holding potential = –40 mV) was significantly larger in male myocytes (-7.41 ± 0.8 pA pF^{–1}, $n = 12$) than in day 0 (-5.5 ± 0.5 pA pF^{–1}, $n = 11$) and day 4 (-4.1 ± 0.3 pA pF^{–1}, $n = 10$) female myocytes. Moreover, I_{Ca} differed significantly between day 0 and day 4 female myocytes, strongly suggesting that I_{Ca} density varies around the oestrus cycle. The density of inward rectifier K⁺ current (I_{K1}) measured at –120 mV was also significantly greater in male myocytes than in female myocytes either at day 0 or day 4. Delayed rectifier (I_K) tail currents measured following pulses to +20 mV were significantly different between male and female day 4 myocytes, but not between male and female day 0 myocytes. Action potential duration at 90% repolarisation (APD₉₀) was significantly shorter in male myocytes than in female myocytes at day 0, but not at day 4, consistent with the combined differences in I_K and I_{Ca} between the three groups.

Taken together, these findings demonstrate that there are sex-related differences in ventricular repolarisation in guinea-pig ventricular myocytes.

Bazett H (1920). *Heart* **7**, 353–370.James AF & Hancox JC (2003). *Cardiovasc Res* **57**, 1–4.

Pham TV & Rosen MR (2002). *Cardiovasc Res* **53**, 740–751.

All procedures accord with current UK legislation

PS P30

Arachidonic acid inhibits spontaneous waves of Ca^{2+} release in rat ventricular isolated myocytes without inhibiting the Ca^{2+} release mechanism

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During myocardial ischaemia free fatty acids accumulate in the ischaemic tissue, released by phospholipase A2 (Ford *et al.* 1991). The $n-3$ class of polyunsaturated fatty acids (PUFAs) are known to have anti-arrhythmic properties at least part of which is due to direct inhibitory effects on the Ca^{2+} release mechanism of the sarcoplasmic reticulum (SR) and a consequent reduction in the frequency of arrhythmogenic waves of Ca^{2+} -induced Ca^{2+} release (CICR; O'Neill *et al.* 2002). However, $n-3$ PUFAs can only be released if they have been previously present in the diet. In their absence the $n-6$ PUFAs are released in greater amounts. The anti-arrhythmic properties of $n-6$ PUFAs are much less clear than those of the $n-3$ class of fatty acid. Some reports have shown inhibition of delayed after-depolarizations but the mechanism is not yet known (Mamas & Terrar, 2001). We have used the $n-6$ PUFA arachidonic acid to determine if there is an effect on arrhythmogenic waves of CICR and, if so, where it is exerted.

In voltage clamp experiments using the perforated patch, we have measured transient inward currents activated by propagation of waves of CICR in Ca^{2+} overloaded rat ventricular myocytes isolated following humane killing. The frequency of these waves is lower in the presence of $50 \mu\text{M}$ arachidonic acid. Much previous work has shown that changes in wave frequency brought about by modulation of the sensitivity of the Ca^{2+} release mechanism are associated with changes in SR Ca^{2+} content (e.g. Overend *et al.* 1997). In the presence of arachidonic acid, SR Ca^{2+} content rises by $12.3 \pm 4.0\%$ (mean \pm S.E.M., $n = 5$; $P < 0.03$ Student's paired t test). Although this increase is statistically significant, it is moderate in size. The $n-3$ class of PUFA can increase SR content by 50% at lower concentrations than those used here for arachidonic acid.

We conclude that $n-6$ PUFAs have two effects that reduce the frequency of waves of CICR: a weak inhibitory effect on the ryanodine receptor and, more importantly, a reduction of Ca^{2+} influx. These two mechanisms are also involved when $n-3$ PUFAs reduce the frequency of waves of Ca^{2+} release but the majority of the effect is due to inhibition of CICR (O'Neill *et al.* 2002).

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Overend CL *et al.* (1997). *J Physiol* **502**, 471–479.

All procedures accord with current UK legislation

PS P31

Is CellTracker Blue a good tool for measuring reduced glutathione levels in single isolated and perfused cardiomyocytes?

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Following ischaemia and reperfusion of isolated rat hearts, the level of tissue damage and functional recovery is aggravated by glutathione depletion (Blaustein *et al.* 1989) and improved by glutathione addition (Seiler *et al.* 1996). This suggests a strong correlation between glutathione level and outcome and also highlights the importance of having an accurate method of monitoring the glutathione content of heart cells during various insults and interventions. In this study we have assessed the ability of a new fluorescent dye, CellTracker Blue, for measuring the concentration of reduced glutathione (GSH) in single isolated and perfused cardiomyocytes.

Male Wistar rats were humanely killed by cervical dislocation and the hearts dissected. Ventricular cardiomyocytes were isolated as described previously (Williams *et al.* 2001). Loading of $2.4 \mu\text{M}$ CellTracker Blue was achieved by incubating the cells for 10 min with the dye in the dark at room temperature, followed by centrifugation and suspension of the pellet in fresh dye-free solution. Changes in CellTracker Blue fluorescence during oxidative stress were measured in Tyrode solution plus 1 mM H_2O_2 on a fluorescence microscope with excitation at 350 nm and emission at 460 nm. All cells were maintained at 37°C and stimulated at 0.2 Hz. A GSH calibration curve was obtained and the effect of different calcium, pH and glutathione-S-transferase concentration was measured during perfusion with dye-containing solutions.

CellTracker Blue was sensitive to GSH concentration and showed good cellular retention. Assuming a normalised fluorescence of $100 \pm 5\%$ at pH 7.0, the fluorescence was not significantly altered by changes in pH, being $100.4 \pm 5.5\%$ at pH 7.2 and $112.5 \pm 10.9\%$ at pH 7.5 ($n = 3$, means \pm S.E.M., ANOVA). There was also no significant difference in fluorescence when 0.2, 0.4 or 0.8 U ml^{-1} of glutathione-S-transferase or when 0, 100 nM or 10 mM calcium was used. Under normal conditions, assuming a cell volume of 1.9 ml (Boyett *et al.* 1991), the mean GSH concentration of single isolated and perfused rat cardiomyocytes was $1.7 \pm 0.1 \text{ mM}$. During oxidative stress, the GSH concentration decreased significantly to $1.1 \pm 0.2 \text{ mM}$ ($P < 0.02$, Student's paired t test, $n = 7$, mean \pm S.E.M.).

This work suggests CellTracker Blue may make a good tool for monitoring the GSH concentration in isolated cells.

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Boyett MR *et al.* (1991). *Exp Physiol* **76**, 259–270.

Seiler KS *et al.* (1996). *Free Rad Res* **24**, 115–122.

Williams H *et al.* (2001). *J Mol Cell Cardiol* **33**, 2109–2119.

This work was supported by the British Heart Foundation.

All procedures accord with current UK legislation

PS P32

The effects of catecholamines on functional recovery and interleukin-6 synthesis in the intact post-ischaemic rat heart

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Pro-inflammatory cytokines (e.g. interleukin-6) have been implicated in damage associated with cardiopulmonary bypass during open-heart surgery (Zahler *et al.* 1999). Recent evidence has suggested that catecholamines and interleukin-6 (IL6) may play a role in mechanisms leading to cardiac hypertrophy in the post-ischaemic heart (Burger *et al.* 2001). Previous studies performed on isolated neonatal rat cardiac myocytes have shown that sympathetic stimulation at supraphysiological levels increases the release of IL6 during reperfusion (Yamauchi-Takahara *et al.* 1995). In the present study we examined the effect of physiological concentrations (0.1, 1 or 5 ng ml⁻¹) of noradrenaline (NA) and adrenaline (Adr) on functional recovery and the concentration of IL6 post-ischaemia in the intact rat heart.

Male wistar rats were humanely killed, and the hearts were removed and perfused in the Langendorff mode. Hearts were exposed to 45 min global normothermic ischaemia followed by 60 min reperfusion in the presence and absence of NA or Adr. Left ventricular developed pressure (LVDP) was measured throughout. Enzyme-linked immunosorbent assay was used to measure the concentration of IL6 protein in heart tissue. All results are expressed means \pm S.E.M. and analysed using ANOVA with Fischer's *post hoc* test (PLSD). $n \geq 3$.

In the absence of any drug, ischaemia resulted in 43 % recovery in LVDP. Reperfusion with NA resulted in no recovery but treatment with Adr (1 ng ml⁻¹) produced a 95 % recovery ($P < 0.05$ versus control). Ischaemia and reperfusion caused no change in IL6 protein with 2.504 ± 0.176 pg ml⁻¹ μ g⁻¹ in the perfusion control and 2.619 ± 0.116 pg ml⁻¹ μ g⁻¹ with reperfusion. There was an increase in the presence of Adr to 4.242 ± 0.729 pg ml⁻¹ μ g⁻¹ ($P < 0.01$) when reperfusing with Adr.

This work shows that NA and Adr have differential effects on recovery following an ischaemic insult. Adr significantly improved functional recovery, which was associated with increased levels of IL6 in the rat heart. Whether the production of IL6 contributes to protection requires further investigation.

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All procedures accord with current UK legislation

PS P33

The effect of systemic perfusion temperature on cytokine release in paediatric cardiac surgery

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Normothermic systemic perfusion (35–37 °C) during cardiopulmonary bypass has been shown to lead to reduced

inflammatory response in adults undergoing cardiac surgery (Birdi *et al.* 1999). Paediatric cardiac surgery is routinely performed under hypothermic conditions (18–28 °C). We studied the effects of cardiopulmonary bypass perfusion temperature on inflammatory (interleukins 6 and 8) and anti-inflammatory (interleukin 10) cytokine response in paediatric cardiac surgery. This cytokine release has previously been documented (Brix-Christensen, 2001) and is known to jeopardise postoperative outcome (Seghaye *et al.* 1993) but the link with perfusion temperature remains unclear.

Having obtained ethical approval and informed consent, 30 patients, matched for age and with similar underlying pathology, were assigned to normothermic or hypothermic (28 °C) cardiopulmonary bypass. Anaesthesia was induced with midazolam (200 to 500 μ g kg⁻¹) or sevoflurane and pancuronium (200 μ g kg⁻¹) and then maintained using a fentanyl infusion of 15 μ g kg⁻¹ h⁻¹. Cytokine release was assessed by measuring interleukins 6, 8 and 10 pre-, intraoperatively and up to 24 h post-operatively using an ELISA assay (Amersham Biosciences).

Cardiopulmonary bypass times were similar in the two groups as were preoperative values of all interleukins for both groups. There was a time-dependent release of IL-6 that was similar in both groups except at 24 h where the level (mean \pm S.E.M.) was significantly higher in the normothermic group (16.16 ± 5.90 vs. 5.34 ± 1.94 pg ml⁻¹, $P = 0.049$, Mann-Whitney *U* test). The release of interleukin 8 was higher overall in the hypothermic group, especially at 6 h after bypass where it tended towards statistical significance (22.79 ± 4.44 vs. 13.16 ± 2.65 pg ml⁻¹, $P = 0.057$). The release of interleukin 10 was no different for the two groups. Maintaining normothermia leads to less inflammatory cytokine release both intra- and post-operatively. This is likely to lead to better functional recovery post-paediatric cardiac surgery.

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Brix-Christensen V (2001). *Acta Anaesthesiol Scand* **45**, 671–679.

Seghaye MC *et al.* (1993). *J Thorac Cardiovasc Surg* **106**, 978–987.

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All procedures accord with current local guidelines and the Declaration of Helsinki

PS P34

Effect of cardiopulmonary bypass perfusion temperature on oxidative stress in paediatric cardiac surgery

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Paediatric cardiac surgery is performed under systemic hypothermic (28 °C) conditions. The reason for this is to protect organs against the damaging effects of cardiopulmonary bypass (Brix-Christensen, 2001). However, the link between temperature and oxidative stress is not known. In this study we compared oxidative stress as measured by 8-iso-prostane release (Morrow *et al.* 1997) in paediatric patients undergoing cardiac surgery, randomised to either normothermic (35–37 °C) or hypothermic systemic perfusion (cardiopulmonary bypass).

Having obtained ethical approval and patients' informed consent, 15 patients scheduled for corrective cardiac surgery, matched for age and with similar underlying pathology, were recruited. Anaesthesia was induced with sevoflurane or

midazolam ($200\text{--}500\text{ }\mu\text{g kg}^{-1}$), supplemented with pancuronium ($200\text{ }\mu\text{g kg}^{-1}$). Blood samples were collected before, during and after cardiopulmonary bypass (up to 24 h later). These were immediately centrifuged, the plasma frozen in liquid nitrogen, and then stored at -80°C for no more than 2 weeks, prior to analysis (8-iso-prostane EIA, Caymen Chemicals).

Cardiopulmonary bypass duration was similar in the two groups. There was a time-dependent release of 8-iso-prostane in the hypothermic group, peaking at the end of cardiopulmonary bypass. The overall release was much higher in the hypothermic group (23.25 ± 6.713 vs. $8.47 \pm 2.64\text{ pg ml}^{-1}$, means \pm S.E.M., $P = 0.16$, Mann-Whitney U test). The result was not statistically significant due to the large standard errors and small sample size, but this represents preliminary data. With further patients recruited it is expected that this result will tend towards significance.

Normothermic, compared to hypothermic, systemic perfusion was associated with overall lower 8-iso-prostane release and hence decreased oxidative stress in paediatric patients undergoing corrective cardiac surgery. This will lead to less postoperative morbidity and quicker recovery.

Brix-Christensen V (2001). *Acta Anaesthesiol Scand* **45**, 671–679.

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PS P35

Experimental and computer models of mechanically heterogeneous myocardium

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The physiological significance of the mechanical heterogeneity of myocardium in the regulation of its contractile function is not yet clear. We developed a new experimental approach to investigate this question, an In-Series Hybrid Duplex, which joined the family of earlier implemented duplexes of different configurations (Markhasin *et al.* 2003).

The hybrid duplex combines a biological cardiac muscle preparation with a 'virtual muscle' (a mathematical model), which interact in real-time, mimicking mechanical connection in-series. In-series elements actually influence each other during the isometric mode of duplex contractions, where the special control algorithm supports the equality of forces of natural and virtual muscles, at the constant sum of their lengths. Parameters of virtual muscles were selected to make uncoupled duplex elements different in their mechanical characteristics of contractions (Fig. 1A, 'fast' and 'slow' muscles), such as time to peak force.

For experiments all animals were anaesthetized with a lethal dose of pentobarbital (150 mg kg^{-1}). Results obtained in hybrid duplexes were compared with the activity of pure virtual combinations. In addition to the duplex settings, 1D models of heterogeneous cardiac tissue composed of serial chains of virtual elements have also been studied. Mechanical parameters of the chain elements varied between extreme values, which were used for the fast and the slow element of corresponding duplexes. Two types of heterogeneity were considered – (i) a regular pattern (gradual change in mechanical properties from the fast boundary element to the slow one), and (ii) a random array (uniform distribution of properties within the chain).

Mechanical effects of a time lag between stimulation of the elements imitating excitation propagation in heart tissue were investigated. Hybrid duplexes typically showed a stable (even increasing) contractile response on the stimulation delay of a faster element. On the contrary, the stimulation delay of a slower partner caused a steep decrease in contractility. The results confirmed predictions, which were made in virtual duplexes merging fast and slow elements (Fig. 1B). Mechano-dependent changes in Ca^{2+} handling (kinetics of Ca^{2+} -troponin C complexes, Ca^{2+} transients) occurring in virtual elements of hybrid duplexes due to the stimulation sequences were very similar to those obtained in the corresponding elements of virtual duplexes, thus validating model assumptions (Fig. 1C). 1D models of cardiac tissue with 'regular' mechanical heterogeneity revealed stable contractility with an increasing delay (entrance-phase) in the activation of boundary elements if excitation had propagated from the slowest element to the fastest one. Inverted excitation sequence of the same models led to a decrease in force generation (Fig. 1D). Isometric force produced by the random distribution models fell between the forces generated by homogeneous samples, which consisted of either only fast or only slow elements (Fig. 1D). In these models, an increase in the time lag in stimulation of boundary elements was followed by decreasing peak force.

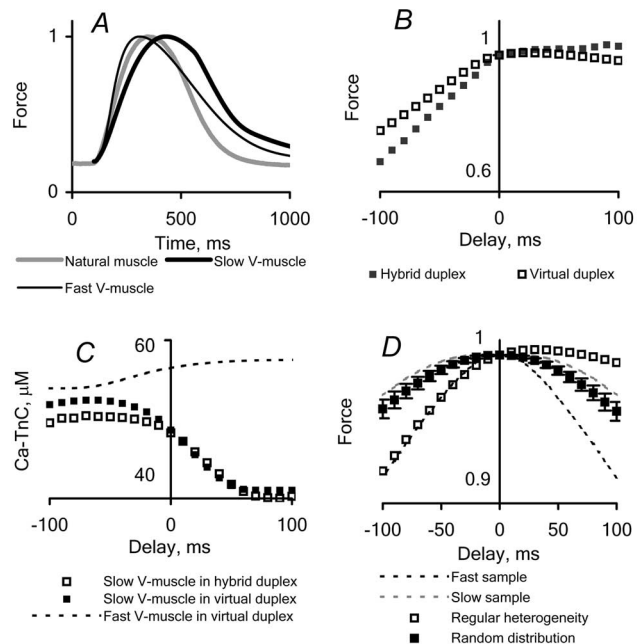


Figure 1. Effect of excitation sequence on inotropic characteristics of heterogeneous myocardium models. A, time courses of isometric force development in the individual elements used to form duplexes. B, peak force generation in heterogeneous duplexes during a series of stimulation delays (positive delay time is for a faster element, and vice-versa). The Hybrid duplex = the Natural muscle (trabeculum from rat right ventricle) and the Slow Virtual (V) muscle, and the Virtual duplex = the Fast and the Slow V-muscles. C, peak $[\text{Ca-TnC}]$ in the elements of the heterogeneous duplexes. D, peak force generation in 1D models of cardiac tissue with either regular or random heterogeneity patterns in comparison with homogeneous samples.

The results obtained show that contractile elements in heterogeneous myocardium do not act as 'independent' generators of tension/shortening because their inotropic characteristics change dynamically due to mechanical

interaction. We conclude that special spatio-temporal heterogeneity is a *physiological necessity* to optimize cardiac performance. Alterations of the physiological heterogeneity (including inverted sequence of activation) will have pathological consequences.

Markhasin VS *et al.* (2003). *Prog Biophys Mol Biol* **82**, 207–220.

All procedures accord with current national and local guidelines and the Declaration of Helsinki

PS P36

Myocyte/fibroblast 2D structured cardiac tissue models

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Cultures of neonatal cardiac myocytes are widely used as *in vitro* cardiac tissue models. Fibroblasts, which constitute one of the largest cell populations in the heart, are usually considered a 'nuisance' in these models, even though they are important in biochemical, mechanical and, possibly, electrical signalling *in situ*. In rabbit sino-atrial node tissue, fibroblasts express connexin40 (Cx40) and connexin45 (Cx45), and the latter appears to be involved in fibroblast–myocyte coupling (Camelliti *et al.* 2002). In neonatal cell culture, myocytes and fibroblasts also form functional gap junctions, which have been attributed to Cx43 (Rook *et al.* 1992). A systematic study, however, of *in vitro* fibroblast–myocyte and fibroblast–fibroblast coupling has not yet been performed, and would require both connexin labelling and cell type identification.

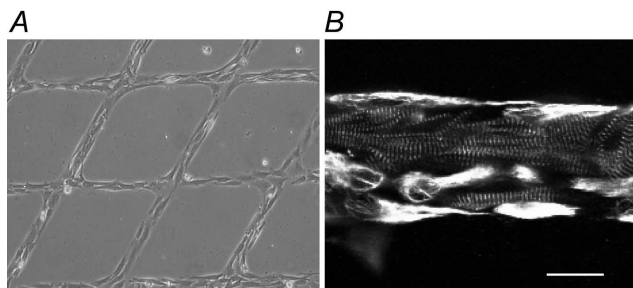


Figure 1. A, 2D micro-patterned neonatal rat myocytes (line width 28 μm). B, cell type identification by immunolabelling for myomesin (striated pattern – myocytes) and vimentin (bright full cells – fibroblasts). Scale bar: 20 μm .

In the present study, we use a micro-fluidic technique, developed by the UCSD lab of Andrew McCulloch (Gopalan *et al.* 2003), to deposit a 2D criss-cross pattern of intersecting lines (28 μm wide) of extracellular matrix proteins (here type I collagen). Cell suspensions from heart of humanely killed neonatal rats containing either preferentially fibroblasts or a mixture of myocytes and fibroblasts are seeded. This gives rise to a 2D cardiac tissue model (Fig. 1A) with improved cyto-architectural properties of both myocytes and fibroblasts (Fig. 1B). Using triple immunocytochemical labelling (anti-vimentin antibodies to mark fibroblasts, anti-myomesin antibodies for myocytes, and anti-Cx40, anti-Cx43 or anti-Cx45 antibodies for gap junctions) and confocal microscopy we studied gap junction localisation relative to coupled cell types. We found sparse punctate Cx40, Cx43 and Cx45 labelling between homologous cell types, both in fibroblast cultures and fibroblast myocyte co-cultures. Cx43 and, to a lesser extent, Cx45 appeared to also be involved in heterologous fibroblast–myocyte coupling.

Thus, cardiac fibroblasts *in vitro* express multiple connexin isoforms and form gap junctions at the point of contact both with fibroblasts and myocytes, via Cx43 or Cx45. Taking into account the overall low density of connexin immunolabelling found in this study, we cannot rule out contributions by other connexin isoforms to cell communication in this 2D structured cardiac tissue models.

Camelliti P *et al.* (2002). *J Physiol* **544**, P, 21S.

Gopalan SM *et al.* (2003). *Biotechnol Bioeng* **81**, 578–87.

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We thank Prof. Andrew McCulloch for access to micro-patterning tools and the BBSRC for financial support. PK is a Royal Society Research Fellow.

All procedures accord with current UK legislation

PS P37

Pulse wave analysis of β_2 -agonist-mediated response in hypertension

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β_2 -Adrenoreceptors activate the L-arginine–nitric oxide pathway in human vasculature and may play an important role in the regulation of vascular function.

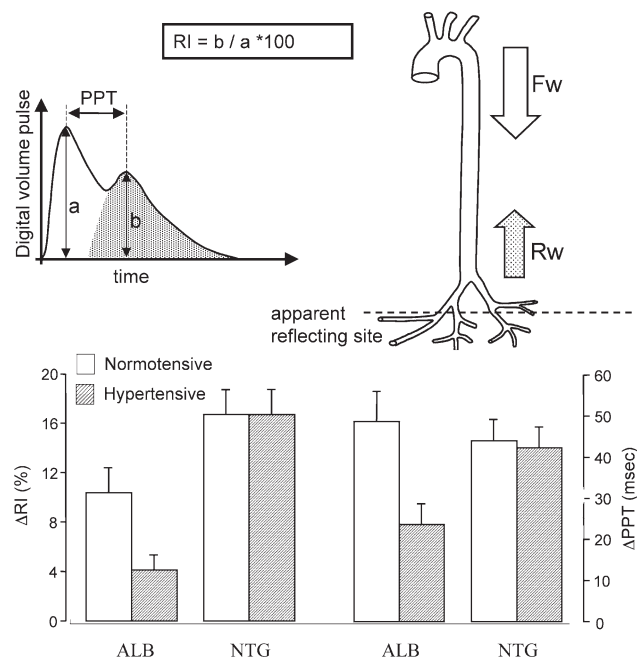


Figure 1. Characteristics of the digital volume pulse. Mean change from baseline in reflection index (DRI) and peak to peak time (DPPT) of the digital volume pulse in response to intravenous infusion of albuterol (ALB 5 $\mu\text{g min}^{-1}$) and nitroglycerine (NTG, 5 $\mu\text{g min}^{-1}$) in normotensive ($n = 19$) and hypertensive subjects ($n = 22$).

We examined effects of systemic administration of the selective β_2 -agonist albuterol on the contour of the digital volume pulse

(DVP) waveform in subjects with essential hypertension and normotensive controls. The digital volume pulse is highly sensitive to changes in pressure wave reflection and arterial distensibility. Nitroglycerine was used as a comparator vasodilator. The digital volume pulse was recorded in 22 subjects with essential hypertension and 19 normotensive controls at baseline and during intravenous infusion of albuterol ($5 \mu\text{g min}^{-1}$) and nitroglycerin ($5 \mu\text{g min}^{-1}$). Baseline DVP measurements and responses to drugs were compared by Student's *t* test or analysis of variance for repeated measures using SPSS. All tests were two tailed and $P < 0.05$ was considered significant. The study was approved by King's College Hospital Research Ethics Committee.

Albuterol had no significant effect on blood pressure but induced characteristic changes in pulse contour consistent with a decrease in pressure wave reflection and increase in large artery distensibility. These changes were blunted in hypertensive subjects compared with controls (change in reflection index: 4.1 ± 1.2 vs. $10.4 \pm 2.0\%$, means \pm S.E.M., $P < 0.02$; change in peak-to-peak time: 23.6 ± 5.0 vs. 48.7 ± 7.3 ms, $P < 0.02$). By contrast changes induced by nitroglycerine were similar in both groups (Fig. 1).

Blunting of the pulse wave response to albuterol in subjects with essential hypertension compared with normotensive controls implicates an altered β_2 -adrenoceptor pathway in arteries responsible for wave reflection in hypertensive subjects.

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Wilkinson B *et al.* (2002). *Arterioscler Thromb Vasc Bio* **22**, 147–152.

This work forms part of validation for our main ongoing study funded by the Wellcome Trust.

All procedures accord with current local guidelines and the Declaration of Helsinki

PS P38

Diastolic stretch causes transient increase in sarcoplasmic reticulum Ca^{2+} content without concurrent rise in resting $[\text{Ca}^{2+}]_i$ in isolated guinea-pig ventricular myocytes

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Effects of mechanical stimulation of cardiomyocytes on sarcoplasmic reticulum (SR) Ca^{2+} handling are not well understood. Ion flux through stretch-activated channels (SACs) could cause a net increase in SR Ca^{2+} load, either directly via Ca^{2+} entry, or secondary to Na^+ influx (affecting $\text{Na}^+-\text{Ca}^{2+}$ exchange). In contrast, Gamble *et al.* (1992) report a reduced SR Ca^{2+} content in rat papillary muscle after 60 s of diastolic stretch. The sub-cellular mechanisms of this response are not clear. In this study, we use remote-controlled carbon fibres to axially stretch ventricular myocytes.

Ventricular myocytes were isolated from guinea-pigs (350–400 g) humanely killed by cervical dislocation. Cells were Fura-2 loaded ($1.25 \mu\text{M}$, 5 min), conditioned by field-stimulation (2 Hz), and Ca^{2+} transients and sarcomere length were simultaneously recorded (IonOptix) at 37°C . After obtaining a steady state, pacing was interrupted for between 5 and 60 s either at resting length or during stretch by 5–10%.

Post-rest potentiation in contractile activity and Ca^{2+} transients was studied in the presence and absence of $40 \mu\text{M}$ streptomycin to block cation non-selective SACs. One-way ANOVA was used for statistical analysis and $P < 0.05$ was considered to indicate a significant difference between means.

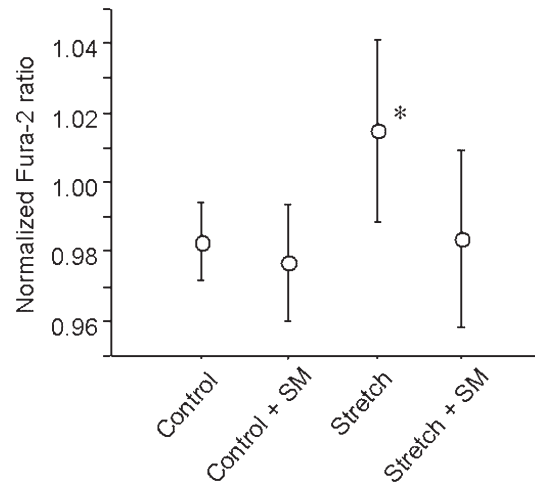


Figure 1. Peak values of Fura-2 ratio during post-rest (5 s) potentiation. Values are normalized to the average of the last 10 regular beats of the conditioning phase. SM: streptomycin. Values are expressed as means \pm S.D. ($n=7$)
* $P < 0.01$ vs. all three other states.

There was no detectable difference in diastolic $[\text{Ca}^{2+}]_i$ (baseline Fura-2 signal at the end of rest period) between control and stretched states. Stretch caused a transient increase in post-rest potentiation of Ca^{2+} transients and contraction, which was significant after 5 s (Fig. 1). Longer rest periods were accompanied by an overall reduction of post-rest potentiation in both control and stretched cells. The initial increase in peak Ca^{2+} transient was prevented by application of streptomycin (Fig. 1).

The present results suggest that stretch transiently increases SR Ca^{2+} content in guinea-pig ventricular myocytes. This effect occurs in the absence of a notable increase in diastolic $[\text{Ca}^{2+}]_i$, and appears to be mediated via activation of streptomycin-sensitive SACs. The decrease in Ca^{2+} transient amplitudes after rest of 10 s or more could be attributable to Ca^{2+} leaks from SR and/or cell, and further studies are required to address this.

Gamble J *et al.* (1992). *Cardiovasc Res* **26**, 865–870.

We thank Dr Michiel Helmes and Prof. David Eisner for help and advice. This work was supported by the BHF. P.K. is a Royal Society Research Fellow.

All procedures accord with current UK legislation

PS P39

Cytosolic calcium attenuates HERG channel currents expressed in HEK 293 cells via a protein kinase C-dependent pathway

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The human *ether-a-go-go* related gene (HERG) encodes a voltage gated potassium (K^+) channel widely expressed in the nervous

system and heart. Attenuation of the cardiac HERG channel current, either resulting from inhibition by drugs or inherited mutations in HERG causes abnormal action potential repolarisation and cardiac arrhythmias. Cyclic AMP and several kinases modify HERG channel activity, but little is known about its modulation by Ca^{2+} -dependent pathways. Here, we have investigated the mechanisms for the decrease of HERG currents in response to elevation of intracellular $[\text{Ca}^{2+}]$ ($[\text{Ca}^{2+}]_i$).

HEK 293 cells either stably or transiently expressing HERG were superfused with a NaCl-based extracellular solution containing 4 mM K^+ and 2 mM Ca^{2+} , at 37 °C. Currents were recorded using whole cell patch clamp. Cells were dialysed with KCl-based, Ca^{2+} -free intracellular solution and currents elicited by stepping from -80 mV to 0 mV for 5 s. Data are expressed as means \pm S.E.M. from more than five cells and were compared with Student's unpaired *t* test, with the level of significance taken as $P < 0.05$.

Elevating $[\text{Ca}^{2+}]_i$ by application of the Ca^{2+} ionophore ionomycin (5 μM) or methacholine (1 mM) to stimulate $\text{G}\alpha_{q/11}$ -coupled muscarinic M_3 receptors resulted in a sustained decrease of HERG current of $38 \pm 1\%$ and $30 \pm 5\%$, respectively. In both cases, the decrease of HERG current could be partially blocked by prior application of 300 nM bisindolylmaleimide-1 (bis-1), a selective protein kinase C (PKC) inhibitor. Higher concentrations of bis-1 had an inhibitory effect on HERG currents. As an alternative approach to reduce PKC activity, cells were treated with 1 μM PMA for 24 h to down-regulate PKC. PMA resulted in a marked decrease in the levels of PKC α , β and δ in Western blots of PKC isoforms and the inhibition of HERG currents by ionomycin and M_3 receptor stimulation was abolished. However, HERG currents from cells pre-treated with an inactive analogue of PMA (4 α PMA) remained sensitive to ionomycin and methacholine. To further characterise the effect of PKC, HERG currents were recorded before and after application of 10 μM 1-oleoyl-2-acetyl glycerol (OAG). OAG reduced peak currents at 0 mV by $37 \pm 3\%$, shifted the voltage dependence of activation by +5.8 mV, but had no significant effect on the voltage dependence of inactivation.

Taken together these results suggest there is no direct or calmodulin-mediated effect of Ca^{2+} on HERG channels under our recording conditions. However, Ca^{2+} can modulate HERG channel activity through stimulation of PKC. Ca^{2+} or diacylglycerol stimulated PKC activity causes a reduction of HERG current and positive shift of activation.

This work was supported by the British Heart Foundation