C1

Ca^{2+}-dependent modulation of L-type Ca^{2+} current in rat ventricular myocytes: importance of location

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Immunocytochemical and functional studies suggest that L-type Ca^{2+} channels are located predominantly in the t-tubules of cardiac ventricular myocytes. The purpose of the present study was to compare modulation of L-type Ca^{2+} current (I_{CaL}) by Ca^{2+} in the t-tubules (i.e. in control cells) and surface sarcolemma (i.e. in detubulated (DT) cells.

Wistar rats were killed humanely using a Schedule 1 method. Ventricular myocytes were enzymatically isolated, and detubulated using formamide as described by Kawai *et al.* (1999). The whole cell configuration of the patch clamp technique was used to record I_{CaL}. TEA/Cs-containing solutions were used, to avoid contamination by other currents; membrane potential was held at −80 mV and test pulses to 0 mV applied. Experiments were performed at 22–24 °C.

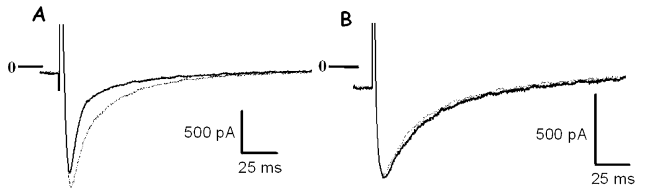

Figure 1. Representative traces of I_{CaL} from a control (*A*) and detubulated (*B*) myocyte. The continuous traces show steady-state I_{CaL} at a stimulation frequency of 0.1 Hz; the dotted traces show the 4th current after increasing stimulation frequency to 1 Hz. Note the faster inactivation and frequency-dependent facilitation in control cells.

The rapid component of inactivation of I_{CaL} was significantly faster in control cells ($\tau = 9.5 \pm 0.6$ ms, mean ± S.E.M., $n = 10$) than in detubulated cells (16.8 ± 2.8 ms, $n = 7$, $P < 0.05$, Student's unpaired *t* test), and frequency-dependent facilitation was present in control cells (10/12) but not in detubulated cells (12/12, see Fig. 1). Inactivation and facilitation of I_{CaL} are known to depend on local Ca^{2+} entry and release. Ryanodine (30 μM) slowed the fast inactivation phase of I_{CaL} in control cells so that it was no longer statistically different from in DT cells (control τ: 22.7 ± 2.1 ms, $n = 10$; detubulated: 23.8 ± 2.0 ms, $n = 7$; NS, unpaired *t* test) and abolished frequency-dependent facilitation in control cells. Formamide treatment of atrial myocytes, which lack t-tubules, did not change the fast inactivation phase of I_{CaL} (control τ: 9.8 ± 1.4 ms, $n = 7$; formamide-treated: 10.0 ± 1.6 ms, $n = 7$; NS, unpaired *t* test).

It appears likely that Ca^{2+}-dependent modulation of I_{CaL} is due to local Ca^{2+} entry and release rather than bulk cytoplasmic Ca^{2+} (see for review Dirksen, 2002). Thus the observation that modulation of I_{CaL} by Ca^{2+} released from the SR is most marked at the t-tubules (i.e. in control cells) suggests that this difference may be due to differences in the structure of the dyad at the t-tubule and surface membrane.

Dirksen RT (2002). *Front Biosci* **7**, d659–d670.

Kawai M *et al.* (1999). *Am J Physiol* **277**, H603–609.

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All procedures accord with current UK legislation.

C2

Effect of detubulation on the contraction–frequency relationship of isolated rat ventricular myocytes

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Increasing stimulation rate increases intracellular Na^+ ($[Na^+]_i$) which, via sodium–calcium exchange (NCX), increases Ca^{2+} transient amplitude, and hence the strength of contraction of cardiac ventricular myocytes (e.g. Harrison *et al.* 1992). Previous work has shown that NCX is located predominantly in the t-tubules of ventricular myocytes (Yang *et al.* 2002); the aim of the present study was to investigate the effects of detubulation on the response to increasing stimulation rate.

Wistar rats (200–250 g) were humanely killed and ventricular myocytes isolated by enzymatic digestion. Cells were detubulated (DT) using formamide as described previously (Yang *et al.* 2002). Intracellular Ca^{2+} ($[Ca^{2+}]_i$) and $[Na^+]_i$ were monitored using fura-2 and SBFI, respectively, and cell length monitored optically. Experiments were performed at 22–24 °C.

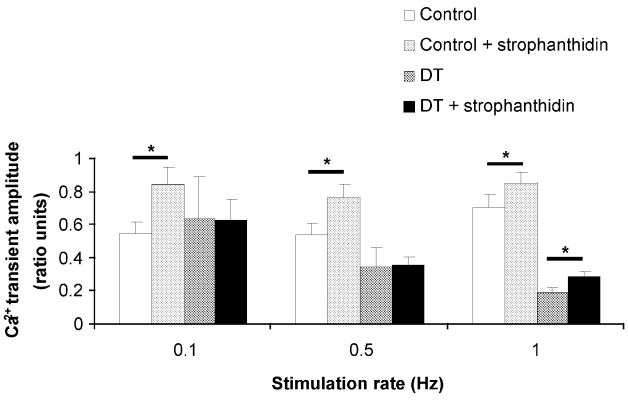

Figure 1. Ca^{2+} transient amplitude at different stimulation rates in control ($n = 7$) and DT ($n = 7$) cells in the absence and presence of strophanthidin. * $P < 0.05$ for effect of strophanthidin in control and DT cells.

Figure 1 shows the relationship between Ca^{2+} transient amplitude and stimulation frequency in control and DT myocytes. This relationship was slightly positive in control cells but negative in DT cells. However the change in $[Na^+]_i$ caused by changing from 0 to 1 Hz stimulation was not significantly different (Student's unpaired *t* test) in control (3.5 ± 1.2 mM, $n = 8$) and DT (4.7 ± 1.6 mM, $n = 7$) cells.

In the presence of strophanthidin (100 μM), to reduce Na^+ efflux via the Na^+–K^+-ATPase, the stimulation-induced increase in $[Na^+]_i$ was significantly greater ($P < 0.05$; paired *t* test) in control (10.6 ± 1.2 mM, $n = 8$) and DT (9 ± 1.8 mM, $n = 7$) cells, but the increase was not significantly different (unpaired *t* test) between control and DT cells. In control cells, this was associated with a significant increase in the Ca^{2+} transient at each frequency resulting in a flatter Ca^{2+} transient–frequency relationship. In DT cells, strophanthidin only increased Ca^{2+} transient amplitude at 1 Hz, and the Ca^{2+} transient–frequency relationship was still negative.

These data show that ion regulation in the t-tubules is an important determinant of the Ca^{2+} transient/contraction–frequency relationship. Although $[Na^+]_i$ rose to the same extent in control and DT cells when stimulation rate was increased and when strophanthidin was added, this was not translated into an increased Ca^{2+} transient in DT cells, consistent with the t-tubules being an important site of NCX activity. However the observation that strophanthidin increased $[Na^+]_i$ in detubulated cells, and caused a small increase in $[Ca^{2+}]_i$ at 1 Hz suggests that some Na^+–K^+-ATPase and NCX are located on the surface membrane.

Harrison SM *et al.* (1992). *J Physiol* **449**, 517–550.
Yang Z *et al.* (2002). *Circ Res* **91**, 315–322.

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All procedures accord with current UK legislation.

C3

The involvement of the PtdIns-3-OH kinase–Akt–NO axis and the sarcoplasmic reticulum in the slow increase in contraction seen in single rat myocytes stretched by carbon fibres

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A slow increase in Ca^{2+} and contraction (the slow response) is seen when cardiac myocytes and muscle are stretched. Vila-Petroff *et al*. (2001) recently proposed that activation of the PtdIns-3-OH kinase–Akt–NO axis leading to enhanced RyR activity underlies the slow potentiation of the $[Ca^{2+}]_i$ transient in rat ventricular myocytes stretched within an agarose gel. We have examined the role of the PtdIns-3-OH kinase–Akt–NO axis and the sarcoplasmic reticulum (SR) in the slow response evoked by stretch of myocytes with carbon fibres.

Rats were killed humanely, and enzymatically isolated ventricular myocytes were stretched following attachment to two carbon fibres. The slow response (force at 5 min as a percentage of that at 10 s after stretch) was recorded under control conditions and after pharmacological intervention in the same cell. Sarcomere length was used as the index of stretch.

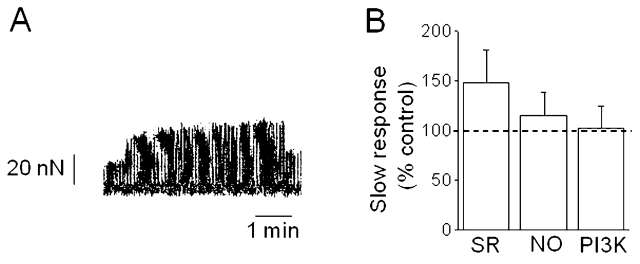

Figure 1. *A*, representative response to stretch recorded using a sample and hold circuit in a rat ventricular myocyte. *B*, the effect of inhibition of SR function, NO synthase and PtdIns-3-OH kinase (PI3K) on the magnitude of the slow response (means + S.E.M.).

Figure 1*A* shows the biphasic increase in force upon stretch observed in a representative cell. No reduction in the magnitude of the slow response was observed when SR function was inhibited with 1 μM thapsigargin and ryanodine (6.4 % stretch; $n = 4$; $P > 0.05$, Student's paired t test; Fig. 1*B*). The NO synthase inhibitor L-NAME (1 mM) had no impact on the slow response (8.3 % stretch; $n = 7$; $P > 0.05$; Fig. 1*B*). Furthermore, unloaded myocytes showed only negative inotropic responses to exogenous supply of NO using the donor *S*-nitroso-*N*-acetylpenicillamine (SNAP; 1–100 μM), although the reduction in shortening observed with 100 μM SNAP was absent in the presence of 1 mM L-NAME. Inhibition of PtdIns-3-OH kinase with 10 μM LY294002 had no effect on the slow response (8.2 % stretch; $n = 6$; $P > 0.05$; Fig. 1*B*).

We find that the slow response observed in single rat myocytes stretched by carbon fibres does not depend on activation of PtdIns-3-OH kinase, generation of NO, or a functional sarcoplasmic reticulum. We have previously shown that the stretch-activated channel plays a major role in the slow response observed in cells stretched by carbon fibres (Calaghan & White, 2002). The present data contrast with those of Vila-Petroff *et al.* (2001) who proposed the RyR as the final effector underlying the slow response. The method by which cells are stretched may have profound consequences for the activation of intracellular signalling cascades, perhaps depending on the extracellular connections/support of the cell.

Calaghan SC & White E (2002). *J Physiol* **544.P**, 23*S*.
Vila-Petroff MG *et al.* (2001). *Nat Cell Biol* **3**, 867–873.

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All procedures accord with current UK legislation.

C4

Characterisation of the electrophysiological effects of diadenosine tetraphosphate and pentaphosphate on guinea-pig sub-endocardial myocardium

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Diadenosine polyphosphates (ApnA) are platelet-derived compounds with potent cardiac electrophysiological effects. We have described their effects on cardiac electrophysiology manifest as changes to action potential duration and refractoriness in sub-epicardial myocytes (Stavrou *et al.* 2001). The purpose of this study was to characterise the action of diadenosine tetraphosphate (Ap_4A) and pentaphosphate (Ap_5A) on sub-endocardial tissue to determine if changes to action potential configuration are variable in different regions of the heart. Such variability has been shown to be an important factor in exacerbating the development of cardiac arrhythmias.

The experiments used left ventricular trabeculae or papillary muscles from male guinea-pigs (300–400 g); the animals had been killed humanely by cervical dislocation. Preparations were superfused with a HCO_3^-/CO_2-Tyrode solution at 36°C, pH 7.35. Muscles were field stimulated with 0.5 ms pulses at either 1 Hz or 3.3 Hz, to compare data with our previous studies; action potentials were recorded with 3 M KCl-filled microelectrodes. Control data were recorded before and after addition of 1 nM or 1 μM Ap_4A or Ap_5A for 30 min. All data are shown as means ± S.D., n is number of experiments, and the differences between data sets were tested for significance ($P < 0.05$) using Student's unpaired t test.

At 3.3 Hz stimulation Ap_4A increased action potential duration (APD) by only a small amount: 1 nM, 105 ± 2 % of control (178 ± 2 to 187 ± 2 ms, $n = 7$) and 1 μM 107 ± 3 % of control (178 ± 2 to 190 ± 3 ms, $n = 8$). By contrast the increase of APD

by 1 μM Ap_4A at 1 Hz stimulation rate was proportionately much greater (134 ± 18 % of control; 198 ± 8 to 290 ± 14 ms, $n = 9$), although there was no significant effect at 1 nM. Similar results were seen with Ap_5A. No significant effect was observed at 1 nM using 1 Hz or 3.3 Hz stimulation. However, at 1 μM concentration a modest prolongation was recorded at 3.3 Hz (109 ± 3 % of control; 173 ± 3 to 188 ± 4 ms, $n = 12$) but a much greater effect at 1 Hz stimulation (130 ± 8 % of control; 204 ± 14 to 295 ± 27 ms, $n = 5$).

These data are different from those observed on sub-epicardial cells where at 3.3 Hz stimulation frequency 1 nM Ap_4A and Ap_5A increased slightly APD. With 1 μM of the respective compounds only Ap_4A generated a small prolongation; Ap_5A had no effect. These data therefore show that the effects of Ap_4A and Ap_5A on action potential configuration are frequency and regionally dependent. Thus these agents would contribute to heterogeneity of conduction in the ventricle and exacerbate the likelihood of arrythmogenesis.

Stavrou BM *et al.* (2001). *J Cardiovasc Pharmacol* **37**, 571–584.

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All procedures accord with current UK legislation.

C5

Mechanisms contributing to the inotropic effects of sevoflurane in rat ventricular myocytes

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In electrically stimulated cells, sevoflurane (Sevo) initially decreases contraction followed by partial recovery during the exposure. On removal of Sevo, contractions transiently increase above control (Davies *et al.* 2000). Hannon & Cody (2002) reported that Sevo increased sarcoplasmic reticulum (SR) Ca^{2+} content in ferret ventricle (via inhibition of sarcolemmal Ca^{2+}-ATPase), which could underlie the increase in contractions on wash-off. However, Davies *et al.* (2000) reported no change of SR Ca^{2+} content at the end of an exposure to Sevo in rat ventricle, tissue where the sarcolemmal Ca^{2+}-ATPase plays a much smaller role in Ca^{2+} regulation. We have investigated other possibilities which could contribute to the inotropic profile of Sevo including changes in myofilament Ca^{2+} sensitivity and the sensitivity of the SR Ca^{2+} release process.

Wistar rats (200–250 g) were humanely killed, the heart removed and ventricular myocytes isolated by enzymatic dispersion. Contraction and intracellular Ca^{2+} (with fura-2) were recorded optically in both unstimulated cells and paced cells (1 Hz). Cells were superfused with a normal Tyrode solution (1 or 2 mM Ca^{2+}) and exposed to 0.6 mM Sevo for a period of 1 or 4 min at 30 °C. Data are presented as means ± S.E.M. and Student's paired t test was used to assess significance.

In unstimulated cells, raising bathing Ca^{2+} to 2 mM induced regular spontaneous Ca^{2+} transients and contractions (frequency of 0.09 ± 0.01 Hz, $n = 8$; see Fig. 1). During the first 30 s of a 4 min exposure to Sevo the frequency of spontaneous Ca^{2+} release was significantly reduced (to 0.03 ± 0.01 Hz, $P = 0.024$) but returned to control (0.08 ± 0.01 Hz) over the next 30–60 s suggesting a transient decrease in the sensitivity of the SR Ca^{2+} release process. Removal of Sevo significantly increased spontaneous activity (to 0.1 ± 0.01 Hz, $P = 0.02$) before returning to control (0.075 ± 0.01 Hz) over the next 30 s, similar to that observed with tetracaine (Overend *et al.* 1997). However, no significant changes in the amplitude of spontaneous Ca^{2+} transients were observed either before, during or after exposure to SEVO.

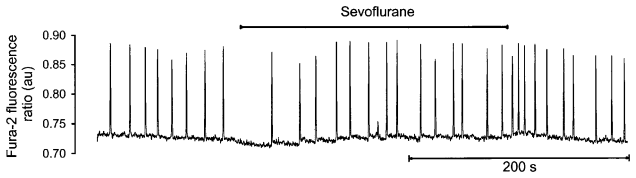

Figure 1. Record of fura-2 fluorescence ratio (Fr) showing spontaneous Ca^{2+} release from the SR. Sevo was applied for the duration of the bar. Frequency and amplitude of release were calculated every 30 s throughout the trace.

Contraction and Ca^{2+} transients were recorded in electrically stimulated cells (1 Hz) before, during and following a 1 min exposure to sevoflurane. From plots of shortening *vs* fura-2 Fr from individual twitches, regression lines were fitted to the final phase of relaxation, the slope of which provides an index of myofilament Ca^{2+} sensitivity (Spurgeon *et al.*, 1992). In 12 cells, the control slope was 121.1 ± 7.2 μm (Fr unit)$^{-1}$. At the point of maximum negative inotropy of Sevo (after ~5 s) the slope was significantly ($P < 0.001$) reduced (to 117.1 ± 6.1 μm (Fr unit)$^{-1}$) but recovered partially during the exposure (slope 118.5 ± 6.7 μm (Fr unit)$^{-1}$). On wash-off, the slope was significantly increased above control (to 122.6 ± 7.3 μm (Fr unit)$^{-1}$; $P < 0.001$) before returning to its original value (121.5 ± 7.2 μm (Fr unit)$^{-1}$).

These data suggest that changes in the sensitivity of (i) the SR Ca^{2+} release process and (ii) the myofilaments to Ca^{2+} contribute to the negative and positive inotropic actions of sevoflurane.

Davies LA *et al.* (2000). *Anesthesiology* **93**, 1034–1044.

Hannon JD & Cody MJ (2002). *Anesthesiology* **96**, 1457–1464.

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

Spurgeon HA *et al* (1992). *J Physiol* **447**, 83–102.

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All procedures accord with current UK legislation.

PC1

Effect of actin disruption and de-t-tubulation on the volume response of adult rat ventricular myocytes to hypo-osmotic challenge

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Cell swelling, which occurs during episodes of ischaemia and reperfusion in the heart, can be mimicked experimentally by exposing myocytes to hypo-osmotic solution. We are interested in the mechanisms which underlie cell swelling. In neonatal chick cardiac myocytes, swelling is potentiated by disruption of the actin cytoskeleton (Zhang *et al.* 1997), and in adult rabbit ventricular myocytes, swelling is reduced by pharmacological blockade of cationic stretch-activated channels (SACs) (Suleymanian *et al.* 1995), which may be located within the t-tubules (Zeng *et al.* 2000). In this study we have investigated the involvement of the actin cytoskeleton and SACs in the volume response of adult rat ventricular myocytes by using the actin disruptor cytochalasin-D (cyto-D) and by removing t-tubules.

Adult Wistar rats were killed humanely and ventricular myocytes isolated enzymatically. Cells were incubated in either 10 μM cyto-D or vehicle solution (dimethyl sulphoxide 0.1 % v/v) for 1 h prior to swelling. Some myocytes were de-t-tubulated (d-Tub) (Brette *et al.* 2002). Cells were superfused with physiological Hepes-based solution (284 ± 1 mosmol l^{-1}, mean ± S.E.M., $n = 3$) and then swollen by a 10 min exposure to a hypo-osmotic version of this solution (191 ± 1 mosmol l^{-1}) at 22–24 °C. Cell volume was calculated from a video image of the cell.

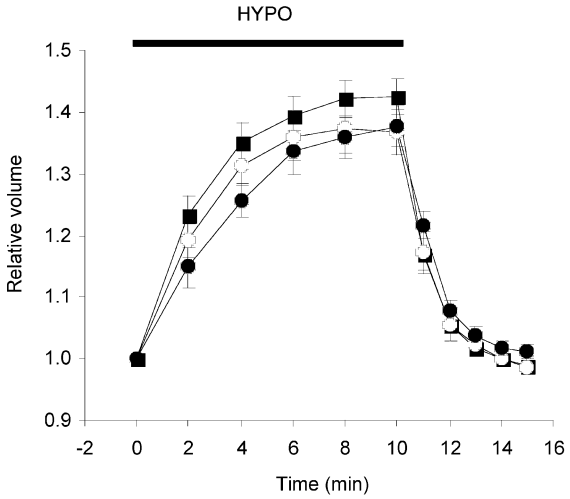

Figure 1. Changes in cell volume (means ± S.E.M.) relative to pre-swollen volume. Hypo-osmotic solution (HYPO) was applied as shown in vehicle (■), cyto-D treated (○) and vehicle + d-Tub (●) myocytes. $n = 12$ cells in each case.

The mean maximal increase in cell volume of cyto-D-treated or d-Tub cells was not significantly different from that in vehicle-treated cells ($P > 0.05$, one-way ANOVA, see Fig. 1). However, the time constant of the swelling response was significantly increased in d-Tub cells (4.21 ± 0.48 min), compared to vehicle (2.58 ± 0.09 min) or cyto-D-treated cells (2.62 ± 0.3 min) ($P < 0.05$ ANOVA with *post hoc* analysis by Tukey's test).

We conclude that, in contrast to neonatal myocytes, the swelling response of adult cardiac myocytes is not modulated by the actin cytoskeleton. In addition, our findings suggest that the magnitude of the swelling response is not dependent upon ion channels specifically located within the t-tubules but that the absence of t-tubules slows the swelling response.

Brette F *et al.* (2002). *Am J Physiol* **283**, H720–728.
Suleymanian MA *et al.* (1995). *J Mol Cell Cardiol* **27**, 721–728.
Zeng T *et al.* (2000). *Am J Physiol* **278**, H548–557.
Zhang J *et al.* (1997). *Am J Physiol* **273**, C1215–1224.

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All procedures accord with current UK legislation.

PC2

Cellular statistics of heterogeneity within the rabbit right atrium and sinoatrial node

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We have previously identified many examples of heterogeneity within the sinoatrial node (SAN) in terms of electrical activity, intracellular calcium handling and protein expression. Parameters often correlate significantly with cell size. To confirm our hypothesis that these correlations reflect spatial distribution within the node we have performed selective isolation of cells from different parts of the SAN structure.

Single cells were isolated from right atrial appendage (RAA), crista terminalis (CT) and peripheral and central SAN tissue obtained from New Zealand White rabbits which were humanely killed by intravenous overdose of anaesthetic ($n = 6$). Samples of tissue of 2 mm^2 were taken, the central sample being centred on the leading pacemaker site and the peripheral sample from SAN tissue overlying the CT. Isolated cells were loaded with calcein using the acetoxymethyl ester form of the dye (5 μM, 30 min). Using a confocal microscope a series of images at 0.5 μm intervals on the z-axis were taken of single cells producing a complete 3-dimensional image of each cell. From these images the dimensions of the cells were obtained. Cell volumes were calculated by producing a rendered 3D volume image using Bitplane Imaris Measurement Pro (Bitplane AG, Zurich).

	RAA	CT	Peripheral SAN	Central SAN
Length (μm)	96 ± 5	127 ± 8	100 ± 5	63 ± 3
Width (μm)	12.9 ± 0.8	10.5 ± 0.6	8.8 ± 0.6	7.9 ± 0.4
Proj. area (μm^2)	1252 ± 138	1072 ± 71	704 ± 36	411 ± 28
Surf. area (μm^2)	622 ± 39	583 ± 29	431 ± 16	272 ± 12
Volume (pl)	17.5 ± 2.2	14.9 ± 1.6	8.1 ± 0.4	4.7 ± 0.3
SA/vol. ratio	0.40 ± 0.04	0.41 ± 0.02	0.53 ± 0.03	0.59 ± 0.01

Table 1. Morphometric measurements of cells isolated from the right atrial appendage (RAA), crista terminalis (CT) and peripheral and central sinoatrial node. Data are shown as means ± S.E.M.

A summary of the data is shown in Table 1, which shows values for the length, width, projected area, surface area, volume and surface area to volume ratio for cells from the RAA, CT, periphery of the SAN and central SAN. Projected area refers to the total area covered by the cell, as would be observed during normal light microscopy, a dimension we have previously used as a measurement of cell size. Data were analysed using one-way ANOVA followed by Tukey comparisons.

Cells from the central SAN were shorter than cells from the periphery, and had a smaller projected area, surface area and volume ($P < 0.05$). Central cells did not differ from peripheral cells in terms of cell width or surface area to volume ratio. Peripheral cells were narrower than atrial cells, and had a smaller projected area, surface area and volume ($P < 0.05$). The cells of the CT were in many respects similar to atrial cells although narrower and longer ($P < 0.05$). The longer length of CT cells perhaps reflects optimisation of this tissue as a rapid conduction pathway within the heart. The surface area to volume ratio of cells from within the SAN is greater than that of other cells ($P < 0.05$), although there is no significant difference between cells from the periphery and centre of the SAN. This reflects the more spindle-shaped or even more complex morphology of the SAN cells compared with the more regular, almost rectangular atrial and CT cells.

Previous studies have endeavoured to divide SAN cells on the basis of cell shape into spindle-shaped, elongated, and spider cells. This study attempts to provide a more quantitative definition of this varied morphology. Regarding spider cells, which are SAN cells with multiple processes, of the 25 central cells studied 7 showed spider type morphology. None were identified from the peripheral SAN tissue. This suggests the spider cell type is concentrated within the central region of the SAN, the initiation site of the cardiac action potential. A more detailed knowledge of the cellular composition of the node will improve our understanding of SAN function and factors affecting the propagation of the cardiac action potential out of SAN and to the rest of the heart.

The authors would like to acknowledge the British Heart Foundation who funded this work.

All procedures accord with current UK legislation.

PC3

Age-dependent spatial re-modelling of connexin43 protein in guinea-pig atrial tissue

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Clinical observations have linked atrial fibrillation, the most common cardiac arrhythmia, with reduced electrical coupling of cardiac myocytes. During atrial fibrillation, spatial re-modelling of the gap junctional protein, connexin43 (Cx43), which is primarily responsible for electrical coupling at the intercalated discs, has been observed in human right atrial tissue (Kostin *et al.* 2002). In this study, we have shown expression of the Cx43 protein changes with age within the right atrium.

Guinea-pigs of 1 month and 38 months of age were humanely killed by anaesthetic overdose. The right atrium was dissected from the heart, then embedded in cryo-media and frozen until sectioned on a cryostat for immunocytochemistry, or punch biopsy samples (4 mm diameter) were frozen until Western blot. Sections of 14 μm were cut and labelled using the antibodies anti-Cx43 (Chemicon International, USA) plus anti-IgG conjugated to FITC (Dako, Denmark) and then imaged using confocal laser scanning microscopy. Data are presented as means ± S.E.M., and were analysed by ANOVA, followed by Student's unpaired *t* test.

Intercalated disc area significantly increased from 33 ± 2.8 μm^2 at 1 month to 83 ± 6.3 μm^2 at 38 months. The dimensions of the intercalated disc significantly increased at the minor axis from 5.2 ± 0.2 μm at 1 month to 8.78 ± 0.3 μm at 38 months, and at the major axis from 7.8 ± 0.4 μm at 1 month to 12.3 ± 0.5 μm at 38 months ($n = 30$, $P < 0.0001$ in both cases). Within the intercalated discs, the area positive for Cx43 protein significantly decreased over 35 % with age ($n = 30$, $P < 0.0001$).

For Western blot, samples were ground in liquid nitrogen, prepared in protease inhibitors, separated by 10 % SDS–PAGE, transferred to nitrocellulose and processed with anti-Cx43 plus anti-IgG conjugated to HRP (Dako, Denmark). Bands of Cx43 protein at 43 kDa were detected by chemiluminescence. The band density of each sample was standardised to a Cx43 peptide control. Connexin43 expression of right atrium tissue significantly decreased 69 %, from 6.25 ± 1.2 μg at 1 month to 1.9 ± 0.5 μg at 38 months ($P = 0.018$, $n = 5$).

Therefore, we have shown a correlation between ageing and a decline in expression of Cx43 protein within the right atrium. This could explain the increased incidence of atrial arrhythmias in the elderly population.

Kostin S *et al.* (2002). *Cardiovasc Res* **54**, 361–379.

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All procedures accord with current UK legislation.

PC4

Effect of trans-sarcolemmal Na^+ gradient on spontaneous contractile activity in detubulated isolated rat ventricular myocytes

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Previous work has shown that Na^+–Ca^{2+} exchange activity appears to occur mainly within the t-tubules of cardiac ventricular myocytes (Yang *et al.* 2002). We have, therefore, investigated the effect of detubulation on the Na^+–Ca^{2+} exchange-mediated increase of spontaneous contractile activity that occurs when extracellular Na^+ concentration ($[Na^+]_o$) is decreased, or intracellular Na^+ concentration ($[Na^+]_i$) increased using strophanthidin.

Ventricular myocytes were isolated from the hearts of Wistar rats killed humanely. Cells were detubulated as described previously (Yang *et al.* 2002). Spontaneous contractile activity was assessed visually in control solution (140 mM Na^+), and in solutions with reduced $[Na^+]$ (replaced with Li^+) and in the presence of strophanthidin (100 μM); observations were started 1 min after decreasing $[Na^+]_o$ from 140 mM. Cells were considered spontaneously active if they showed spontaneous contractile activity within 5 s of being observed.

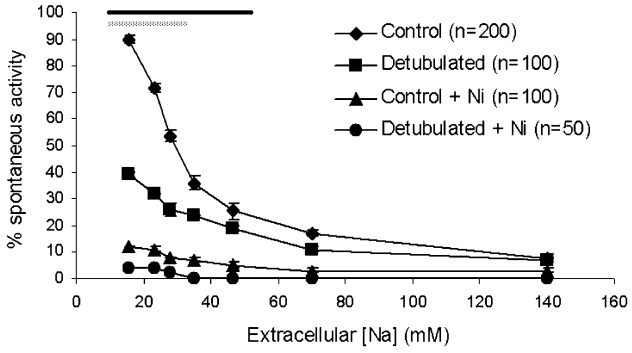

Figure 1. The effect of lowering $[Na^+]_o$ on mean (± S.E.M.) spontaneous contractile activity in control and detubulated rat ventricular myocytes in the absence and presence of 5 mM Ni^{2+}. The grey line above the graph indicates the range of $[Na^+]_o$ over which detubulation significantly ($P < 0.05$; χ^2 test) reduced spontaneous activity in the absence of Ni^{2+}. The black line indicates the range over which Ni^{2+} significantly decreased spontaneous activity in control and detubulated myocytes. Experiments carried out at 22–24 °C.

Figure 1 shows that reducing $[Na^+]_o$ increased spontaneous contractile activity in control cells. Calculation of $E_{Na/Ca}$ ($[Na^+]_i = 10$ mM, $[Ca^{2+}]_i = 100$ nM, $[Ca^{2+}]_o = 1$ mM) suggests that the exchanger will reverse, to produce Ca^{2+} influx, when $[Na^+]_o$ is less than ~70 mM, the concentration below which spontaneous contractile activity increased significantly ($P < 0.05$). Figure 1 also shows that the increase in spontaneous activity caused by decreasing $[Na^+]_o$ was significantly reduced in detubulated cells. In both normal and detubulated cells, the increase in spontaneous activity was inhibited by Ni^{2+} (which inhibits

Na^+–Ca^{2+} exchange; Fig. 1) and the sarcoplasmic reticulum inhibitor ryanodine (1 μM), but was unaffected by the Ca^{2+} channel blocker nifedipine (20 μM; not shown). The increase in spontaneous contractile activity caused by strophanthidin was also reduced in detubulated cells.

These data provide functional evidence for the loss of some, but not all, Na^+–Ca^{2+} exchange activity in detubulated ventricular myocytes.

Yang Z *et al.* (2002). *Circ Res* **91**, 315–322.

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All procedures accord with current UK legislation.

PC5

Localization of Na^+–K^+-ATPase activity in rat ventricular myocytes

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A recent study has shown that Na^+–K^+-ATPase activity is present on the surface sarcolemma of cardiac ventricular myocytes (Fowler *et al.* 2003). However the fraction on the surface sarcolemma compared to that within the t-tubules is unknown. The aim of the present study was to investigate this distribution.

Wistar rats were anaesthetised by I.P. injection of Nembutal (1 mg g^{-1}). The heart was removed and ventricular myocytes enzymatically isolated, and detubulated as described by Kawai *et al.* (1999). Na^+–K^+ pump current (I_{pump}) was measured, using the whole cell patch clamp technique, as the outward current activated by 4 mM K^+ at a holding potential of −20 mV in the presence of inhibitors of contaminating currents. The same protocol, but using high resistance electrodes, was used to investigate the $[Na^+]_i$ dependence of I_{pump} after ~10 min Na^+–K^+ pump inhibition, in cells loaded with sodium-binding benzofuran isophthalate (SBFI) to monitor $[Na^+]_i$ (Despa *et al.* 2003). All experiments were performed at 23–25 °C.

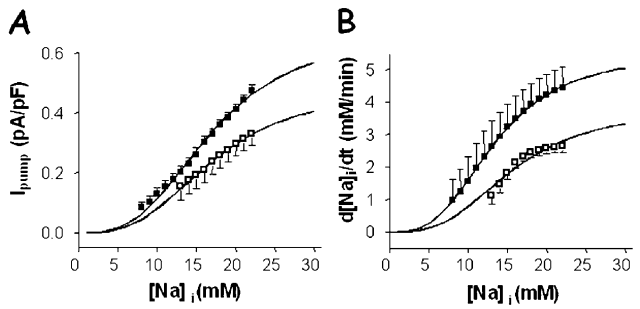

Figure 1. *A*, mean (± S.E.M.) $[Na^+]_i$ dependence of I_{pump} in 7 control (filled symbols) and 8 detubulated (open symbols) myocytes. *B*, $[Na^+]_i$ dependence of the rate of $[Na^+]_i$ decline in control and detubulated myocytes (same symbols).

Detubulation induced a 32 % decrease in cell capacitance (control: 156 ± 7 pF, mean ± S.E.M., $n = 24$; detubulated: 106 ± 5 pF, $n = 19$; $P < 0.05$, Student's unpaired t test) and a 39 % decrease in I_{pump} density (control: 0.28 ± 0.02 pA pF^{-1}, $n = 14$; detubulated: 0.17 ± 0.03 pA pF^{-1}, $n = 16$; $P < 0.05$), indicating concentration of I_{pump} in the t-tubules. Pump re-activation resulted in a rapid outward shift in the membrane current, followed by a decay. The initial rapid phase of this decay occurred with little decrease of bulk $[Na^+]_i$ and may be due to local subsarcolemmal $[Na^+]_i$ depletion by the pump (Despa *et al.* 2003). The subsequent slower phase was accompanied by a decrease of $[Na^+]_i$; detubulation decreased I_{pump} density and the rate of decrease of $[Na^+]_i$ at a given $[Na^+]_i$ during this phase (Fig. 1). Fitting these data using the Hill equation showed that detubulation decreased V_{max} to ~70 % of control, but did not alter the K_m for $[Na^+]_i$ (control: 17.0 ± 0.3 mM, $n = 7$; detubulated: 16.9 ± 0.4 mM, $n = 8$; NS, unpaired t test).

We conclude that the functional density of Na^+–K^+ pump in the t-tubules is ~3-fold higher than in the surface sarcolemma, but that the $K_{m,Na}$ of the pump in the t-tubules is the same as that on the surface membrane.

Despa S *et al.* (2003). *Biophys J* **84**, 4157–4166.

Fowler MR *et al.* (2003). *J Physiol* **551.P**, C2.

Kawai M *et al.* (1999). *Am J Physiol* **277**, H603–609.

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All procedures accord with current National and local guidelines.

PC6

mRNA encoding sarcoplasmic reticulum Ca^{2+} handling proteins decreases *in vitro* in rat cardiac muscle

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The factors that affect the expression of genes coding for Ca^{2+} handling proteins in the heart are not well understood. We have, therefore, investigated the use of the muscle culture system described by Janssen *et al.* (1998) to study changes in mRNA levels in rat cardiac muscle.

Male Wistar rats (~300g) were killed humanely. A thin (< 1 mm) papillary muscle was dissected from the right ventricle and either frozen immediately in liquid N_2, or after 8 h mounted in a muscle bath as described by Janssen *et al.* (1998), bathed in M199 culture medium containing 1.75 mM Ca^{2+} and equilibrated with 95 % O_2–5 % CO_2, stretched to L_{max} and stimulated at 0.33 Hz using a stimulus ~10 % above threshold.

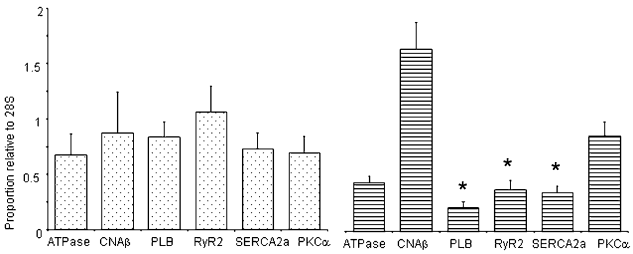

Figure 1. Mean (± S.E.M.) relative amounts of mRNAs in freshly isolated (stippled bars, $n = 5$) and maintained (hatched bars, $n = 6$) cardiac muscle. * Significant difference ($P < 0.05$; unpaired t test) in mRNA in cultured *versus* freshly isolated muscles.

RNA was extracted using a Quiagen kit and reverse transcription carried out using Superscript II (Life Technologies) and random hexamers. Quantitative RT-PCR was performed using a Lightcycler (Roche) for the cDNAs of the following transcripts: 28S, sarcolemmal calcium ATPase (ATPase), calcineurin Aβ (CNAβ), phospholamban (PLB), ryanodine receptor (RyR2),

sarcoplasmic reticulum ATPase (SERCA2a) and protein kinase Cα (PKCα). The amounts of mRNA were expressed relative to a standard sample and corrected for input with the value of 28S cDNA.Figure 1 shows that in freshly isolated muscle, the levels of the different mRNAs investigated were relatively uniform. However in the muscles maintained for 8 h, the levels of mRNAs coding for the Ca^{2+} handling proteins in the sarcoplasmic reticulum were significantly decreased.

These data suggest that this system provides a useful tool for study of gene expression in cardiac muscle, which will allow the effect of changes in environmental factors on gene expression to be investigated. The data also show that gene expression can change in 8 h following muscle isolation, which may have important implications for experiments on isolated tissue or cells maintained for 8 h or more. The factors causing the observed changes of expression are currently unknown, but the changes may represent reversion to a fetal pattern of gene expression, which is associated with the development of hypertrophy.

Janssen PM *et al.* (1998). *Am J Physiol* **274**, H1481–1488.

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All procedures accord with current UK legislation.

PC7

The effect of cytochalasin-D on the morphology of isolated rat ventricular myocytes

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L-type Ca^{2+} channels are located predominantly at the t-tubules of rat ventricular myocytes, co-localised with the Ca^{2+} release channels of the sarcoplasmic reticulum (Scriven *et al.* 2000). The mechanism by which the L-type Ca^{2+} channels are trafficked and targeted is unknown, although it has been suggested that the cytoskeleton may play an important role. We have, therefore, investigated the effect of the actin depolymerising agent cytochalasin-D on L-type Ca^{2+} channel distribution.

Wistar rats were killed humanely. Ventricular myocytes were enzymatically isolated, and then maintained in long-term ($\leqslant$ 8 days) culture in serum-free medium on laminin-coated coverslips (Mitcheson *et al.* 1996). Cultured cells were probed with an anti-calcium channel α_1 subunit antibody.

In control cells, staining the cell membrane with di-8-ANEPPS showed regular transverse staining (t-tubules) at ~2 mm intervals at 0 h; t-tubule density subsequently decreased to 44 % by 96 h. During this time the cells lost their characteristic rod-shaped morphology and staining actin with phalloidin showed disorganisation of its normal staining pattern. After fixing in 4 % (v/v) paraformaldehyde, antibody staining of the α_1 subunit of the L-type Ca^{2+} channel, using an avidin–biotin immunocytochemical staining procedure (Maier *et al.* 2002), revealed a shift from a linear transverse to a punctate longitudinal staining pattern during this time, with concentration of punctate staining in the perinuclear region after 96 h. Ca^{2+} transients, elicited by field stimulation of fluo-3-loaded myocytes, showed loss of synchronous Ca^{2+} release across the cell width on stimulation and the appearance of asynchronous Ca^{2+} sparks (Fig. 1).

In contrast, cells cultured for 96 h in 40 μM cytochalasin-D retained 84 % of their t-tubular structure, their rod-shaped morphology, orderly actin staining, and linear transverse anti-α_1 subunit antibody staining. Furthermore, synchronous Ca^{2+} transients could be elicited for longer by field stimulation (Fig 1). Cells incubated in the presence of 20 μM cycloheximide, a protein synthesis inhibitor, showed a similar stabilisation of morphology and function up to 48 h, suggesting that once inserted in the membrane the turnover of the α_1 subunit is relatively slow.

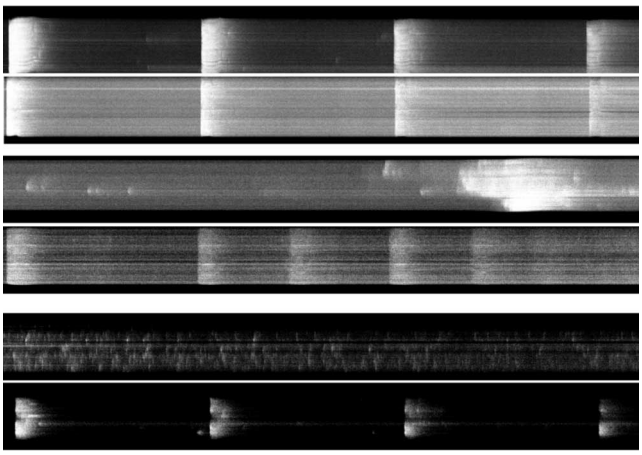

Figure 1. Line scan images across the width of representative fluo-3-loaded ventricular myocytes after 24 h (top), 48 h (middle) and 96 h (bottom) culture in the absence (top trace of each pair) and presence (bottom trace of each pair) of cytochalasin-D. Stimulation rate 1 Hz, 24 °C.

These data show that cytochalasin-D appears to stabilise actin and cell morphology during culture. The loss of the normal α_1 staining pattern in control cells, despite its slow turnover, and the accumulation of perinuclear α_1 staining suggest that normal actin structure is required for anchoring and trafficking the α_1 subunit.

Maier SK *et al* (2002). *Proc Natl Acad Sci U S A* **99**, 4073–4078.

Mitcheson JS *et al* (1996). *Pflugers Arch* **431**, 814–827.

Scriven DR *et al* (2000). *Biophys J* **79**, 2682–2691.

This work was supported by the Wellcome Trust and British Heart Foundation

All procedures accord with current UK legislation.

PC8

Confocal imaging of Ca^{2+} transients and Ca^{2+} sparks in cardiac myocytes from rainbow trout

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The role of the sarcoplasmic reticulum (SR) during excitation–contraction (E–C) coupling in trout ventricle is unclear. Similar to amphibians, trout ventricular myocytes are long and thin, devoid of t-tubules and rely primarily on extracellular Ca^{2+} entry to initiate myofilament contraction. Similar to mammals, trout myocytes have a fairly well developed SR, capable of accumulating large Ca^{2+} stores which can contribute to E–C coupling under certain conditions.

Our aim in this study was to better understand the role of the trout SR during E–C coupling by investigating temporal and spatial co-ordination of the Ca^{2+} transient and the occurrence of Ca^{2+} sparks in intact, contracting trout ventricular myocytes using confocal microscopy. Ca^{2+} sparks and Ca^{2+} transients have not been reported for fish hearts. Therefore, in addition to

trout, experiments were conducted on rat ventricular myocytes as a positive control.

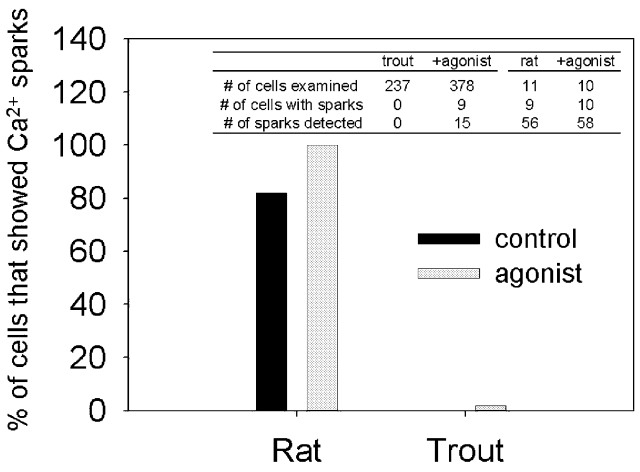

Figure 1. Percentage of cells exhibiting Ca^{2+} sparks in trout and rat ventricular myocytes. Inset shows the number of cells examined, those of which displayed sparks and the total number of sparks observed. Control conditions are 2 mM Ca^{2+}. Agonist conditions are either 6 mM Ca^{2+} or 100 nM Bay K.

Wistar rats and rainbow trout were humanely killed. Myocytes from both species were loaded with 4–10 μM Fluo 4 and were examined with repetitive line scans (1000 lines of 512 pixels, 4–7 ms intervals) across the width of the cell. All values are means ± S.E.M. Differences were considered significant at $P < 0.05$ as assessed with Student's unpaired t test and RM ANOVA with SNK *post hoc*.

In contrast to de-tubulated rat ventricular myocytes which show a pronounced delay (>100 ms) of initiation in the Ca^{2+} wave-front in the centre of the cell, trout myocytes show a delay of only ~8 ms between cell periphery and cell centre. Additionally, the time to peak of transient was longer ($P = 0.034$, $n = 7$) and the rate of rise was slower ($P = 0.016$, $n = 7$) in the centre *versus* the periphery of the trout myocyte.

Spark-like events were observed in only a very small number of the trout myocytes and then only after agonist stimulation (Fig. 1). Decreasing temperature from 21 °C to 14 °C had no effect on spark frequency. In general, trout ventricular spark-like events were narrower and shorter, with faster rise and 50 % decay times than those observed in rat ($P < 0.05$).

In summary, in trout ventricular myocytes, the Ca^{2+} wave-front is initiated at the cell periphery and diffuses rapidly to the cell centre probably due to the narrow width (8.1 ± 1.9 μm) and depth (6.5 ± 0.5 μm) of the cell. Ca^{2+} spark-like events were not found under control conditions which may relate to the density or type of ryanodine receptor present.

This work was supported by the BHF (E.W.) and NSERC Canada (H.S.).

All procedures accord with current UK legislation.

PC8b

Testosterone inhibits the recombinant human L-type Ca^{2+} channel α_{1C} subunit stably expressed in HEK 293 cells

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Clinical studies indicate that testosterone replacement therapy is beneficial to the cardiovascular system, and reduced levels of testosterone are associated with conditions such as coronary heart disease (English *et al.* 2000*a,b*). In addition, *in vitro* studies have shown that testosterone acts as a vasodilator in vessels from various vascular beds (reviewed by Jones *et al.* 2003). Since Ca^{2+} influx through L-type Ca^{2+} channels is a major determinant of vascular tone, we have investigated whether testosterone may act via blockade of L-type Ca^{2+} channels. To do this, we have employed whole-cell patch clamp recordings (using 20 mM Ba^{2+} as the charge carrier) from HEK 293 cells stably expressing the α_{1C} subunit of a human L-type Ca^{2+} channel (hHT splice variant) of the cardiovascular system (see Fearon *et al.* (2000) for further experimental details).

Bath application of testosterone (1–10 000 nM) caused a concentration-dependent inhibition of L-type Ca^{2+} current amplitudes with an estimated IC_{50} of 61.0 nM ($n = 4$–9 cells at each of 5 concentrations tested). At the lowest concentration examined (1 nM), currents were reduced by 25.4 ± 2.6 % (mean ± S.E.M., $n = 4$ cells). This inhibition did not achieve statistical significance (Student's paired t test), but the effects of testosterone were partially reversible. At all higher concentrations, the inhibitory effects of testosterone were always irreversible for at least 10 min of recording. At 1000 nM, testosterone inhibited currents maximally by 82.7 ± 3.8 % ($P < 0.001$, $n = 9$). Current–voltage relationships indicated that testosterone caused a similar degree of inhibition at all activating test potentials, suggesting that its inhibitory effect was voltage-independent. No significant effects of testosterone were seen on channel kinetics.

Our results suggest that testosterone – at physiologically relevant concentrations – acts as an inhibitor of human L-type Ca^{2+} channels via a direct (i.e. non-genomic) mechanism, raising the intriguing possibility that it may act as an endogenous dihydropyridine antagonist. If true, this would in turn suggest testosterone binds to the α subunit of the channel, since no auxiliary subunits were co-expressed in this study. Such an action may account for its beneficial cardiovascular effects *in vivo*.

English KM *et al.* (2000*a*). *Eur Heart J* **21**, 890–894.

English KM *et al.* (2000*b*). *Circulation* **102**, 1906–1911.

Fearon, IM *et al.* (2000). *Circ Res* **87**, 537–539.

Jones RD *et al.* (2003). *Br J Pharmacol* **138**, 733–744.

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