

**Influence of Connexin Expression/Co-Expression Levels on
Electrical Impulse Propagation Investigated in the HL-1 Cell
Model**

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by

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For my family

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Abstract

In the myocardium action potentials are transmitted from cell-to-cell through gap junctions. These specialised junctions play a pivotal role in regulating the speed and safety of impulse propagation by controlling the amount of depolarised current that is passed from excited to non-excited regions of the heart. In mammalian hearts gap junction proteins connexin43, connexin40 and connexin45 are co-expressed in distinctive combinations and relative quantities in functionally specialised subsets of cardiac myocyte. The functional consequences of these connexin expression/co-expression patterns in modulating impulse propagation are poorly understood. To study the relative importance of membrane excitability and electrical coupling in relation to propagation velocities, clones of the HL-1 mouse atrial myocyte tumour line were used as an *in vitro* cell model. Five clones were characterised for expression of myocytic markers, calcium handling proteins and connexins, two of which (#2 and #6) displayed large differences in conduction velocities using microelectrode arrays. To ascertain which factor(s) were the main determinants of speed of conduction, the membrane excitability (voltage-gated channels) and electrical coupling (gap junctions) between the two clones were compared. Sodium, L- and T-type calcium channels were present in both clones but no significant differences were found in the current densities. However, large differences were seen in expression levels of connexin43, connexin40 and connexin45. RNA interference combined with microelectrode arrays was employed to establish the relative importance of each connexin in impulse propagation. The results indicate that electrical coupling by gap junctions is a major determinant of conduction velocities in HL-1 cell lines. Further experiments using RNA interference to suppress the expression of proteins thought to play a role in the action potential parameters should help in defining the part played by either the active or passive electrical properties in action potential propagation.

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CHAPTER 1.

Introduction

1.1. Intercellular communication

Intercellular communication is of paramount importance in multicellular organisms and tissues permitting individual cells to interact with neighbouring cells and with cells at a distance in a coordinated manner through a variety of modes of transmembrane signalling (Yeager 2009; Herve et al. 2007). Direct cell-to-cell signalling requires intercellular junctions to be formed so mediating different types of interactions between cells (Severs 1995). Intercellular junctions consist of specialised microdomains in the apposing plasma membranes of neighbouring cells. The three main classes of junction are: occluding junctions (tight junctions), anchoring junctions (desmosomes and adherens junctions) and communicating junctions (gap junctions) (Severs 1995).

The main focus of our study is on gap junctions which are clusters of transmembrane channels that directly link the cytoplasmic compartments of neighbouring cells allowing the passage of electrical impulses and exchange of various compounds such as regulatory molecules less than 1kDa including metabolites, ions and second messengers (Gros and Jongsma 1996; Sohl and Willecke 2004). Gap-junctional communication plays a key role in tissue homeostasis and regulation of growth, development and differentiation (Severs et al. 2008). In the heart gap junctions mediate the orderly spread of current flow from one cardiac myocyte to the next on which the sequential contraction of the cardiac chambers depends on (Severs et al. 2004a). Alterations in electrical coupling brought about by changes in connexin expression and gap junction organisation referred to as 'gap junction remodelling' are key contributors to rhythm disturbances (arrhythmias) and contractile dysfunction. Thus a detailed knowledge of the role of gap junctions is essential to our understanding of function in the healthy and diseased heart.

1.2. Molecular and structural characteristics of gap junction channels

1.2.1. Identification of gap junctions and connexins

The first structural evidence of gap junctions emerged from thin-section electron microscopy between Mauthner cell synapses in goldfish brains described as a honey comb like hexagonal arrays of closely packed subunits (Robertson 1963). Further work by Benedetti and Emmelot (1968) revealed hexagonal arrays across membrane sheets in

negatively stained preparations of isolated liver membranes but mistook them as components of tight junctions (Figure 1.1 (A)). It was work by Revel and Karnovsky (1967) that distinguished gap junctions from tight junctions by using an extracellular tracer composed of oxidised lanthanum salts which identified extracellular spaces in electron microscope specimens. They were able to visualise cell junctions with a minute gap between the external membranes in both the liver and heart. It is this gap that gives gap junction its name. Figure 1.1 (B) shows a thin-section view of a gap junction.

The development of freeze-fracture electron microscopy whereby the biological sample is frozen and physically broken apart (fractured) revealing the internal organisation of the exposed membrane (Severs 2007a) unequivocally recognized tight junctions and gap junctions as distinctive structures. A replica of the fractured membrane is made by vacuum-deposition of platinum carbon. Subsequent studies of these replicas demonstrated that gap junctions are organised in patches of closely-packed particles corresponding to earlier work by thin-section microscopy and negative staining studies as seen in Figure 1.1 (C) (Kreutziger 1968; Goodenough and Revel 1970). Freeze-fracture combined with the complementary replica technique in which the matching replicas of each side of the fracture plane are viewed (Severs 1995) revealed two sets of membrane spanning structures, one in each of the two junctional membranes (Chalcroft and Bullivant 1970; Steere and Sommer 1972; De Mazière et al. 1987). Since the original identification of gap junctions, several procedures have been developed such as the use of detergents (e.g. sarkosyl deoxycholate) and biochemical analysis (e.g. sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)) to isolate and purify gap junctions (Evans and Gurd 1972; Goodenough and Stoeckenius 1972; Fallon and Goodenough 1981). These developments led to the first identification of connexins- the protein components of gap junctions, in the liver (32 kiloDaltons (kDa)) and heart (43kDa) (Manjunath and Page 1985; Revel et al. 1985; Green et al. 1988). In the next section I will present the essential nomenclature applied to connexins and detail the genes that encode them.

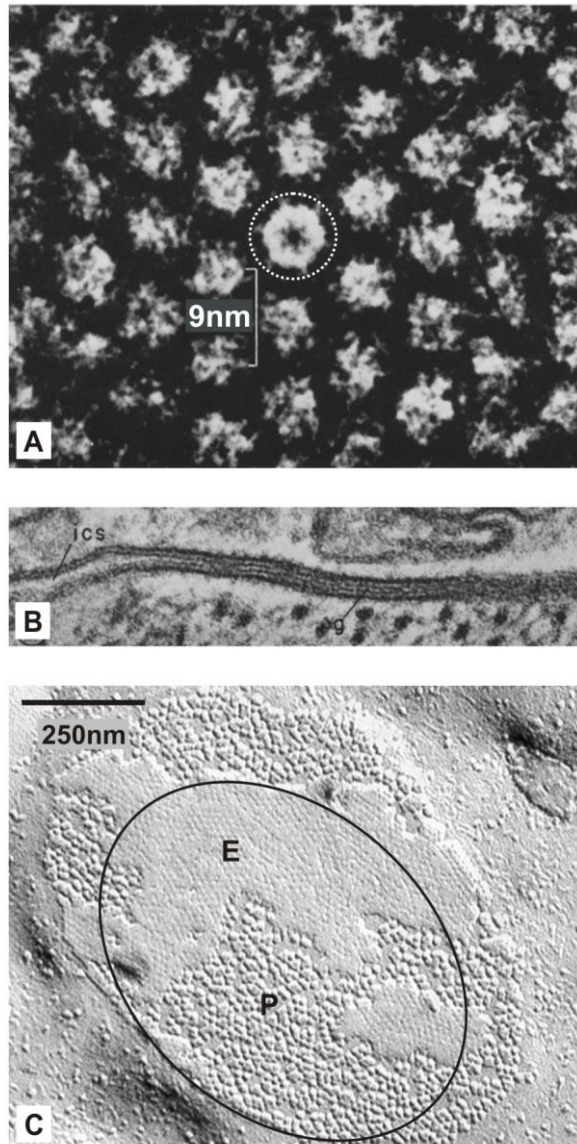


Figure 1.1: Early views of gap junction channels by electron microscopy and freeze-fracture

Frontal views of gap junctions from rat liver plasma membranes negatively stained with phosphotungstate at 37° C. Gap junctions are seen as hexagonally packed subunits with a centre-to-centre distance of ~9 nm. (Image enhanced by rotation; x 1,667,000). Image reproduced from Benedetti and Emmelot (1965). Image (B) is a high magnification view of a gap junction. The normal intercellular space (ics) is reduced to a gap (g) of ~2nm in the junctional area. Image reproduced from Revel and Karnovsky (1967). The aggregated particles in the circled area in (C) represent closely-packed gap junction hemichannels from the rat heart. As freeze-fracture splits the membrane along its hydrophobic core, two types of views may be seen: clusters of particles attached to the protoplasmic (P) face and corresponding pits attached on the exoplasmic (E) face. Image reproduced from Severs et al. (1993).

1.2.2. Connexin nomenclature

The family of connexin genes comprises of 20 members in the mouse and 21 in the human genome (Table 1.1) (Sohl and Willecke 2004; Laird 2006; Beyer and Berthoud 2009). The connexin family members serve a common purpose of permitting the intercellular exchange of small molecules but the functional channel properties are attributed to the subset of connexins expressed in any one cell type (Laird 2006). For example in the myocardium, Cx43 plays a key role in action potential propagation (Severs et al. 2008) whereas in the lens, which is avascular, Cx43 shares the responsibility in maintaining homeostasis and transparency (White et al. 1994; Shakespeare et al. 2009).

A new nomenclature system for connexin genes was decided at the International Gap Junction Conference 2007 that recognises the sub-divisions of the gene family based on DNA sequence comparisons (Kidder 2009). A key feature of this system is that orthologous genes in all mammalian species will have the same symbols whereas non-orthologous genes are assigned unique numbers. In brief, the new terminology retains the use of the connexin protein name written as the abbreviation Cx followed by a suffix indicating the molecular mass in kDa. Many of the connexin genes have identifiable orthologous pairs in both human and mouse (Table 1.1). There are exceptions with certain connexins expressed only in the mouse (e.g. Cx33) or the human genome (e.g. CX25). Furthermore, orthologous connexins in different species may not only differ in their molecular mass (e.g. mouse Cx30.2 is the ortholog of human CX31.9) but also in expression (e.g. human CX31.9 could not be detected in the atrioventricular node when its mouse counterpart Cx30.2 is expressed) (Sohl and Willecke 2004; Kreuzberg et al. 2009).

For identification of connexin genes a Greek letter classification is used. The genes begin with a three letter code starting with *Gj* (gap junction) and a sub-group derived from the Greek letter classification (α , β , γ , δ and ϵ). This is then followed by a number according to the order of discovery (e.g. Cx43 the first identified connexin gene of the α -group is encoded as *Gja1*). Although the connexin genes carry the same abbreviation irrelevant of the species in human and mouse, to distinguish human connexins from other species connexin genes and proteins are given capital letters such as *GJA1* and

CX43 respectively. Throughout my thesis for clarity and convenience I will use the term Cx where the species is irrelevant.

Table 1.1: Nomenclature of connexin proteins and genes in human and mouse

Human		Mouse		Tissue expressing the connexins
Protein	Gene	Protein	Gene	
CX43	<i>GJA1</i>	Cx43	<i>Gja1</i>	Heart, Skin, Lung, Lens, Bone*
CX46	<i>GJA3</i>	Cx46	<i>Gja3</i>	Lens
CX37	<i>GJA4</i>	Cx37	<i>Gja4</i>	Blood vessels
CX40	<i>GJA5</i>	Cx40	<i>Gja5</i>	Heart, Skin
-----	-----	Cx33	<i>Gja6</i>	Testes
CX50	<i>GJA8</i>	Cx50	<i>Gja8</i>	Lens
CX59	<i>GJA9 (GJA10)</i>	-----	-----	
CX62	<i>GJA10</i>	Cx57	<i>Gja10</i>	Retina
CX32	<i>GJB1</i>	Cx32	<i>Gjb1</i>	Liver, Nervous system
CX26	<i>GJB2</i>	Cx26	<i>Gjb2</i>	Liver, Skin
CX31	<i>GJB3</i>	Cx31	<i>Gjb3</i>	Skin
CX30.3	<i>GJB4</i>	Cx30.3	<i>Gjb4</i>	Skin
CX31.1	<i>GJB5</i>	Cx31.1	<i>Gjb5</i>	Skin
CX30	<i>GJB6</i>	Cx30	<i>Gjb6</i>	Skin
CX25	<i>GJB7</i>	-----	-----	Skin
CX45	<i>GJC1 (GJA7)</i>	Cx45	<i>Gjc1</i>	Heart, Skin
CX47	<i>GJC2 (GJA12)</i>	Cx47	<i>Gjc2</i>	Nervous system
CX30.2/CX31.3	<i>GJC3 (GJE1)</i>	Cx29	<i>Gjc3</i>	Brain
CX36	<i>GJD2 (GJA9)</i>	Cx36	<i>Gjd2</i>	Retina, Nervous system
CX31.9	<i>GJD3 (GJC1)</i>	Cx30.2	<i>Gjd3</i>	Heart
CX40.1	<i>GJD4</i>	Cx39	<i>Gjd4</i>	Developing muscle
CX23	<i>GJE1</i>	Cx23	<i>Gje1</i>	N/A

The introduction of the new system led to same gene names having the same abbreviation. These were changed and their former names are in brackets. The table is adapted from (Laird 2006; Beyer and Berthoud 2009). *Cx43 is the most ubiquitously

expressed connexin in mammals present in more than 35 different cell types including astrocytes and smooth muscle cells.

1.2.3. Structure of the connexin gene and protein

Connexin gene structure

The genomic structure of connexin genes is relatively simple. The gene consists of a 5'-untranslated region (5'-UTR) designated as exon 1 (E1) that is separated from a second exon (exon 2) by an intron of variable size (Sohl and Willecke 2004; Oyamada et al. 2005; Beyer and Berthoud 2009). Exon 2 contains the complete coding sequence and the 3' untranslated region (3'-UTR) (Figure 1.2 (A)). Since the initial identification of connexin gene structure many exceptions have been identified. For examples *Gjb1* the gene for Cx32, which contains multiple 5'-UTR exon 1 sequences that are alternatively spliced to exon 2 after transcription (Figure 1.2 (B)) (Neuhaus et al. 1995; Sohl et al. 1996). Alternatively spliced variants of *Gjb1* are driven by two tissue specific promoters: (1) in hepatocytes and acinar cells Cx32 mRNA is made from promoter 1 upstream of exon 1 and (2) in Schwann cells transcription is initiated from promoter 2 upstream of exon 1b (Oyamada et al. 2005). Another example is *Gjc1* (encoding Cx45) as it contains three exons including two 5'-UTR exons with the complete reading frame located in exon 3 (Figure 1.2 (C)) (Baldrige et al. 2001; Jacob and Beyer 2001). Since *Gjc1* contains multiple exons, transcripts can be differentially spliced containing the 5'-UTR generated from either exon 1 or exon 2 or only exon 2. Studies on *Gja1* (encoding Cx43) have identified six exons, five of which correspond to the 5'-UTR (exons 1A to 1E) and one containing the coding region (exon 2) and three alternative promoter regions (Figure 1.2 (D)) (Pfeifer et al. 2004). As a result nine different mouse *Gja1* mRNA have been generated through the use of different promoters and alternative splicing. The differing 5'-UTR could provide a major site for translational regulation by providing a site for interaction with other proteins that can increase or reduce the efficiency of translation initiation (Pickering and Willis 2005). For example approximately 10% of all mRNA contain a long 5'-UTR that increases the propensity for the formation of a stable secondary structure (e.g. hairpin structure). This structure could prevent the progress of the ribosome along the mRNA thereby inhibiting translation initiation.

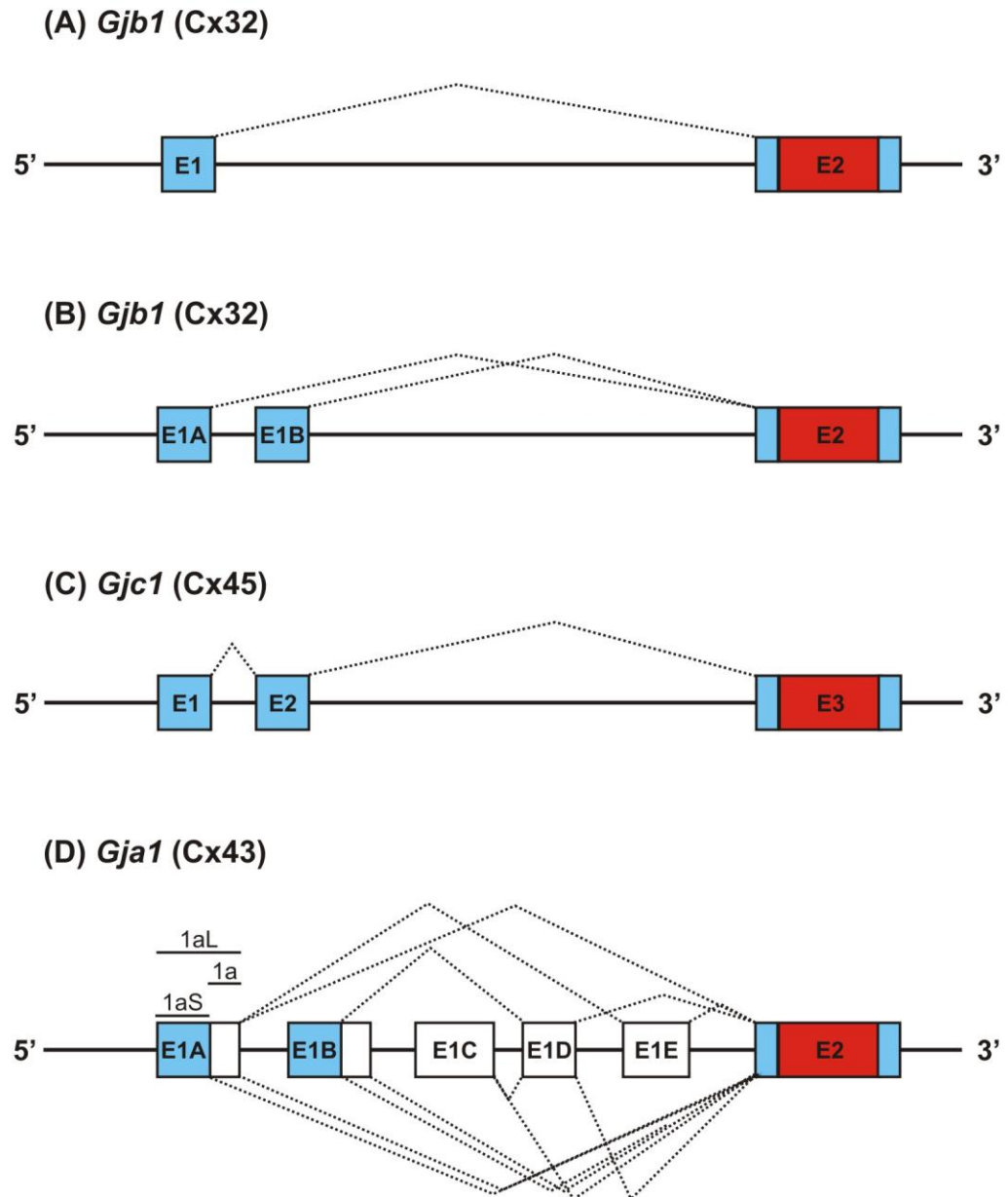


Figure 1.2: Connexin gene structure

(A) is the most common gene structure which contains only one 5'-UTR designated at exon 1 (E1) and an uninterrupted coding region in exon 2 (E2) using *Gjb1* as an example. Some connexin genes contain two or more 5'-UTR exon 1 sequences that are alternatively spliced to exon 2 due to transcription from tissue-specific promoters (B). *Gjc1* consists of two 5'-UTR exons (1 and 2) that may be present with the coding region (exon 3) in the mature mRNA (C). Nine different *Gja1* mRNA can be generated by the presence of five 5'-UTR (exons 1A to E) and three alternative promoter regions (D). The connexin protein encoded by the gene is in brackets. Image modified from Beyer and Berthoud (2009) and Oyamada et al. (2005).

In many connexin genes the promoter is located ≤ 300 base pairs upstream from the first exon containing the transcriptional initiation site (Beyer and Berthoud 2009). Within

this region binding sites for transcription factors are present. Examples of ubiquitous transcription factors include specificity protein family (Sp1/Sp3), TATA box binding protein and activator protein-1 (AP-1). Binding of these factors are important in the basal transcriptional process and may also be important for large changes in expression (Oyamada et al. 2005; Beyer and Berthoud 2009). For instance at the onset of labour a marked increase in the myometrial expression of *Gja1* mRNA may occur through *c-fos* production which interacts with AP-1 (Piersanti and Lye 1995).

Connexin gene promoters bind transcription factors specific to the tissue type. These include Nkx2.5, GATA4 and Tbx5 which regulate *Gja5* (Cx40) expression in the heart. Nkx2.5 is also an important regulator of *Gja1* and *Gja7* (Dupays et al. 2005). Transcription factors can be specific for the cell type or more ubiquitous as demonstrated for the differential expression of *Gjb1* (Cx32). For example the P1 promoter contains binding sites for cell independent factors Sp1/Sp3 together with hepatocyte nuclear factor-1, a cell type specific promoter highlighting the different mechanisms of transcript regulation (Koffler et al. 2002).

Transcription regulation is also governed by biological substances (e.g. cyclic adenosine monophosphate (cAMP) and retinoids) or through the manipulation of signal transduction pathways (e.g. Wnt/ β -catenin/T-cell factor/lymphocyte enhancer binding factor and Ras-Raf-MAPK pathway). The majority of the work regarding gene regulation has been carried out using *Gja1*. For instance cAMP has been shown to affect *Gja1* expression by not only enhancing the gene promoter activity but also the redistribution of Cx43 to the membrane (Oyamada et al. 2005). cAMP has been shown to work in parallel with the Wnt pathway in enhancing the activity of rat *Gja1* promoter P1 thereby increasing mRNA expression (van der Heyden et al. 1998). Connexin transcription could also be suppressed through epigenetic mechanisms such as hypermethylation of DNA or histone acetylation of the DNA in promoter regions (Beyer and Berthoud 2009).

Recently the role of microRNA (miRNA), which are non coding RNA (~22 nucleotides in length) that mediate post-transcriptional gene silencing by either reducing translation or mRNA stability through annealing to complementary sequences in the 3'-UTR of target mRNA, has been highlighted in regulating connexin expression (Bushati and

Cohen 2007). Anderson et al. (2006) demonstrated that during muscle differentiation *miR-206* and *miR-1* forms base pairs with *Gja1* in the 3'-UTR thereby inhibiting the translation of Cx43 mRNA. In addition overexpression of *miR-1* may function as a arrhythmogenic factor in humans with coronary heart disease and in rats with myocardial infarctions by post-transcriptionally suppressing *Gja1* and *KCNJ2* (which is a gene encoding the inward rectifying potassium (K^+) channel Kir2.1) affecting cardiac conduction (Yang et al. 2007).

To conclude, connexin expression is a complex process controlled by multiple regulatory mechanisms including transcription and post-translation processes depending on the tissue type and biological need of the cell.

Connexin protein structure

Connexins are classified as a family of proteins based on sequence similarity and inferred transmembrane topology (Beyer et al. 1990). Sequence analysis revealed a significant amount of similarity (35-65%) between connexins (Bruzzone et al. 1996). In certain cases (i.e. Cx43) a high degree of conservation across species is maintained. The organisation of connexins was first proposed by Zimmer et al. (1987) using a combination of proteolytic digestion and site-directed antibodies and this has remained largely unchanged since.

Connexins are integral membrane proteins spanning the membrane four times to form four hydrophobic transmembrane domains (designated M1, M2, M3 and M4) separated by one cytoplasmic loop between M2 and M3 and two extracellular loops (E1 and E2) (Figure 1.3) (Goodenough et al. 1996; Sohl and Willecke 2004; Laird 2006). The carboxyl- and amino-termini of the protein are located in the cytoplasm (Harris 2001; Laird 2006). This topology has only been validated for Cx32 (Zimmer et al. 1987; Goodenough et al. 1988; Milks et al. 1988), Cx43 (Yancey et al. 1989) and Cx26 (Zhang and Nicholson 1994) but no exceptions have been identified to date and so this model has been extrapolated to all the family members (Beyer and Berthoud 2009).

Extracellular space

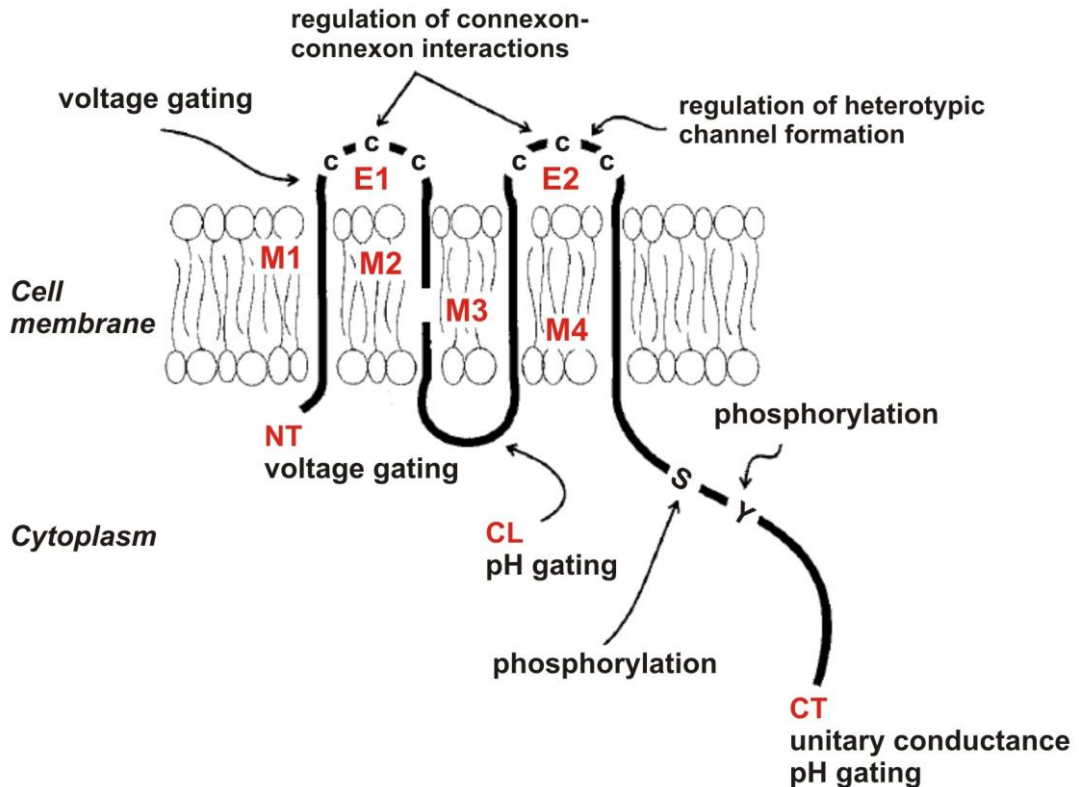


Figure 1.3: Membrane topology of connexin proteins

A connexin is composed of four transmembrane domains (M1, M2, M3 and M4), two extracellular loops (E1 and E2) with three characteristically spaced cysteine residues on each loop, a cytoplasmic loop (CL) an amino-terminus (NT) and carboxyl-terminus (CT). Each of the domains has specific functions in gating, phosphorylation and channel make-up, some of which are shown here. Image modified from Bruzzone et al. (1996).

Amino acid sequence alignment of different connexins has revealed that the four transmembrane domains, two extracellular domains (only exception is rat Cx31) and the amino-terminus are conserved between connexin family members (Laird 2006). The sequences and the length of the cytoplasmic loop and carboxyl-terminus between connexins are not conserved and are responsible for the diversity and differences in molecular mass observed between connexins (Harris 2001; Laird 2006).

There are different domains within the connexin structure that have specific roles: (1) each of the extracellular loops contain three cysteine residues that are involved in the docking of connexons in apposing membranes by disulphide bridge formation; (2) transmembrane domains form the channel and are critical determinants of the correct

membrane topology of connexins; (3) cytoplasmic loops influence pH and chemical gating properties; (4) amino-terminus influence the voltage gating and channel opening and closure (Maeda et al. 2009) and (5) carboxyl-terminus influence pH, voltage gating and unitary conductance and provides sites for post-translational modifications such as phosphorylation (Bruzzone et al. 1996; Harris 2001).

Based on their sequence homologies connexins are divided into subgroups (e.g. α , β and γ) with respect to gene structure, degree of sequence identity and length of the cytoplasmic loop as shown in Figure 1.4 (Sohl and Willecke 2004; Koval 2006). For example: (1) the length of the cytoplasmic loop of the α connexins is approximately double that of the β connexins (Bruzzone et al. 1996) and (2) α connexins have a larger cytoplasmic loop and carboxyl-terminal tail (Yeager 1998).

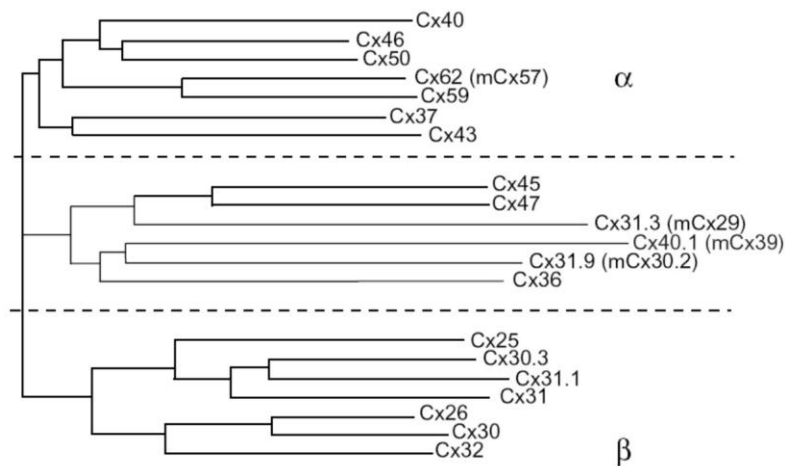


Figure 1.4: Connexin sequence homology

Phylogram of 20 human connexins based on the sequence homology. Connexins are divided into two main subgroups α and β with an additional group with intermediate homology. Originally proposed as a scheme for terminology of connexin proteins, this type of classification remains useful in the context of compatibility of different connexin types to form gap junction channels. The mouse connexin names that differ from the human orthologs are in brackets. Image reproduced from Koval (2006).

1.2.4. From connexins to connexons and gap junction channels

Six connexin subunits oligomerise into a connexon or hemichannel. A single connexon is composed of 24 α -helical bundles suggestive of the four transmembrane helices per connexin subunit with a central aqueous cavity (Figure 1.5 (A)) (Unger et al. 1997;

1999; Verselis 2009). Connexons from apposing plasma membranes interact across a narrow extracellular space ('gap') to form the complete gap junction channel. The individual gap junction channels are arranged in hexagonal arrays that are often referred to as 'gap junction plaques' (Goodenough et al. 1996).

The structure of gap junction channels has been studied using nuclear magnetic resonance, atomic force microscopy and two- and three-dimensional image processing by electron cryomicroscopy (Yeager 2009). In brief, atomic force microscopy (Hoh et al. 1993; Lal and Lin 2001) and three dimensional crystallography (Perkins et al. 1997) revealed that the extracellular surface topology of a homomeric connexon consists of six alternating peaks and valleys due to structural asymmetry between the extracellular domains (Figure 1.5 (B)). From this it was proposed that the peak of one connexon will fit into the valley of the other which requires a 30° rotational stagger to dock (Figure 1.5 (C)) (Unger et al. 1997; Perkins et al. 1998). This interdigitation of the peaks and valleys significantly increases the potential number of bonds (i.e. hydrogen bonds, hydrophobic interactions and ionic interactions) creating a tight interlocking structure necessary for a functional channel and stability (Sosinsky and Perkins 2000).

Maeda et al. (2009) determined the crystal structure of gap junction channels composed of Cx26 at a 3.5Å resolution, which further highlighted key structural determinants in channel regulation. The study identified the first transmembrane helix to be the major pore lining region in each connexin by forming a funnel at the channel entrance thereby restricting the charge and size of the molecules entering it. In addition the N-terminal region was shown to be crucial in regulating the gating of the channel (Oshima et al. 2008; Maeda et al. 2009). For example Cx26 channels are known to close in response to a positive potential, which in turn results in the movement of the helical portion of the N-terminus forming a plug at the cytoplasmic entrance thereby closing the channel.

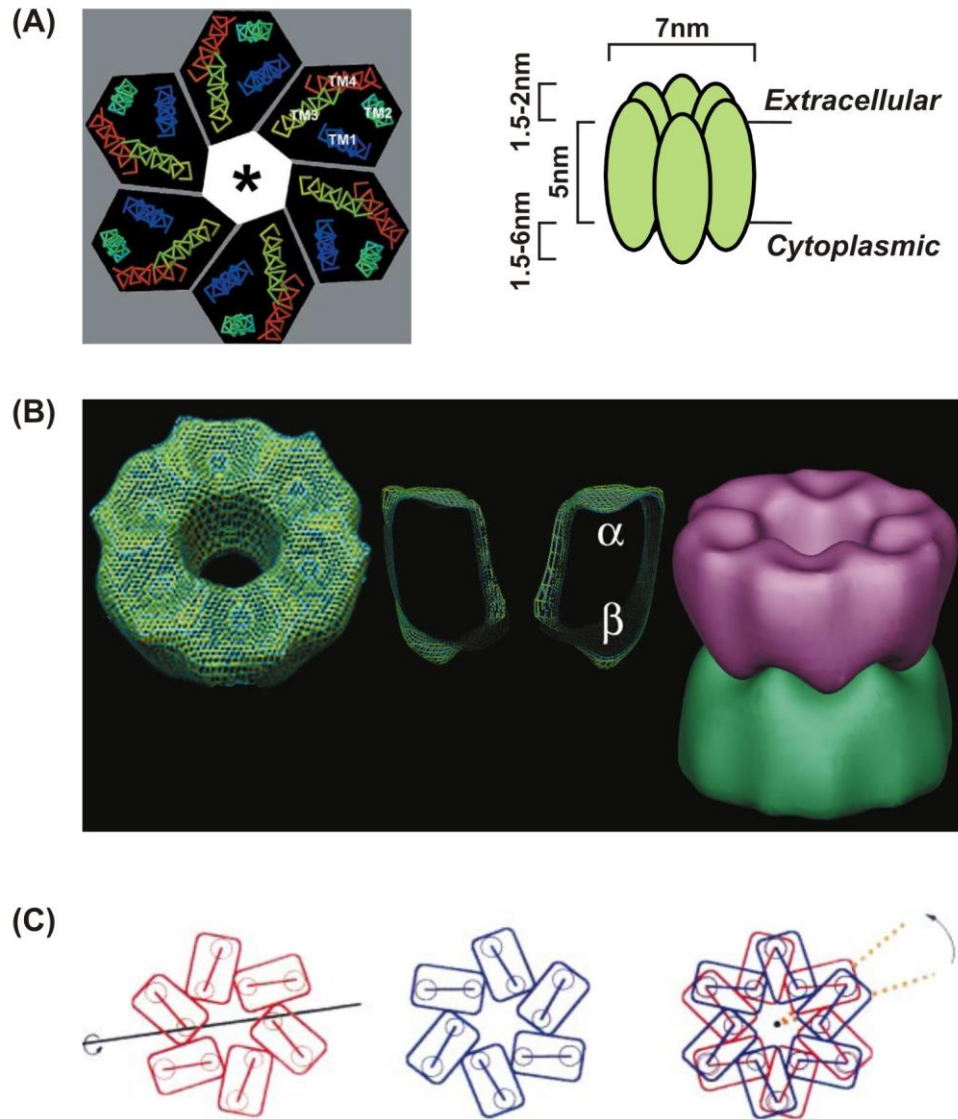


Figure 1.5: Connexin channel structure

Image A, left panel is an *en face* view of a connexon showing only the transmembrane helical domains as viewed from outside the cell looking into the permeability pore (asterisk). The transmembrane domains correspond to M1 (blue), M2 (green), M3 (yellow) and M4 (red). Image reproduced from Koval (2006). Image A, right panel depicts a connexon structure within the plane of the plasma membrane. Approximate sizes of the extracellular loops, transmembrane domains and cytoplasmic regions are indicated. Image (B) is a reconstruction of split gap junction channels of Cx32 from 2D crystals. From left to right, cytoplasmic, cross section, and modelled docked structure of a channel. The peak and valley topology of connexons permits the formation of a channel sealed sufficiently to enable electrical communication. Image (C) is the alignment of two connexons to form junctional channels. Connexins in the apposed plasma membranes (red and blue) are represented as rectangles, and two α -helices within each connexin as circles. The figure on the right illustrates the 30° rotational stagger between the two connexons, causing the α -helices of each connexon to align. Images (B) and (C) are reproduced from Harris (2001).

As mentioned earlier the intermolecular interaction between two connexons occurs through disulphide bonds formed between the conserved three cysteine residues on the extracellular loops (CX₆CX₃C in extracellular loop 1; CX₄CX₅C in extracellular loop 2) (Dupont et al. 1989; Hoh et al. 1991; John and Revel 1991; Rahman and Evans 1991; Foote et al. 1998). Studies on isolated hepatocyte junctions showed that mutations in any of the six cysteine residues completely blocks gap-junctional conductances highlighting their importance in channel function (Dahl et al. 1991; 1992).

Different connexins may be present within a given cell type. For example rodent hepatocyte gap junctions contain both Cx32 and Cx26. These observations raise the possibility that naturally occurring gap junction channels could be formed from more than one connexin subtype (Sosinsky 1995). Figure 1.6 illustrates the different connexon types that could be formed when two different connexin subtypes are co-expressed: homomeric connexons composed of a single connexin; heteromeric connexons composed of at least two different connexins; homotypic channels when two identical connexons dock and heterotypic connexons formed by the docking of two non-identical connexons (Cottrell and Burt 2005). In cells that express two connexin subtypes, fourteen different homomeric and heteromeric connexons may form (Brink et al. 1997). And if each of these fourteen connexons were to form channels with each other then this could yield as many as 196 (14²) different channels. These structural variants could provide a variety of channels with different permeabilities and gating sensitivities (Harris 2001).

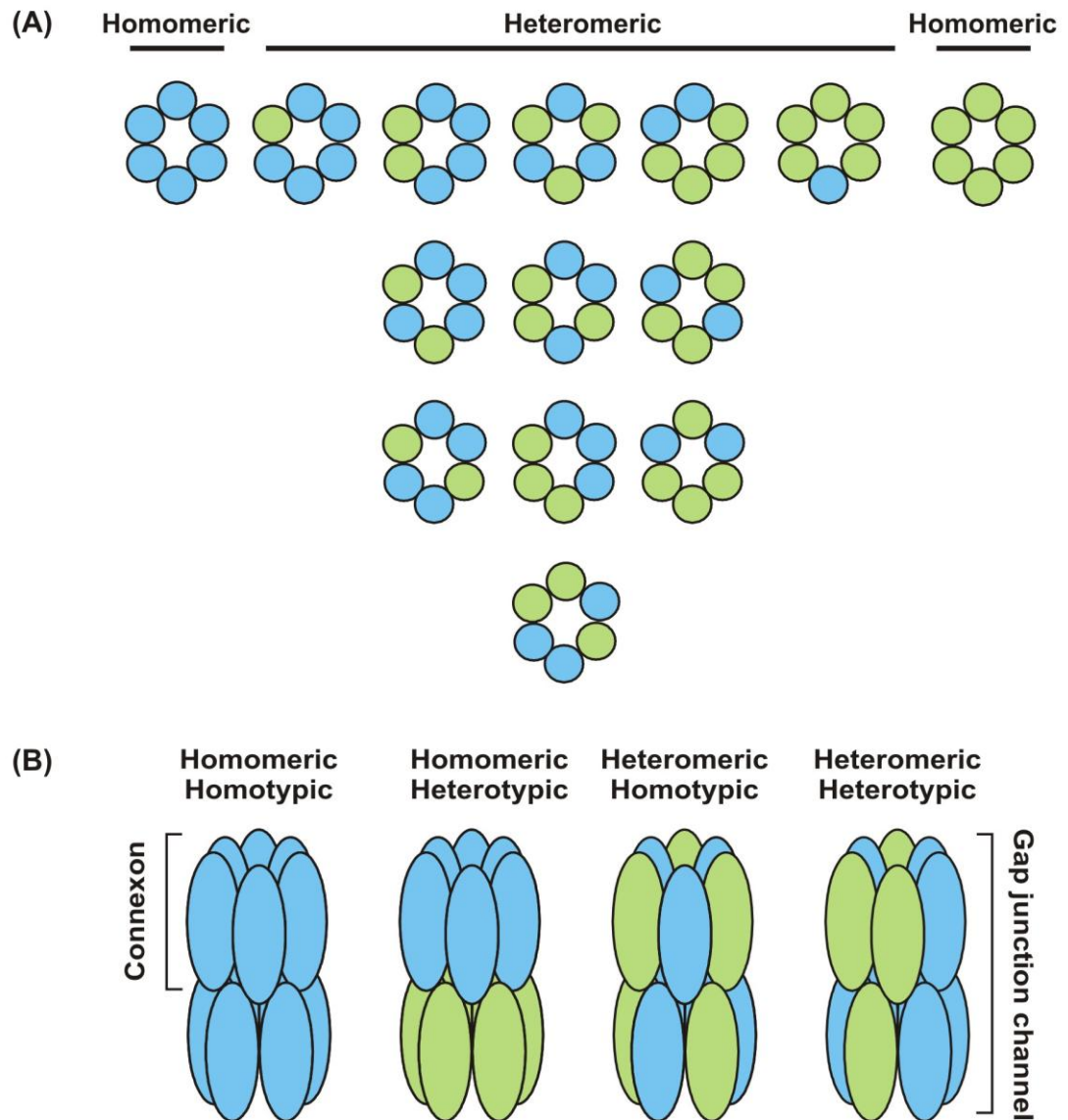


Figure 1.6: Predicted configurations of connexons and gap junction channels formed from two different connexins

When two different connexins are expressed, theoretically fourteen different connexons can be formed, two homomeric connexons consisting of one type of connexin and twelve heteromeric connexons consisting of two types of connexins (A). Image adapted from Brink et al. (1997). The docking of two homomeric connexons results in a homomeric/homotypic channels. Two homomeric connexons of different connexin composition produce a homomeric/heterotypic channel and two heteromeric connexons form a heteromeric/heterotypic channel. Image was kindly gifted by Stephen Rothery.

Not all connexins are compatible and have the ability to form functional gap junction channels. To study compatibility a range of techniques has been used. The majority of evidence for heterotypic and heteromeric channels have been based on *in vitro*

electrophysiological experiments using the dual voltage clamp technique on paired *Xenopus* oocytes (Bruzzzone et al. 1993; White et al. 1995) and transfected mammalian cell lines (Elfgang et al. 1995; Haubrich et al. 1996; Elenes et al. 2001; Desplantez et al. 2004) expressing different connexin subtypes.

Cells expressing a single connexin subtype are co-cultured together and the gap-junctional conductance measured between the cell pairs. Fluorescence imaging using different fluorescent markers (e.g. green fluorescent protein (GFP) and cyan fluorescent protein (CFP)) has been used to distinguish cells expressing two different connexin subtypes and this has been followed by confocal microscopy to visualise gap junction formation (Hopperstad et al. 2000; Rackauskas et al. 2007a). Gap junction channel assembly and distribution from the different connexin types have also been studied in living cells using fluorescent tracers (Falk 2000).

Evidence for heterotypic channels *in vivo* comes from scanning transmission electron microscopy of intact and split liver junctions between Cx26 and Cx32 (Sosinsky 1995). Other biological settings in which heterotypic channels have been identified include Cx45-Cx36 in the retina (Dedek et al. 2006) and Cx32-Cx26 in the central nervous system (Altevogt and Paul 2004). Studies on chimeric connexins whereby the different domains (e.g. extracellular loops 1 and 2 and cytoplasmic loop) between two different connexins are swapped indicated that compatibility between homomeric connexons likely depends on the specific motifs in the extracellular loops (White et al. 1995; Haubrich et al. 1996).

Experimental data of multiple connexin co-expression and co-localisation at the cell and tissue level have come from double and triple labelling techniques. Examples include Cx40, Cx43 and Cx45 in the atria and Purkinje fibres (Vozzi et al. 1999; Severs et al. 2001), Cx40, Cx43 and Cx37 in aortic endothelial cells (Yeh et al. 2000), Cx45 and Cx36 in the rodent retina (Li et al. 2004b) and Cx30, Cx26 and Cx31 in the rat cochlea (Nickel et al. 2009). It should be noted that multiple labelling by confocal microscopy is only indicative of a possible co-localisation within the same gap junction plaque. Electron microscopy is required for definitive evidence of co-localisation.

Co-immunoprecipitation and electrophysiological experiments using cell pairs provided evidence for heteromeric channels in endogenously co-expressing tissue such as Cx32-Cx26 in the liver (Bevans et al. 1998), Cx46-Cx50 in the lens (Jiang and Goodenough 1996) and Cx43-Cx46 in the lung (Das et al. 2001) and transfected cell models such as Cx40-Cx43 and Cx45-Cx43 (Brink et al. 1997; Valiunas et al. 2001; Martinez et al. 2002a; Desplantez et al. 2004). Heteromeric compatibility between connexins loosely correspond to the α and β subgroups (Figure 1.4) although there are exceptions to the rule (Koval 2006). For instance Cx29 that does not belong to either subgroup can form heteromers with β connexin Cx32. Using site-directed mutagenesis it was found that motifs that determine heteromeric compatibility include amino acid residues at specific locations in the amino-terminus and the cytoplasmic end of the third transmembrane domain (Lagree et al. 2003). Although the formation of heterotypic and heteromeric channels give rise to gap junctions with unique biophysical properties their roles *in vivo* are still unknown (Cottrell and Burt 2005).

1.3. Gap junction life cycle

1.3.1. Connexin turnover

Connexins are dynamic short-lived proteins with a half-life of only a few hours, exceedingly short compared to typical integral plasma membrane proteins (Laird 2010). The classical approach in studying connexin turnover involves pulse-chase studies whereby cells are exposed to labelled metabolic precursors (e.g. [³⁵S] methionine) for short periods of time (pulse). This labelled compound is incorporated into the connexins. The introduction of this label is followed by unlabelled compound (chase). The label is tracked and the connexin formation and trafficking followed thereafter (Laird et al. 1991; Laing and Beyer 1995; Beardslee et al. 1998; Musil et al. 2000). From such studies in rat cardiac myocytes the half-lives of Cx43 and Cx45 are 1-2 hours and 2-3 hours respectively (Laird et al. 1991; Darrow et al. 1995). However there are exceptions such as in cultured cells of the lens where two different kinetic pools of Cx56 were identified, with half-lives of 2-3 hours and of 2-3 days (Berthoud et al. 1999). It is likely that the function of the longer half-life pool is to maintain connexin stability. The rapid turnover normally retained by connexins ensures cells are able to govern the level of intercellular communication by modulating their assembly and

degradation and rapidly respond to physiological stimuli that prolongs or enhances their function (Laird 2006; Musil 2009).

1.3.2. Connexin synthesis, oligomerisation and transport to the plasma membrane

Gap junction channels are oligomeric and the connexin subunits must assemble before delivery into the plasma membrane (Segretain and Falk 2004; Koval 2006). The early stages of connexin biogenesis examined using a cell-free coupled transcription-translation *in vitro* approach showed that connexin polypeptides are co-translationally integrated into the endoplasmic reticulum (ER) (Falk et al. 1994; 1997; Ahmad et al. 1999). The only identified exception to this rule is Cx26, which has been shown to be post-translationally imported to the ER membrane (Zhang et al 1996; Ahmad et al 1999) and directly imported into the plasma membrane bypassing the Golgi network (Diez et al. 1999; George et al. 1999; Ahmad and Evans 2002; Martin et al. 2001). *In vitro* studies using *N-glycosylation* site tagging of connexins combined with protease protection assays demonstrated that connexins integrated *in vitro* are in the correct topological orientation (Falk and Gilula 1998; Martin and Evans 2004). Although no clear mechanism in connexin membrane integration has been identified it is likely that a molecular chaperone facilitates the process as connexins reach their folded and stable state (Falk and Gilula 1998; Laird 2006). The formation of inter-disulphide bridges between the extracellular loops suggests that connexins are under the surveillance of disulphide-isomerases (Laird 2006).

The precise location of connexin oligomerisation remains the subject of some debate. Studies from *in vitro* cell-free systems have reported that connexins assemble into connexons within the ER (Falk et al. 1994; 1997; Ahmad et al. 1999; Das et al. 2002; Maza et al. 2003; 2005). However treatments that block the ER-to-Golgi transport (e.g. brefeldin A disrupts the Golgi apparatus by vesiculating the membrane) did not interfere with oligomeric assembly thereby identifying a novel site for multiunit assembly at the *trans*-Golgi network for Cx43 and Cx46 (Musil and Goodenough 1993; Koval et al. 1997; Musil 2009). A schematic diagram of the life cycle of connexins is shown in Figure 1.7. Differences in the intracellular location of oligomerisation may differ between connexins and even cell types (Martin and Evans 2004; Musil 2009). There is also experimental evidence (Musil 2009) that the site of connexon assembly may be

driven by connexin protein concentration such that overexpression shifts the compartment in which assembly is initiated from the *trans*-Golgi network to the ER (Musil 2009; VanSlyke et al 2009). For instance in baby-hamster kidney cells in which Cx32 is overexpressed, electron microscopy revealed areas of unusual intercellular distribution with double-membrane gap junctions in the ER and the nuclear envelope (Kumar et al. 1995) which was later confirmed by VanSlyke et al. (2009). This could help explain inconsistent findings in different experimental settings such as exogenous overexpression in comparison to endogenous expression (Laird 2006; Musil 2009). It is also possible that connexon assembly is a progressive event where subunits begin to express in the ER and the ER-Golgi intermediate compartment with stable oligomers being established in the late-Golgi networks (Laird 2006; VanSlyke et al. 2009).

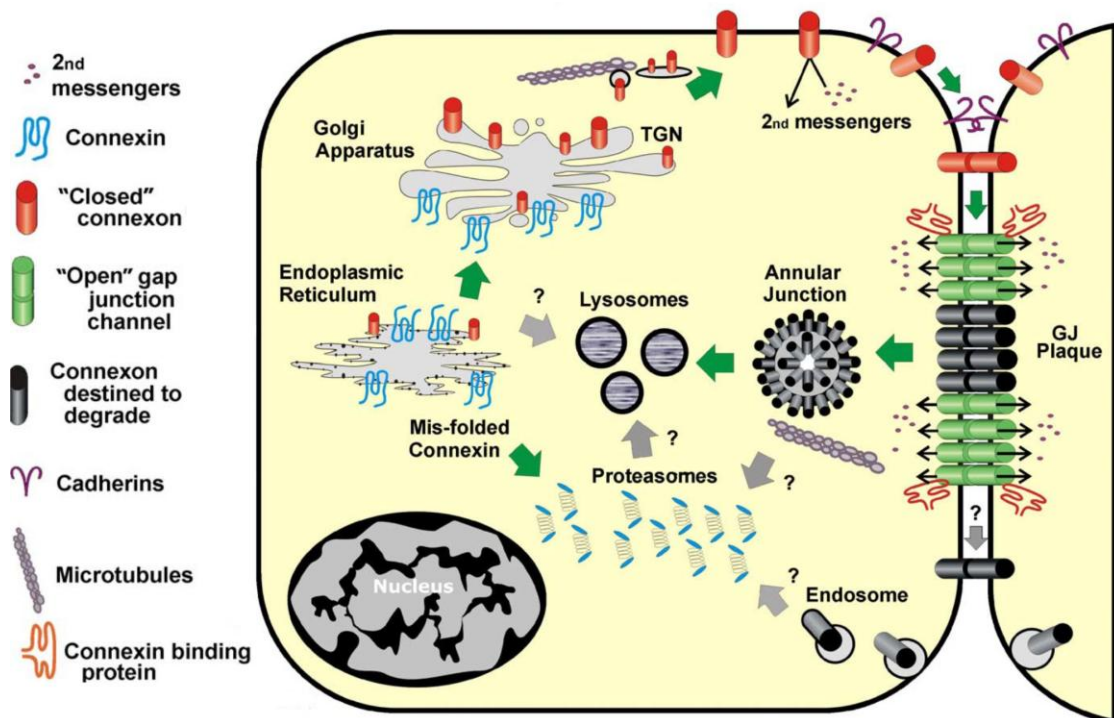


Figure 1.7: Life cycle of gap junctions

The gap junction life cycle is a multi-step pathway involving connexin synthesis, trafficking to the plasma membrane, channel formation, gap junction maintenance, gap junction internalisation and connexin degradation. Connexins furthermore interact with many protein partners, some of which are indicated in this overview. Image reproduced from Laird (2005).

The plasma membrane is mainly composed of hexameric connexons confirming that oligomerisation is completed in the Golgi and is a key determinant for the trafficking of

connexons from the Golgi to the plasma membrane (Li et al. 1996; George et al. 1999). This suggests a precise intercellular mechanism that distinguishes between proteins for further trafficking and dispensable proteins must exist. The major component of 'quality control' of newly synthesised proteins occurs at the stage of the ER (Musil 2009). It is also likely there are quality control systems function in Golgi network that complements the ER quality control pathway since in some connexins, like Cx43, oligomerisation takes place after exiting the ER (Arvan et al. 2002). Proteins that are incorrectly synthesised or misfolded are degraded through an ER-associated degradation (*ERAD*) process (Figure 1.7) (Ellgaard and Helenius 2003; Meusser et al. 2005). Since *ERAD* is an important mechanism of connexin turnover either up- or down-regulation of this process would have a major effect on connexin protein levels and gap junction function (Musil 2009).

Transport of connexons to the cell surface generally follows the conventional secretory pathways: passage from the ER to the ER-Golgi intermediate compartment and from there to the cis-, medial- and *trans*-Golgi cisternae, the *trans*-Golgi network and thence to the plasma membrane (VanSlyke and Musil 2000; Laird 2006; Musil 2009). Intracellular trafficking is usually monitored using newly synthesised connexins that are labelled with metabolic precursors or fluorescent markers such as chemiluminescent aequorin, GFP and its cyan and yellow (YFP) variants (George et al. 1999; Falk 2000; VanSlyke and Musil 2000; Martin et al. 2001; Lauf et al. 2002). Application of drugs that disrupt protein trafficking (e.g. brefeldin A) and the cytoskeleton (e.g. nocodazole that disrupts microtubules) have also been used. Connexins exit the Golgi predominantly in vesicular carriers heterogeneous in size and shape that traffic along microtubules to reach the plasma membrane (Lauf et al. 2002; Thomas et al. 2004; Thomas et al. 2005). Microtubules are not essential in facilitating connexin delivery but rather improve the efficiency of the delivery process (Thomas et al. 2001; Johnson et al. 2002b; Laird 2006). Recently the role of ER-associated chaperone protein 29 (ERp29) has been highlighted in the trafficking process by binding to and stabilising Cx43 oligomers facilitating its exit from the ER to the *trans*-Golgi network (Das et al. 2009).

It is still discussed whether the fusion of vesicles occurs to all parts of the plasma membrane or whether the vesicles are specifically transported to the vicinity of existing gap junction plaques. Studies using fluorescent tagged vesicles showed that insertion of

connexons appears random with movement of connexons laterally in the plane of the plasma membranes and thus joining the periphery of pre-existing gap junction plaques (Lauf et al. 2002; Thomas et al. 2005). This is also supported by observations that connexons are distributed as single particles or small groups rather than large aggregates (Lauf et al. 2002). Pulse-chase labelling demonstrated that new gap junction channels accumulate preferentially at the outer margins of gap junction plaques, with older ones accumulating in the centre destined for internalisation and degradation (Gaietta et al. 2002; Lauf et al. 2002). Support for direct insertion was described by Shaw et al. (2007) who noted that connexin-containing vesicles are transported directly along microtubules arriving closely at the edge of gap junction plaques. During ischemia and oxidative stress displacement of the end binding 1 protein from the microtubule plus ends not only limited the microtubule growth pattern but also reduced connexin delivery to the plasma membrane (Smyth et al. 2010). Most likely, the degree of specificity of targeting may depend on the cell type (e.g. polarised epithelial cells versus non-polarised cells), physiological conditions (e.g. ischemia) or connexin subtype (e.g. Cx26) (Martin and Evans 2004).

In the life cycle, connexins interact with numerous proteins of the cell machinery (Figure 1.7) (Martin and Evans 2004; Giepmans 2006; Laird 2010). This is particularly apparent in the heart where connexin interacting proteins such as zonula occludens-1 (ZO-1) has been associated with regulating the formation and size of Cx43 gap junctions (Hunter et al. 2005; Laing et al. 2007; Bruce et al. 2008). Other examples include the interaction of Cx43 with tubulin that links this connexin directly to the microtubule network (Giepmans et al. 2001) and interactions with calcium (Ca^{2+}) dependent cell-adhesion molecules such as N- and E-cadherin to help facilitate connexon diffusion into the plasma membrane (Jongen et al. 1991; Meyer et al. 1992; Kostin et al. 1999; Wei et al. 2005). Wu et al. also demonstrated that Cx43 delivery to the plasma membrane requires an interaction between α -or β -catenin with N-cadherin (Wu et al. 2003). It is likely that these adhesion molecules facilitate the docking of connexons by bringing the membranes close enough to initiate connexon interaction (Segretain and Falk 2004).

The structure of the carboxyl-terminus in particular, gives the opportunity for intracellular proteins to interact with connexins, creating a platform for connexin subtype or cell type specific regulation mechanisms (Severs 2007b). To date numerous connexin interacting proteins have been identified; for detailed reviews of this topic see Giepmans (2006) and Herve et al. (2007). The major categories of interaction are recognised as: (1) *inter-connexin interactions* that can lead to heteromeric or heterotypic channel formation; (2) *post-transcriptional modifiers* such as protein kinases and phosphatases that participate in regulation of channel gating events and connexin life cycle; (3) *scaffolding and cytoskeletal proteins* that bridge the connexins to microfilaments, microtubules and molecules such as cadherins and claudins to form a junctional nexus; (4) *trafficking regulators* that regulate or facilitate connexin delivery, assembly or internalisation such as caveolin 1 and 2 and ERp29, and (5) *growth regulators and other molecules* that facilitate growth, modulate channels structure and closure (Laird 2010).

1.3.3. Hemichannels and gap junctions

Once connexons are in the plasma membrane they can either form gap junction channels by docking to another connexon or function as hemichannels (Li et al. 1996; Segretain and Falk 2004; Evans et al. 2006). After connexin oligomerisation is complete the resulting hemichannel is thought to be in a closed configuration after insertion into the plasma membrane (Evans et al. 2006; Laird 2006; Goodenough and Paul 2003). Studies on Cx46 expressed in *Xenopus* oocytes revealed that high concentrations of Ca^{2+} on the cytoplasmic side maintained hemichannels in their closed state but this effect could be reversed in solutions with a low extracellular Ca^{2+} (Ebihara et al. 1995; Evans et al. 2006).

The use of connexin mimetic peptides (e.g. Gap 26 and Gap 27) which are peptides identical to a short amino acid sequence on the connexin subunit revealed that blocking hemichannels inhibited Ca^{2+} oscillations in smooth muscle cells (Dora et al. 1999) and electrical coupling (Isakson and Duling 2005). There is also evidence that hemichannels are utilised by cells in response to cell injury and metabolic inhibition (Evans et al. 2006; Goodenough and Paul 2003). For example, in the heart during a stress response ATP crosses the hemichannels and vasodilates the blood vessels facilitating the increase

in amount of oxygen being delivered (Headrick et al. 2003). Depending on the physiological condition of the cell, a connexon becomes docked into a gap junction channel or remains a hemichannel (Saez et al. 2005).

Typically a single gap junction plaque is less than a 100nm to several micrometers in diameter and contains a varying number of channels (less than a dozen to many thousands) (Forge et al. 2003; Segretain and Falk 2004). The mechanisms that mediate gap junction channel aggregation are still unclear. It has been shown that connexons are inserted into the membrane randomly through lateral diffusion. Time-lapse microscopy of gap junctions assembly shows that as the connexons move closer together in the plane of the membrane plaque they suddenly fuse over their entire length indicating some intrinsic affinity for each other (Falk 2000).

Structural studies have investigated the composition of gap junction channels and how channels composed of the different connexin subtypes are distributed within the plaque. Simultaneous visualisation of co-expressed connexins revealed that Cx32 and Cx26 from the same β subgroup were homogenously expressed throughout the plaque while Cx43 of the α subgroup when paired with either Cx32 or Cx26 segregated into well-separated domains. One of the mechanisms for segregation is that not all connexins are compatible and compatibility is dependent on the structural motifs encoded by the connexin polypeptide (Haubrich et al. 1996; Foote et al. 1998; Lagree et al. 2003; Koval 2006). Another mechanism would be through the different affinities connexins have for surrounding lipids or connexin binding proteins (Segretain and Falk 2004; Cascio 2005; Koval 2006). For example connexins (e.g. Cx43) have shown to associate with lipid rafts, which are specialised domains, enriched in cholesterol, sphingolipids (Simons and Toomre 2000) and co-localisation with lipid raft marker protein caveolin-1 (Schubert et al. 2002; Mograbi et al. 2003) portioning into microdomains. It is also reported that depending on the connexin its preference for certain lipid environments may vary resulting in channel aggregation, mixing or segregation.

1.3.4. Gap junction internalisation and connexin degradation

Since connexins have short half-lives an efficient mechanism must be in place for their internalisation and degradation (Musil 2009). Electron micrographs identified double-

membrane vesicular structures termed 'annular junctions' in the cytoplasm of cultured cells and in tissues which are proposed to contain fragments of gap junctions en route to degradation (Larsen and Hai 1978; Mazet et al. 1985; Severs et al. 1989). These findings were confirmed by immunogold labelling using specific connexin antibodies to establish that each vesicle contained substantial quantities of connexin protein (Figure 1.7) (Naus et al. 1993; Murray et al. 1997). Further evidence from fluorescent-tagged connexins in live cells demonstrated the internalisation of Cx43 in only one of the two cells from the plasma membrane into the cytoplasm (Jordan et al. 1999; 2001; Gaietta et al. 2002; Falk et al. 2009). Similar studies of Cx43-GFP transfected HeLa cells showed that internalisation of gap junction vesicles also involves clathrin-coated endocytosis (Piehl et al. 2007; Gumpert et al. 2008). The intracellular vesicles of gap junction membrane are thought to form by the invagination and pinching off of both junctional membranes into the cytoplasm of one of the two cells without dissociating channels into their component connexons (Segretain and Falk 2004; Salameh 2006). Once inside the cells the junctions are subdivided into smaller vesicles that are targeted by endo/lysosomal pathways (Piehl et al. 2007). However there are indications that additional pathways exist for gap junction removal. For instance annular junctions do not facilitate the internalisation of small gap junctions from the plaque centre as observed by Lauf et al. (2002) and Gaietta et al. (2002) and are even absent in certain cell types (e.g. hepatocytes). It is also possible that annular junction formation is dependent on the physiological state of the cell rather than being a constitutive 'housekeeping' process (Musil 2009).

Proteins are also degraded by lysosomes and proteosomes (Laird 2005). Electron microscopy has shown that annular junctions may be in close proximity and possibly fuse to lysosomes for degradation by lysosomal enzymes (Qin et al. 2003). Inhibition of proteosomal degradation through *N*-acetyl-leucyl-leucyl-norleucinal treatment also leads to the accumulation of Cx43 (Laing and Beyer 1995; Laing et al. 1997; Musil et al. 2000) and in the perfused rat adult heart (Beardslee et al. 1998). Inhibiting proteosome activity also resulted in accumulation of connexins particular in the cytoplasm where majority of the proteosome-dependent degradation normally takes place (VanSlyke and Musil 2002). It is likely that the proteosome pathway is responsible for ER-associated degradation while the lysosomal pathway is primarily responsible for degradation of membrane connexins (Laird 2006).

1.3.5. Connexin mutations in human disease

Germline mutations of several different connexins are known to cause a number of human diseases. Disruptions in intercellular communication due to faulty connexin folding, oligomerisation, trafficking or docking have been identified as the underlying factors that lead to the manifestation of these diseases. I will briefly introduce a few selected samples here; more comprehensive information and detailed clinical features are available in recent reviews (e.g. Laird (2008)).

Mutations can occur anywhere in the connexin gene. Mutations in the non-coding regions could affect elements that regulate transcription or interfere with the splicing to generate mature mRNA. An example of this is Chromosome X-linked Charcot-Marie Tooth disease, an inherited neurological disorder characterised by a loss of sensation and progressive weakening and atrophy of the distal limbs. This is associated with mutations in *GJB1* which encodes CX32 (Bergoffen et al. 1993; Bondurand et al. 2001; Martin et al. 2000). CX32 is expressed in Schwann cells and oligodendrocytes that provide the cell-to-cell communication in conjunction with other myelin-associated proteins essential for the functional and structural integrity of the myelin sheath and underpin nervous conduction (Martin et al. 2000). Site-directed mutagenesis of Cx32 resulted in aberrant trafficking and disrupted oligomerisation (Martin et al. 2000; VanSlyke et al. 2009). This leads to the failure of transfer of ions and small molecules across the myelin sheaths and demyelination (Abrams and Rash 2009). The hemichannel open probability can also be affected by mutations. For instance a mutant that increases the probability of hemichannel opening may cause cell death similar to that suggested for CX32 (Abrams et al. 2002).

The majority of known connexin mutations are located within the protein coding region (Beyer and Berthoud 2009). Non-sense mutations or frameshift mutations result in the loss of normal full-length protein because mRNA translation ends prematurely. An example in the 35delG mutation of *GJB2* (encoding CX26) resulting in deafness (Carrasquillo et al. 1997). Over 90 mutations in the *GJB2* gene are associated with inherited skin diseases and/or hearing loss (Laird 2006; Aasen and Kelsell 2009). Transfection of corresponding mutated forms of Cx26 all lead to reduced intercellular

coupling either due to faulty assembly (Martin et al. 2000; Forge et al. 2003) or failure to dock (Thomas et al. 2003).

Gap junction channel properties including the permeability, selectivity and gating could be also be altered by mutations (Beyer and Berthoud 2009). Dominant-negative mutations may alter the specificity and compatibilities of the co-expressed connexin. In the skin Cx43 and Cx26 do not form heteromeric channels but several Cx26 mutants associated with skin disease can inhibit Cx43 function via heteromerisation (Rouan et al. 2001). More than 20 autosomal dominant mutations in the *GJA1* gene are known to cause the rare disorder, oculodentodigital dysplasia, in which patients show craniofacial abnormalities and malformations in fingers and teeth (Paznekas et al. 2009). In patients with oculodentodigital dysplasia, gap junction communication is thought to be markedly reduced despite a functional CX43 wild-type allele (Roscoe et al. 2005). In addition to germline mutations, disease may be produced by the development of connexin mutations within somatic cells (Beyer and Berthoud 2009). Several mutant forms of *GJA5* (encoding CX40) have been isolated from the hearts of patients with atrial fibrillation suggesting somatic mutations of *GJA5* may be responsible for some cases of arrhythmia (Gollob et al. 2006).

Taken together, these findings highlight that mutated connexins may cause disease through disrupting the connexin life cycle such that intercellular communication is markedly reduced.

1.4. Regulation of gap junction function

1.4.1. Electrical properties

Gap junction channels are voltage sensitive. Macroscopic gap-junctional current (I_j) is sensitive to: (1) transjunctional voltage defined as the potential difference between the two cell interiors and (2) the transmembrane potential which is the voltage difference between the cell interior and the external medium, depending on the species (Bukauskas and Verselis 2004; Gonzalez et al. 2007).

Development of the dual whole-cell voltage clamp technique has allowed accurate resolution of the electrical properties of gap junction channels. This technique permits the cells to be clamped at a fixed membrane potential and the gap junction current to be recorded (Desplantez et al. 2004). To begin with the membrane potential of both cells is clamped to the same value. Thereafter the membrane potential of only one of the cells is depolarised or hyperpolarised in a stepwise fashion to establish a voltage gradient or gap-junctional potential. From the current recorded is possible to calculate the macroscopic junctional conductance. This value is influenced by: (1) the number of gap junction channels (N); (2) the open probability (P_0) and (3) the channel specific unitary conductance (Van Veen et al. 2006; Moreno and Lau 2007).

Dual voltage clamp analysis has shown that, in homotypic channels, steady-state junctional conductance is usually maximal when transjunctional voltage is zero. As one cell of a cell pair is depolarised or hyperpolarised, conductance relaxes from the main-state to lower values. Junctional current is rarely abolished, even at high voltages a voltage-insensitive component of junctional conductance remains. This relationship of macroscopic voltage sensitivity is commonly visualised by plotting all normalised gap-junctional current values measured at each depolarising and hyperpolarising voltage step, fit to a Boltzman equation.

To establish the unitary conductance, the number of channels between cell pairs has to be reduced. To do so, treatments with gap-junctional communication inhibitors such as heptanol, octanol or halothane permitted the recordings of single channel behaviours. After removing the agent, the channels open gradually enabling single currents to be recorded at a given voltage over time. By analysis of these single channel recordings, unitary open conductances for individual channels can be determined. These values are connexin isotype and channel specific. The electrical properties of connexins (e.g. Cx40, Cx43 and Cx45) have been studied most commonly on *Xenopus* oocytes and cultured mammalian cells such as HeLa, N2a and RIN cells transfected with different connexin subtypes (Dahl et al. 1987; Elfgang et al. 1995; Valiunas et al. 2000; 2001; Elenes et al. 2001). From these experiments it was determined that homotypic channels composed of Cx45 exhibited the smallest unitary conductance at 30-40pS, whereas Cx43 and Cx40 homotypic channels have values of 60-120pS and 150- 200pS (Gros and Jongsma 1996; Moreno 2004).

The electrical properties of heteromeric and heterotypic channels have also been studied in co-transfected cells to identify the functional significance of connexin co-expression patterns *in vivo*. Heterotypic docking of Cx43-Cx45 channels produced conductances that were intermediate between that of the single unitary conductance for homotypic Cx43 or Cx45 indicating a good association between the two connexins (Elenes et al. 2001; Desplantez et al. 2004; Moreno 2004). Similar results were obtained for Cx40-Cx45 heterotypic channels (Moreno 2004) and Cx30.2-Cx45 (Kreuzberg et al. 2006; Rackauskas et al. 2007b). The formation of heterotypic channels between Cx40 and Cx43 remains controversial with conflicting results (Elfgang et al. 1995; Haubrich et al. 1996; Rackauskas et al. 2007a). However work by Valiunas et al. (2000) and Cottrell et al. (2002) have reported the presence of heterotypic channels in cell pairs with considerably reduced coupling compared with the homotypic conductances for Cx43 or Cx40.

It has also been shown that Cx45, Cx43 and Cx40 can form heteromeric connexons producing channels with intermediate conductances or lower than what was observed for their respective homotypic conductances. For example Cx40 and Cx43 form heteromeric channels in co-expressing HeLa cells with an average conductance that is lower than values for homotypic channels of either connexin (Valiunas et al. 2001). In the A7r5 smooth muscle cell line that naturally co-express Cx40 and Cx43, a range of unitary conductances distinct from homomeric-homotypic Cx40 or Cx43 channels were observed (He et al. 1999). Thus there is considerable scope for electrical properties to vary according to the precise connexin make-up of the channel.

1.4.2. The role of phosphorylation

Phosphorylation is a key regulatory element in the lifecycle of connexins and gating of hemichannels and gap junction channels (Warn-Cramer and Lau 2004; Laird 2005; Solan and Lampe 2005). The carboxyl-terminus of connexins contains consensus phosphorylation sites for multiple kinases such as protein kinase A, protein kinase C and mitogen activated protein kinase (Solan and Lampe 2005; Herve et al. 2007). Phosphorylation of connexins has been primarily identified through shifts in their electrophoretic mobility when analysed by SDS-PAGE and the direct incorporation of ³²P (Musil and Goodenough 1991; Laird 1991; Laing et al 1994; Cooper et al 2000).

Cx43 phosphorylation has been the most extensively described to date. Cx43 is typically detected as three bands derived from different phosphorylation states; the fastest migrating band represents the newly synthesised connexin which is non-phosphorylated (NP) and two slower migrating bands (P_1 and P_2) which represent the phosphorylated forms (Musil and Goodenough 1991). Treatment with alkaline phosphatase confirmed that differences in molecular weight were due exclusively phosphorylation. Pulse-chase experiments showed that the NP form is trafficked to the membrane where it is then phosphorylated and integrated into functional gap junction plaques. It should be noted that phosphorylation does not exclusively take place at the membrane as some studies have shown (Puranam et al. 1993; Solan et al. 2003). Using phosphospecific and epitope-specific antibodies it has been established that differences in the P_1 and P_2 mobility forms are derived from phosphorylation at different sites of the protein (Musil et al. 1990; Solan et al. 2003; Solan et al. 2007; Solan and Lampe 2009).

Regulators of phosphorylation such as cAMP can mediate gap junctional communication via an increase in synthesis, trafficking and assembly at the plaque (Atkinson et al. 1995; Darrow et al. 1996; Paulson et al. 2000). cAMP has been shown to mediate its effects by adenylyl cyclase and protein kinase A leading to enhanced assembly of gap junction plaques (Paulson et al. 2000). Activation of kinases is cell type and cell stage specific and is highly sensitive to altered physiological conditions such as the presence of growth factors such as platelet derived growth factor and vascular endothelial growth factor (Warn-Cramer and Lau 2004).

Phosphorylation also plays a very important role in disease particularly in ischemia. During ischemia changes in phosphorylation include a rapid shift (5 minutes) from the $P_{1/2}$ isoforms to the faster migrating NP isoform in Cx43 (Solan and Lampe 2009). This loss in phosphorylation has been shown to occur at various sites (e.g. S325, 328 and 330) and is typically observed in the gap junction plaques. In contrast to the decrease in phosphorylation at sites S325, 328 and 330, an increase in phosphorylation at S368 has also been reported at the cell membrane and this is likely to contribute to a change in channel permeability (Solan and Lampe 2009). Due to the increasing evidence that Cx43 plays an important role in ischemia, its function in cardioprotection has been illustrated in ischemic preconditioning (Li et al. 2004a; Srisakuldee et al. 2009). In these studies, ischemic preconditioning preserved the phosphorylation of Cx43

phosphorylation by activating protein kinase ϵ whereas hearts from Cx43 deficient mice could not be preconditioned (Li et al. 2004a; Rodriguez-Sinovas et al. 2009). The emergence of Cx43 as a therapeutic target will be discussed further in section 5.5 of this chapter.

Phosphorylation has been correlated with an increase or decrease in hemichannels and gap junction channels (Moreno 2005). For instance the opening of a variety of hemichannels (e.g. Cx43, Cx46) can be blocked by the activation of protein kinases (e.g. protein kinase C) (Saez et al. 2003). Phosphorylation of rat Cx43 by protein kinase C causes a shift in the channel unitary conductance favouring a lower conductance and lower permeability to Lucifer yellow (Moreno et al. 1992; 1994; Kwak et al. 1995; Lampe et al. 2000).

1.4.3. Molecular and ionic permeability

Gap junctions are permeable to many biologically relevant molecules. Using a technique called transport specific fractionation Bevens et al. (1998) demonstrated the different permeabilities of connexins to biological molecules. Cyclic nucleotides such as cGMP and cAMP readily pass from one cell to the next through gap junction channels. However depending on the connexin type certain molecules are more efficiently transferred than others. For instance cGMP passes more efficiently through heteromeric channels composed of Cx32 and Cx26 than channels composed of Cx32 only (Bevens et al. 1998).

Components of cellular signalling pathways are also spread through gap junctions. The second messenger inositol 1,4,5-triphosphate (Niessen et al 2002) is transferred with connexin subtype specific permeabilities. Gap junctions are also permeable to siRNA molecules of a specific connexin subtype from cell-to-cell thereby influencing gene expression in neighbouring cells (Valiunas et al. 2005). Moreover Wallner et al. (2009) also demonstrated the transfer of miRNA via gap junctions in HeLa cells which potentially represents a new mechanism for the control of gene expression by the cellular environment.

Differences in ion selectivity by the connexin subtypes to dyes of varying molecular mass and charge (e.g. Lucifer yellow, ethidium bromide and Alexa dyes) were shown in HeLa cells and *Xenopus* oocytes transfected with different connexins (Elfgang et al. 1995; Cao et al. 1998; Valiunas et al. 2002; Weber et al. 2004). The flux of fluorescent dye through gap junctions is then measured by light microscopy to determine whether cells are coupled by gap junctions. These studies revealed that channels composed of each connexin display distinctive permeability properties.

Compared to classical ion channels, gap junctions are not selective for specific species although they discriminate between anions and cations. They seem to work as an aqueous pore that enables ion exchange. Cx45 channels show equal permeability to caesium and K^+ cations, but reduced flux for chloride ions indicating charge selectivity (Veenstra et al. 1995). Further studies demonstrate that Cx40 channels are cation selective (Beblo and Veenstra 1997), while channels composed of Cx43 exhibit no overall selectivity for either anions or cations (Wang and Veenstra 1997).

1.4.4. Chemical gating

The role of Ca^{2+} in cell uncoupling had initially been established from experimental data correlating loss of electrical and dye coupling to increased intracellular Ca^{2+} $[Ca^{2+}]_i$ monitored by the Ca^{2+} indicator aequorin (Rose and Loewenstein 1975; 1976). This was later confirmed in a number of cell types including cardiac cells, cultured lens cells and osteoblasts (Peracchia 2004). Its role in uncoupling may be particularly important in protecting intact cells from membrane depolarisation and leakage of metabolites through gap junctions by disconnecting them from damaged cells (Rackauskas et al. 2010). This process is called 'healing-over'. Ca^{2+} -mediated inhibition of intercellular communication has been confirmed in a number of cell models (De Mello 1975; Dahl and Isenberg 1980; Neyton and Trautmann 1985). Although it is established that Ca^{2+} affects channel gating, the $[Ca^{2+}]_i$ that activates the gating mechanism is still unclear and may depend on the connexin and cell type ranging from a concentration of 500nM-400 μ M (Peracchia 2004). The wide concentration range could be due the variation in effectiveness of the internal buffering systems and sometimes the increase in $[Ca^{2+}]_i$ is too brief to activate the relatively slow gating mechanism. To enable a functional interaction between Ca^{2+} and connexins, clusters of negatively charged amino-acid

residues would be required to face the cytoplasm. Among connexins only a single amino acid residue (glutamate) faces the cytoplasm located at the transition between the fourth transmembrane (M4) domain and carboxyl-terminus. Despite there being six of these residues per connexon to bind to Ca^{2+} this may not be sufficient to mediate channel gating. It is likely that Ca^{2+} acts through an intermediate and one potential candidate is calmodulin (Peracchia et al. 1983; Torok et al. 1997).

Alterations to intracellular pH can also affect junctional conductance (Turin and Warner 1977; Spray et al. 1981). Cellular uncoupling through pH dependent gating would protect neighbouring cells from the potentially damaging effects of intracellular acidification. Sensitivity to pH varies among cell types and is in part related to the type of connexin expressed. By using a method that monitors junctional conductance and pH at different levels of acidification, studies demonstrated that Cx32 is the least sensitive to changes in pH and Cx50 was the most sensitive (Liu et al. 1993; Stergiopoulos et al. 1999). Connexin co-expression could also affect the pH sensitivity with properties unique to those of the individual connexins. For example Gu et al. (2000) demonstrated that mixing of Cx40 and Cx43 yields channels with increased sensitivity to pH. The molecular mechanism of pH gating is likely to involve the connexin cytoplasmic loop and carboxyl-terminal. Cx43 truncation abolishes pH sensitivity (Liu et al. 1993) but co-expression of the mutant connexin with the missing carboxyl-terminal sequence restores pH sensitivity and gating (Morley et al. 1996). Histidine residue at position 95 in the cytoplasmic loop has been proposed to be involved in pH gating since substitution with a basic or uncharged residue reduces pH sensitivity (Ek et al. 1994). The proposed mechanism is that low-pH facilitates interaction of the carboxyl-terminal with a receptor domain elsewhere in the connexin molecule causing the channel to close (Liu et al. 1993; Ek et al. 1994; Morley et al. 1996).

1.5. Gap junction function in cardiac tissue

Flow of electrical current between cardiac muscle cells is facilitated through low resistance pathways formed by gap junctions ensuring mechanical responses are orderly and synchronous in the heart (Green and Severs 1993). Action potential propagation is initiated in the sinoatrial node where it spreads through the atria (~1.2m/sec) to the atrioventricular node (~0.05m/sec). This conduction delay in the atrioventricular node

ensures the ventricles contract after the atria. After this, conduction is accelerated through the His bundle and Purkinje fibres ($\sim 2\text{-}4\text{m/sec}$) before entering the ventricles ($\sim 0.6\text{m/sec}$) resulting in contraction. The precise orchestration of electrical flow through the different tissue compartments is governed by: (1) cell morphology and size and (2) specific expression and co-expression connexin patterns. Disruptions in any of these factors contribute to conduction abnormalities in the adult heart.

1.5.1. Gap junction organisation in the normal adult heart

In the working (contractile) myocardium, gap junctions are located at the intercalated disc in discrete zones of interaction between adjacent plasma membranes together with two types of adhesion junctions: (1) fascia adherens, to which myofibrils of adjacent cells are joined and (2) desmosomes, that serve as anchoring sites for intermediate filaments that form a mechanical scaffold within the cell (Figure 1.8 (A)) (Green and Severs 1993; Severs et al. 2008). The intercalated discs are situated where the cells abut in a step-like structure (Figure 1.8 (A)). This ensures that myofibrils of end-to-end cells are mechanically coupled via the fascia adherens junctions. Thin-section electron microscopy revealed that gap junctions occupy intervening portions of the disk step that lie parallel to the long axis of the cell. It should be noted that depending on the cell type gap junction organisation may differ slightly as shown in Figure 1.8 (B) and (C). For example in the normal atrial myocardium, gap junction organisation is variable with some confined to the intercalated disc as in ventricles while others show a spread along the lateral aspects of the cell (Severs et al. 2004a; 2008). Depending on the myocyte subtype gap junction size may vary. For instance in nodal cells the gap junctions composed of Cx45 are sparse and small in size (Masson-Pevet et al. 1979). Collectively this organisation of intercellular junctions at the intercalated disc together with tissue architecture (i.e. cell size and shape) results in the anisotropic propagation observed in the heart ensuring conduction is faster in the longitudinal direction than in the transverse axis.

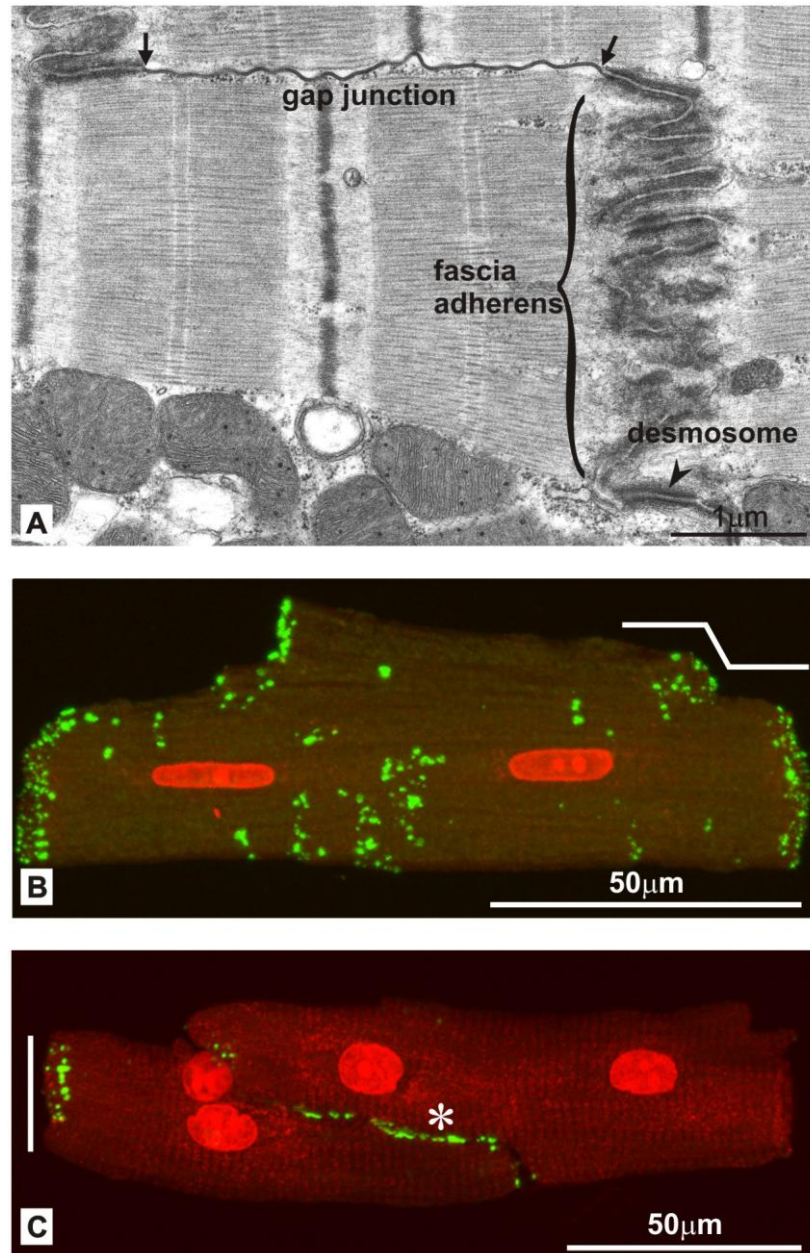


Figure 1.8: Gap junction organisation at the intercalated disc

The intercalated disc in a cardiac myocyte shown by thin section electron microscopy (A). The three junction types are indicated. Fasciae adherens occupy vertical zones of the disc, gap junctions and desmosomes are located mainly at the lateral facing zones. Image reproduced from Severs (2000). Reconstruction of serial optical sections through an isolated ventricular myocyte labelled for Cx43 (green) illustrating the organisation of gap junctions in clusters at the intercalated disc in the classic step-like configuration indicated by the line at top left hand corner (B). Image reproduced from Severs et al. (2004b). Atrial cells are a lot slender than their ventricular counterparts. (C). Gap junction organisation is also a lot more varied while some are present at the ends of the intercalated disk and others are spread laterally (asterisks). Image reproduced from Severs et al. (2008). The nuclei are counterstained with propidium iodide in images (B) and (C).

1.5.2. Connexin co-expression in the healthy heart

In mammalian hearts the main connexins expressed are Cx40, Cx43, Cx45 and Cx37 (Figure 1.9) (Gros and Jongsma 1996; Severs et al. 2008). However Cx37 is restricted to vascular endothelium where it is co-expressed with Cx40 and as a result does not participate in conduction (Vozzi et al. 1999; van Kempen and Jongsma 1999; Simon and McWhorter 2002). Therefore this investigation will be focussed on Cx40, Cx43 and Cx45 and this sub-group will be referred to as the ‘cardiac connexins’ for the remainder of my thesis.

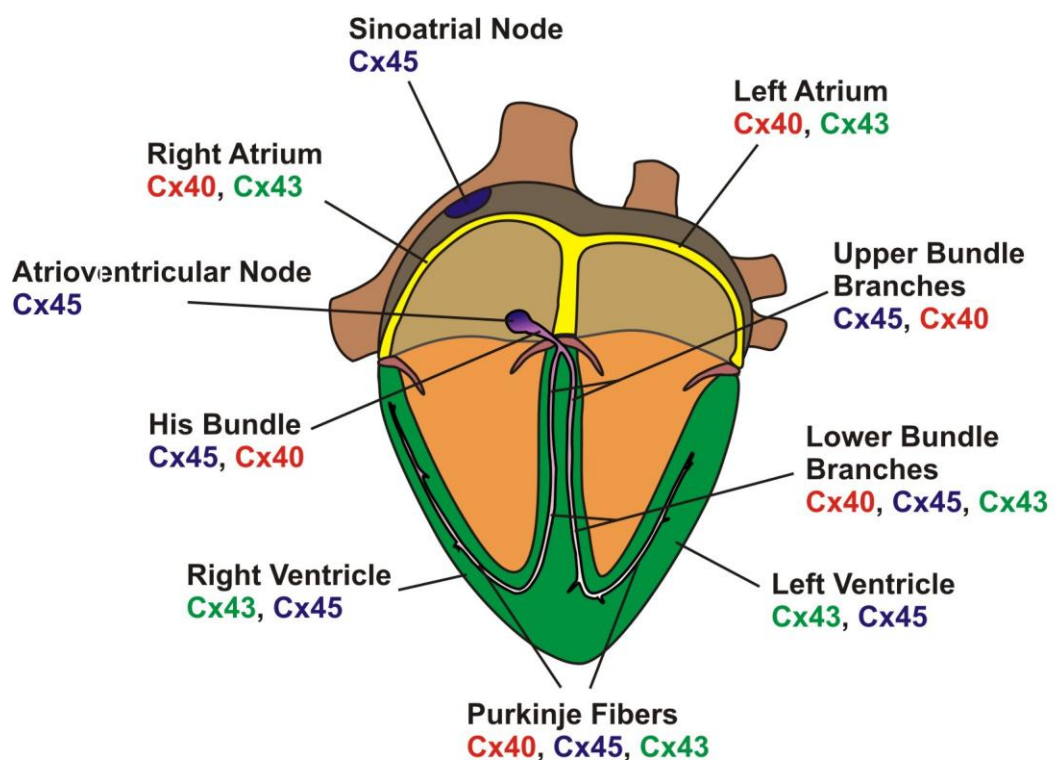


Figure 1.9: Connexin co-expression patterns in the adult healthy heart

Connexins are co-expressed in a tissue-specific manner. Cx43 is predominant throughout the working myocardium, Cx45 is mostly found within the specialised conduction system, commonly co-expressed with Cx40. Image adapted from Severs et al. (2008).

In the adult heart Cx43 is the most abundant isoform expressed in myocytes of the working atrial and ventricular myocardium with exceptions being the sinoatrial and atrioventricular nodal cells (Figure 1.9 and Figure 1.10 (A)) (Vozzi et al. 1999; van Veen et al. 2001). In the atria Cx43 is co-expressed with Cx40 with low amounts of Cx45 also present. Double label immunogold labelling demonstrated that CX40 and CX43 are co-localised within the same gap junction plaque (Figure 1.10 (B)) (Severs et

al. 2001; Kanagaratnam et al. 2002). Cx40 expression is highly variable with levels in the right atrium 2 to 3 fold higher than the left (Vozzi et al. 1999; Dupont et al. 2001a). Working ventricular myocytes lack detectable Cx40 despite its high levels of expression during embryonic development (Gros et al. 1994; Vozzi et al. 1999; Coppen et al. 2001b). However in the His-Purkinje conduction system Cx40 is strongly expressed where it forms high conductance channels with large gap junctions correlating with the fast conduction properties of this system (Gourdie et al. 1993; Gros and Jongsma 1996; Severs et al. 2001).

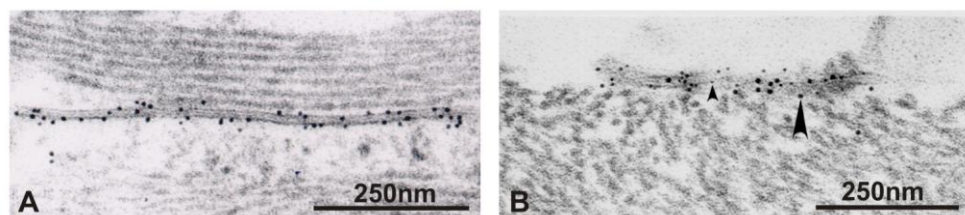


Figure 1.10: Immunogold labelling of CX40 and CX43 in the different myocyte subtypes

Immunogold thin-section electron microscopy demonstrated the presence of CX43 in the human ventricles (A) and both CX40 and CX43 in human atrial myocyte gap junctions (B). CX40 and CX43 were differentiated by using gold particles of different width, 10nm and 5nm for CX40 and CX3 respectively highlighted by the arrows. Image reproduced from Severs et al. (2005)

Cx45 is expressed at low levels throughout the myocardium including the His-Purkinje conduction system where it co-localises with Cx40 (Davis et al. 1995; Coppen et al. 1998b; 1999; Vozzi et al. 1999; von Maltzahn et al. 2009). Cx45 is also the sole connexin expressed in the sinoatrial and atrioventricular node forming small dispersed gap junctions (Coppen et al. 1999; Severs et al. 2001). These features together with the low conduction properties of Cx45 channels indicate relatively poor coupling in these areas (Elenes et al. 2001; Desplantez et al. 2004). A fourth connexin, Cx30.2 has been discovered in the mouse heart specifically at the sinoatrial and atrioventricular node but its human ortholog CX31.9 was not detected in the working myocardium and conduction system (Kreuzberg et al. 2005; 2009). This also highlights species differences in connexin expression particularly important when comparing small and large mammals possibly reflecting the need for impulse delay according to heart size (Severs et al. 2008). These differences need to be borne in mind when extrapolating functional data from animal models to the human.

1.5.3. Gap junction remodelling and cardiac disease

Cardiovascular disease is the leading cause of death and disability in most industrialized countries of the developed and developing worlds. Disturbances in cardiac rhythm (arrhythmias) are a common, serious and often fatal complication of many forms of heart disease. Electrophysiological studies have shown the most dangerous disturbances of heart rhythm are re-entry arrhythmias in the ventricle, caused by slowed conduction and conduction block. A re-entrant circuit develops when the wavefront of propagation is partially blocked at a given position, causing rotation around this point and re-entry into the site of original excitation in a repetitive cycle (Kleber and Rudy 2004). Since gap junctions govern the electrical flow that leads to contraction of the heart, the question, first posed by Professor Severs and Green, was whether alterations of gap junction organisation and connexin expression could play a role in abnormal impulse propagation (Smith et al. 1991). Since that time, numerous studies have been conducted to examine this hypothesis and have identified gap junctions and connexin expression to be key contributors to the arrhythmic substrate alongside factors such as membrane excitability (i.e. voltage-gated ion channels), cell size and tissue architecture (Severs et al. 2008).

Gap junction remodelling can be divided into two main categories: (1) altered distribution or organisation of gap junctions and (2) alterations in the amount and type of connexin expressed (Severs 2009).

Gap junction remodelling in the ventricle

Myocardial infarction results in a significant loss of cellular communication in the affected cells (Figure 1.11). Ultrastructural examination of the myocardial zone bordering the infarct scar tissue showed extensive loss of CX43 expression and organisation of gap junctions over the lateral surfaces of the cells first reported in patients with end-stage ischemic heart disease by Smith et al. (1991) (Figure 1.11). Similar areas of gap junction disarray are also associated with end-stage heart failure due to idiopathic dilated cardiomyopathy (Kostin et al. 2003) and in compensated hypertrophy due to valvular aortic stenosis (Kostin et al. 2004). These patterns of gap junction organisation have also been reported in animal models of ventricular heart disease including myocardial infarction in rat (Matsushita et al. 1999) and dog (Luke

and Saffitz 1991; Peters et al. 1997; Kieken et al. 2009), heart failure in the dog (Akar et al. 2007) and hypertrophied ventricles in rats (Uzzaman et al. 2000; Emdad et al. 2001). Structural remodelling such as this is strongly associated with arrhythmia and slowing of conduction which could potentially increase the risk of re-entrant circuits (Peters et al. 1997; Jongsma and Wilders 2000; Severs 2009).

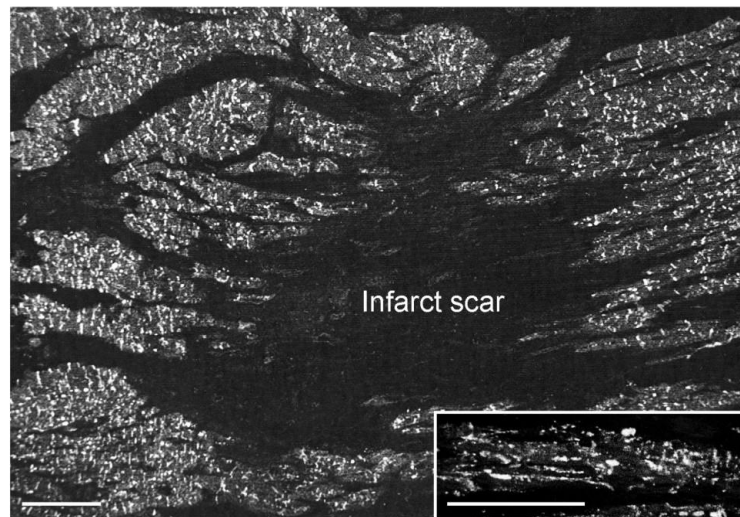


Figure 1.11: Immunofluorescence microscopy of human ventricular myocardium from a patient with end-stage ischemic heart disease

No CX43 signal can be detected within the infarct scar. In the surviving myocardium at the border of the infarct scar CX43 immunolabel is dispersed over the lateral borders of the cells rather than showing the normal polarisation at the intercalated disk highlighted by the enlarged picture. Image reproduced from Severs (2009).

Alterations in gap junction organisation are often accompanied by changes in Cx43 expression. Reduced CX43 protein and transcript levels have consistently been reported in patients with heart failure irrespective of whether the condition is due to idiopathic dilated cardiomyopathy (Dupont et al. 2001b), artery stenosis (Kostin et al. 2004), hibernating myocardium (Kaprielian et al. 1998) or other etiologies (Kitamura et al. 2002). In addition alterations in gap junction size and density have consistently been identified as major features of gap junction remodelling. In the diseased human heart reduced gap junction size has been reported in the hibernating myocardium (Kaprielian et al. 1998) and hypertrophy (Kostin et al. 2004) together with the decrease in overall amount of CX43 immunolabelling (Peters et al. 1993; Kitamura et al. 2002; Kostin et al. 2003). Alongside the reduction in CX43, elevated levels of CX45 (Yamada et al. 2003)

and CX40 have been shown in ventricles of patients with congestive heart failure due to ischemic cardiomyopathy (Dupont et al. 2001b). The significance of increased CX40 and CX45 expression is still unclear but could represent a compensatory response to failing levels of CX43.

Gap junction remodelling in the atria

The most common atrial arrhythmia is atrial fibrillation, a condition in which wavelets of electrical activity propagate in multiple directions leading to disorganised depolarisation and ineffective atrial contraction (Zipes 1997). The condition is associated with progressive electrical, contractile and structural remodelling, including altered cell size and mitochondrial shape, loss of sarcomeres and perinuclear accumulation of glycogen (Severs et al. 2004a). Alterations of gap junctions and connexin expression, in particular that involving Cx40, are reported to feature in the remodelling process (Severs et al. 2004a).

Studies on human atrial samples have revealed inconsistent results concerning the exact nature of gap junction remodelling in atrial fibrillation. In comparing patient samples with normal sinus rhythm to those with atrial fibrillation different studies report CX40 protein levels to be elevated (Polontchouk et al. 2001), significantly reduced expression of transcript, protein and immunosignal (Nao et al. 2003; Wilhelm et al. 2006) or no change in CX40 when examined by immunofluorescent labelling (Kanagaratnam et al. 2004) and western blot (Takeuchi et al. 2006). With regard to structural remodelling, gap junction lateralisation, heterogeneity and alterations in gap junction number have been reported in various studies (Polontchouk et al. 2001; Kostin et al. 2002; Takeuchi et al. 2006). Changes in junctional proteins such as N-cadherin and desmoplakin are reported to show similar changes in distribution to those of CX40 (Kostin et al. 2002) suggesting a spatial association between gap junctions and adhesive junctions during their re-organisation at the plasma membrane (Severs et al. 2004a). Studies in goat models of pacing-induced atrial fibrillation also identified heterogeneity in Cx40 distribution with areas of low density Cx40 next to areas of higher (normal) density Cx40 and a reduced ratio of Cx40/Cx43 (van der Velden et al. 1998; 2000; Ausma et al. 2003). However no lateralization of Cx40 observed in human samples was noted.

Discrepancies between studies on atrial fibrillation arise from the use of different animal models and species, different time courses when comparing samples, lack of antibody specificity, use of different patient sub-groups with different associated pathological conditions and interpretation of the data (Polontchouk et al. 2001; Kanagaratnam et al. 2002; 2004; Duffy and Wit 2008; Severs et al. 2008). While it is recognised that gap junctions are contributing factors to the occurrence of atrial fibrillation, deciphering how their precise role requires more rigorous experimental approaches as proposed by Duffy and Wit (2008).

One aspect not always fully appreciated is the need to distinguish between gap junction remodelling brought about as part of atrial fibrillation which potentially contribute to the perpetuation of the condition and gap junction features that may increase susceptibility to atrial fibrillation in the first place. For example, analysis of patient samples undergoing coronary artery bypass surgery without previous history of arrhythmias revealed elevated levels of CX40 are associated with a higher risk of the development of post-operative atrial fibrillation (Dupont et al. 2001a). In these samples no difference in protein and mRNA for CX43 and CX45 were detected between the atrial fibrillation and non-atrial fibrillation groups. To correlate connexin expression with conduction velocities (CV) in the human atrium, Kanagaratnam et al. (2002) compared CX40 and CX43 expression quantified by immunofluorescent labelling to atrial CV in patients undergoing cardiac surgery. Results showed that an increase in CX40 relative to the total connexin signal ($CX40/[CX40+CX43]$) is associated with a decrease in CV. The reason for this decrease in CV when a connexin of high conductance is expressed is not yet clear.

1.5.4. Transgenic mouse models of cardiac connexins

Transgenic mice have been developed in which the expression of specific connexins is ablated or one connexin type is substituted for another to study their roles in impulse propagation and cardiac morphogenesis.

Transgenic Cx45 models

The expression of Cx45 is critical for the normal development of the heart. Replacement of Cx45 with the lacZ reporter gene in transgenic mice resulted in heart failure and

embryonic death at day 10 (Kumai et al. 2000). Furthermore the mice displayed severe defects in cardiac looping, vascular developments and the endocardial cushion (also known as atrioventricular canal or septal defects) (Kruger et al. 2000; Kumai et al. 2000). When deletion of Cx45 is restricted to the myocardium, development of the endocardial cushion is not disrupted but the mice still die from conduction block similar to those observed in non-conditional knockouts (Nishii et al. 2003). In the developing heart Cx45 is the earliest detectable connexin particularly in the conduction tissue and is the only connexin to be continuously expressed throughout the conduction system in the adult heart (Alcolea et al. 1999; Coppen et al. 2001a). As a result functional compensation by other cardiac connexins cannot occur in Cx45 knockout models. Over-expression of cardiac specific Cx45 in transgenic mice did not result in structural malformations and contractile dysfunction but did show enhanced susceptibility to spontaneous and inducible arrhythmias and altered intercellular coupling (Betsuyaku et al. 2006). These results confirm the requirement of Cx45 in early cardiogenesis and the maintenance of electrical coupling in adult hearts.

Transgenic Cx40 models

Cx40 is a major determinant of cardiac development and electrical impulse propagation in the cardiac conduction system. To examine the function of Cx40 in the developing and adult mouse heart two independent approaches have been undertaken to generate Cx40 knockout mice (Kirchhoff et al. 1998; Simon et al. 1998). Cx40 knockout mice often die just after birth or as young adults due to developmental malformations; however many of the animals survive to adulthood (Kirchhoff et al. 2000; Gu et al. 2003). The type of abnormalities observed may be dependent on the genetic background differences in the mice used to generate the models (Gu et al. 2003). Cx40 null mice displayed a range of malformations such as tetralogy of Fallot, endocardial cushion defects and myocardial hypertrophy (Kirchhoff et al. 2000; Gu et al. 2003). Structural abnormalities were also observed in heterozygous knockout mice but generally incidence of malformations was lower and less severe than for homozygous knockout. Thus it appears that although Cx40 is not essential for cardiac morphogenesis, its absence or limited expression increases the probability of cardiac malformations.

Animals which lack Cx40 show delays in impulse conduction in the atria, atrioventricular node, His bundle and bundle branches (Kirchhoff et al. 1998; Simon et

al. 1998; Hagendorff et al. 1999; Verheule et al. 1999; VanderBrink et al. 2000; Leaf et al. 2008) in agreement with the distribution patterns of Cx40 (Gros et al. 2004). Furthermore, increased incidence of spontaneous and/or pacing induced atrial arrhythmias is apparent (Kirchhoff et al. 1998; Hagendorff et al. 1999; Verheule et al. 1999; Bagwe et al. 2005). Recordings of electrical activation by optical mapping showed an abnormal pattern of excitation and propagation in the right atria of adult Cx40 knockout mice most likely due to the intraatrial conduction slowing and the high incidence of cardiac malformations found in these animals (Bagwe et al. 2005). Transgenic mice in which Cx40 was replaced by Cx45 resulted in slow conduction in the left atrium but normal CV elsewhere suggesting a chamber-specific role for Cx40 (Alcolea et al. 2004). In Cx40 knockout mice no up-regulation was detected in transcripts and protein levels of the other cardiac connexins Cx43, Cx45 and Cx37 (Kirchhoff et al. 1998; Bagwe et al. 2005).

Results on impulse propagation differed from those obtained in the whole heart and *in vitro* cell models. To examine the functional relevance of Cx40/Cx43 co-expression Beauchamp et al. (2006) generated cultured strands of atrial myocytes derived from Cx40 and Cx43 knockout mice and measured CV using optical mapping. In contrast to observations made in the intact heart genetic ablation of Cx40 led to an increase in CV with a higher amount of Cx43 expression at the plasma membrane although the total Cx43 protein content remained unchanged. Kanagaratnam et al. (2002) also reported similar findings in human atria i.e. that decreased CX40/CX43 co-expression is accompanied by a paradoxical increase in CV. Differences in CV measurements are likely to depend on the experimental design and the connexin co-expression pattern. It is therefore plausible to obtain different results in a setting with reduced number of cells (Beauchamp et al. 2006) or in a restricted area of the atria (Kanagaratnam et al. 2002) in comparison to the whole heart (Kirchhoff et al. 1998).

Transgenic Cx43 models

The first transgenic mouse to be established was the germline deletion of both Cx43 alleles (Reaume et al. 1995). The mice survive to term but die at birth from severe malformations of the right ventricular outflow tract which prevents blood flow to the lungs (Reaume et al. 1995). Despite morphological abnormalities, the foetal Cx43 knockout heart is still able to function normally most likely due to the presence of Cx40

and Cx45 during embryonic development since conduction does not exclusively require Cx43 channels (Reaume et al. 1995; Van Kempen et al. 1996; Delorme et al. 1997; Alcolea et al. 1999). Studies on electrical impulse at various stages of embryonic development revealed no difference in CV at embryonic day 12.5 in Cx43 knockout and control mice (Vaidya et al. 2001). At embryonic day 15.5 there is gross reduction in CV in the right ventricle of the mutants and in both ventricles at embryonic day 17.5 which corresponds to a down-regulation of Cx40 expression. There also does not appear to be any significant alterations to total Cx45 expression in Cx43 knockout mice (Johnson et al. 2002a).

Electrophysiological analysis of intercellular communication in cultured Cx43 knockout mice showed a reduction of 60% in macroscopic junctional conductance, impaired contractility and loss of dye coupling compared to wild type cells (Vink et al. 2004). Quantification of Cx40 and Cx45 mRNA transcripts showed no alterations in compensation to the lack of Cx43. A study by Beauchamp et al. (2004) on ventricular myocyte strands from germline Cx43 knockout mice provided further information on CV after the loss of Cx43. In this system, impulse propagation was reduced by 97% corresponding to a reduction in transjunctional conductance on cell pairs by a similar amount. Analysis of the single channel properties identified the presence of Cx45 to be the sole conductor in these myocytes after Cx43 knockout. Data from this work confirm that Cx43 channels are the principal conductors in ventricles of the mouse heart but conduction can still be maintained by low levels of Cx45 in the absence of Cx43 albeit at slow velocity.

To investigate the role of Cx43 specifically in the myocardium, Gutstein et al. (2001a) generated mice with a cardiac-restricted inactivation of Cx43 using the Cre/Lox system. The mice were born with a normal heart and contractile function. Despite the 90% loss in Cx43, CV was markedly slow but propagation was still maintained until the onset of spontaneous ventricular tachycardia leading to premature death at two months after birth (Gutstein et al. 2001a). This suggests that although basic heart function can be maintained for a limited period through the expression of Cx45, Cx43 is critical in the formation of the arrhythmogenic substrate.

Heterozygous Cx43 mice have also been developed in neonatal and adult hearts which survive and breed normally (Guerrero et al. 1997). The level of Cx43 in heterozygous mice is approximately 50% of the normal amount of Cx43 (Guerrero et al. 1997; Thomas et al. 1998; Beauchamp et al. 2006). The levels of Cx40 and Cx45 were unaltered in Cx43 heterozygous hearts (Guerrero et al. 1997; Thomas et al. 1998; Beauchamp et al. 2004). However Johnson et al. (2002a) did report a reduction in the amount immunosignal area occupied by Cx45 in Cx43 both in heterozygous and homozygous knockout mice. Data on ventricular CV in Cx43 heterozygous mice vary considerably with studies either reporting slowed ventricular conduction (Guerrero et al. 1997; Thomas et al. 1998; Gutstein et al. 2001b) while others detected no change in CV (Morley et al. 1999). Furthermore Thomas et al. (1998) reported no alteration in CV in the atria of Cx43 heterozygous mice suggesting that Cx43 conduction plays a subordinate role in atrial conduction. Effects of diminished Cx43 expression accelerate the onset and increase the incidence of arrhythmias when exposed to acute regional ischemia (Lerner et al. 2000).

Using outbred cardiac-restricted knockout mice it was possible to study the gradual loss of Cx43 in the heart (Danik et al. 2004). No change in propagation was detected in animals expressing $\geq 40\%$ of Cx43. Reductions in CV (50%) were only observed when Cx43 protein content was decreased to 18% of the control levels and 80% of these mice were inducible to lethal ventricular arrhythmias. Further studies in these cardiac-restricted knockout mice suggested that a mismatch in the coupling between the Purkinje fibres (unaltered levels of Cx40 and Cx45) and the Cx43 deficient ventricular myocytes lead to altered activations and wavefront collisions (Morley et al. 2005).

Taking these findings together, there is now clear evidence that gap junction remodelling and altered patterns of connexin expression do occur in certain categories of human heart disease and corresponding changes in animal models cause arrhythmia. In particular, reduced expression of ventricular Cx43 and hence alterations in the ratio of co-expression of different connexin types is of critical importance. Changes in connexin expression/co-expression ratios will be explored in greater depth in the work reported in this thesis.

1.5.5. Role of antiarrhythmic peptides on gap junction communication

As discussed in the section above, gap junctions play a pivotal role in facilitating action potential propagation and disturbances in their expression and distribution contribute to conduction abnormalities (Dhein et al. 2010). Current antiarrhythmic therapy (e.g. ion channel blockers) is hindered through a lack of efficiency and side effects, creating the need for a new generation of drugs (De Vuyst et al. 2011). In this section I focus on the key compounds that have been developed to improve gap junction communication.

In 1980, Aonuma et al. identified a natural antiarrhythmic peptide, AAP in bovine atrium, which increased synchronization between embryonic chicken cardiac myocytes (Aonuma et al. 1980). Since this discovery numerous derivatives have been synthesised of which AAP10 was chosen as the lead peptide. AAP10 (10-50nm) functions by increasing gap junctional conductance as shown in dual whole-cell voltage clamp studies performed on guinea pig (Dhein et al. 2001) and human atrial cardiac myocytes (Hagen et al. 2009). This increase in conductance was via: (1) preventing the dephosphorylation of Cx43 and promoting the phosphorylation of Cx43 at serine 368 through protein kinase C- α leading to the opening of the gap junction channels (De Vuyst et al. 2011) and (2) inhibit the removal of Cx43 from the cell membrane and/or enhances the formation of functional channels (Dhein et al. 2001). In addition to increased conductance AAP10 enhanced metabolic coupling, assessed by Lucifer yellow dye transfer (Hagen et al. 2009). The influence of AAP10 on intercellular communication was not a sudden effect but rather a progressive increase in conductance (5-10 minutes). It should be noted that AAP10 is connexin specific and only improves electrical and metabolic coupling in Cx43 and Cx45 coupled cells but not in Cx40 coupled cells (Hagen et al. 2009).

Since the discovery of AAP10, the compound has been chemically modified through a rotation-inversion to produce ZP123 (rotigaptide). Rotigaptide improves electrical coupling in a similar manner to AAP10 by increasing Cx43 synthesis and phosphorylation and by decreasing its degradation. Studies on animal models showed that rotigaptide prevented: (1) increased dispersion of action potential duration during ischemia and acidosis; (2) conduction slowing in ischemia; (3) re-entrant ventricular

tachycardia one to four hours after left anterior descending ligation in the open chest dog model and (4) reperfusion-induced arrhythmias due to the re-opening of the occluded artery (De Vuyst et al. 2011). The effects of both AAP10 and ZP123 on infarct size have also been studied on a rat and dog model. Treatment with the peptides resulted in a significant reduction in the infarct size together with increased gap junction expression in the diseased area (Haugan et al. 2006; Hennen et al. 2009).

More recently GAP-134 a small dipeptide analog of ZP123 has been developed which has reduced atrial fibrillation with no effect on heart rate and arterial pressure in a dog model. However the mechanism of improved gap junction communication is unclear since there is no change in Cx43 and Cx40 transcript levels. GAP-134 is currently under clinical trials (phase 1 is complete) to be the first orally available gap junction modifier (De Vuyst et al. 2011). It should be taken into consideration that experimental and theoretical cell models show that action potential propagation can fail in well coupled cells if these form a large mass receiving a limited amount of current from a smaller source; under these conditions, reducing coupling in the 'sink' can actually overcome a conduction block (Rohr et al. 1997; Rudy and Shaw 1997). Thus the reduction of Cx43 to decrease coupling could be part of a protective response that increases the safety of conduction in the diseased heart (Severs et al. 2008). Collectively these results illustrate the potential of antiarrhythmic peptides to improve gap junctional communication.

1.5.6. Action potential generation and propagation and gap junction remodelling in theoretical models

The passage of electrical impulses through the myocardium can be considered as a reaction-diffusion system whereby action potentials generated by individual cells in response to a suprathreshold current stimulus creates a voltage difference between the excited and resting tissue. This potential difference then drives the action potential to excite the resting tissue thereby supporting the spread of excitation in a wave-like manner (Kleber et al. 2001; Kleber and Rudy 2004).

Action potential generation

In the cells of the sinoatrial and atrioventricular node the action potential shape is different to those observed in atria and ventricles (Figure 1.12 (A)). The action potential

upstroke is slow since Ca^{2+} ions are the major contributor for excitation since these cells express little or no sodium (Na^+) current (Dobrzynski et al. 2007). The resting membrane potential is more depolarised (-50mV) compared with atrial and ventricular cells (~-80mV). Nodal cells undergo periodic oscillations of their membrane potential giving a slow depolarisation phase which drives the membrane voltage from the end of the repolarisation phase of an action potential towards the threshold potential where upon the next action potential is initiated (Mangoni et al. 2006). This pacemaker potential provides the sinoatrial node with automaticity and the capacity for spontaneous rhythmical self excitation. The hyperpolarisation-activated 'funny' current (I_f) has been implicated as the primary source of slow diastolic depolarising current (Herrmann et al. 2007; Verkerk et al. 2007) which activates at the termination of an action potential and deactivates during the upstroke of an action potential (Barbuti et al. 2007; DiFrancesco 2006; Bucchi et al. 2006). T-type Ca^{2+} channels expressed in all cardiac tissue displaying automaticity including the sinoatrial could also be a potential candidate in providing the automaticity (Mangoni et al. 2006; Takebayashi et al. 2006; Vassort et al. 2006).

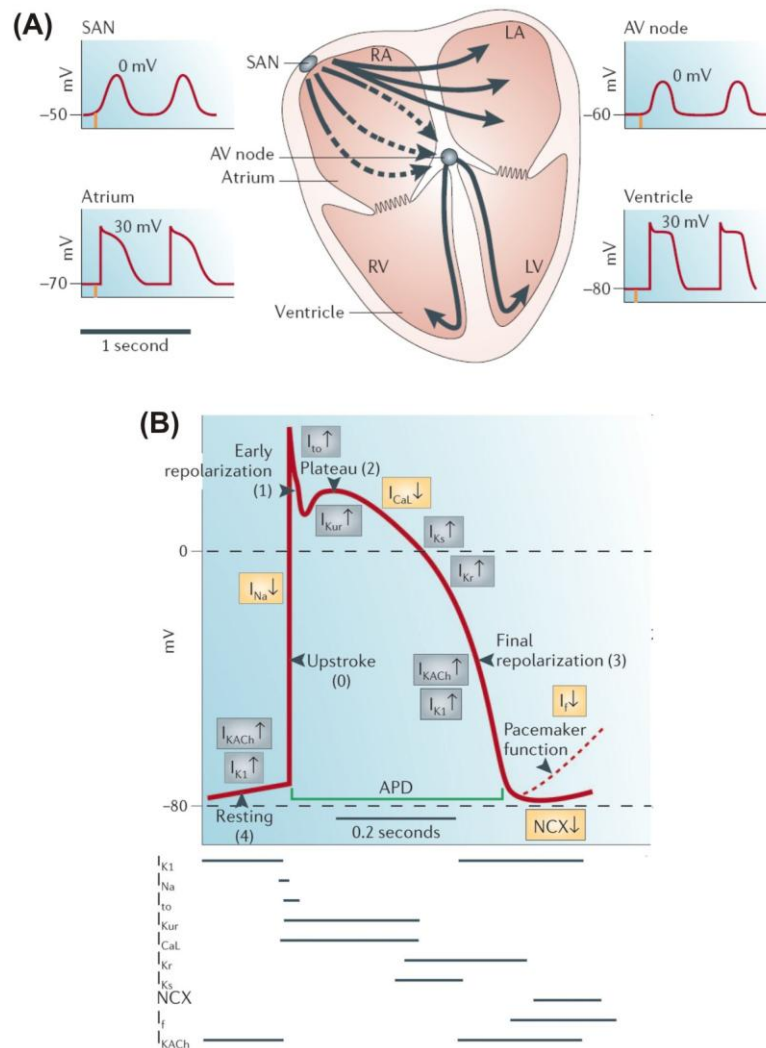


Figure 1.12: Membrane excitability currents that generate an action potential

Heart rhythm is initiated in the sinoatrial node (SAN), the natural pacemaker region in mammals which produces electrical impulses that spread across the right (RA) and left atrium (LA) and towards the atrioventricular (AV) node where its velocity is reduced before passing on to the left (LV) and right (RV) ventricles (A). Representative action potentials are shown from sinoatrial node and atria (left) and from atrioventricular node and ventricles (right), respectively (A). Voltage-gated ion channels responsible for generating a cardiac action potential (B). The four main phases of the action potential are the resting (4), upstroke (0), plateau (2) and final repolarisation (3) phase. Rapidly activating K^+ currents such as I_{to} and I_{Kur} are responsible for very rapid initial repolarization (phase 1). The slow upstroke at the end of phase 3 in pacemaker cells, such as the sinoatrial node, is shown as a broken line. The inward currents, I_{Na} , I_{Ca} , and I_f , are shown in yellow boxes; the sodium-calcium exchanger (NCX) is also shown in yellow. The outward currents, I_{KACh} , I_{K1} , I_{to} , I_{Kur} , I_{Kr} , and I_{Ks} are shown in gray boxes. Image reproduced from Nattel and Carlsson (1997b).

In the atria and ventricles Na^+ is the main current responsible for the initial phase of the action potential and in membrane excitability and conduction (Figure 1.12 (A) and (B))

(Shaw and Rudy 1997). The influx of Na^+ ions into the cells depolarises the cell membrane and is responsible for the very rapid upstroke of the cardiac action potential. The speed at which Na^+ enters the cells determines the upstroke velocity (dV/dt_{max}). Membrane depolarisation activates the L-type Ca^{2+} channels which not only contributes to the maintenance of the action potential plateau but is an essential component for muscle contraction (Fozzard 2002). Under normal conditions the intracellular Ca^{2+} $[\text{Ca}^{2+}]_i$ is low in the cell. Upon membrane depolarisation Ca^{2+} entry through the L-type Ca^{2+} channels triggers Ca^{2+} release from the sarcoplasmic reticulum (SR) through the ryanodine receptor (RyR) a process termed Ca^{2+} induced Ca^{2+} release (CICR). The combination of Ca^{2+} influx and release raises the $[\text{Ca}^{2+}]_i$ sufficiently for a large amount of Ca^{2+} to bind to the myofilament protein troponin C, which then ‘switches on’ the contractile machinery (Bers 2002). The plateau phase of the action potential is followed by the repolarisation phase. This process relies on the slow inactivation of the Ca^{2+} channels and the opening of the K^+ channels (Widmaier et al. 2006; Grant 2009). The outward flow of K^+ ions from the cell returns the membrane potentials to its resting state. During this final phase muscle relaxation also takes place via a decrease in $[\text{Ca}^{2+}]_i$ allowing Ca^{2+} to dissociate from troponin. Ca^{2+} removal from the cell is facilitated by: (1) the sarco-endoplasmic reticulum Ca^{2+} -ATPase (SERCA) that pumps Ca^{2+} back into the SR; (2) Na^+ - Ca^{2+} exchanger (NCX) that pumps Ca^{2+} out of the cell in exchange for importing Na^+ into the cell at a ratio of 1:3; (3) plasma membrane Ca^{2+} ATPase (PMCA) that removes Ca^{2+} from the cell and (4) mitochondrial Ca^{2+} uniport that uptakes Ca^{2+} into the mitochondria (Bers 2002). Figure 1.12 (B) is a schematic diagram of the ionic currents involved in generating the action potential.

Action potential propagation

After electrical excitation the transmission of action potential is facilitated through gap junction channels. In a theoretical, multicellular one-dimensional fibre of myocytes, impulse propagation can be defined in terms of ‘source-and-sink’ (Kleber and Rudy 2004). During propagation an excited cell acts as a ‘source’ of electrical charge to the neighbouring cell which acts as a ‘sink’ to receive the electrical charge. A sufficient amount of charge must pass from the source to the sink in order to bring the latter to the threshold of depolarisation to trigger an action potential. At threshold, the sink becomes the source to a further cell downstream thereupon perpetuating the process of impulse propagation.

The source-and-sink relationship can be described in terms of the safety factor (SF) of conduction. The SF is defined as '*a dimensionless parameter that indicates the margin of safety with which the action potential propagates relative to the minimum requirements for sustained conduction*' (Shaw and Rudy 1997). Successful propagation requires a SF of 1 whereby the source charge matches the current required by the sink to reach the threshold of depolarisation. If more current is generated by the source than is needed by the sink, the SF increase above 1 and conduction continues efficiently. However if SF falls below 1, conduction is impaired and block will occur.

To elucidate the precise relationship between ion channel fluxes (Na^+ and L-type Ca^{2+}) and gap junction modulation of SF and excitability Luo and Rudy developed a useful ventricular cell model (Luo and Rudy 1994). The three main parameters used to characterise conduction were: (1) CV; (2) SF for conduction and (3) the $dV_m/dt_{\max}$. Figure 1.13 (A) presents the response to reduced membrane excitability by decreasing the availability of Na^+ current. The upper plot in Figure 1.13 (A) shows that decreased membrane excitability reduced CV and the SF of conduction. The lower plot in Figure 1.13 (A) shows that reducing the maximal conductance of the Na^+ current results in a steep decrease in $dV_m/dt_{\max}$. By reducing the Na^+ conductance (g_{Na}) only a modest drop in CV (1/3 of control values) was observed before conduction failure.

When the intercellular coupling was reduced initially there was a significant increase in SF (Figure 1.13 (B), upper plot) although there is a decrease in intercellular coupling causing a concurrent reduction in CV. The $dV_m/dt_{\max}$ also increased in a similar fashion to the SF with the progressive uncoupling of cells (Figure 1.13 (B), lower plot). The Na^+ channel kinetics remained largely unchanged indicating that alteration to depolarising membrane current was not the cause of the elevated SF and $dV_m/dt_{\max}$. Rather, these data demonstrate that intercellular coupling is a prime determinant of the source-and-sink relationship within a multicellular fibre, which directly feedback on the properties of the membrane current.

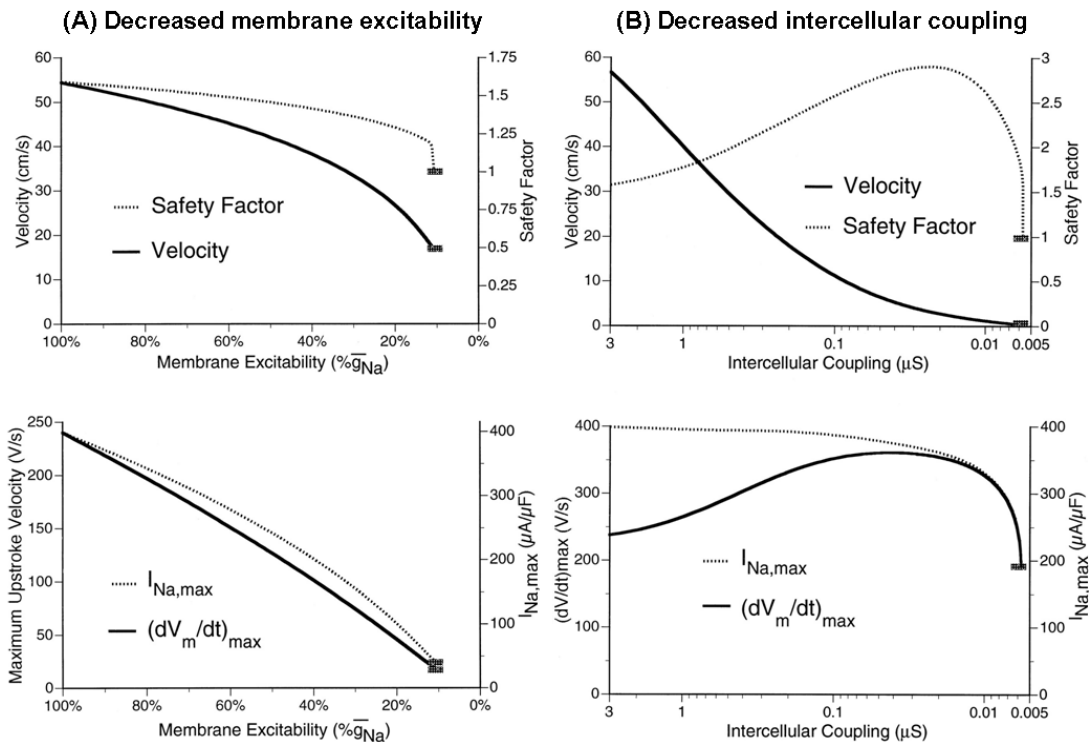


Figure 1.13: Effects of reduced membrane excitability and intercellular coupling on conduction, SF and maximum upstroke velocity

Image (A), top panel shows the relationship between velocity (solid line) and safety factor (broken line) with reduced membrane excitability. CV decreases threefold to 17cm/sec before conduction block. Image (A) bottom panel shows the steep decrease in action potential upstroke velocity (dV_m/dt_{max}) as the g_{Na} is reduced. Image (B), top panel shows the relationships between velocity (solid line), safety factor (broken line) and reduced gap-junctional conductance. Conduction velocity decreases 200-fold to 0.26cm/s before conduction block occurs. Image (B), bottom panel shows the relationships between action potential maximum upstroke velocity, Na^+ currents and gap-junctional conductance. Reduced coupling had little effect on membrane currents, but significantly increased action potential upstroke velocity. Images reproduced from Shaw and Rudy (1997).

Upon entering a cell at the sink location, the source current will depolarise the membrane and initiate an action potential. By reducing the intercellular coupling, the proportion of charge that remains in the sink cell increases in relation to that which may pass further downstream i.e. the sink volume is decreased. This elevates the amount of current available for local depolarisation, whereby SF and dV_m/dt_{max} increase. At much higher levels of uncoupling, the resultant delay of charge transfer from the source to the sink means that more of the Na^+ and Ca^{2+} channels responsible for action potential generation will inactivate before threshold is reached. This leads to the sharp reduction in SF and dV_m/dt_{max} shown in Figure 1.13 (B). When uncoupling is sufficiently high

enough, conduction block will therefore occur. During reduced coupling the L-type Ca^{2+} channel plays a major role in sustaining conduction whereby there is a switch of intrinsic membrane processes from a Na^{+} based upstroke to a Ca^{2+} based one. This interplay between membrane and gap junction factors may mean that under some conditions one change (reduced coupling) may compensate for another (reduced excitability) (Shaw and Rudy 1997).

One of the key features of the model proposed by Shaw and Rudy (1997) is that conduction can persist following profound reductions in intercellular coupling albeit at much lower velocities due to the increase in SF. This could also explain the observations in transgenic mouse hearts which have shown that almost complete ablation of connexin expression is required to promote arrhythmias. The theoretical model suggests that reductions in intercellular communication could enhance safe propagation by diminishing source-and-sink mismatches. This was proven by Rohr et al. (1997) using a very small volume of cells (source) connected to a large mass cells (sink) (Figure 1.14). Under control conditions the source was unable to depolarise the large sink due to a source-and-sink mismatch resulting in a unidirectional block. However the same source is freely able to depolarise the sink when partial uncoupling is induced so diminishing the source-and-sink mismatch.

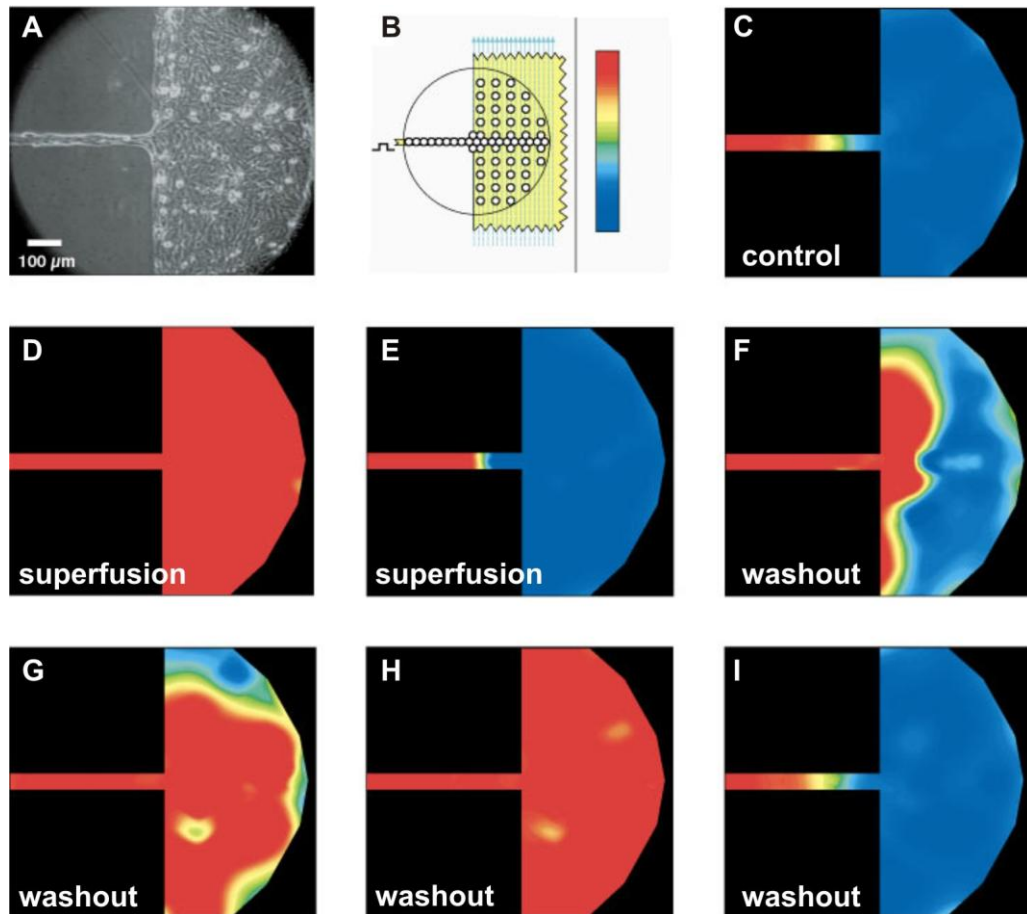


Figure 1.14: Optical mapping of successful conduction across a source-and-sink mismatch through reduced intercellular coupling

(A) video micrograph of the patterned growth myocyte culture used in this experiment. A narrow myocyte strand (source) neighbours a large rectangular myocyte monolayer (sink). (B) depicts the recording system used to measure conduction. The graded-colour scale represents the strength of an action potential fired by cells. Red colour denotes full-sized action potential; dark blue means there is an absence of depolarisation. (C) shows that under normal conditions, substantial source-and-sink mismatch causes conduction block within the myocyte strand. In (D) cells have been partially uncoupled by brief (1.5 minutes) superfusion with the gap junction inhibitor palmitoleic acid. Conduction is now successful. (E) further superfusion with inhibitor, causes complete uncoupling and conduction block. (F-H) demonstrate that as the inhibitor is removed and coupling is slowly restored, conduction is again successful and dependent on the degree of coupling. (I) after washout period, complete coupling is regained and unidirectional conduction block is re-established. Image modified from Rohr et al. (1997).

In vivo, prominent examples of source-and-sink mismatches are the sinoatrial node-atrial tissue and the Purkinje fibre-ventricles. At these locations, a small source must provide a large enough charge to activate depolarisation of a large mass. A

characteristic feature of the diseased human ventricle is a heterogeneously reduced level of Cx43 expression (Severs et al. 2004b; Dupont et al. 2001b). This may lead to premature activation through dispersion of action potential durations, mismatches of source-and-sink relationships and local conduction delays or block (van Rijen et al. 2006). Although heterogeneously altered levels of connexin expression contribute to conduction abnormalities by causing large differences in electrical coupling, the exact relationships between intracellular resistance, CV and the specific expression of Cx40, Cx43 and Cx45 at these locations are yet to be determined.

1.6. HL-1 cell model to study impulse propagation

The functional correlates of connexin co-expression are difficult to elucidate *in vivo* due to: (1) the various cell morphologies/physiology in the conduction system; (2) the technical difficulties of measuring CV in specific regions of the heart and (3) the impossibility of changing levels of expression. Although work on transgenic mice has given valuable insight on the functional consequences of connexin expression in the healthy and diseased heart, the inability to modulate connexin co-expression ratios makes their uses limited. In addition computer simulations relating intercellular coupling and conduction cannot resolve the precise contributions of gap junction channels which display a variety of functional properties. Therefore in parallel with such studies *in vitro* cell models that permit the manipulation of connexin expression (e.g. RNA interference) to mimic co-expression patterns found *in vivo* would open new opportunities to advance our knowledge.

In my work the *in vitro* system used to determine the role of each connexin and combination of co-expressed connexins that occur in the normal heart was the cardiac cell line HL-1.

1.6.1. Development of the HL-1 atrial cell line

The growth of cardiac muscle cells is classified into two stages of development: (1) embryogenesis during which the cells divide and; (2) postnatal development when functional demand on the heart is met by cell enlargement rather than proliferation with a complete loss in the ability of the cells to divide (Steinhilber et al. 1990; Borisov and

Claycomb 1995). As a result isolated neonatal or adult cells are unable to survive passaging in culture while retaining their differentiated phenotype. In attempts to develop a cardiac cell line that maintained long-term proliferation, several studies have looked into the transfection of large T oncogene of the monkey papovirus simian virus 40 (SV40) (Amsterdam et al. 1988; Pfeifer et al. 1993). It is thought that the SV40 large T oncogene induces cell proliferation by its ability to complex with and repress the activity of tumour suppressor proteins pRb and p53 involved in regulating the cell cycle (Lanson, Jr. et al. 2000). Using this technique Claycomb et al. produced transgenic mice expressing the SV40 T oncogene under the control of a tissue specific human atrial natriuretic factor (ANF) promoter resulting in atrial hyperplasia (Steinhelper et al. 1990; Borisov and Claycomb 1995). ANF, a peptide hormone synthesised and stored in cardiac atria functions in regulating extracellular fluid volume and electrolytes (Field 1988).

The atrial cells under the control of an ANF promoter termed AT-1 were highly differentiated and exhibited rhythmic contractile activity (Steinhelper et al. 1990; Lanson, Jr. et al. 1992). However as the cells were tumour-derived the hyperplastic growth (up to 30-50% of the host body mass) was lethal to the mice. In addition AT-1 cells could only be maintained through sub-cutaneous transplantation into syngenic animals and could not be passaged in culture or recovered from frozen stocks (Field 1988; Steinhelper et al. 1990). To overcome these difficulties the HL-1 cell line was isolated from an AT-1 sub-cutaneous tumour and cultured (Claycomb et al. 1998). Cells from the excised tissue were plated in gelatin-fibronectin coated flasks and grown in an atmosphere of 5% CO₂ at 37°C. Once cells reached confluency, cultures were split 1:3 and under selective conditions (e.g. retinoic acid and norepinephrine) were highly proliferative and allowed at least 240 passage while retaining a differentiated phenotype (Claycomb et al. 1998).

HL-1 cells share several characteristics with primary cultures of cardiac myocytes. Electron microscopy revealed the cells were abundant in nascent myofibrils and areas rich in glycogen typical of mitotically active cells. At the various passages tested all cells possessed perinuclear ANF-containing granules, α -type myosin heavy chain, muscle specific desmin intermediate filaments, α -type cardiac actin and Cx43 (Claycomb et al. 1998). Electrophysiological studies confirmed the presence of

functional voltage-gated ion channels (e.g. inward and delayed rectifier K^+ , L- and T-type Ca^{2+} and I_f), $[Ca^{2+}]_i$ release from the SR and spontaneous action potentials (Sartiani et al. 2002; Claycomb et al. 1998; Akhavan et al. 2003; Xia et al. 2004). The genes expressed in the HL-1 cells are depicted diagrammatically in Figure 1.15. These features, together with their ability to retain or maintain their differentiated characteristics during continuous passage in culture, make them an ideal *in vitro* model to study various aspects of cardiac cell biology (White et al. 2004).

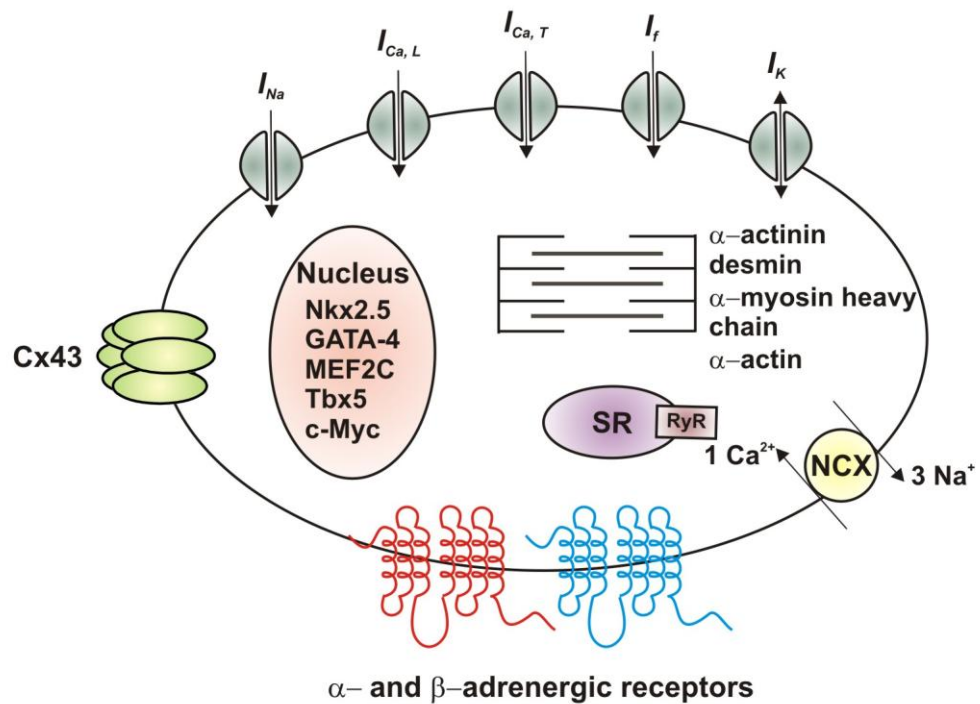


Figure 1.15: Schematic depiction of the key characteristic features in a HL-1 cell

The HL-1 cells display a cardiac phenotype with the expression of a number of cardiac myocyte markers such as α -myosin heavy chain and α -actin, both key components of the contractile apparatus. Cells expressed ANF granules normally secreted by atrial cells *in vivo* in response to stressful stimuli such as hypoxia and hyperglycemia. They were also able to generate action potentials showing that they express the necessary ion channels required for excitability (sodium (I_{Na}), L- ($I_{Ca,L}$) and T-type ($I_{Ca,T}$) Ca^{2+} , potassium (I_K) and hyperpolarising-activated ‘funny’ current (I_f)). Cells displayed $[Ca^{2+}]_i$ oscillations indicative of a functional SR and Ca^{2+} handling proteins. HL-1 could be a useful model to study the role of various receptors in cell signalling pathways such as α - and β -adrenergic receptors. A number of transcription factors are also expressed such as Nkx2.5, GATA-4, c-Myc, Tbx-5 and MEF2C. Collectively these features make the HL-1 cell line a useful *in vitro* model to study cardiac physiology and pathophysiology. Image modified from White et al. (2004).

1.7. Scope of thesis

I set out to investigate the functional significance of distinctive connexin co-expression patterns characteristic of the different myocyte subtypes. Using the HL-1 cell model we were able to manipulate connexin expression using RNAi technology and assign CV to each of the different connexin combinations using microelectrode arrays (MEA). To address the role of connexin expression in impulse propagation this investigation was performed with the following objectives in mind:

1. To sub-clone the HL-1 cells due to heterogeneity of the original cell line
2. To characterise voltage-gated ion channels mainly involved in cell depolarisation and $[Ca^{2+}]_i$ release which are key components in action potential generation and contractile activity.
3. To examine connexin distribution patterns, co-expression ratios and investigate the possible gap junction channel types in terms of connexin composition.
4. To determine CV in the HL-1 clones, homogeneity of wavefront propagation and pacemaker location.
5. To manipulate connexin expression levels and determine the effects of altered expression on CV.

General methods that were routinely used to fulfil the goals of the investigation are described in Chapter 2. These include cell culture procedures, immunofluorescent labelling and confocal microscopy, western and northern blotting. The main body of the experimental work is presented in Chapters 3-7. These chapters each contain a focused introduction, a description of the specific methods undertaken for the particular investigations, the experimental results and a concise discussion. Some of the techniques that were specific to the work in a chapter are described in more detail when used.

The HL-1 cells needed to be cloned to create more homogenous populations in order to relate experimental data to the chosen gene expression and exclude differences due to a heterogeneous cell line. It was important to establish that the HL-1 clones were able to express functional voltage-gated ion channels and the relevant Ca^{2+} handling proteins determined by whole cell patch-clamp and northern blotting respectively confirming the clones were of cardiac myocyte origin (Chapter 3). Gap junction expression levels and

connexin co-expression patterns were analyzed by western and northern blotting and immunofluorescence confocal microscopy (Chapter 4). Action potential propagation was measured in all the HL-1 clones using MEA in combination with optical mapping (Chapter 5). To correlate connexin expression to CV, RNAi using short interfering RNA (siRNA) was used to decrease connexin expression (Chapter 6). The functional consequences of altered connexin expression levels were assessed by MEA. A summary of the main findings, their significance and future directions for this study are discussed in Chapter 8.

CHAPTER 2.

General methods

2.1. Introduction

This chapter gives a detailed description of all the general procedures used throughout the investigation such as immunocytochemistry, western blotting, and northern blotting for the characterisation and functional assessment of the HL-1 clones. Specific methodological detail such as the optimisation of the microelectrode arrays and RNA interference are given in the relevant individual chapters. All chemicals and reagents were supplied by Sigma-Aldrich and VWR unless otherwise stated and were of the highest analytical grade.

2.2. Cell culture techniques

2.2.1. Culture maintenance of the HL-1 clones

All sterile procedures were carried out in a laminar flow safety cabinet. The HL-1 clones were cultured in Claycomb medium supplemented with 10% foetal bovine serum (FBS), 4mM L-glutamine, penicillin (100 units/ml), streptomycin (100mg/ml) and 10 μ M norepinephrine maintained in an atmosphere of 5% CO₂ at 37°C. A list of the reagents used to prepare the Claycomb medium is presented in Table 2.1. Cultures were maintained at high density and split every four to five days when confluency was reached and cells were visibly contracting under microscope examination with the exception of clone 2. Flasks were pre-coated with 0.02% gelatin, 5mg/ml fibronectin in distilled water (dH₂O) to help cells adhere to the surface and be passaged repeatedly while maintaining their contractile phenotype (White et al. 2004). To passage a 25cm² flask, the cell monolayer was washed with phosphate-buffered saline (PBS) and incubated with 2ml of 1x trypsin-EDTA (5mg/ml trypsin, 2mg/ml EDTA, PBS) for approximately 3 minutes at 37°C for partial detachment of cells. The trypsin together with cellular debris produced by the cells was removed by aspiration and replaced with fresh trypsin (0.5ml). The flask was returned to the incubator for a further 5 minutes for complete cell detachment. Trypsin activity was blocked by 1x trypsin inhibitor from glycine max (soya bean) at a ratio of 10 μ l per 1cm² of cells. Flasks were rinsed thoroughly with Claycomb medium to ensure high cell recovery and a new flask seeded approximately by a 1:2 or 1:3 dilution.

For experiments where a specific cell density was required, cells were trypsinised as above, centrifuged (130gmax, 5 minutes room temperature) in serum free Dulbecco's Modified Eagle Medium (DMEM). The cell pellet was re-suspended in the appropriate volume of Claycomb medium and seeded in the suitable culture vessels (e.g. 24 well-plate and 35mm dishes).

Table 2.1: List of reagents used to prepare the Claycomb medium

Reagent	Concentration
Total protein	261mg/l
Bovine albumin	48.85mg/l
Non-essential amino acids	0.1mM
Fetuin	165mg/ml
Transferrin	31.8mg/ml
Retinoic acid	300µg/l
Human insulin (recombinant)	15µg/l
Long R ³ IGF-1 (recombinant)	0.1µg/l
Long EGF (recombinant)	0.1µg/l
Cholestrol	1.96mg/ml
Linoleic acid	0.78mg/ml
γ-Oleyl-β-pal-α-phosphatidylcholine	1.96mg/ml
Ascorbic acid	0.3mM

Table modified from White et al. (2004)

2.2.2. Cryopreservation of the HL-1 clones

For freezing, sub-confluent cell monolayers in 75cm² flasks were detached as above with the proportion and volumes of trypsin and trypsin inhibitor adjusted; centrifuged (130gmax, 5 minutes room temperature) and the cell pellet re-suspended in 5ml FBS containing 5% dimethyl sulphoxide (DMSO) (v/v). Aliquots of 1ml were transferred into cryovials before freezing at -80°C for a maximum of 24 hours and subsequent long-term storage in liquid nitrogen. For revival, cryopreserved cells were thawed at 37°C, diluted in Claycomb medium and plated in a 25cm² flask. Medium was changed 3-4 hours post-seeding to remove any dead cells and prevent any DMSO toxicity. Thawing

of each aliquot resulted in an approximately 30-40% confluent 25cm² flask after one day in culture.

2.3. DNA Engineering to obtain probes for northern blot

2.3.1. Preparation of freshly saturated *E. coli* ER2925 cells

Freshly saturated cells were prepared by inoculating 20ml of Luria-Bertani (LB) broth (10g/l bacto tryptone w/v, 5g/l bacto yeast extract w/v, 10g/l sodium chloride w/v, 100µl 10M sodium hydroxide v/v) with 500µl of frozen ER2925 cells (New England BioLabs) overnight at 37°C with shaking at 350rpm using a rotary shaker. For future use and long-term storage freshly saturated bacteria were frozen at -80°C in 1ml aliquots after addition of glycerol at a final concentration of 15% (v/v).

2.3.2. Preparation and transformation of competent *E. coli* ER2925 cells

The calcium chloride method originally described by Cohen et al. (1972) was used to prepare and transform competent *E. coli* to allow the cloning of various DNA fragments. The principle behind this technique is that bacterial cell membranes are permeable to chloride ions but impermeable to Ca²⁺ ions. Thereby as the chloride ions enter the cell, water molecules accompany the charged particles causing the cells to swell. Through this alteration of the cell wall, bacteria are more likely to incorporate plasmid DNA and such cells are said to be 'competent'. A freshly saturated ER2925 culture stored at -80°C was thawed and 250µl of cells dispensed into a 50ml falcon tube containing 25ml LB broth. The culture broth was incubated at 37°C on a rotary shaker at 350rpm for approximately 2 hours. When an optical density of 0.3 when read at 600nm was reached, the culture was immediately cooled on ice for 10-20 minutes. This was to ensure the number of viable cells per 1ml did not exceed 10⁸ therefore preventing the bacteria from entering the late log phase of growth which decreases transformation efficiency. Cells were pelleted by centrifugation at 1500gmax for 10 minutes at 4°C, rinsed thoroughly with 0.1M ice-cold calcium chloride (0.45µm filtered) and re-centrifuged. From this stage the bacteria were competent and all plastic ware used during experiments had to be ice-cold to maintain competency. The resulting pellet was re-suspended in 1ml of 0.1M calcium chloride using ice-cold tips and incubated

overnight on ice at 4°C to reach maximal competency and increase transformation efficiency.

50ng of purified plasmid DNA was used for each transformation, re-suspended in a maximum volume of 10µl Tris-EDTA (TE) (10mM tris w/v, 1mM EDTA v/v) pH 8. DNA was first transferred to a sterile 14ml round-bottomed tube (Falcon 2059) and stored on ice for 30 minutes. Using ice-cold pipette tips, 200µl of competent cells were added to the DNA, gently mixed and incubated on ice for 30-45 minutes. Cells were heat shocked for exactly 90 seconds at 42°C. 800µl of LB broth was added to the cells and incubated for a further 45 minutes at 37°C with agitation at 250rpm to allow cells to recover and initiate expression of the ampicillin resistance gene encoded by the plasmid. Transformed cells were selected by plating up to 200µl of the transformed mixture onto LB-agar plates (1.5% agarose w/v, LB broth) containing 100µg/ml ampicillin and incubated overnight at 37°C. Positive controls were prepared with cells transformed with 25ng ultra clean plasmid DNA (pcDNA 3.1 V5/His A, 5.5kB). This protocol typically yields $1-3 \times 10^6$ transformed colonies per 1µg of supercoiled plasmid DNA. Cells transformed without plasmid DNA were used as negative controls. The resistant colonies were transferred using a bacteriological transfer loop to 50ml falcon tubes containing 15ml LB broth and incubated overnight at 37°C with agitation at 250rpm to obtain a freshly saturated culture. Cultures were screened for successful transformation by small-scale DNA preparations (mini-prep) and/or used to inoculate larger cultures for large-scale plasmid DNA production (maxi-prep).

2.3.3. Small-scale preparations of plasmid DNA (mini-prep)

For small-scale isolations and rapid screening of the clones, mini-preps were used to yield up to 50µg DNA. The alkaline lysis method initially described in 1979 by Birnboim and Doly was used for isolating plasmid DNA. 2ml of culture was transferred to a 2ml Eppendorf tube and spun at 22,000gmax for 2 minutes at room temperature. This step was repeated twice using a total of 6ml of the freshly saturated culture. The bacterial pellet was re-suspended in 200µl of alkaline lysis solution I (25mM tris pH 8 w/v, 50mM glucose w/v and 10mM EDTA v/v) by vigorous vortexing to give a homogenous suspension. 400µl of alkaline lysis solution II (2M sodium hydroxide v/v, 1% sodium dodecyl sulphate (SDS) w/v) was added to the cell suspension and

incubated for 30 minutes with gentle rocking. At alkaline pH and in the presence of SDS the cellular membranes and proteins are solubilised and the plasmid DNA denatured. The cellular debris and genomic DNA was precipitated by the addition of 300µl alkaline lysis solution III (5M potassium acetate w/v, glacial acetic acid v/v) with gentle shaking and incubation on ice for 5-10 minutes. Potassium acetate helped neutralise the alkaline solution enabling the re-naturing of single-stranded DNA to double-stranded DNA. This is a selective process whereby only small circular plasmid DNA can re-nature and dissolve easily in solution and precipitate the dodecyl sulphate protein complexes. A white insoluble precipitate forms consisting of chromosomal DNA and SDS/protein complexes that were removed by centrifugation at 22,000 gmax for 5 minutes. The supernatant containing the plasmid DNA was transferred to a fresh tube.

Plasmid DNA was extracted with 1 volume of phenol buffered to pH 8 and then with 1 volume of chloroform:isoamyl alcohol (25:1) v/v. Samples were vortexed vigorously to denature the protein and remove contaminants that would interfere with the restriction digestion step later in the procedure. The chloroform helped separate the phenol and aqueous phases. The aqueous phase was collected after centrifugation at 22,000gmax for 5 minutes. Plasmid DNA in the aqueous phase was precipitated by mixing with an equal volume of propan-2-ol v/v followed by incubation at -20°C for 1 hour. After centrifugation the DNA/RNA pellet was washed with 1ml of 75% ethanol v/v and vortexed. Pellets were recovered by centrifugation, air-dried and re-suspended in 30µl of TE pH 8 with 25µg/ml RNase A to digest contaminant RNA. Clones were screened using restriction endonuclease digestion and agarose gel electrophoresis as described in section 5 of this chapter.

2.3.4. Large-scale preparations of plasmid DNA (maxi-prep)

To obtain large amounts of pure plasmid DNA maxi-preps were used giving a yield of 1-3mg from a 500ml culture. 100µl of cells from a freshly saturated culture containing the plasmid either kept at 4°C or freshly thawed from -80°C was seeded into 500ml LB broth with ampicillin 100µg/ml and incubated overnight at 37°C on a rotary shaker at 250rpm. Cells were collected by centrifugation (1500gmax, 15 minutes) in 50ml falcon

tubes with the supernatant discarded after each spin and replaced with a further fresh 50ml culture until all the culture was collected in a single tube. The final pellet was re-suspended, lysed and debris precipitated in alkaline lysis solutions I (10ml), II (20ml) and III (15ml) respectively. Debris was removed by centrifugation at 1500gmax for 15 minutes at 4°C and the supernatant divided into 2 fresh 50ml falcon tubes. Nucleic acids were precipitated using 0.6 volume (of the 45ml of alkaline lysis solutions) of propan-2-ol with incubation at room temperature for 10-15 minutes and centrifugation at 4000gmax for 15 minutes at 4°C.

Pellets were washed in 10ml 75% ethanol and re-centrifuged (4000gmax, 15 minutes 4°C). The alcohol was carefully aspirated and pellets briefly air-dried before re-suspension in 2.5ml TE pH 8. 2.5ml of 5M lithium chloride was used to precipitate high molecular mass RNA. After centrifugation at 4000gmax for 10 minutes at 4°C the supernatant was transferred to a fresh 15ml falcon tube, and nucleic acids were precipitated with 1 volume (5ml) of propan-2-ol and centrifuged (4000gmax, 10 minutes 4°C). Pellets were rinsed in 75% ethanol and re-centrifuged. To remove any remaining RNA contaminants, pellets were re-suspended in 500µl TE pH 8 containing 25µg/ml RNase A and incubated for 45 minutes at room temperature. To purify plasmid DNA, samples were precipitated with 600µl of polyethylene glycol (13% PEG v/v, 1.6M sodium chloride) for 15 minutes and centrifuged (22,000gmax, 10 minutes 4°C). PEG selectively precipitates plasmid DNA leaving short RNA and DNA fragments in the supernatant. Small pellets were re-suspended in 400µl TE pH 8 and extracted with phenol pH 8 (1 volume). Samples were thoroughly mixed by vortex and centrifuged (22,000gmax, 10 minutes). The upper aqueous phase containing the DNA was collected, purified with 400µl phenol:chloroform (1:1 v/v ratio) and re-centrifuged (22,000gmax, 10 minutes). The aqueous phase was further purified with 1 volume of chloroform. After centrifugation the upper aqueous phase was recovered and the plasmid DNA was precipitated with 100µl of 10M ammonium acetate and 500µl of propan-2-ol with incubation on ice for 15 minutes. Pellets were recovered by centrifugation at 22,000gmax for 10 minutes at 4°C, washed in 750µl 75% ethanol, re-centrifuged for 5 minutes and air-dried. Purified plasmid DNA was re-suspended in 200µl of TE pH 8 and the optical density determined at a 1:250 dilution. Samples were measured at an absorbance of 260nm and 280nm. An optical density of 1.0 at 260nm

(A_{260}) is equivalent to 50 μ g of DNA and purity of the sample was assessed from the ratio of A_{260}/A_{280} . Pure plasmid DNA preparation gave a ratio of approximately 1.8.

2.3.5. Restriction endonuclease digestion

Analysis of the plasmids obtained from mini- and maxi-preparations was carried out using restriction endonuclease digestion and the resulting bands visualised using agarose gel electrophoresis.

As a general rule in enzymatic digestions the concentration of enzyme used should never exceed 10% of the total volume and 1 unit of enzyme will cut 1 μ g of a 3Kb plasmid in 1 hour at 37°C. For mini-prep analysis ~1 μ l (1 μ g) of DNA from the 30 μ l of mini-prep culture was digested in a total volume of 20 μ l containing 10 units of the appropriate enzyme (Roche) in the corresponding restriction enzyme buffer (up to a maximum of 2 μ l) (Roche) and dH₂O for 1-2 hours at 37°C. The restriction enzyme was the last component added to the mixture preventing any possible damage caused by contact with the undiluted buffer.

2.3.6. Agarose gel electrophoresis

Analysis of the restriction digest was carried out by agarose gel electrophoresis. An agarose gel of 1% shows good resolution of DNA fragments of between 500bp and 5000bp in size. A 1% agarose (LE, Analytical grade, Promega) w/v gel containing 1x Tris acetate-EDTA (TAE) buffer (40mM tris-acetate pH 8, 1mM EDTA) v/v and 0.5 μ g/ml ethidium bromide v/v was prepared. Ethidium bromide was used to visualise the DNA during electrophoresis because of its ability to intercalate between the stacked bases of DNA. 4 μ l of (~0.2 μ g) DNA was loaded per lane from a 20 μ l restriction-digestion mixture to allow visualisation of the electrophoretic pattern. Samples were prepared in 0.1 volume of 10x loading buffer (0.1% xylene cyanole w/v, 0.1% bromophenol blue w/v, 50% glycerol v/v, 0.5% SDS w/v, 1x TAE v/v) and electrophoresed in running buffer containing 1x TAE v/v and 0.5 μ g/ml ethidium bromide v/v at a voltage of 100V. DNA markers of known fragment size were also run on the gels to analyse accurately the size of the digest products. Two sets of markers were used. Lambda DNA cut with EcoRI and HindIII (Sigma) gives clear bands

between 21,226bp and 125bp in size. pBR332 DNA cut with HaeIII gives bands between 587bp and 123bp in size. DNA bands were visualised using an UV transilluminator and recorded on Polaroid film.

2.3.7. Recovery of DNA to be used as probes

To purify DNA inserts to be used as probes coding sequences were removed from the plasmid DNA by restriction enzyme digestion and isolated by agarose gel electrophoresis. As an example, preparation of the probe used to detect mRNA for the L-type Ca^{2+} channel is described in Figure 2.1. In brief the plasmid map for L-type Ca^{2+} channel had two restriction sites flanking the coding sequence for EcoR1 and EcoRV. Each enzyme cut the plasmid at those single sites. Digestion was carried out using 9µg of pCI-neo L-type, 10 units of each EcoR1 and EcoRV and a restriction enzyme buffer that was compatible with both enzymes.

Digested fragments were resolved by agarose gel electrophoresis. The gel was prepared using a 1% lower melting point (~65°C) agarose w/v in 1x TAE and 0.5µg/ml ethidium bromide v/v. DNA fragments were visualised using a UV transilluminator and the bands of correct size were cut out of the gel using a clean razor blade. The gel band was transferred to an Eppendorf tube, diluted to 500µl TE pH 8 and melted at 75°C for ~10 minutes. Samples were vortexed extensively to ensure all the DNA was solubilised and snap-frozen in liquid nitrogen. Samples were thawed at room temperature with vigorous vortexing and the agarose removed by centrifugation (22,000gmax, 10 minutes 4°C). In this condition agarose forms a tight pellet and the DNA remains in solution. DNA was precipitated with 0.1 volume 3M sodium acetate pH 5.2 and 1 volume of propan-2-ol and recovered with centrifugation (22,000gmax, 10 minutes 4°C). The DNA pellet was washed with 500µl 75% ethanol, centrifuged 22,000gmax, 10 minutes 4°C) and solubilised in 30µl TE pH 8. There is an approximate 25% loss of DNA during the probe recovery that needs to be taken into account to ensure the final concentration of DNA is at 2.5µg/µl.

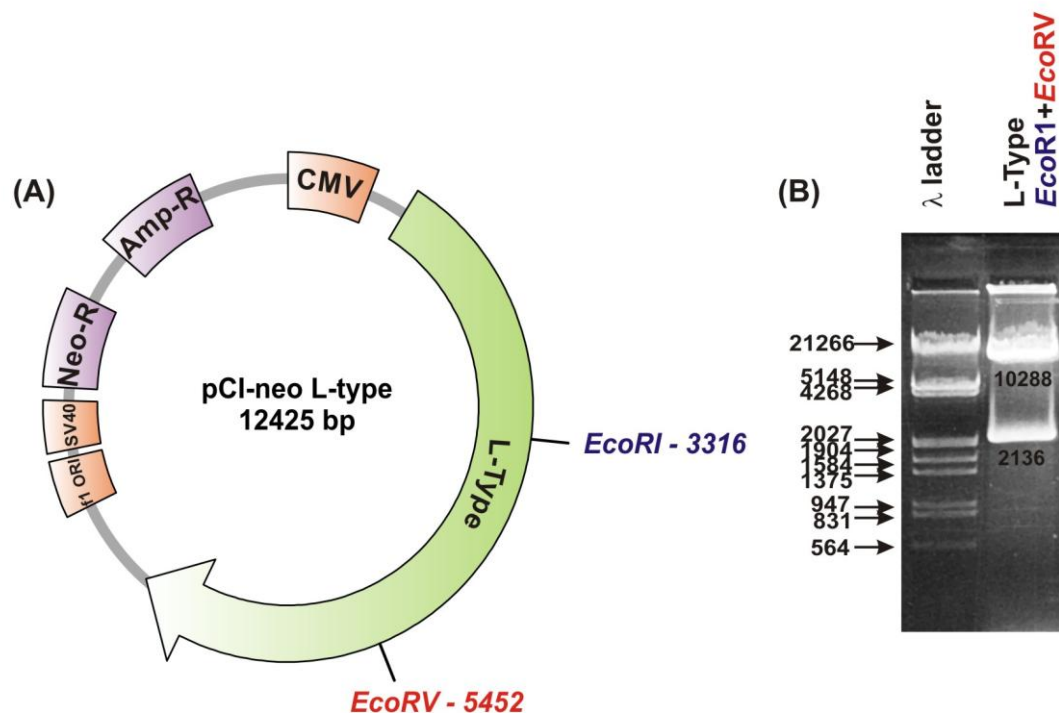


Figure 2.1: Plasmid map of L-type Ca^{2+} channel

The plasmid map containing cDNA for the L-type Ca^{2+} channel has two restriction sites flanking the coding sequence for EcoRI and EcoRV (A). Plasmid digestion was carried out using restriction enzymes EcoRI and EcoRV in the enzyme buffer compatible for both enzymes and the fragments resolved in low melting point gel electrophoresis (B). The smaller of the two DNA fragments (2136bp) in the gel was cut with a sharp blade and precipitated for northern blot analysis.

2.4. Analysis of mRNA expression by northern blotting

Northern blotting was used to detect and quantify the mRNA transcribed in the HL-1 clones in relation to Ca^{2+} handling proteins and connexin expression.

2.4.1. Sample preparation

Cells were grown in 100mm dishes and cultured to the required densities. Cultures were washed in PBS and lysed in 750 μl of Solution D (4M guanidinium thiocyanate w/v, 25mM sodium citrate pH 7 w/v, 0.5% sodium lauryl sarcosinate w/v, 1% 2-mercaptoethanol v/v) to solubilise all cellular components and denature endogenous RNases that are released during cell lysis. Samples were scraped into 2ml Eppendorf tubes and stored at -20°C until further use.

2.4.2. RNA extraction and quantification

All glassware used for RNA extraction was baked overnight at 180°C to remove any contamination by RNases.

RNA was extracted using the acid guanidium-phenol-chloroform (AGPC) technique (Chomczynski and Sacchi 1987) with modifications. After cell lysis in Solution D, RNA was acidified with a 0.1 volume of 2M sodium acetate pH 4 and precipitated with 1 volume phenol pH 4.5 and 0.2 volume chloroform:isoamyl alcohol (25:1 v/v). This step separates the RNA into the aqueous phase from DNA and protein which is retained in the phenol phase at acidic pH. Samples were vortexed extensively, left to stand on ice for 15 minutes and centrifuged at 22,000gmax for 15 minutes at 4°C. The RNA was precipitated from the aqueous phase with 1ml propan-2-ol for 1 hour or overnight at -20°C. The pellets were recovered by centrifugation (22,000gmax, 15 minutes 4°C) and solubilised in 400µl of Solution D. RNA was re-precipitated with 0.1 volume of 3M sodium acetate pH 5.2 and 1 volume of propan-2-ol and incubated for 1 hour at -20°C. Samples were centrifuged (22,000gmax, 15 minutes 4°C) and the pellets washed in 750µl 75% ethanol v/v. The RNA pellets were recovered by centrifugation and re-suspended in 100% formamide (30-60µl). Formamide provides a chemically stable environment for RNA and protection against degradation by RNases. Samples were stored at -20°C for long-term storage.

To measure the concentration and purity of the RNA extracted, samples were diluted 1:400 in TE pH 8 and the absorbance measured at 260nm and 280nm. A reading of 1.0 optical density at A_{260} is equivalent to 40µg/ml of RNA. The absorbance ratio of A_{260}/A_{280} is used to assess RNA purity and an A_{260}/A_{280} ratio of 2.0 is indicative of highly purified RNA.

2.4.3. Northern blotting

RNA was denatured and separated according to size in a denaturing formaldehyde agarose gel. For each sample, 5µg of total RNA was diluted in a volume of 5µl 100% formamide and mixed with an equal volume of RNA loading buffer (40mM MOPS pH 7-7.5 w/v, 10mM sodium acetate v/v, 36 % formalin v/v, 2mM EDTA v/v, 0.02µg ethidium bromide v/v, 10% tracking dye v/v) and denatured at 75°C for 10 minutes.

Samples (10 μ l) were loaded on a 1% agarose gel (1% agarose w/v, 20mM MOPS pH 7-7.5 w/v, 5mM sodium acetate v/v, 18% formalin v/v, 1mM EDTA v/v) and run at 70V in running buffer (20mM MOPS pH 7-7.5 w/v, 5mM sodium acetate v/v, 18% formalin v/v, 1mM EDTA v/v). Formaldehyde maintains RNA in a denatured state by forming unstable Schiff bases with single imino groups of guanine residues. After migration the 18S and 28S ribosomal RNA subunits on the gel were visualised under the UV transilluminator and recorded on Polaroid film to quantify the amount of nucleic acid present. The gel was equilibrated twice in a plastic container with 10x saline sodium citrate (SSC) (1.5M sodium chloride, 150mM sodium citrate pH 7-7.5) on a rocker for 30 minutes each. The RNA gel was capillary blotted overnight in 10x SSC onto a Hybond N nylon membrane (Amersham) using the set-up illustrated in Figure 2.2. After northern blotting the membrane was rinsed briefly in 2x SSC (300mM sodium chloride, 30mM sodium citrate pH 7-7.5) and dried thoroughly at 37°C. RNA was cross-linked to the membrane by UV irradiation on a transilluminator for 5 minutes before further processing. This was the optimal time developed in our lab to ensure each membrane could withstand more than one round of hybridisation and washing without the significant loss of the bound nucleic and subsequent hybridisation signal.

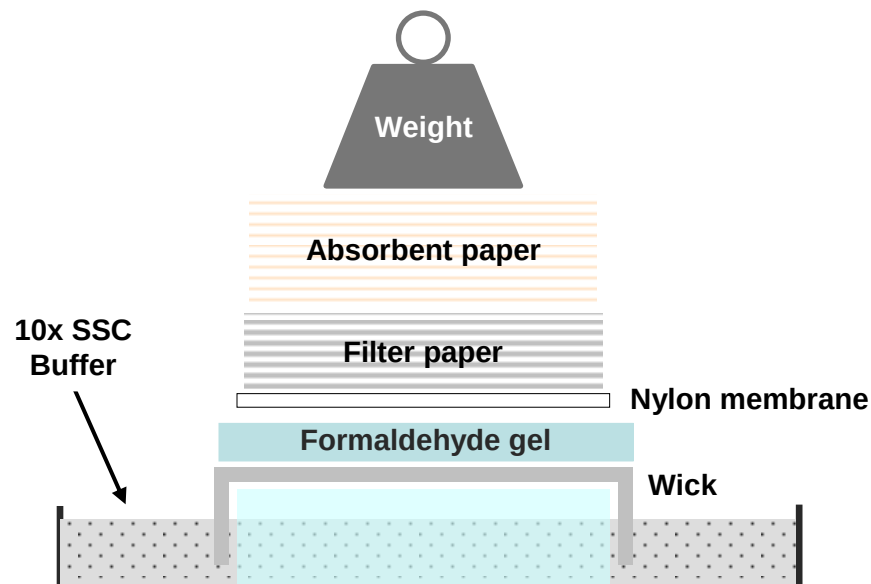


Figure 2.2: Apparatus set-up for northern blotting procedure

2.4.4. Probe synthesis and hybridisation

For each hybridisation 25-50ng of DNA probe was diluted in 30µl ultra-pure water (MilliQ) and denatured at 95°C for 10 minutes. The DNA was used as a template to synthesise radioactive probes using a Mega Prime DNA Labelling kit (Amersham, GE Healthcare) and α -³²P-labelled dCTP radionucleotides prepared according to manufacturer's instructions. Unincorporated nucleotides were removed by gel filtration chromatography using a Micro Bio-Spin 30 chromatography column (prepared according to manufacturer's instructions from Bio-Rad). The hybridisation buffer was prepared containing 7% SDS, 0.5M sodium phosphate, and 1mM EDTA. To prevent any SDS from precipitating the buffer was pre-warmed at 65°C for a minimum of 30 minutes in a hybridisation oven with gentle rocking. Membranes were blocked in the warmed buffer for a further 30 minutes to which the radiolabelled probe was added and incubated overnight at 65°C. After hybridisation, membranes were briefly rinsed in 2x SSC, washed twice with 0.5x SSC (75mM sodium chloride, 7.5mM sodium citrate pH 7/7.5) and 0.1% SDS at 65°C for 1 hour per wash and placed in clear a plastic envelope. Membranes were exposed using X-OMAT imaging films (Kodak) in intensifying screen cassettes (Amersham) at -80°C.

Membranes were dehybridised in a boiling solution of SDS 0.1% in ultra-pure water (MilliQ) and left on a rocker until room temperature was reached. This step was repeated until no, or minimal, radioactivity was detected using a Geiger counter and the membranes ready to be re-hybridised with a new probe.

2.5. Analysis of protein expression by SDS-PAGE and western blotting

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and western blotting was used to detect:

- (1) Total connexin expression
- (2) The ratio of junctional to non-junctional connexins
- (3) Connexin co-expression ratio
- (4) Co-immunoprecipitation studies on connexon interaction and
- (5) Effects of RNA interference on connexin expression

This technique utilizes the specific binding of SDS to protein through its hydrophobic alkyl chain at a ratio of 1.4g SDS per 1g of protein to form a rod-like micelle structure.

This unique property gives the denatured protein a large net negative charge approximately proportional to its mass. The denatured proteins are then separated according to size using gel electrophoresis, which are then immunoblotted onto a membrane and the target proteins detected using the appropriate primary and secondary antibodies.

2.5.1. Sample preparation

Total connexin expression

Cells were seeded in either 60mm dishes or 6-well plates and grown to the required confluency. Cultures were washed twice in PBS and lysed in solubilisation buffer SB₂₀ (20% SDS w/v, 0.1M Tris pH 6.8 v/v, 10mM EDTA v/v) at a ratio of 10µl per 1cm² of cells. Cell lysates were sonicated (LSL sonicator, Heat systems-Ultrasonics, Inc) at high power for 10-15 seconds to shear the genomic DNA and reduce viscosity. The solution was allowed to clear and 10-20µl was removed for protein quantification before storage at -20°C.

Differential solubilisation of junctional and non-junctional connexins

To assess the ratio of junctional to non-junctional connexins, cells were initially treated with mild non-ionic detergent Triton X-100 that was able to solubilise single connexons and molecular connexins without causing them to dissociate into their component connexins (Musil and Goodenough 1991). Junctional connexons that are still docked as channels are resistant to solubilisation with Triton X-100. The final step was to treat the remaining cellular material with SDS that solubilises the remaining connexins, which represent the connexin present at the junction.

Cells were seeded in six-well plates and grown to full confluency. Samples were scraped directly from the medium in which they were cultured using a cell scraper and transferred to a 15ml falcon tube. The wells were rinsed thoroughly with complete DMEM (10% FBS, 4mM L-glutamine, penicillin (100units/ml) and streptomycin (100mg/ml)) to recover any remaining cells and added to the falcon tube. Cells were centrifuged at 700gmax for 5 minutes at 4°C and the supernatant carefully aspirated with minimal disruption to the pellet. Cell pellets were re-suspended in 2ml of complete DMEM and transferred to a 2ml Eppendorf and centrifuged (22,000gmax, 5 minutes

4°C). The medium was aspirated and the cell pellet was washed in PBS and centrifuged (22,000gmax, 5 minutes 4°C). Cell pellets were directly re-suspended in solubilisation buffer containing Triton X-100 (1% Triton X-100 v/v, 40mM Tris pH 8 v/v, 150mM sodium chloride v/v, mammalian protease inhibitor cocktail 1:250 v/v (Sigma)) at a ratio of 5µl per 1cm² of cells. Samples were homogenised by vortexing and sonication at low power.

To solubilise the non-junctional connexons samples were incubated on ice for 30 minutes with vortexing every 5-10 minutes. Samples were centrifuged at 22,000gmax for 30 minutes at 4°C after which the supernatant containing the non-junctional connexons was removed and kept on ice. The remaining pellet was lysed directly in SB₂₀ in exactly the same volume that was used to solubilise the non-junctional connexons. Samples containing the junctional connexons were sonicated at low power until the pellet was dispersed and left to clear at room temperature. To provide an estimate for total protein content an equal volume of the junctional and non-junctional samples were mixed together which was used later for protein estimation. For ease of use later during SDS-PAGE sample preparation, each the samples obtained from the two extraction steps were mixed with an equal volume of detergent (solubilisation buffer containing Triton X-100 or SB₂₀) to give a final concentration of 1 volume SDS: 1 volume Triton X-100. For example 30µl of sample from the Triton X-100 extraction was mixed with 30µl SB₂₀. This was crucial because a difference in detergent concentration will affect the migration of protein during gel electrophoresis.

Co-immunoprecipitation

For co-immunoprecipitation to study connexon interaction the extraction procedure used was very similar to that used for the differential solubilisation technique described above. Cells were initially treated with mild detergent Triton X-100 followed by lysis in urea that causes the gap junctions to split into their component membranes, thereby dissociating gap junction channels into their respective connexons while preserving their structure (Manjunath et al. 1984; Zimmer et al. 1987; El Aoumari et al. 1990).

In brief cells were scraped directly in the medium, washed in PBS and lysed in solubilisation buffer containing Triton X-100 as described above. Samples were

centrifuged (22,000gmax, 30 minutes 4°C) and the supernatant removed. At this stage 10µl of sample was taken for protein estimation. The insoluble pellet was re-suspended in solubilisation buffer containing urea (4M urea v/v, 1% Triton X-100 v/v, 40mM Tris pH 8 v/v, 150mM sodium chloride, and mammalian protease inhibitor cocktail 1:250 v/v). The volume used was exactly the same as that used to solubilise the non-junctional connexons. The pellet was dispersed by sonication and the sample incubated with gentle vortexing for 1 hour at room temperature. Any denatured connexin was removed by centrifugation (22,000gmax, 30 minutes 4°C). The supernatant was retained for co-immunoprecipitation described in Chapter 2, section 6.

2.5.2. Estimation of protein concentration

Protein concentration in the lysed samples was measured using the Bio-Rad DC colorimetric assay based on the original method by Lowry et al. (1951). The reaction is based on two reactions; (1) the protein reacting with the alkaline copper tartrate (solution A) and (2) the subsequent reduction of Folin reagent (solution B) by the copper treated protein. Colour development is primarily due to amino acids tyrosine and tryptophan producing a blue colour with an absorbance of 750nm.

All samples were diluted either 1:10 or 1:20 (v/v) with dH₂O giving a final SDS concentration of 1 or 2%. For the standard curve, 1mg/ml bovine IgG standard was prepared from a stock solution of 2.5mg/ml in the appropriate IgG standard buffer to give a final detergent concentration equivalent to that of the samples. Using this 1mg/ml solution, serial dilutions of 0.5, 0.25, 0.125 and 0.0625mg/ml were prepared in dilution buffer. A blank (0 mg/ml) was also prepared using 20µl of dilution buffer. The IgG standard and dilution buffer used was dependent on the extraction procedure and the exact volumes for each of the detergents are listed in Table 2.2.

Table 2.2: Buffers used for protein estimation

Extraction procedure	IgG standard buffer	Dilution buffer
Total protein	40µl IgG sol. + 55µl dH ₂ O + 5µl SB ₂₀	950µl dH ₂ O + 50µl SB ₂₀
Differential solubilisation	40µl IgG sol. + 50µl dH ₂ O + 5µl Triton X-100* + 5µl SB ₂₀	900µl dH ₂ O + 50µl SB ₂₀ + 50µl Triton X-100*
Co-immunoprecipitation	40µl IgG sol. + 55µl dH ₂ O + 5µl Triton X-100*	950µl dH ₂ O + 50µl Triton X-100*

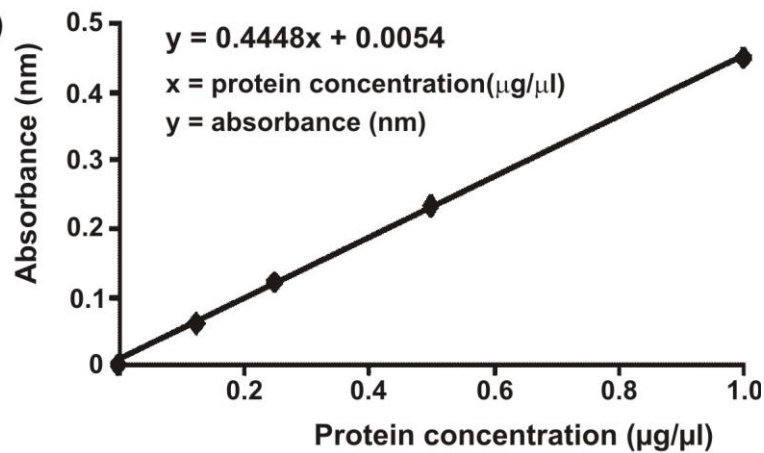
*Triton X-100 = solubilisation buffer containing Triton X-100

For the assay, 20µl of the diluted standard or sample was added to 100µl of Solution A' (prepared by mixing 20µl of Reagent S with 1ml Solution A) followed by 800µl of Solution B. Samples were mixed thoroughly and the colorimetric reaction was left to develop for 30-45 minutes at room temperature. The absorbance of each standard and sample was read at 750nm. An example of how the quantification was performed on the samples using the IgG protein standard solutions is described in Figure 2.3.

(A)

Standard (µg/µl)	Absorbance (nm)
0	0
0.125	0.061
0.25	0.121
0.5	0.232
1	0.447

(B)



(C)

Sample	Absorbance
1	0.142

$$x = (0.142 - 0.0054) / 0.4448$$

protein concentration of sample 1 = 0.307 µg/µl

Figure 2.3: Typical standard curve for the Bio-Rad DC assay using the IgG standard

Using the Bio-Rad DC colorimetric assay, absorbances of the known standard protein concentrations (1-0.125 µg/µl) (A) were measured at 750nm. This data was used to plot a linear regression with protein concentration on the 'x' axis and absorbance on the 'y' axis (B). The resulting equation ($y = 0.4448x + 0.0054$) from the linear regression was used to calculate the protein concentration in sample 1 as illustrated in (C).

2.5.3. SDS-PAGE

Sample preparation

All samples were prepared in loading buffer, as shown in Table 2.3 and incubated for 45 minutes at 37°C to ensure complete protein denaturation. SDS functions by disrupting non-covalent interactions within a polypeptide chain releasing the individual protein molecules and solubilising them in a detergent solution. Samples were also treated with reducing agent 2-mercaptoethanol to break all the disulphide bridges formed between

cysteine residues of the protein. Bromophenol blue loading dye allowed protein migration to be visualised since it moves through the gel at approximately the same speed as glycine the smallest known amino acid.

Table 2.3: Loading buffers used for sample preparation

Extraction procedure	Loading buffer
Total protein extraction/ Co-immunoprecipitation	965µl SB ₂₀ + 25µl 2-mercaptoethanol + 10µl bromophenol blue
Differential solubilisation	500µl SB ₂₀ + 465µl Triton X-100* + 25µl 2-mercaptoethanol + 10µl bromophenol blue

*Triton X-100 = solubilisation buffer containing Triton X-100

Gel preparation

Gels were prepared according to Laemmli (1970) using the Hoefer Mini Gel system. The key advantage of using this system is that it concentrates all of the protein complexes within a sample into a very small volume which greatly increases the gel's resolution. SDS was present in all gels and buffers at a concentration of 0.1% to ensure the proteins retain a rod-like structure during migration.

The percentage of acrylamide used for gel electrophoresis was determined by the molecular mass of the target proteins. For example proteins of smaller size that would move rapidly through a gel require a higher percentage of acrylamide with a greater degree of cross-linking thereby slowing protein migration. To detect connexin expression a 12.5% separating gel (12.5% acrylamide v/v, 375mM tris pH 8.8 v/v, 0.1% SDS v/v) was prepared to which 0.05% ammonium persulphate v/v and 0.005% N,N,N',N'-tetramethylethylenediamine (TEMED) v/v was added to polymerise the gel. The preparation was poured between 1mm glass and metal plates leaving a gap of approximately 3.5cm from the top of the glass plate for the stacking gel. The separating gel was layered with 1ml of propan-2-ol on top of each gel to flatten it. After polymerisation the alcohol was removed and the gels were rinsed thoroughly with dH₂O. A 4.5% stacking gel (4.5% acrylamide v/v, 125mM tris pH 6.8 v/v, 0.1% SDS v/v) with 0.1% ammonium persulphate v/v and 0.01% TEMED v/v was poured directly on top of the separating gel and 15- or 18- well combs inserted. The individual gels

were separated and transferred to the Hoefer gel running apparatus (Mighty Small II SE250/SE260). The upper and lower reservoirs were filled with 1x electrophoresis running buffer (0.192mM glycine w/v, 25mM trizma base w/v, 0.1% SDS v/v). To estimate the molecular weight of protein samples coloured molecular markers (Precision Plus Protein Kaleidoscope standards, Bio-Rad) diluted in the same loading buffer as the samples were run on each gel. Figure 2.4 shows the distribution of these markers on a typical gel. Unused lanes were filled with identical volumes of loading buffer to ensure all samples migrated at the same rate and the width of the bands was constant. Electrophoresis was initiated at 60V (~15mA per electrophoresis chamber) until the dye front reached the separating gel when the voltage was increased to 100-150V (~50mA per electrophoresis chamber) and terminated when the bromophenol blue front was approximately 0.5cm from the bottom of the separating gel.

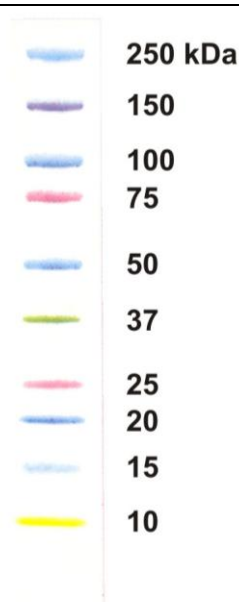


Figure 2.4: Precision plus molecular mass standards for western blot procedure

2.5.4. Western blotting and immunodetection of protein expression

Protein transfer

Protein samples were electrophoretically transferred onto polyvinylidene fluoride (PVDF) membranes (Millipore). While gel electrophoresis was taking place PVDF membranes were cut to a similar size of the gel, wetted in methanol and equilibrated in transfer buffer (192mM glycine w/v, 25mM trizma base w/v, 10% methanol v/v, 0.01%

SDS v/v) for a minimum of 1 hour. Prior to assembly of the transfer apparatus the nylon scouring pads, blotting paper and equilibrated membranes were immersed in transfer buffer that had been pre-cooled on ice. To ensure efficient transfer the PVDF membranes were gently rubbed to remove any bubbles. The gel was rinsed quickly in transfer buffer, set up in a sandwich (Bio-Rad) as shown in Figure 2.5 and placed in a pre-filled tank containing the pre-cooled transfer buffer. Protein transfer was carried out overnight at 6V/cm with stirring in the presence of a cooling device.

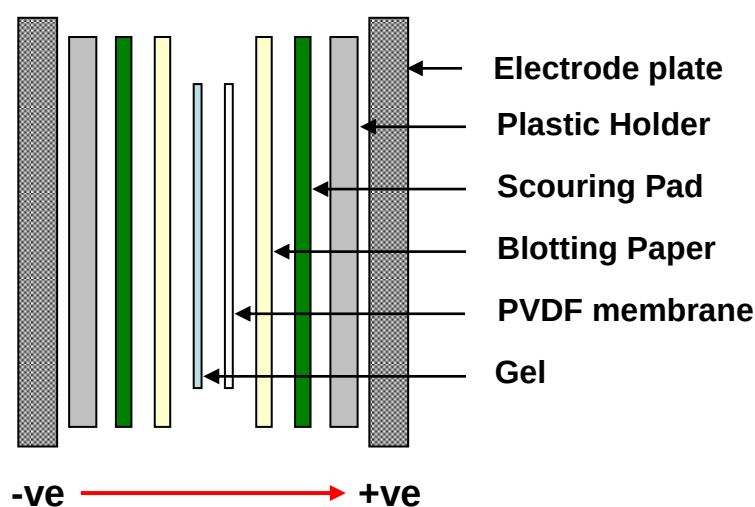


Figure 2.5: Apparatus set-up for western blotting procedure

The direction of current flow is indicated below the apparatus diagram.

Immunodetection

After transfer, membranes were briefly washed with dH₂O. Membranes were incubated in blocking solution (20mM tris pH 7.6 v/v, 150mM sodium chloride v/v, 0.1% tween₂₀ v/v, 4% non fat dried milk w/v) for 1 hour at room temperature. All primary and secondary antibodies were prepared in blocking solution. Membranes were incubated with the relevant primary antibody, as shown in Table 2.4, for 2 hours with the exception of the Cx45 antibody which was left overnight at 4°C. Membranes were washed (three times for 10 minutes) with tris-buffered saline (TBS)-Tween buffer (20mM tris pH 7.6 v/v, 150mM sodium chloride v/v, 0.1% tween₂₀ v/v) to remove any unbound primary antibody.

Alkaline phosphatase-conjugated secondary antibodies, as shown in Table 2.5, were added to the membranes and incubated for 1 hour. Membranes were washed (three times for 10 minutes) with TBS-Tween followed by a final wash (three times for 15 minutes) in TBS (20mM tris pH 7.6 v/v, 150mM sodium chloride v/v) to remove the tween₂₀ that causes a smearing effect of the insoluble precipitate. Membranes were developed using an alkaline phosphatase buffer (100mM tris pH 9.5 v/v, 100mM sodium chloride v/v, 5mM magnesium chloride v/v) containing 165µg/ml 5-bromo-4-chloro-3-indolyl-phosphate (Promega) and 330µg/ml nitro-blue-tetrazolium (Promega) for 45 minutes at room temperature. The reaction was terminated by washing the membranes in dH₂O and placed between filter paper to dry.

Table 2.4: Primary antibodies used in western blot studies

Primary antibody	Company	Host organism	Dilution	Sample protein required
Cx40 (polyclonal)	Santa Cruz	Goat	1:500	0.125-0.5µg
Cx43 (polyclonal)	Sigma	Rabbit	1:1500	0.5-1µg
Cx45 (monoclonal)	Q14E (MAB19-11-5)	Mouse	1:50	15-20µg
ZO-1	Invitrogen (61-7300)	Rabbit	1:1000	10-15µg

Table 2.5: Secondary antibodies used in western blot studies

Secondary antibody	Company	Host Organism	Dilution
Rabbit IgG-alkaline phosphatase conjugated	Pierce	Donkey	1:2000
Goat IgG-alkaline phosphatase conjugated	Pierce	Rabbit	1:2000
Mouse IgG-alkaline phosphatase conjugated	Pierce	Goat	1:2000

Coomassie blue staining

Equal loading of protein samples was verified using a total protein content staining. For staining, gels were loaded with 3-5µg of protein and dried between sheets of gel drying film for long-term storage. After electrophoresis, the polyacrylamide gel was immersed in a coomassie blue solution (0.1% coomassie blue w/v, 50% methanol v/v, 5% acetic acid v/v) overnight with gentle rocking at room temperature. The following day the gel was de-stained (50% methanol v/v, 10% acetic acid v/v) to remove any background dye and washed in dH₂O until all protein bands were visible and the remainder of the gel was transparent.

Membranes and coomassie blue gels were scanned and digital images recorded. Protein quantification was performed using Sigma gel (Jandel Scientific).

2.6. Quantification of Cx40 and Cx43 co-expression ratio using the V5 tag

To quantify the relative expression levels of Cx40 to Cx43, a system was established in our laboratory by Dr N. Thomas and Dr G. Rowlinson that used HeLa cells transfected either with Cx40, Cx43 or with a V5 ‘tagged’ version of these connexins (Rowlinson et al. 2007; Thomas 2007). The V5 tag encodes two separate components: (1) a V5 epitope, which is a 14 amino acid sequence derived from proteins of the Simian virus type 5 (SV5) and (2) a polyhistidine peptide stretch. The V5 tag provided a common epitope enabling the same antibody to detect different molecules. The amounts of tagged connexins were then standardised using the V5 signal intensity and thereafter the tagged and untagged connexins were detected together using specific anti-connexin antibodies. This allowed us to calculate the levels of native Cx40 and Cx43 in these transfectants. The HeLa cell lysates were then diluted such that each sample contained an identical number of connexin units per unit volume. These standardised lysates were loaded alongside the test samples for detection and quantification. This quantification technique also permitted the comparison between membranes blotted at different times. As an example, quantification of the Cx40 and Cx43 co-expression ratio in clone 6 (total, junctional and non-junctional fractions) is described in Figure 2.6.

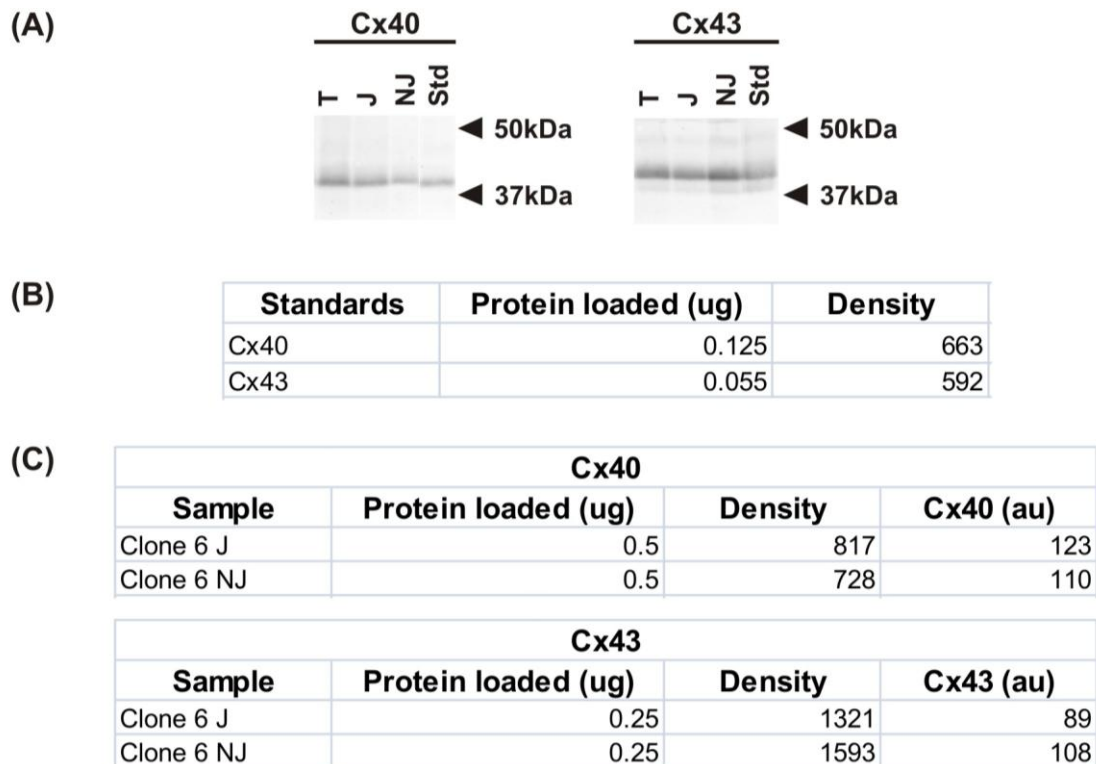


Figure 2.6: Quantification of Cx40 and Cx43 co-expression ratio in clone 6

Two western blot gels were prepared loaded with the same sample (clone 6) detecting for Cx40 and Cx43 in the junctional (J) and non-junctional (NJ) fractions (A). In both the gels 0.125µg of the Cx40-V5tag and 0.055µg of Cx43-V5tag were also loaded, which ensured each of the standards contained an identical number of connexin molecules per unit volume (A). These values for protein loading were established from work previously done in the lab (Rowlinson et al. 2007; Thomas 2007). Densitometric analysis was performed on the samples and standards (B and C). The density values obtained from the standards (Cx40: 712 arbitrary units (au) and Cx43: 996au) were then used to calculate the relative expression levels of Cx40 (C, top panel) and Cx43 (C, bottom panel) in the samples (e.g. Cx40 clone 6 junctional: $((817 \times 100) / 663 = 123\text{au})$).

2.7. Protein interaction studies using co-immunoprecipitation

Co-immunoprecipitation is a biochemical technique used to determine protein-protein interactions. The technique uses an antibody to precipitate the target protein and co-immunoprecipitate interacting proteins. Antibodies that are specific for the protein they are raised against are bound to protein A or G (Pierce) immobilized on cross-linked agarose resin. Protein A and G are proteins of microbial origin that bind to the fragment crystallizable (Fc) region of mammalian immunoglobulin molecules. The main difference between the two molecules is in their binding specificities and affinities. In

our studies we used protein A for polyclonal rabbit antibodies (anti-Cx43 and anti-ZO-1) and protein G for the polyclonal goat Cx40 antibody and monoclonal mouse Cx45 antibody. Beads with the bound antibodies are added to a cell lysate and the proteins specific to the antibodies are precipitated. After desorption of the protein-antibody complex, the co-immunoprecipitated protein(s) can be identified by SDS-PAGE and western blot. All co-immunoprecipitation experiments were performed with Dr K. Grikscheit.

Immunoprecipitation protocol

Proteins were extracted into their junctional and non-junctional fractions and the protein content estimated. For co-immunoprecipitation, the junctional fraction extracted in solubilisation buffer containing urea was desalted using the Bio-Rad® P-6DG desalting gel. Desalting was necessary to remove the urea as it significantly reduces the binding efficiency of the antibody to both the beads and any specific interacting proteins in the lysate. 1ml of the Bio-Rad desalting gel (packed volume) was added to Zeba spin columns (Pierce) and centrifuged at 1000gmax for 4 minutes. To equilibrate the columns, solubilisation buffer containing Triton X-100 was added and the column centrifuged for 2 minutes at 1000gmax. Buffer that had passed through the column was discarded. An additional 2 minutes of centrifugation (1000gmax) was required to ensure complete removal of the dead volume of buffer. This ensured when the samples were added to the column the starting volume was equal to the final volume. The junctional sample was added to the column and collected after centrifugation (4 minutes, 1000gmax).

All samples (junctional and non-junctional) were pre-cleared before use in co-immunoprecipitation experiments in order to reduce non-specific binding to Protein A or Protein G resin. For pre-clearing 25µl of Protein A or G resin was washed twice with PBS and once with solubilisation buffer containing Triton X-100. The sample was added to the beads and incubated at 4°C for 2 hours on a rotary mixer. Samples were separated from the beads by centrifugation (2 minutes, 1000gmax 4°C) and were then ready for co-immunoprecipitation.

For each co-immunoprecipitation reaction, 25µl of Protein A or Protein G was washed with PBS and recovered by centrifugation (1000gmax, 2 minutes 4°C). This step was

repeated twice. After washing, Protein A or G resin was added to the relevant primary antibody (2.5µg of antibody per reaction) in 1ml PBS and mixed on a rotary shaker for 1.5 hours at room temperature. When using the monoclonal Cx45 antibody that was produced in our laboratory, a volume of 1ml culture supernatant from the antibody producing hybridoma was used per co-immunoprecipitation reaction. The mixture containing the beads and antibody was washed twice with PBS and once with solubilisation buffer containing Triton X-100. Any residual solubilisation buffer containing Triton X-100 was removed by centrifugation (1000gmax, 2 minutes 4°C) to prevent dilution of the mixture. 100-200µl of pre-cleared sample was incubated with the beads-antibody mixture overnight at 4°C on a rotary mixer. The following day samples were centrifuged (1000gmax, 2 minutes 4°C) and the supernatant (unbound fraction) collected. Before the protein complexes (bound fraction) were desorbed, the samples were washed five times with solubilisation buffer containing Triton X-100. Loading buffer was added to the beads and vortexed for 30 seconds, heated at 95°C for 5 minutes and the beads removed by centrifugation (1000gmax, 5 minutes). The bound fraction was collected in the supernatant. The volume of electrophoresis loading buffer (containing 20% SDS) used was dependent on the volume of lysate originally added to the beads. For example 30µl of lysate will be mixed with 30µl of loading buffer.

Proteins were detected by SDS-PAGE and western blot as described in section 5 of this chapter. For each blot negative controls were loaded containing: (1) only the beads; (2) antibody with the beads and (3) beads with the sample. This was necessary to help identify bands derived from the beads (Protein A or G) and immunoglobulin chains of the antibodies.

2.8. Morphological characterisation using immunofluorescent labelling and confocal laser scanning microscopy

Immunoconfocal microscopy was used to study the expression patterns of various cardiac phenotypic markers and the localisation/co-localisation of gap-junction proteins Cx40, Cx43 and Cx45.

2.8.1. Preparation and fixation of cells

Cells were cultured and grown to the required density on gelatin-fibronectin coated 13mm plastic coverslips (Thermanox) in a 24-well plate. Plastic coverslips were used as an alternative to glass to improve cellular attachment and distribution across the surface. Immunolabelling requires four major steps: (1) fixation of cells; (2) blocking of non-specific binding sites; (3) application of primary antibodies and (4) application of secondary antibodies.

Fixation: Cells were washed twice in PBS containing 1.5mM calcium chloride to help maintain cellular adhesion through Ca^{2+} dependent adhesion molecules. Depending on the antigen being examined and the properties of the antibody required for labelling two methods of fixation were used: (1) application of ice-cold methanol (~0.5ml per well for 10 minutes at room temperature) fixes cells by precipitating cellular proteins and permeablising the membranes simultaneously and (2) addition of 2% paraformaldehyde (freshly prepared in PBS) for 20 minutes at 4°C fixes cells by forming covalent bonds between the formaldehyde groups from the reagent and the free amino groups of the cellular proteins thereby creating a scaffold. Formaldehyde has an advantage over alcohol fixation as it provides a greater preservation of cellular architecture and intracellular markers. After formaldehyde fixation cells were washed in PBS and required a membrane permeabilisation step using 0.5% Triton X-100 in PBS for 15 minutes at room temperature.

Blocking: Following fixation cells were washed thoroughly with PBS and treated with blocking solution (1% bovine serum albumin (BSA) w/v in PBS) for 1 hour at room temperature to prevent non-specific antibody binding.

Primary antibody incubation: Cells were incubated with the appropriate primary antibody diluted in blocking solution at either 37°C for 2 hours or overnight at 4°C. For the Cx45 antibodies, incubation was always overnight at 4°C. See Table 2.6 for information regarding the primary antibodies used in this study.

Immunolabelling using two or three primary antibodies on the same sample was also applied in the study, which included: 1) two or three connexin subtypes and 2) zonula

occludens-1 (ZO-1) with a single connexin subtype. For the connexin triple labelling (Cx40/Cx43/Cx45) the connexin antibody was applied first followed by the appropriate species-specific secondary antibodies linked to fluorophores of different spectra (e.g. FITC and Cy3). The following protocol for labelling was used.

- (1) Cx45 antibody
- (2) Guinea pig IgG- CY3 antibody
- (3) Cx43 antibody
- (4) Mouse IgG- FITC antibody
- (5) Cx40 antibody
- (6) Rabbit IgG- CY5 antibody

For the ZO-1/ connexin labelling, the connexin antibody was applied first followed by the anti-ZO-1.

Secondary antibody incubation: Cells were washed thoroughly with PBS and incubated in the corresponding fluorophore-conjugated secondary antibody diluted in blocking solution at either 37°C for 1 hour or overnight at 4°C. For the double labelling a mixture of the appropriate species-specific secondary antibodies were applied simultaneously and incubated overnight at 4°C. See Table 2.7 for information regarding the secondary antibodies used. Coverslips were washed extensively in PBS and left overnight at 4°C before mounting.

Coverslips were removed from the wells using clean forceps and the excess PBS was removed by pressing the edges of the coverslips against a paper towel. For mounting plastic coverslips were sandwiched between a glass slide and plastic coverslip with drop of Citifluor (Agar Scientific) mounting medium on either side and sealed with nail varnish ready for analysis.

Controls: To confirm the specificity of labelling, omission of the primary antibody in the presence of the secondary antibody was inspected for background fluorescence. For multiple labelling negative controls included using each primary antibody with matching and non-matching secondary antibodies. All secondary antibodies were confirmed to be species specific for their individual primary antibody. A variety of experiments were also conducted for each multiple labelling protocol to optimise the

order and combination for primary and secondary antibody application and the incubation conditions.

2.8.2. Confocal laser scanning microscopy

Immunolabelled sections were examined using a Leica TCS 4D confocal laser-scanning microscope. Indocarbocyanine (CY3), cyanine 5.18 (CY5) and fluorescein isothiocyanate (FITC) fluorophores were excited by wavelengths of 568nm, 647nm and 488nm channels respectively. Images were recorded by single- or sequential dual/triple-channel scanning. For multiple labelling, signals were assigned corresponding colours and co-localisation of these signals resulting from the superimposition of the two colours gave a third colour. For example co-localisation of red and green resulted in a yellow signal. Each image was presented as projection views of a number of optical sections of 0.5µm thickness (when using a 63x lens), encompassing signal from the whole depth of the sample.

2.9. Statistical analysis

Statistical analyses were carried out using Microsoft Excel and GraphPad Prism version 4.00. Comparison of a pair of data sets was performed by an unpaired t-test, and comparison between groups of data with one-way analysis of variance, Tukey's multiple comparison test. Statistical differences were judged significant at $p < 0.05$. Data was expressed as mean $\pm$ standard error of the mean and n signifies the number of times an experiment was repeated.

Table 2.6: Primary antibodies used for immunocytochemistry

Primary antibody	Company	Host organism	Dilution
Cx43 (monoclonal)	Chemicon	Mouse	1:250
Cx40 (polyclonal)	Santa Cruz	Goat	1:250
Cx40 (polyclonal)	S15C (R84)	Rabbit	1:250
Cx45 (monoclonal)	Q14E (MAB19-11-5)	Mouse	1:25
Cx45 (polyclonal)	Q14E (GP42)	Guinea Pig	1:250
ZO-1 (polyclonal)	Santa Cruz	Rabbit	1:250
α -actinin (monoclonal)	Sigma	Mouse	1:250
Desmin (polyclonal)	Sigma	Rabbit	1:250

Table 2.7: Secondary antibodies used for immunocytochemistry

Secondary antibody	Company	Host organism	Dilution
Mouse IgG- CY3	Jackson	Goat	1:250
Rabbit IgG- CY3	Jackson	Donkey	1:250
Goat IgG- CY3	Jackson	Rabbit	1:250
Mouse IgG- FITC	Jackson	Sheep	1:250
Rabbit IgG- CY5	Chemicon	Donkey	1:250
Rabbit IgG- FITC	Jackson	Donkey	1:250
Guinea pig IgG- CY3	Chemicon	Donkey	1:250

CHAPTER 3.

Do the HL-1 clones behave like atrial myocytes?

3.1. Introduction

The understanding of cardiac action potential generation and propagation has been hindered by a lack of cellular models that allow simple molecular interventions such as plasmid transfection. Cardiac function is commonly studied in isolated cardiac myocytes, including those from transgenic animals. However long-term cultures of myocytes isolated from adult hearts lead to progressive changes in phenotype, as the cells move through de-differentiated and re-differentiated states, limiting their uses (Mitcheson et al. 1998; White et al. 2004). Some of these limitations can be overcome by using the cardiac muscle cells HL-1 derived from proliferating atrial myocytes AT-1 (Field 1988). The HL-1 cells have the ability to divide and retain a cardiac myocyte phenotype (Claycomb et al. 1998). Several studies have illustrated the biological and electrophysiological features of the original HL-1 cell line including the ability to generate action potentials and to express a number of cardiac myocyte markers such as α -myosin heavy chain, desmin and connexin43 (Cx43) (Claycomb et al. 1998; Sartiani et al. 2002; Akhavan et al. 2003; Xia et al. 2004). The generation of the HL-1 cell line is described in greater detail in Chapter 1, section 6.

The HL-1 cells are tumour-derived and are likely to contain cells blocked at various developmental stages of differentiation (Trosko et al. 1993) and therefore cultures are functionally heterogeneous. To overcome this problem Dr Dupont selected clones from low-density HL-1 cultures that were visually beating and therefore showed evidence of electrical automaticity and functional cellular Ca^{2+} regulatory systems. More homogenous cell lines were established in this way. This was essential so that experimental data could be reliably attributed to the chosen variation of gene expression and to exclude differences due to a heterogeneous cell line.

The purpose of the work described in this chapter was to produce a general description of the newly generated HL-1 clones to ensure they displayed phenotypic characteristics consistent with cardiac cells that could be used to study impulse propagation. We show that the HL-1 clones: (1) display cardiac specific filamentous proteins α -actinin and desmin which contribute to cell shape and morphological integrity; (2) express the transcripts for Ca^{2+} handling proteins including L-type Ca^{2+} channel, sodium-calcium exchanger (NCX), ryanodine receptor 2 (RyR2) and sarco-endoplasmic reticulum Ca^{2+} -

ATPase (SERCA) which are key components of cellular Ca^{2+} homeostasis and excitation-contraction (EC) coupling; (3) display rhythmic release of intracellular Ca^{2+} ($[\text{Ca}^{2+}]_i$) and have caffeine-releasable stores indicating a functional sarcoplasmic reticulum (SR); (4) express functional ion channel proteins involved in the upstroke and plateau phases of an action potential and (5) display the hyperpolarisation-activated ‘funny’ current (I_f), involved in the generation and control of the slow diastolic ‘pacemaker potential’.

3.2. Materials and methods

Cell culture procedures were routinely followed as described in Chapter 2, section 2. Northern blot was used to measure the mRNA levels of the Ca^{2+} handling subunits (Chapter 2, section 4). Immunofluorescence microscopy was performed to analyse the expression patterns of various cardiac phenotypic markers (Chapter 2, section 8).

3.2.1. Sub-cloning

To obtain homogenous cell lines, clones from the original HL-1 cell line were selected for their ability to spontaneously contract. Cells were split at very low density (1:250, 1:500) and although most of the cells never divided, some could be visualized as colonies after 2-3 weeks in culture. Further daily microscopical examination revealed several colonies of cells that were spontaneously contracting. These were isolated using cloning rings, grown and passaged at a ratio of 1:3. Of the six clones obtained (clone 1 to clone 6), clone 5 did not survive the cloning procedure, clones 1 and 2 stopped contracting upon passaging and clones 3, 4 and 6 maintained a stable contractile phenotype. The appearance of spontaneous contraction was dependent upon the time in culture, with clone 6 displaying spontaneous contraction within 15-20 hours after seeding while cells from clones 3 and 4 required growth to almost full confluency (3-4 days). The sub-cloning was performed by Dr E. Dupont.

3.2.2. Patch-clamp technique

The patch-clamp technique in the whole-cell configuration was used to study the active membrane electrical properties (ionic currents, action potential) in clones 2 and 6. There

are two modes in patch-clamp: (1) voltage clamp and (2) current clamp which will be discussed later in this section.

Preparation of cells

Cells were seeded at low density in Claycomb medium on gelatin-fibronectin coated 35mm glass bottom dishes with a 14mm microwell in the centre (MatTek Corporation). It was important to seed the cells at low density to ensure single cells were obtained. This was achieved by re-suspending a 25cm² flask of confluent cells in 5ml Claycomb medium and adding 50-100µl of the cell suspension to every 35mm dish prepared. Experiments were always performed 24-48 hours after seeding to allow the cells time to recover from the trypsin and no later than 72 hours. This is because after a few days cells seeded for patch-clamp experiments become flatter and more fragile.

Electrophysiological apparatus

All electrophysiological experiments were performed using an Axopatch-2B system (Axon Instruments). Pipettes were pulled from borosilicate glass capillaries (GC150F-15, Harvard Apparatus LTD) using a horizontal puller (DMZ Universal, Zeitz-Instruments GmbH). They were then fire polished and when filled with pipette solution had a tip resistance of 2-6 MΩ. The filled pipette was connected to a x0.1 gain headstage amplifier (HS-2, Axon Instruments) by a silver-silver chloride (Ag/AgCl) wire. The reference earth electrode was also made using an Ag/AgCl wire and placed in the superfusion chamber while connected to the headstage amplifier ground terminal. The Ag/AgCl wires were made from polytetrafluoroethylene-coated silver wire (Goodfellow, Cambridge Cambs). The chlorided Ag wire functions as a redox electrode with a reaction between silver metal (Ag) and its salt, silver chloride (AgCl). To chloride the wires some of the insulation coating was removed and the wire exposed to concentrated domestic bleach overnight. The electrodes are simple to produce, inexpensive and are generally not toxic to the biological preparations and have stable potentials. They allow the current to be transformed from a flow of electrons at the electrode to a flow of ions in solution by means of a reversible chemical reaction completing the circuit (Equation 3.1).

Equation 3.1: Electrode reaction



Ag = silver; Cl^{-} = chloride ions; $AgCl$ = silver chloride; e^{-} = electrons

A Faraday cage was used to isolate the microscope and the pipette head stage from electrical noise (usually 50Hz) from various sources (e.g. computer and power cables).

Whole-cell configuration in single cell and cell pairs

There are a variety of patch-clamp configurations that can be used to study ion channels. See Figure 3.1 for a summary of the patch-clamp configurations available. In this investigation all experiments were performed using the whole-cell configuration, which allows the cytoplasmic and extracellular ionic environments of the cell to be controlled and modified.

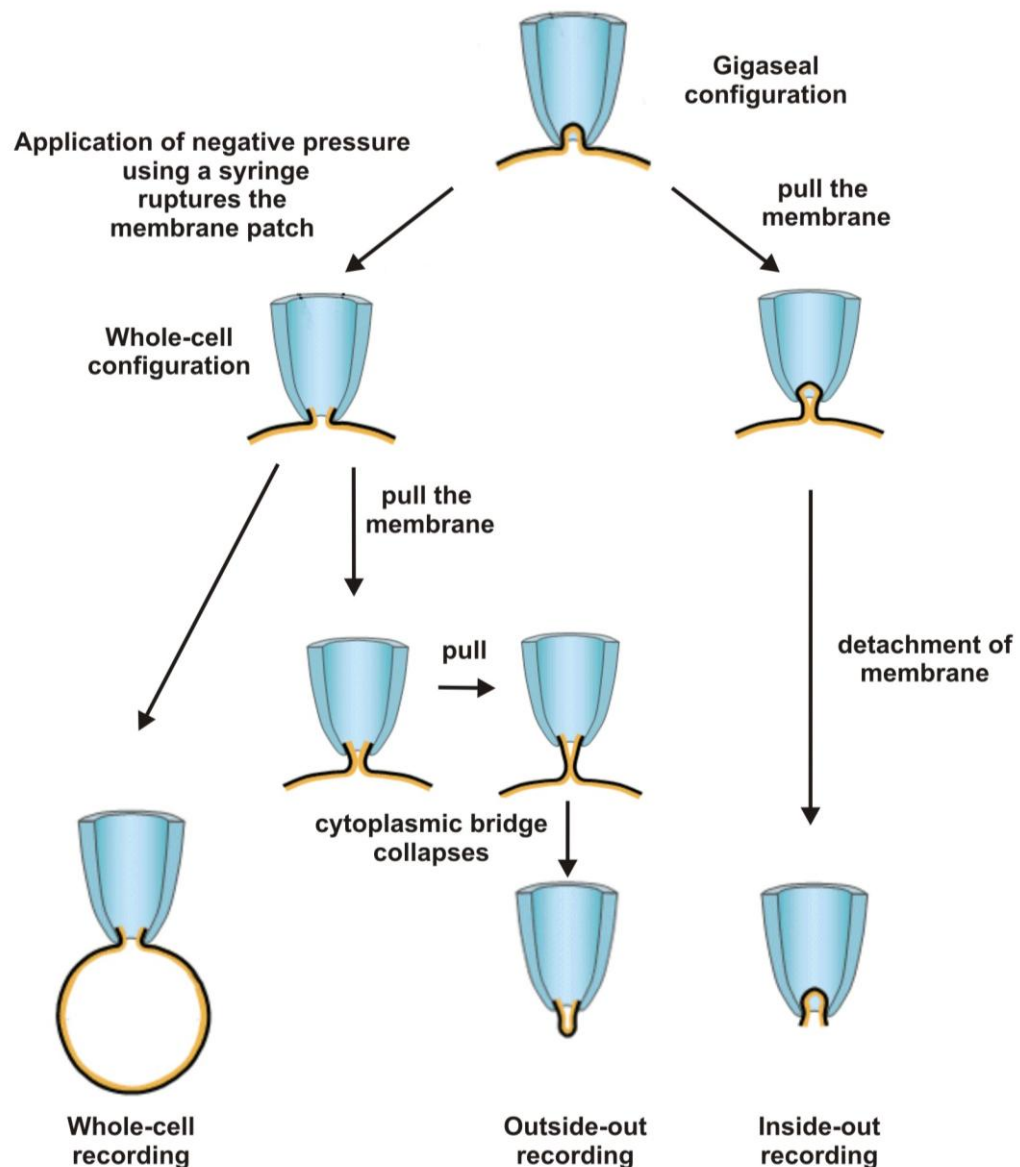


Figure 3.1: A summary of the patch-clamp configurations available to study ion channels

After a gigaseal configuration has been established a variety of patch-clamp configurations can be formed depending on what you want to study. For example activity of all the ion channels in a cell membrane can be studied using whole-cell configuration when the membrane under the pipette tip in gigaseal formation is ruptured. Outside-out and inside-out configurations refer to the extracellular and intracellular side of the membrane, respectively, and out refers to the bath. In these configurations only the patch of membrane attached to the pipette can be studied, which reduces the amount of ion channels studied.

The cell preparation was first placed onto the stage of the inverted microscope (Nikon Diaphot-TMD). The pipette connection to the amplifier was achieved by a pipette holder that allowed a syringe to be attached. This could provide small positive pressures to the pipette fluid to remove any contaminants from its tip and ensure a clean contact

with the cell membrane. Using a coarse manipulator, the pipette was lowered into the bath solution and a small oscillating test pulse of 10mV for 10ms was delivered down the patch electrode resulting in a visible square current pulse on the oscilloscope (Figure 3.2 (A) and (B)). At this stage the junctional potential caused by the difference in ion composition in the pipette and bath solution was compensated and then assumed not to change during the course of the experiment.

Using Ohm's law (Equation 3.2) the pipette resistance was calculated.

Equation 3.2: Ohm's law

$$V = R \times I$$

V = voltage; R = resistance; I = current

To form the whole-cell configuration the pipette tip was positioned near the membrane of the cell under study using a fine manipulator. When the tip of the pipette touched the cell membrane, a little suction was applied using a syringe to form a high resistance seal or gigaseal ($G\Omega$). A good seal was crucial for: (1) preventing any leak current passing between the patch pipette and adjacent membrane and (2) decreasing recording noise that may affect data analysis. The pipette current produced by the initial test pulses decreased indicating an increase of the pipette resistance due to the seal between the pipette and the cell membrane (Figure 3.2 (C)). Once the current recording indicated the formation of a good seal, the whole-cell configuration was achieved by rupturing the small patch of membrane in the pipette mouth using a small amount of suction applied by the syringe or a 'zap' of electrical current of 1.5V for 100 μ s. After the whole-cell configuration was formed, the pipette and cell membrane capacitances were compensated by zeroing the capacitive currents (Figure 3.2 (D) and (E)). Current data was expressed in terms of current density, by normalisation to the cell capacitance and expressed as pA/pF. The size of the pipette current is related to the surface area of the cell and as a general rule $\sim 1\mu$ F is equal to 1cm^2 of cell membrane. Peak currents were measured during the voltage protocols and current densities (pA/pF) were obtained by dividing the peak current by the cell capacitance obtained from the Clampex function. Once the whole-cell configuration was established two modes of recording, voltage clamp (to record currents) and current clamp (to record voltage) could be applied.

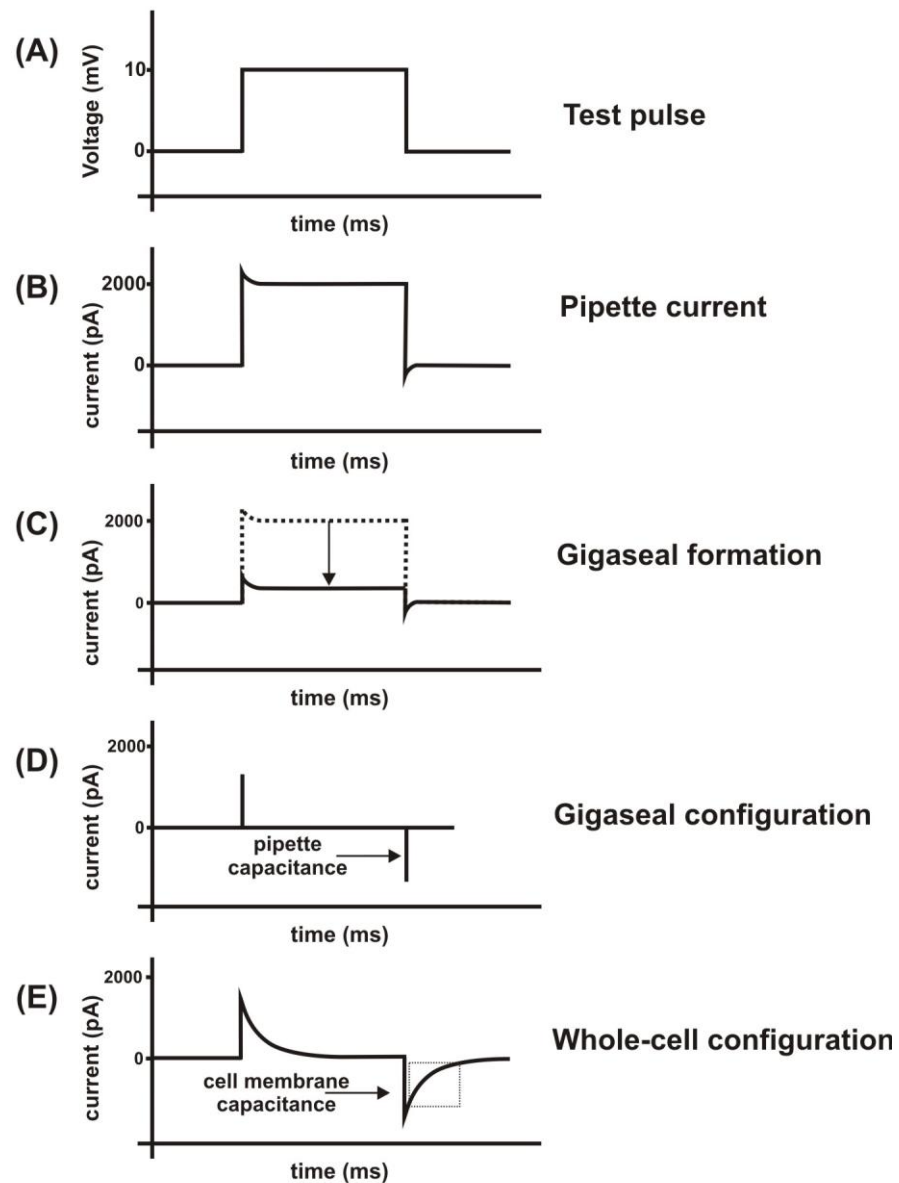


Figure 3.2: Pipette current responses to the voltage test pulse during the different stages of the whole-cell formation

A small oscillating rectangular test pulse of 10mV for 10ms was applied (A) resulting in a pipette current on the oscilloscope (B) when the pipette was placed in the bath. As the pipette neared the cell membrane there was an increase in the current resistance (C) until a gigaseal configuration was formed (D). Whole-cell configuration (E) was established after the application of a little negative pressure.

Single cell voltage clamp

The technique of controlling the voltage across a cell membrane and measuring the transmembrane current was first developed in the 1940s by Cole and Marmont and later used by Hodgkin and Huxley to describe the initiation and propagation of action potentials in neurons (Hodgkin and Huxley 1952). Under voltage clamp, the current

flowing across the cell membrane is measured by means of a feedback system that maintains a set potential difference between the two electrodes (pipette and bath electrode). The current required to maintain the membrane potential at a command voltage is equal and opposite to the sum of the transmembrane ionic fluxes through ion channels (e.g. sodium (Na^+), Ca^{2+} , potassium (K^+) channels) and electrogenic symports, antiports and co-transporters (NCX, Na^+ - K^+ ATPase).

A series of voltage clamp protocols were designed to activate the specific ion channels under investigation. These will be described in greater detail in the results section.

Current clamp: During current clamp experiments cells were at their resting membrane potential. Current pulses (positive or negative) 1-2ms in duration were applied to the cells to elicit action potentials. These were performed by Dr. T Desplantez.

Data acquisition

Experimental protocols for voltage and current clamp were controlled using the Clampex version 6.0.3. Data was analysed using Clampfit 8.2 (Axon Instruments), Microsoft Excel and GraphPad Prism and n signifies the number of cells patched. Data acquisition rates were typically 3.33kHz with the amplifier filter set to record a medium bandwidth (Frequency = 300Hz). Data obtained was acquired simultaneously on an IBM compatible computer running Clampex. Signals were digitised using a 12-bit analogue-digital converter (Digidata 1200b, Axon Instruments) interfacing between the computer and apparatus. For the purposes of illustration membrane currents in the figures were further filtered using a low-pass Gaussian filter for clarity.

3.2.3. $[\text{Ca}^{2+}]_i$ measurements

Ca^{2+} transients were measured in HL-1 clones with the fluorescent Ca^{2+} indicator fluo-4 (Molecular Probes, Invitrogen). The Ca^{2+} measurements were performed with Dr K. T. MacLeod.

Preparation of cells

Cells were seeded at a 1:3 dilution in Claycomb medium, on 35mm glass bottom dishes with a 14mm microcells in the centre. All experiments were performed 48 hours after seeding.

Ca²⁺ staining and confocal laser scanning microscopy

Stock solutions of fluo-4 AM (50µg) were prepared in dimethyl sulfoxide (DMSO) (50µl) and thoroughly mixed. 10µl of the stock solution (5µg/ml of medium) was added to the cell preparation and incubated in an atmosphere of 5% CO₂ at 37°C for 20 minutes. The carboxylate groups on Fluo-4 are transformed into acetoxymethyl acetate esters forming fluo-4 AM, which is membrane permeant. Once inside the cell the acetomethyl acetate groups are hydrolysed by ubiquitous intracellular esterases producing the Ca²⁺-sensitive polyanion, fluo-4. Any dye remaining in the loading solution was washed off with phosphate-buffered saline (PBS) and replaced with fresh Claycomb medium (Ca²⁺ concentration in Claycomb medium: 0.2g/l). Cells were incubated for a further 20 minutes in an atmosphere of 5% CO₂ at 37°C to ensure de-esterification was complete before being mounted on a confocal laser scanning microscope (Bio-Rad Radiance 2000) for analysis. For the controls, cell preparations were perfused with normal Tyrode and fluorescence measured across groups of cells using line scans at an excitation wavelength of 488nm and a recording wavelength of 520nm. When required, cells were also treated with caffeine (10mM) (added as a solid to the normal Tyrode solution) by rapid perfusion to demonstrate caffeine-releasable stores of Ca²⁺. The intensity of fluorescence recorded was dependent on the amount of dye loaded into the cells and the [Ca²⁺]_i.

Confocal data acquired was analysed using ImageJ, Clampfit 8.2, Microsoft Excel and GraphPad Prism.

3.2.4. Experimental solutions

The extracellular solutions used for patch-clamp and [Ca²⁺]_i measurements had an ionic composition that mimicked that of extracellular fluid *in vivo* or in culture. A modified Tyrode solution containing a high K⁺ concentration was also used to measure I_f. During experiments the cells were continuously perfused with Tyrode solution at 37°C and

rapid solution exchange was assured by a gravity-fed multi-solution exchange system. See Table 3.1 for the composition of extra- and intracellular solutions used.

Specific currents flowing through ion channels were identified using pharmacological methods. Current flowing through the cardiac form of the Na^+ channel ($\text{Na}_v1.5$) is sensitive to 1mM procaine hydrochloride, which was added as a powder to normal Tyrode. Mibefradil (5 μM) was used to inhibit the low voltage-gated T-type Ca^{2+} channels ($\text{Ca}_v3.2$) and nifedipine (10 μM) was used to identify L-type Ca^{2+} channels ($\text{Ca}_v1.2$). Mibefradil and nifedipine were prepared as stock solutions in DMSO at a concentration of 20mM. The final concentration of DMSO in the superfusate was less than 0.1%. No DMSO-mediated effect was observed. Caesium (Cs^+) (4mM) was used to block I_f which was added as a powder to the modified Tyrode. Solutions containing the ion channel inhibitors were always freshly prepared before use.

Table 3.1: Ionic composition of the extracellular and intracellular solutions

Reagent	Normal Tyrode (mM) (pH 7.4)	Modified Tyrode (mM) (pH 7.4)	Pipette solution (mM) (pH 7.2)
Sodium chloride	140	140	10
Potassium chloride	4	25	--
Calcium chloride	2	2	1
Sodium pyruvate	2	2	--
Glucose	5	5	--
Magnesium chloride	1	1	--
HEPES	5	5	5
Barium chloride	--	2	--
Nickel chloride	--	2	--
Potassium aspartate	--	--	130
EGTA	--	--	10
Magnesium-ATP	--	--	3

3.3. Results

3.3.1. Morphological characterisation of the HL-1 clones

Figure 3.3 illustrates the cellular morphology of two of the clones 2 (A) and 6 (B) by phase contrast microscopy. Both clones had a similar cell size and shape. As expected for an immortal cell line, the HL-1 clones were able to proliferate in culture. Clones 2 and 6 had an average cell capacitance of 15pA/pF ($n = 26$) and 13pA/pF ($n = 29$) respectively. No significant difference was observed in the cell capacitance between the two clones ($p > 0.05$). Highlighted in image (B) is a cell undergoing mitosis demonstrating that the cells can be serially passaged.

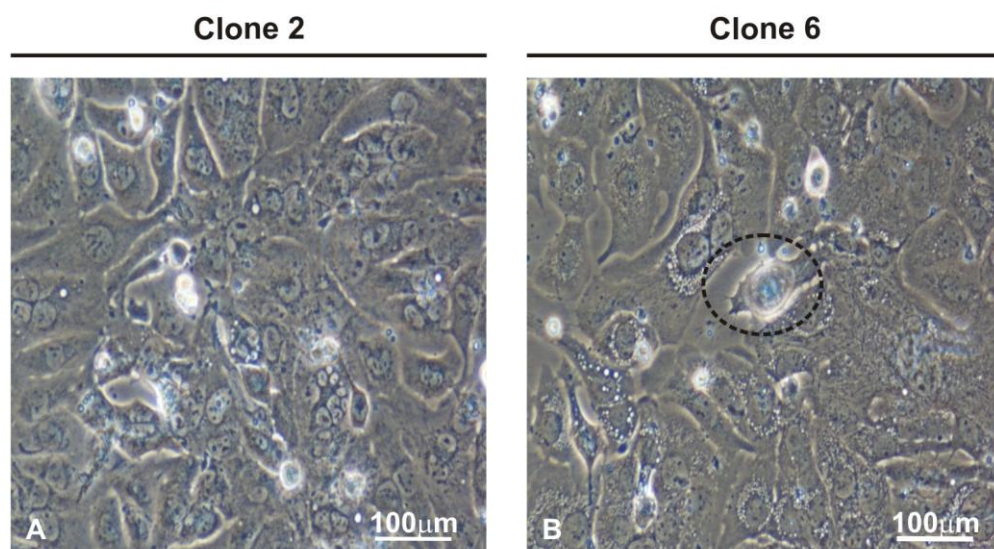


Figure 3.3: Phase contrast micrographs of clones 2 and 6

Phase contrast images of clones 2 (A) and 6 (B) isolated from the original HL-1 cell line. Both clones displayed similar (homogenous) characteristics in cell size and shape. The circle in (B) is a cell undergoing mitosis.

As mentioned above, of the five clones isolated, clones 1 and 2 lost the ability to contract upon passaging. To try to explain these differences in contractility, immunofluorescent labelling was performed to visualise the sarcomeric and cytoskeletal organisation in a non-contracting (clone 2) and contracting (clone 6) clone. Cardiac specific filamentous proteins α -actinin and desmin were expressed at high levels as expected for a cell of myocytic origin (Figure 3.4). These two markers were chosen because both α -actinin (located at the Z-disc of the sarcomeric assembly) and desmin

(plays an essential role in muscle architecture) are specific for muscle cells (e.g. cardiac and skeletal muscle).

There were clear visible differences in the organisation of α -actinin between the two clones. Clone 6 displayed the classic sarcomeric organisation with the clear-banded patterns in comparison to clone 2, where the α -actinin was associated with the cell membrane (Figure 3.4 (A) and (B)). Desmin was present as a fibre around the nucleus, however the intermediate filaments appeared more delicate and finer in clone 2 (Figure 3.4 (C)) than in clone 6 (Figure 3.4 (D)). The fibres in both clones were larger than what is observed in neonatal myocytes (Zhang et al. 2005).

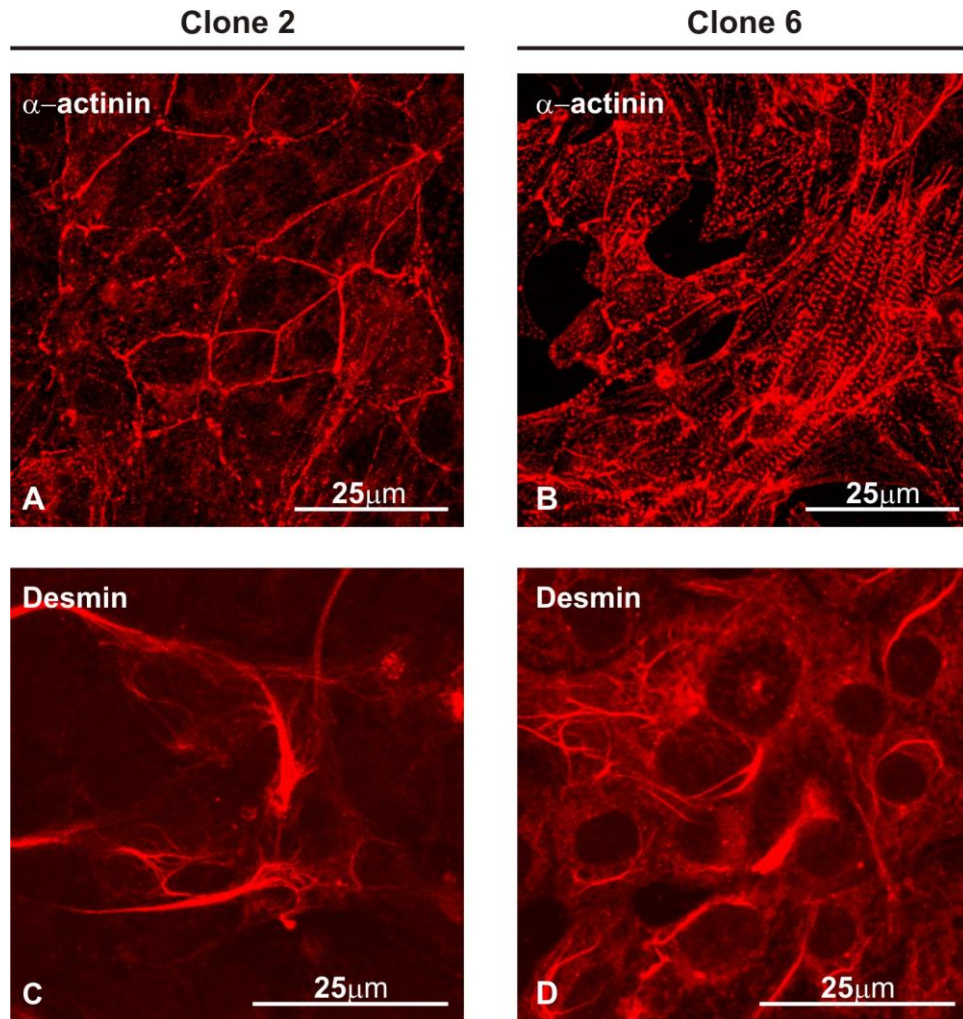


Figure 3.4: Immunofluorescent labelling of α -actinin and desmin in clones 2 and 6

The upper panel are immunoconfocal images of α -actinin in clones 2 (A) and 6 (B). There were visible differences in the organisation of α -actinin between the two clones. Clone 6 displayed the classic sarcomeric organisation in comparison to clone 2, where the α -actinin was associated with the cell membrane. The bottom panel are images for desmin clone 2 (C) and 6 (D). In both clones desmin was associated as a fibre around the nucleus.

3.3.2. Molecular characterisation of Ca^{2+} handling proteins

The amounts of mRNA coding for the major proteins involved in Ca^{2+} handling were analysed by northern blot in all HL-1 clones and compared to RNA extracted from a adult mouse heart (Figure 3.5). Levels of transcript expression for NCX, α_{1C} subunit of the L-type Ca^{2+} channel, α_{1H} subunit of the T-type Ca^{2+} , RyR2 and SERCA2 were similar in clones 1, 3, 4 and 6 and in the mouse heart. In clone 2 transcripts for NCX, α_{1C} subunit of the L-type Ca^{2+} channel, RyR2 and SERCA2 were expressed at lower

levels in comparison to the mouse heart. The probes labelled a single mRNA band at the size expected. Different exposure times were required to obtain similar signal intensities but this also helped to estimate the relative levels of transcript expressed in the HL-1 clones. Normalised expression of mRNA for each of the Ca^{2+} handling proteins is summarised in Table 3.2. The values were compared with the mouse heart noted as 100% and expressed as a percentage of this value ($n = 2$). The RNA samples were stained with ethidium bromide to visualise the 18S and 28S ribosomal RNA bands, check the equivalent loading of samples and measure the molecular mass of the bands obtained.

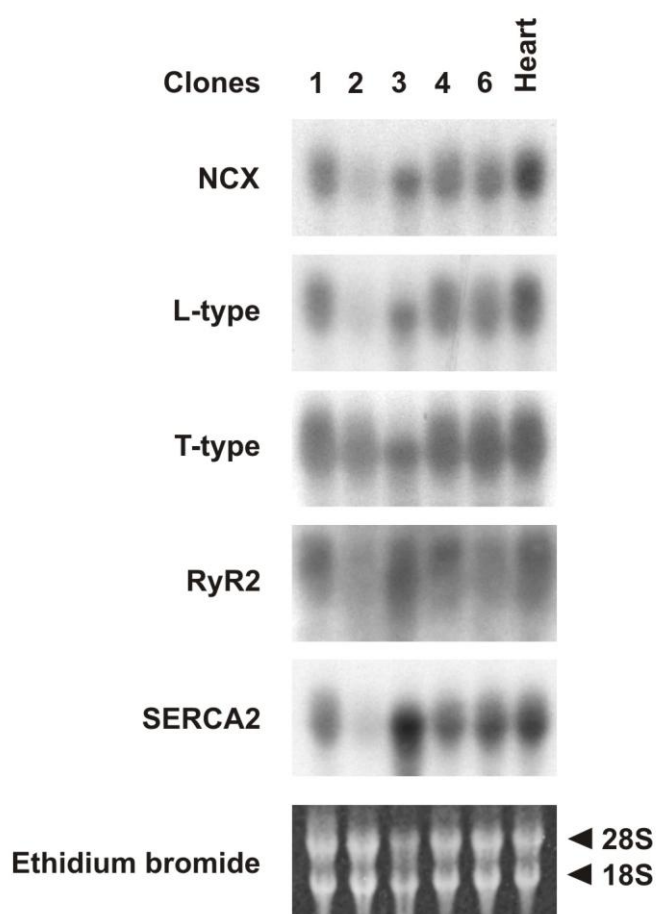


Figure 3.5: Transcript analysis of Ca^{2+} handling proteins in the HL-1 clones

Northern blot analysis of total RNA extracted from the HL-1 clones and mouse heart (indicated on top of the picture). Membranes were hybridised with probes for NCX, L-type Ca^{2+} channel, T-type Ca^{2+} channel, RyR2 and SERCA2 as indicated on the left of the picture. Membranes were stained with ethidium bromide to visualize the ribosomal RNA bands 18S and 28S, check equivalent loading of samples and measure the molecular mass of the bands.

**Table 3.2: Summary of transcript analysis of Ca^{2+} handling proteins
normalised to the adult mouse heart**

Probe	Size of band	Exposure time	1 (%)	2 (%)	3 (%)	4 (%)	6 (%)
NCX	~ 7kB	4 hours	78 ± 10	38 ± 4	70 ± 5	66 ± 4	64 ± 1
L-type	~ 8kB	4 hours	85 ± 14	25 ± 7	61 ± 9	74 ± 9	68 ± 10
T-type	~ 5kB	1.5 hours	109 ± 23	80 ± 11	93 ± 17	81 ± 16	104 ± 6
RyR2	~ 16kB	24 hours	65 ± 4	29 ± 8	95 ± 9	61 ± 9	45 ± 2
SERCA2	~ 4kB	4 hours	58 ± 1	22 ± 1	119 ± 9	75 ± 2	82 ± 7

3.3.3. Rhythmic oscillation of $[\text{Ca}^{2+}]_i$ in clones 2 and 6

Cells were loaded with the Ca^{2+} -sensitive dye fluo-4 and $[\text{Ca}^{2+}]_i$ fluctuations were monitored by confocal imaging in line scan mode. For each experiment a single line scan was placed over a group of cells and the resulting Ca^{2+} transients measured for 13.3s (each line scan was completed in 1.33ms and 10,000 lines were collected). The fluorescence measured was an average pixel value from the cells along the line of interest. Therefore the measurements will be influenced by changes in background fluorescence and cellular debris floating across the line during the recording period. The size of the transients will also be affected by the synchronisation of the cells whereby if in one cell the transient occurs before that in the neighbouring cell the average amplitude will be affected. To circumvent these problems line scans were only performed in stable areas of the culture and on cells beating synchronously.

Figure 3.6 (A) and (B) illustrates the changes in fluorescence intensity of fluo-4 measured in cells displaying spontaneous rhythmic oscillations of $[\text{Ca}^{2+}]_i$ in clones 2 and 6 respectively. Fluorescence changes were normalised to the background (resting) fluorescence (F/F_0). No significant differences was found in the amplitude of $[\text{Ca}^{2+}]_i$ transients in both clones (clone 2: $1.7 \pm 0.05 F/F_0$; clone 6: $1.8 \pm 0.04 F/F_0$; $p = 0.3$; $n = 5$ and 6 respectively).

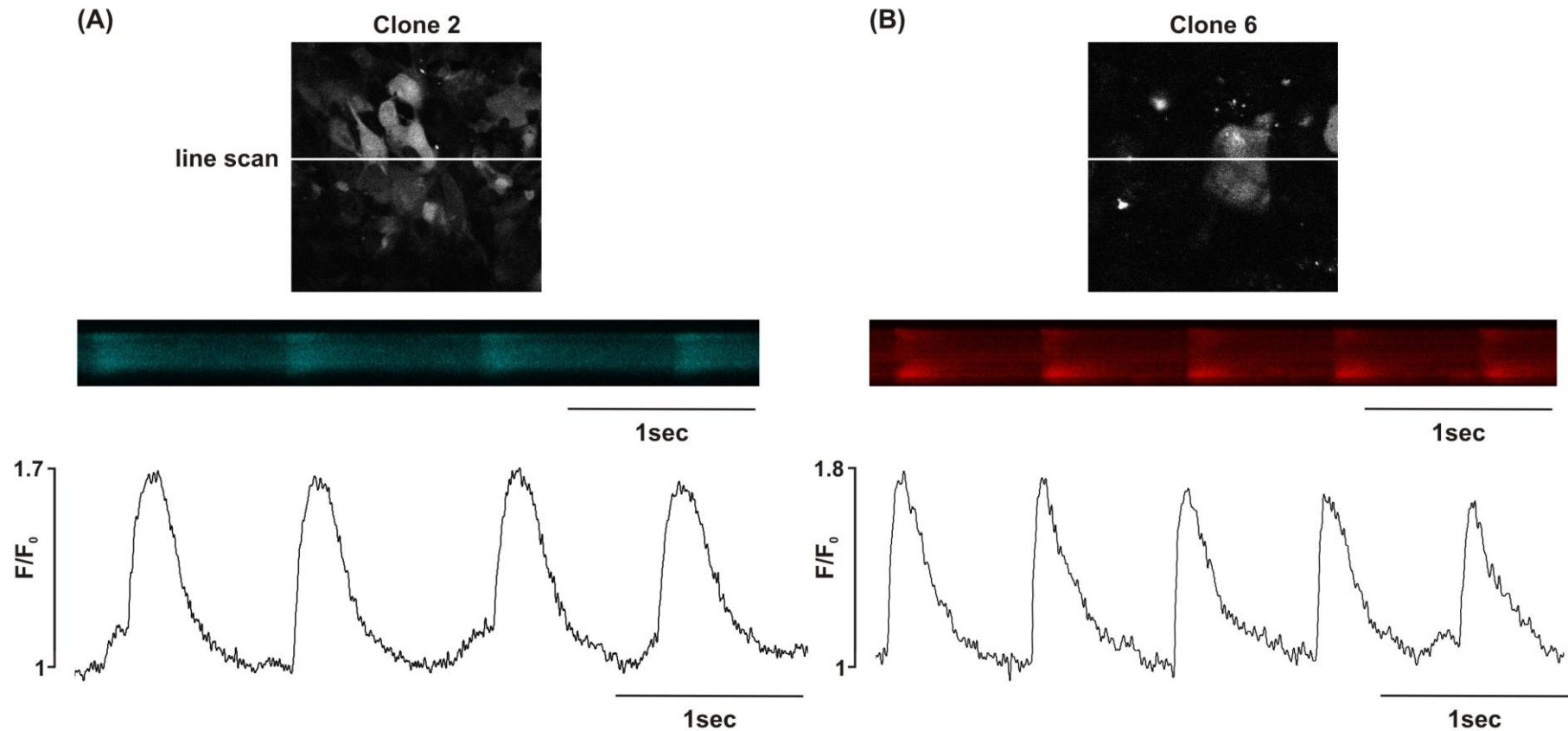


Figure 3.6: Confocal line scan images of Ca^{2+} transients in clones 2 and 6

Traces (A) and (B) show spontaneous rhythmic oscillations of $[Ca^{2+}]_i$ in clones 2 and 6 after staining with fluo-4. Quantification of the traces showed that both clones had a comparable $[Ca^{2+}]_i$ release (clone 2: $1.7 \pm 0.05 F/F_0$; clone 6: $1.8 \pm 0.04 F/F_0$; $p = 0.3$; $n = 5$ and 6 respectively). The values for $[Ca^{2+}]_i$ release were represented as fluorescence changes normalised to the background (resting) fluorescence (F/F_0). For the purposes of illustration, recordings were filtered using the Butterworth (8-pole filter) with a -3dB cutoff filter at 60Hz.

However there were significant differences in the time course of the Ca^{2+} transients between the two clones including the time to peak (clone 2: $222 \pm 27\text{ms}$; clone 6: $59 \pm 2\text{ms}$; $p < 0.0001$; $n = 5$ and 6 respectively); time to 50% relaxation from the peak (clone 2: $382 \pm 40\text{ms}$; clone 6: $157 \pm 6\text{ms}$; $p < 0.0001$; $n = 5$ and 6 respectively) and time to 90% relaxation from the peak (clone 2: $541 \pm 45\text{ms}$; clone 6: $397 \pm 14\text{ms}$; $p = 0.002$; $n = 5$ and 6 respectively).

SR Ca^{2+} content was assessed with the rapid application of 10mM caffeine to the extracellular solution and monitoring the resultant Ca^{2+} release (Figure 3.7). Since all recordings were performed on spontaneous Ca^{2+} transients one caveat of this study was that we were unable to determine if the SR Ca^{2+} was depleted from the previous contraction. In both clones application of caffeine evoked a rapid increase in Ca^{2+} release into the cytoplasm by similar levels (clone 2: $1.8 \pm 0.12 \text{ F/F}_0$; clone 6: $2.0 \pm 0.14 \text{ F/F}_0$; $p = 0.3$; $n = 4$ and 5 respectively). The effects of caffeine were reversible upon perfusion with Tyrode and $[\text{Ca}^{2+}]_i$ returned to control levels.

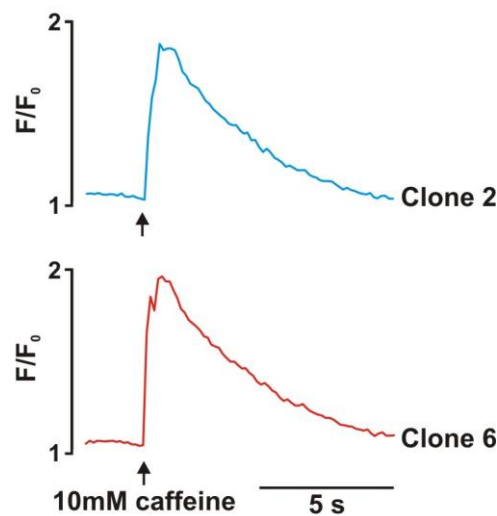


Figure 3.7: Caffeine-releasable stores in clones 2 and 6

Clones 2 and 6 have caffeine-releasable intracellular stores of Ca^{2+} . When 10mM caffeine was applied to the cells marked by the arrow there was an increase in $[\text{Ca}^{2+}]_i$ levels by similar levels.

It should be taken into account that Fluo-4 is a non-ratiometric dye whereby the fluorescence intensity is sensitive to factors such as: (1) calcium buffering within a cell; (2) homogeneity of the dye loading; (3) incomplete hydrolysis of the conjugated cell-permeant ester and (4) photobleaching (Paredes et al. 2008). To eliminate these

variables, ratiometric dyes need to be considered (e.g. Indo-1) for future experiments which function by undergoing a shift in either their excitation or emission wavelength upon Ca^{2+} binding (Paredes et al. 2008). The $[\text{Ca}^{2+}]_i$ is then calculated by ratioing the fluorescence intensities at the two wavelengths.

3.3.4. Action potential characteristics in clones 2 and 6

Action potentials were recorded from cells in clones 2 and 6 under whole-cell current clamp conditions. Figure 3.8 shows a typical action potential recorded upon stimulation of 1Hz. It should be noted that action potentials were recorded in only one cell patched in clone 2 since a large proportion of cells elicited no action potentials even after electrical stimulation. However cells in clone 6 always produced action potentials upon stimulation. Clone 6 had a mean action potential duration measured at 50% repolarisation (APD_{50}) of $17 \pm 6\text{ms}$ with an overshoot of $28 \pm 5\text{mV}$ ($n = 4$). Clone 2 had a resting membrane potential that was more depolarised of $-43 \pm 6\text{mV}$ ($n = 20$) compared with clone 6 at $-67\text{mV} \pm 2\text{mV}$ ($n = 25$).

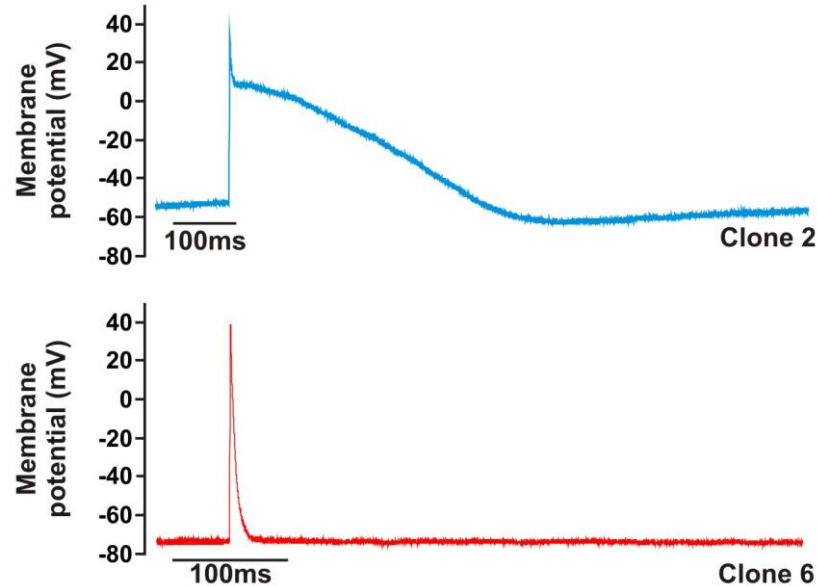


Figure 3.8: Action potential characteristics in clones 2 and 6

A typical action potential recorded from clones 2 and 6 under whole-cell current clamp with stimulation at a frequency of 1Hz.

3.3.5. Characterisation of voltage-gated ion channels in clones 2 and 6

L-type Ca^{2+} currents

To assess the function of some of the ion channels knowing their relative amounts of mRNA we used whole-cell patch-clamp techniques to examine their Ca^{2+} currents. The Ca^{2+} current data was kindly provided by Dr N. Ullrich. A series of voltage steps from a holding potential of -50mV up to 60mV were evoked to elicit the L-type Ca^{2+} current Figure 3.9 (A). The voltage dependence and pharmacological sensitivity of the L-type Ca^{2+} current recorded in clones 2 and 6 is illustrated in Figure 3.9 (B) and (C) respectively. Both clones showed evidence of nifedipine-sensitive inward currents suggesting the presence of L-type Ca^{2+} channels (Figure 3-9 (B)). Subtracted currents were obtained from the normal Tyrode trace minus the nifedipine sensitive trace. The plots of the mean data from the subtracted current that yield a current-voltage relationship (I-V) showed no significant difference in the maximum sizes of the currents in both clones (clone 2: -0.89 ± 0.07 pA/pF; clone 6: -0.86 ± 0.04 pA/pF; $p = 0.7$; $n = 5$ and 14 respectively). However maximal current activation was obtained at different voltages (clone 2: 10mV; clone 6: -10mV). Currents from clone 6 had a more negative reversal potential when compared to clone 2.

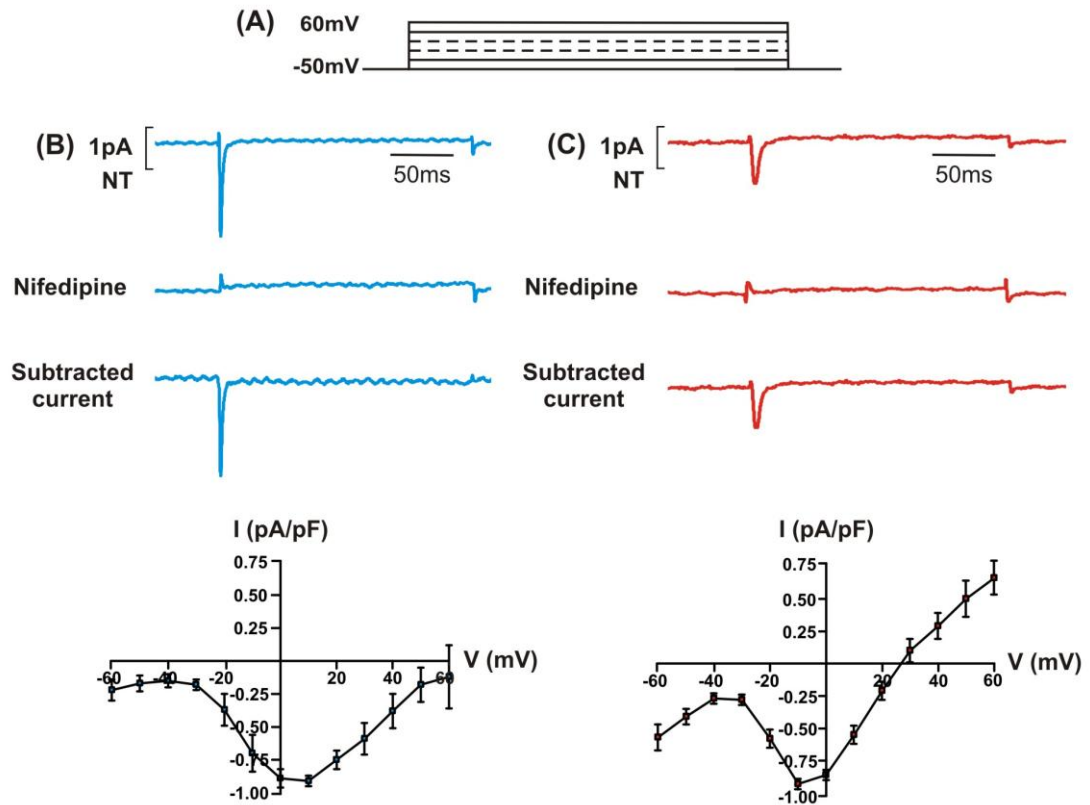


Figure 3.9: Voltage-gated L-type Ca^{2+} currents recorded from clones 2 and 6

Cells were clamped at a holding potential of -50mV and a series of voltage steps were imposed (represented by the dashed lines) to 60mV at 10mV increments (A). Current traces were recorded when cells were perfused with normal Tyrode [NT] and nifedipine in clone 2 (B) and clone 6 (C)). The subtracted current represented the traces recorded in normal Tyrode trace minus the Tyrode containing nifedipine. The current-voltage (I-V) curve was plotted using the subtracted current values expressed as current density (pA/pF). There was no difference in the maximum sizes of the nifedipine-sensitive current (clone 2: $-0.89 \pm 0.07 \text{ pA/pF}$; clone 6: $-0.86 \pm 0.04 \text{ pA/pF}$; $p = 0.7$; $n = 5$ and 14 respectively).

T-type Ca^{2+} currents

T-type Ca^{2+} currents were evoked by voltage clamp steps from a holding potential of -80mV to a pre-step of -40mV and subsequent steps to 40mV in 5mV increments (Figure 3.10 (A)). Clones 2 and 6 both had mibefradil-sensitive inward currents that peaked at -25mV as shown in Figure 3.10 (B) and (C) respectively. This was somewhat more positive than a typical T-type Ca^{2+} current which has a peak current at around -30mV (Vassort et al. 2006) There was no significant difference in the maximum sizes of the mibefradil-sensitive current (clone 2: $-0.94 \pm 0.04 \text{ pA/pF}$; clone 6: $-0.89 \pm 0.03 \text{ pA/pF}$; $p = 0.3$; $n = 8$ and 10 respectively).

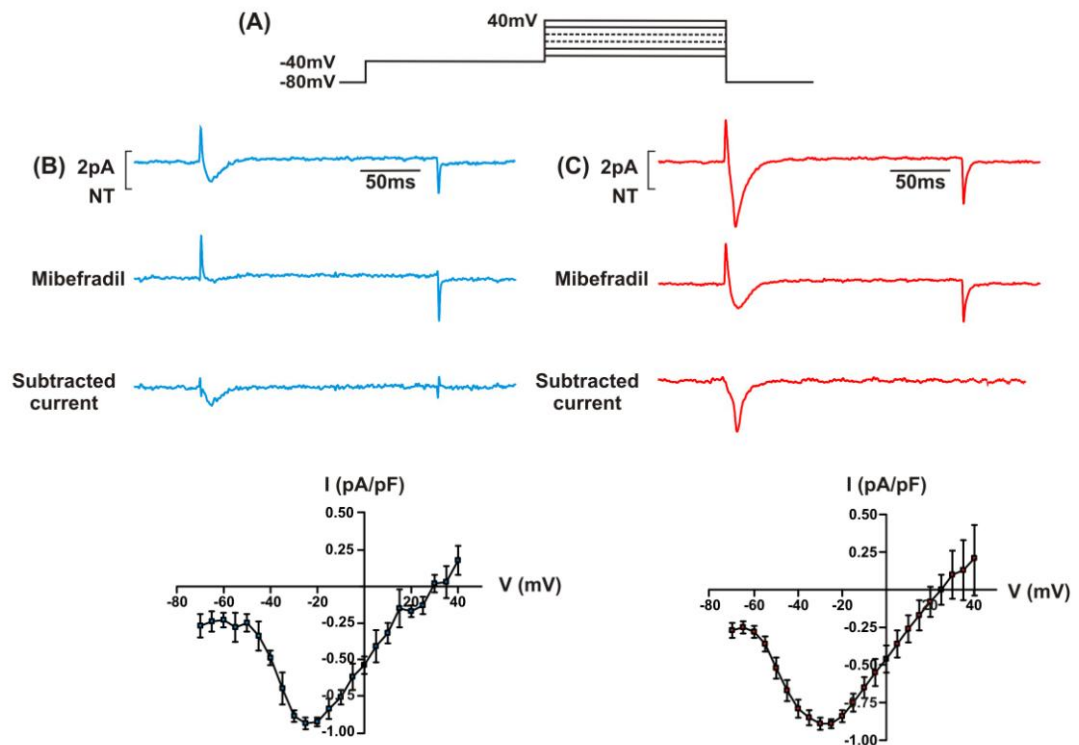


Figure 3.10: Voltage-gated T-type Ca^{2+} currents recorded from clones 2 and 6

Depolarising voltage pulses from -80 to a pre-step of -40mV followed by 5mV increments (represented by the dashed lines) to 40mV (A) was used to evoke T-type Ca^{2+} currents from clones 2 (B) and 6 (C) shown together with the respective current obtained in the presence of mibefradil. A membrane holding potential of -40mV was used to inactivate the Na^{+} channels. I-V curves plotted on the subtracted current (pA/pF) demonstrated a peak inward current obtained at -25mV. There was also no difference in the maximum sizes of the mibefradil-sensitive current (clone 2: -0.94 ± 0.04 pA/pF; clone 6: -0.89 ± 0.03 pA/pF; $p = 0.3$; $n = 8$ and 10 respectively).

Na^{+} currents

Representative Na^{+} currents are shown in Figure 3.11 for both clones 2 and 6. Sequential voltage clamp steps were imposed from a holding potential of -120mV to 50mV in 10mV increments (Figure 3.11 (A)), to fully activate the Na^{+} current. Cells were clamped at -120mV to ensure the Na^{+} channels were closed and ready to be activated. The fast inward current obtained from clones 2 and 6 was sensitive to 1mM procaine resulting in an almost complete block (Figure 3.11 (B) and (C) respectively). In the presence of procaine clone 6 often showed some remnant of an inward current, activating in similar ranges as that observed for Ca^{2+} currents. The I-V curves were plotted from the subtracted current of the normal Tyrode and procaine traces. In both clones Na^{+} currents were activated at a threshold of -60mV. There was no significant difference in the maximum values of the activated current (clone 2: -77.5 ± 6.1 pA/pF;

clone 6: -85.0 ± 14.3 pA/pF; $p = 0.6$; $n = 15$ and 9 respectively). Currents were maximally activated in clone 2 at -30 mV and in clone 6 at -20 mV in clone 6. The reversal potential appeared to be very similar in both clones (clone 2: ~ 55 mV; clone 6 ~ 60 mV).

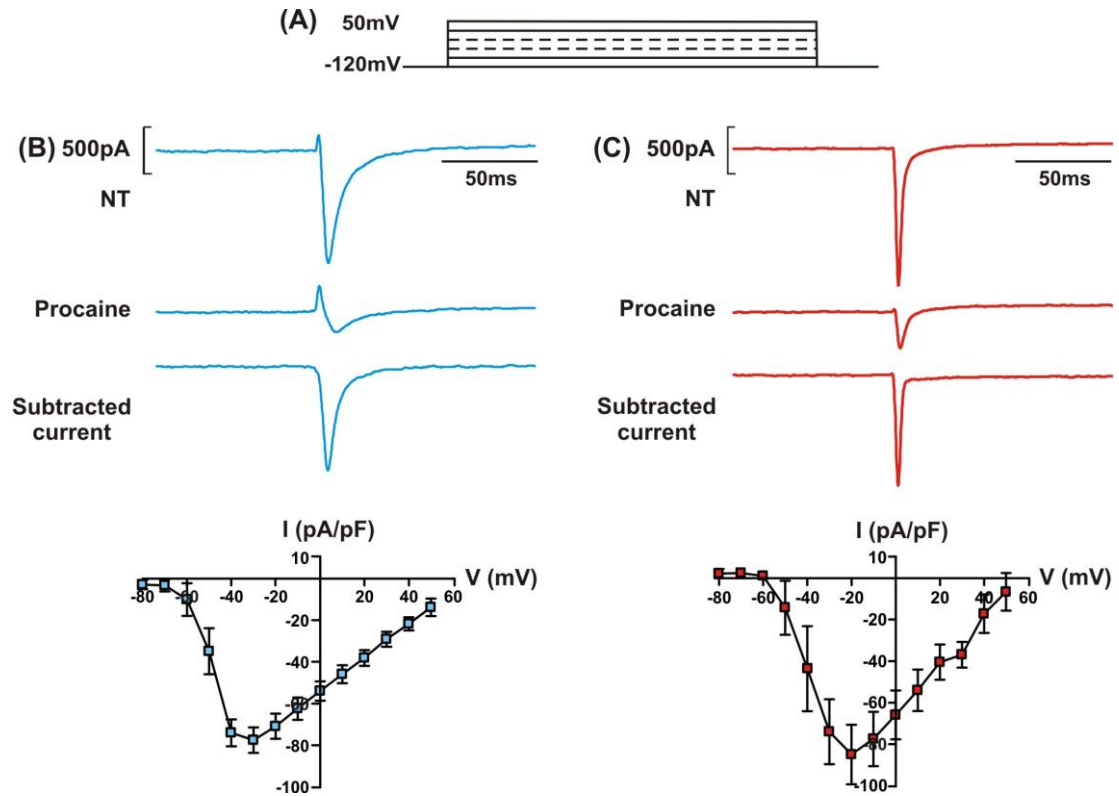


Figure 3.11: Voltage-gated Na^+ currents recorded from clones 2 and 6

Cells were clamped at a holding potential of -120 mV and a series of voltage steps were imposed (represented by the dashed lines) to 50 mV at 10 mV increments (A). Current traces were recorded when cells were perfused with normal Tyrode (NT) and Na^+ channel blocker procaine (blue traces for clone 2 (B) and red traces for clone 6 (C)). The I-V curves were plotted for both clones with no significant difference in the current size of the procaine-sensitive currents (clone 2: -77.5 ± 6.1 pA/pF; clone 6: -85.0 ± 14.3 pA/pF; $p = 0.6$; $n = 15$ and 9 respectively).

***I_f* currents**

Cells were voltage clamped and hyperpolarised for 1.5 s from a holding potential of -40 mV to more negative potentials ranging from -60 to -130 mV in 10 mV increments. Representative current traces recorded from clones 2 and 6 are shown in Figure 3.12 (A) and (B) respectively. I_f current was not present in any of the cells patched in clone 2 ($n = 20$). Clone 6 had an inward current that slowly activated with time upon hyperpolarisation to more negative potentials. We observed a complete block of the

current elicited at peak activation of -130mV by perfusing the cells with 4mM Cs^+ . This effect was reversible by perfusing the cells with modified Tyrode. The I-V curve shows the Cs^+ sensitive current (modified Tyrode current subtracted from the Cs^+ current) as a function of the holding potential and we observed a peak current activation of $-32 \pm 4\text{pA/pF}$; $n = 16$. In clone 6 I_f was not present in every cell, but we did observe a non-inactivating inward current with an amplitude that increased as the potential became more negative similar to those present in the original HL-1 cell line (Sartiani et al. 2002). These currents were confirmed to be Cs^+ insensitive.

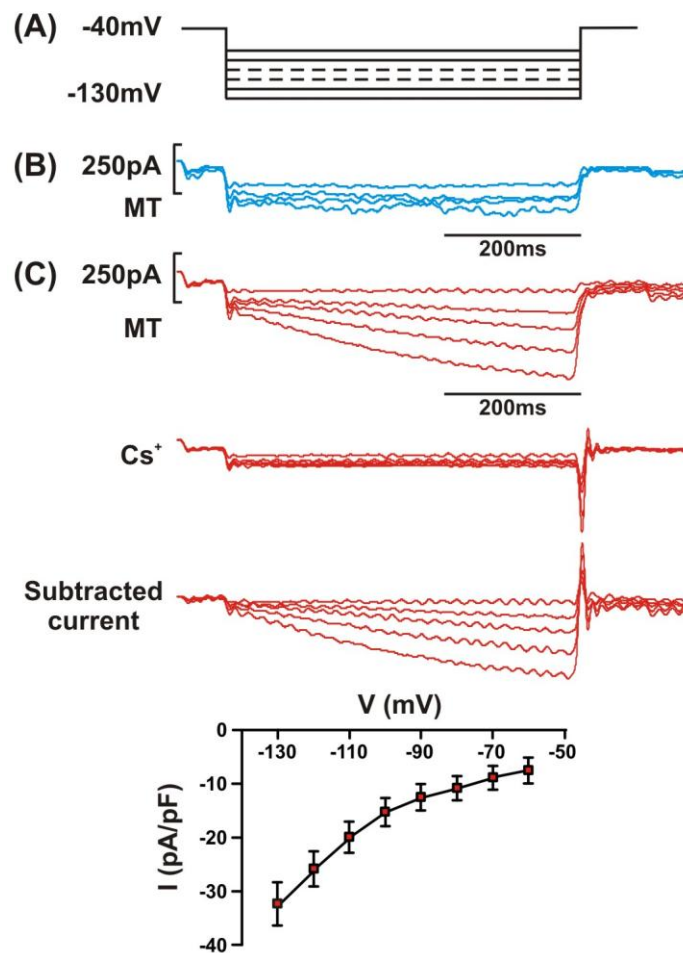


Figure 3.12: Voltage-gated I_f currents recorded in clones 2 and 6

Cells were hyperpolarised for 1.5 seconds from a holding potential of -40mV to more negative potentials from -60 to 130mV (indicated by the dashed lines) in 10mV increments (A). In clone 2 (B) we did not observe I_f in any the groups of cells patched ($n = 20$) in modified Tyrode (MT). Currents elicited in clone 6 perfused with modified Tyrode displayed a Cs^+ sensitive current with an amplitude that increased as the potential became more negative. Mean I_f activation points were obtained at each membrane potential and the values were represented as pA/pF . Clone 6 had a peak current activation of $-32 \pm 4\text{pA/pF}$; $n = 16$.

3.4. Discussion

The HL-1 cells are a unique cardiac myocyte cell line derived from proliferating atrial myocytes of transgenic mice. However, this transformed, tumorigenic line contains cells that are blocked at different stages of differentiation (Trosko et al. 1993). Thus it was necessary to produce a more homogeneous cell line to allow better correlation of change of function with molecular intervention. In this chapter the morphological features and electrophysiological characteristics of the HL-1 clones are described with a particular focus on clones 2 and 6.

After several passages, of the five clones obtained contractile activity in clones 1 and 2 declined with the other clones starting spontaneous contraction at different times in culture. To explain these differences in contraction, we investigated two key features: (1) Ca^{2+} entry in the cell triggering Ca^{2+} induced Ca^{2+} release (CICR) from the SR which directly activates the myofilaments causing contraction (Bers 2002; Endo 2009) and (2) the localisation of myofibrillar and cytoskeletal proteins (e.g. desmin, myosin, and actin) (Kostin et al. 2000).

We started by comparing the cytoskeleton of the two clones and found that in clone 2 the sarcomeric-banded organisation of α -actinin was replaced by a sub-membranous distribution, likely explaining the lack of contractility (Zhang et al. 2005; Skwarek-Maruszewska et al. 2009). Desmin a marker for myofibril integrity and maturation, also appeared to be more delicate and finer in clone 2 as previously described in non-contracting cells (Kostin et al. 2000; Bar et al. 2004; Paulin and Li 2004; Pelloux et al. 2006). The delicate fibres in clone 2 may also be a cause of the lack of contraction as studies on embryonic hearts showed that desmin associated with the Z-line only after the hearts started to beat (Zhang et al. 2005). Desmin knock-out studies have demonstrated that the mice develop hypertrophy with disorganised myofibrils and altered configurations of the intercalated discs. The mitochondria are also abnormal in morphology and function as they appear swollen and accumulate between the myofibrils. Desmin may also be involved in supporting sacromere alignment or force transmission indicating the importance of the protein in maintaining muscle function and structural integrity (Li et al. 1997; Paulin and Li 2004). Morphological alterations of other cytoskeleton proteins (e.g. actin, desmin, titin) associated with reduced

contractile function have been reported in studies on heart failure (Hein et al. 2000). It is likely that both these differences in sarcomeric skeleton (α -actinin) and cytoskeletal proteins (desmin) may contribute to loss of contractility and cellular integrity in clone 2 (Kostin et al. 2000; Zhang et al. 2005).

As mentioned before, $[Ca^{2+}]_i$ plays a key role in the E-C coupling whereby electrical excitation is transduced into contraction (Bers 2002). Since clone 2 does not contract we looked into possible differences in the Ca^{2+} release process between the two clones. Using line scan confocal microscopy both clones displayed spontaneous Ca^{2+} transients with similar amplitudes when monitored using fluo-4. In the heart the amount of $[Ca^{2+}]_i$ released is governed by: (1) Ca^{2+} influx through the L-type Ca^{2+} channel activated upon membrane depolarisation and (2) SR Ca^{2+} release triggered by the initial Ca^{2+} influx (Venetucci et al. 2007). Analysis of current densities for the L-type Ca^{2+} channel and SR Ca^{2+} content assessed by application of caffeine were comparable in both clones. This was despite cells from clone 2 expressing less mRNA coding for L-type Ca^{2+} channel, RyR2, the SR Ca^{2+} release channel. Thus there seemed to be no direct correlation between the amount of messenger expressed and the functional protein perhaps indicating different post-transcriptional regulations between the two clones.

Although the role of T-type Ca^{2+} channels is unclear in atrial cells these channels could contribute to the influx of Ca^{2+} . No difference was observed in the mRNA expression levels and current density in both clones. It is also thought that T-type Ca^{2+} currents contribute very little to the initial Ca^{2+} influx triggering contraction since no expression of functional channels is present in adult ventricular myocardial cells (Hermsmeyer et al. 1997; Vassort et al. 2006). However the existence of T-type Ca^{2+} channels in both clones 2 and 6 does support the view that such channels play a more important role in cell growth (Hermsmeyer et al. 1997), cell cycle progression (Rodman et al. 2005), cell development (Zhang et al. 2003) and pacemaker generation (Mangoni et al. 2006). Evidence for this comes from the prominent expression of T-type Ca^{2+} channels in the developing embryo and foetus but declines during maturation particularly in ventricular myocytes (Vassort et al. 2006). T-type Ca^{2+} channels are also found during ventricular hypertrophy and subendothelial thickening and Ca^{2+} entry through these channels may contribute to arrhythmias (Nuss and Houser 1993; Hermsmeyer et al. 1997; Takebayashi et al. 2006). The presence of T-type Ca^{2+} channels in cardiac tissue

displaying automaticity make them a possible candidate to provide an inward depolarising current during the slow diastolic depolarisation phase in pacemaker generation (Vassort et al. 2006). Although I have not investigated the various roles of T-type Ca^{2+} channels, the HL-1 clones provide an ideal cellular model for such a study.

Another regulator of $[\text{Ca}^{2+}]_i$ content is via NCX which is expressed by all the HL-1 clones. NCX is primarily involved in muscle relaxation in combination with SERCA in lowering $[\text{Ca}^{2+}]_i$ concentration to resting levels (Venetucci et al. 2007). It functions by exporting one Ca^{2+} ion out of the cell in exchange for importing three Na^+ ions into the cell. Since NCX was expressed at high levels in all the HL-1 clones with the exception of clone 2 it could potentially play a key role in pacemaker generation. During diastolic depolarisation SR Ca^{2+} can be released spontaneously through RyR2. This release locally activates NCX, which in turn produces a ‘booster inward current’ thereby depolarising the membrane, allowing threshold to be reached and an action potential to be triggered (Bogdanov et al. 2001). To confirm and study the functional role of NCX in pacemaker generation, further patch-clamp experiments will need to be performed.

Cells from clone 6 had average resting membrane potentials of $-75\text{mV} \pm 3\text{mV}$ as expected for a cell of atrial origin (Shih 1994). However cells in clone 2 were more depolarised with a resting membrane potential of $-43 \pm 6\text{mV}$ similar to values observed in nodal cells (DiFrancesco 2005). We were only able to record a single action potential in clone 2 upon electrical stimulation. This was despite the cells displaying spontaneous Ca^{2+} transients. The lack of action potential generation in clone 2 could be explained by differences in cell density. During experiments for patch-clamp cells are isolated into single cells and for Ca^{2+} measurements cells are seeded in large aggregates. Moreover we also demonstrate in Chapter 5 that cells in clone 2 are able to produce extracellular field potentials detected by MEA when seeded at high density. This factor of seeding density is crucial in the HL-1 cell line since the development of spontaneous contraction is very much dependent on the development stage of the culture and cell density (Claycomb et al. 1998; Hong et al. 2008).

The action potential characteristics of clone 6 in terms of the shape were comparable to those obtained from the original HL-1 cell line (Sartiani et al. 2002) and of mouse atrial cells (Knollmann et al. 2007). The action potential overshoot was considerably higher

than those recorded in the original HL-1 (Sartiani et al. 2002) and HL-5 cell line also derived from the AT-1 cell line (Xiao et al. 2006). Action potential durations (APD_{50}) were also variable between clone 6, the HL-1 cell line and the HL-5 cells. These results are conflicting since one would expect all three cell lines to have similar action potential characteristics as they were originally derived from the AT-1 cell line (Claycomb et al. 1998; White et al. 2004). This illustrates the heterogeneity of the original cell lines and highlights the importance of sub-cloning enabling a more accurate analysis of the experimental results. Compared with clone 6, neonatal mouse hearts displayed similar action potential durations (Nuss and Marban 1994) and with larger overshoots (Nuss and Marban 1994; Xiao et al. 2006). The large overshoot in neonatal mouse hearts could be explained by the presence of larger Na^+ currents when compared with the HL-1 clones (Nuss and Marban 1994).

The action potential amplitude and upstroke in the heart is determined by the Na^+ current (Herfst et al. 2004) through the opening of $Na_v1.5$ channels (Abriel 2010). Current densities in clones 2 and 6 were not significantly different when cells were clamped at -120mV to ensure all the Na^+ channels were in their closed state ready to be activated. However it should be noted in clone 2 resting membrane potentials were more depolarised compared with clone 6. Thus over this voltage range the number of Na^+ channels that are likely to be open are considerably reduced (Grant 2009). This in turn could potentially lower the current density and action potential amplitude affecting action potential propagation since Na^+ currents are primarily responsible for membrane excitability in the heart (Shaw and Rudy 1997).

In clones 2 and 6 recordings of Na^+ sensitive currents were comparable in activation values and reversal potentials mirrored by a similar overshoot in action potentials. In comparison to studies on adult atrial myocytes and neonatal mouse hearts, the HL-1 clones displayed maximum activation ranges at more positive potentials (Nuss and Marban 1994; Feng et al. 1996; Mille et al. 2009). Similarly the current reversal potentials were also a lot more positive in the HL-1 clones compared to neonatal mouse strains (Mille et al. 2009). The currents particularly in clone 2 had a slow inactivation compared with cardiac Na^+ currents. These differences could be due to recording conditions but for a more detailed comparison the inactivation of these currents will be need to studied more systematically. It is possible that splice variants of the normal

cardiac SCN5A gene encoding Na_v1.5 may exist in the HL-1 clones that differ in protein synthesis, assembly and post-translation modifications producing some subtle alterations in their function (Herfst et al. 2004). It is also possible that the HL-1 clones are comparable to an embryonic phenotype unable to express the Na⁺ channels characteristic of adult cardiac myocytes.

We also demonstrated the presence of I_f , that activates upon voltage hyperpolarisation and slowly depolarises the membrane up to threshold for firing a new action potential (DiFrancesco 2005). We were only able to record I_f in clone 6 and not in clone 2. In clone 6, I_f activated at a negative membrane potential of -60mV and its amplitude increased at more negative potentials with maximum activation at -130mV. The electrophysiological characteristics of I_f were similar to those found in the original HL-1 cells (Sartiani et al. 2002) and cardiac pacemaker cells (DiFrancesco 1993). The current was also blocked by Cs⁺ (Denyer and Brown 1990; Sartiani et al. 2002; Xiao et al. 2006). I_f was not always present in clone 6 which was consistent with findings in the original HL-1 cells with I_f present in only 30% of the cells patched (Sartiani et al. 2002) and in 39% of the HL-5 cells (Xiao et al. 2006). This was also confirmed by our action potential data since most of the cells exhibited a flat diastolic potential rather than a spontaneous diastolic depolarisation phase expected in the presence of I_f (Dobrzynski et al. 2007). Cellular heterogeneity with respect to the presence of I_f has been reported in myocytes isolated from native cardiac tissues such as the sinoatrial node (Denyer and Brown 1990) and atrial tissue (Zhou and Lipsius 1992). To correlate the presence of a spontaneous diastolic depolarisation to I_f , recordings of action potentials and I_f will need to be performed on the same cells. Although we did not study the relationship between I_f and automaticity it is unlikely that it is fully responsible for the pacemaker current generated in the HL-1 clones and that other ion currents may contribute to the initiation of action potentials. For example despite I_f being absent in clone 2 these cells were still able to display spontaneous Ca²⁺ transients. Studies by Lakatta et al. have implicated the localised rhythmic oscillations of [Ca²⁺]_i by the SR that cross-talks with other mechanisms (e.g. NCX) to regulate the automaticity of the heart (Lakatta et al. 2003).

In summary we have shown that the HL-1 clones express many of the Ca²⁺ handling proteins, display spontaneous Ca²⁺ transients, have a functional SR and have majority of the cardiac ionic currents. Lack of contractility in clone 2 is most likely caused by the

lack of sarcomeric organisation rather than Ca^{2+} influx triggering cardiac excitation since no difference was found when compared to clone 6. Compared with freshly isolated primary cultures the HL-1 clones exhibit properties that are comparable to a neonatal phenotype. The HL-1 clones provide a useful model to study cardiac channel regulation and functional expression including pacemaker automaticity and the specific roles $[\text{Ca}^{2+}]_i$ and I_f play in this process. Since we have established that the HL-1 clones have the necessary ion channels required to generate action potentials, the next stage of my investigation was to study gap junctions and their component connexins that link together individual myocytes, forming the pathways for action potentials propagation.

CHAPTER 4.
Characterisation and quantification of connexin
expression/co-expression in the HL-1 clones

4.1. Introduction

As in most tissue and organs multiple connexins are expressed in the heart. The three principal cardiac connexins, connexin40 (Cx40), Cx43 and Cx45 are found in a chamber-related and myocyte-specific manner (Gros and Jongsma 1996; Severs et al. 2006). Connexins can form functional gap junction channels by themselves however co-expression of more than one connexin subtype raises the possibility of different channel type formations (White and Bruzzone 1996; Cottrell and Burt 2005). As discussed in Chapter 1, section 3, if a cell co-expressed two connexin subtypes that were free to associate in a random fashion there are fourteen possible connexon types that could be formed i.e. 12 heteromeric connexons composed of both connexin subtypes and 2 homomeric connexons made up of solely one or the other connexin (Brink et al. 1997; Cottrell and Burt 2005).

The ability of connexons to form junctional channels is a selective process dependent on the constituent connexin subtypes (Gros and Jongsma 1996). Several electrophysiological and biochemical studies have shown the formation of heteromeric gap junction channels in a variety of cell expression systems and tissue. Co-immunoprecipitation and cell pair data on A7r5 vascular smooth muscle cells demonstrated that heteromers are formed between Cx40 and Cx43 and exhibit channel and voltage gating properties unique from homomeric-homotypic Cx40 or Cx43 channels (He et al. 1999). This data was confirmed by Beyer et al. in HeLa transfectants (Beyer et al. 2001; Valiunas et al. 2001). Examples of heteromer formation between other connexin subtypes include Cx46-Cx50 in the lens (Jiang and Goodenough 1996), Cx26-Cx32 in the rodent liver (Bevans et al. 1998) and Cx43-Cx30 in astrocytes (Nagy et al. 1997).

Although there is a wealth of data supporting the formation of Cx40 and Cx43 heteromeric formation, studies have reported conflicting results as to whether connexons composed of Cx40-Cx43 form heterotypic channels. For example in *Xenopus* oocytes, there was no gap-junctional communication detected between paired oocytes expressing Cx40 and Cx43 using dual cell patch-clamp (Bruzzone et al. 1993; White et al. 1995). However in the same expression system high levels of conductance were recorded when oocytes expressing Cx40 or Cx43 were paired with Cx37. This

highlights the ability of connexins to distinguish one connexin subtype from another. In HeLa cells there was no Lucifer yellow dye transfer observed after microinjection between Cx40 and Cx43 expressing cells (Elfgang et al. 1995). Lack of gap junction formation between HeLa cells expressing Cx40 and Cx43 on apposed plasma membranes was also demonstrated by immunofluorescence (Haubrich et al. 1996; Rackauskas et al. 2007a). However electrophysiological studies by Valiunas et al. (2000) and Cottrell et al. (2001) did demonstrate the formation of functional heterotypic channels between Cx40 and Cx43 in HeLa and RIN cells transfected with Cx40 and Cx43 respectively albeit with conductance at significantly lower levels than homotypic channels composed either Cx40 or Cx43.

In contrast the presence of functional heterotypic channels between Cx45 and Cx40 or Cx43 have consistently been reported (White and Bruzzone 1996; Rackauskas et al. 2007a). When ROS cells that naturally express Cx43 were transfected with Cx45, there was a reduction in dye permeability by the formation of heteromeric-heterotypic channels (Koval et al. 1995). Similar observations were also made in our laboratory on the rat liver epithelial cell line (RLE) that endogenously express Cx43 and are transfected to co-express Cx45 (RLE Ind45) (Grikscheit 2010). Using fluorescent imaging and electrophysiological data from HeLa transfectants Cx45 readily formed junctional channels with Cx40 or Cx43 (Rackauskas et al. 2007a; Valiunas et al. 2000). Cell pair data in HeLa and N2a transfectants have also demonstrated the presence of heterotypic channels between Cx43 and Cx45 that were functionally distinct from homotypic channels composed of Cx43 or Cx45 (Elenes et al. 2001; Desplantez et al. 2004). Similar findings were also reported by Martinez et al. (2002a) in HeLa cells using Lucifer yellow dye permeability, affinity purification and cell pair data.

Collectively these studies indicate that heteromeric-heterotypic channels characterised by different electrical properties (e.g. unitary conductance, voltage gating, dye transfer) may have the potential to regulate the extent of cell-to-cell communication between cells co-expressing more than one connexin subtype. The ability or inability of certain connexin combinations to form heterotypic channels could also limit or segregate communication between cells in close physical contact (White et al. 1995; Elfgang et al. 1995).

Intercellular communication at the gap junction plaque is also regulated by factors that affect the abundance, size and cellular distribution of gap junction channel aggregates (Hunter et al. 2005). For instance connexin expression may be regulated at the transcriptional (e.g. interaction of transcription factors with target elements in controlling gene expression) or post-transcriptional level (e.g. connexin synthesis, oligomerisation and degradation) (Saffitz et al. 2000; Oyamada et al. 2005). Recently the role of connexin interacting proteins have been highlighted and, in particular zonula occludens-1 (ZO-1) in mediating the formation, extent and size of gap junctions (Hunter et al. 2005; Laing et al. 2005). In HeLa cells inhibition of Cx43 with ZO-1 lead to large extensive gap junctions and this effect was reversed when the capacity to interact was restored (Hunter et al. 2005). It has also been shown in our laboratory that in patients with congestive heart failure there was an increased interaction between Cx43 and ZO-1 associated with a reduction and decrease in size of Cx43 gap junctions (Bruce et al. 2008). These findings raise the potential role of ZO-1 as a mediator in gap junction remodelling in the heart.

From this background we set out to: (1) quantify the connexin expression in the HL-1 clones; (2) quantify the connexin co-expression (Cx40/Cx43) ratio and compare it with human atrial samples; (3) characterise the connexin expression/co-expression patterns of Cx40, Cx43 and Cx45; (3) assess if Cx40 and Cx43 do form heteromers; and (4) assess the level of co-localisation between ZO-1 and Cx40, Cx43 and Cx45.

4.2. Materials and methods

HL-1 clones were routinely cultured as described in Chapter 2, section 2. Connexin transcripts were detected by northern blot technique (Chapter 2, section 4). Expression of connexin protein was detected by western blot technique (Chapter 2, section 5). Connexin co-expression ratios were quantified according to methods described in Chapter 2, section 6. Connexin protein interactions were studied using co-immunoprecipitation (Chapter 2, section 7). The HL-1 clones were also characterised by immunofluorescence to study the distribution of Cx40, Cx43 and Cx45 and co-localisation of ZO-1 with Cx40, Cx43 and Cx45 (Chapter 2, section 8).

4.3. Results

4.3.1. Molecular and biochemical characterisation of connexin expression

Transcripts for Cx40, Cx43 and Cx45 (Figure 4.2 (A)) were present in the HL-1 clones. All the probes labelled a single mRNA band at the size expected. Different exposure times were required to obtain similar signal intensities and helped estimate the relative levels of transcript expressed in the HL-1 clones. The RNA samples were loaded with ethidium bromide to visualise the ribosomal RNA bands (18S and 28S) and verify the samples were loaded equally for quantification. All the HL-1 clones expressed Cx40, Cx43 and Cx45 at varying levels but retained the general characteristics of a cardiac myocyte (n =2). Although the data was not sufficient for a statistical analysis, in all the clones Cx40 transcript levels were higher compared with the adult mouse heart. This was expected since Cx40 in the mouse heart is only expressed in small amounts of the tissue including the atria and endothelial cells (Gros and Jongsma 1996) and we were comparing the whole heart tissue to the HL-1 clones. Cx43 and Cx45 were found at similar levels to those in the mouse heart. A summary of the connexin transcript levels is shown in Table 4.1. The mRNA expression levels were normalised to the mouse heart, which was taken as the control at 100%. To detect and quantify Cx40 and Cx43 expression in clone 2, membranes were exposed for a considerable length of time (Cx40: 24 hours; Cx43: 2 days).

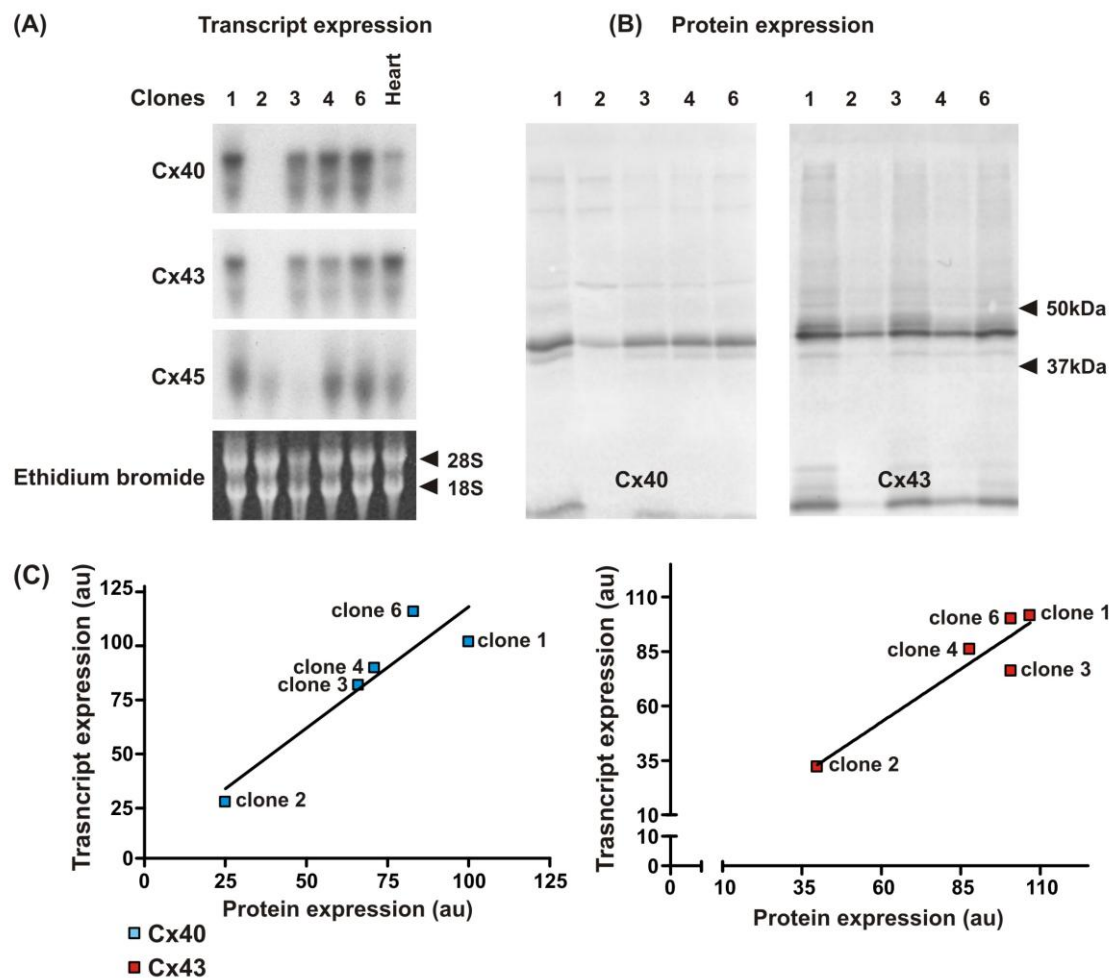


Figure 4.1: Transcript and protein analysis of connexin expression in the HL-1 clones

Northern analysis of connexin mRNA expression in the HL-1 clones and adult mouse heart (A). Membranes were hybridised with probes for Cx40, Cx43 and Cx45 as indicated on the left of the picture. The three connexins were expressed at varying levels by all the HL-1 clones. Membranes were stained with ethidium bromide to visualize the ribosomal RNA bands 18S and 28S. Western blot analysis of Cx40 and Cx43 (B). The Cx40 antibody detected a single band migrating at 40kDa expressed at similar levels in all the HL-1 clones except clone 2. Cx43 analysis obtained a single band migrating at 43kDa. As with Cx40, Cx43 was present at similar levels in all HL-1 clones with the exception of clone 2. The clone numbers for transcript analysis and protein expression are indicated at the top of the picture. There is a positive correlation of connexin transcript to protein expression for Cx40 and Cx43 (C).

Table 4.1: Summary of connexin transcript expression normalised to the mouse heart

Probe	Size of band	Exposure Time	1	2	3	4	6
Cx40	~ 3kB	4 hours	353 ± 14	46 ± 5	190 ± 43	286 ± 21	332 ± 18
Cx43	~ 3kB	24 hours	78 ± 2	18 ± 5	68 ± 12	69 ± 7	87 ± 3
Cx45	~ 2kB	5 days	123 ± 7	51 ± 5	7 ± 3	132 ± 2	145 ± 3

To assess whether connexin expression was regulated at the transcriptional or translational stage, Cx40 and Cx43 protein levels were measured and compared to the corresponding levels of mRNA transcribed in the HL-1 clones (Figure 4.2 (B)). Levels of Cx40 and Cx43 expression were similar in all the HL-1 clones with the exception of clone 2 where the signal was lower. Figure 4.2 (C) shows analysis of the relationship between Cx40 and Cx43 transcript and the corresponding protein expressed. The protein and mRNA expression levels were normalised to clone 1. There was a positive correlation for both Cx40 and Cx43 suggesting that the mRNA steady state levels determines the connexin protein level. Cx45 remained undetectable with our western blot protocol indicating that Cx45 expression levels were at least twenty times lower than Cx40 or Cx43.

4.3.2. Quantification of Cx40/Cx43 co-expression ratio in the junctional and non-junctional fractions

To determine the relative amount of Cx40 and Cx43 that was present within gap junction plaque (the 'junctional' fraction) and elsewhere in the cell (the 'non-junctional' fraction) differential solubilisation extraction using Triton X-100 was performed. The total, junctional and non-junctional samples were loaded for clones 2 and 6 and immunoblotted for Cx40 and Cx43 (Figure 4.2 (A)). The total sample was equivalent to the junctional and non-junctional samples mixed together in an equal volume. The sum of intensities for the junctional and non-junctional bands equalled the respective total samples. Densitometric analysis of the immunoblots revealed that approximately 60% of the total Cx40 expressed was present at the junction for clones 2 and 6 (clone 2: 55 ± 15%; clone 6: 60% ± 2%) and 40% in the non-junctional fraction (clone 2: 45 ± 15%; clone 6: 40% ± 3%) (Figure 4.2 (B)). These differences were not statistically significant ($p > 0.05$). For Cx43, the amount of connexin present in the junctional (clone 2: 53 ±

3%; clone 6: $50 \pm 13\%$) and non-junctional fractions was approximately 50:50 in both clones (clone 2: $47 \pm 15\%$; clone 6: $50 \pm 3\%$) (Figure 4.2 (C)).

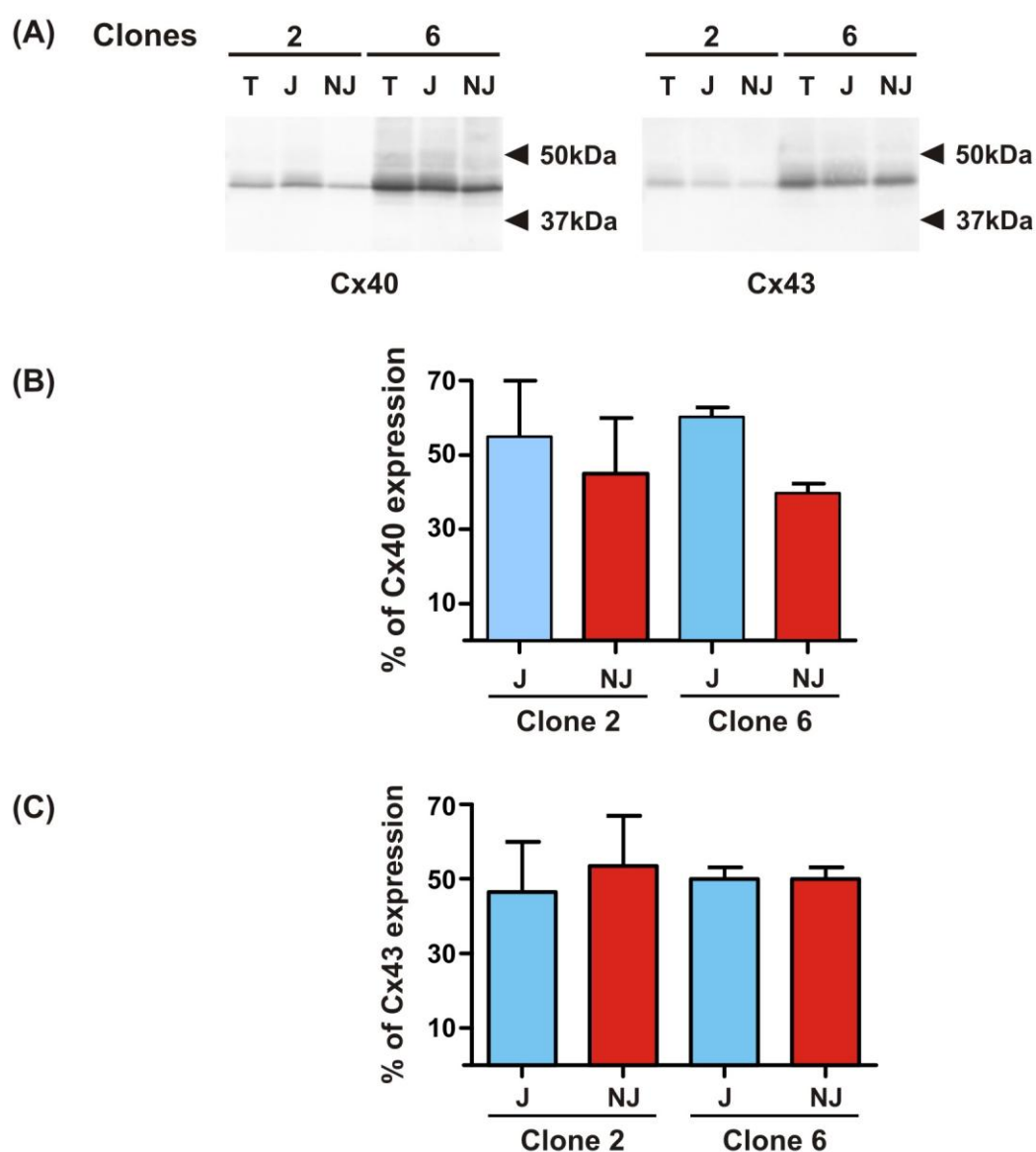


Figure 4.2: Quantification of Cx40 and Cx43 levels in the junctional and non-junctional fractions in clones 2 and 6

Differential solubilisation using Triton X-100 was used to separate the non-junctional (NJ) connexin from the junctional (J). All fractions were compared to the total (T) connexin expression. Immunoblots show analysis of clones 2 and 6 probed for Cx40 and Cx43 (A). Quantification of the junctional and non-junctional fractions indicated that a greater proportion of Cx40 was present in the junctional membrane in both the clones (B) but the differences were not statistically different ($p > 0.05$). For Cx43, there was approximately an equal amount of connexin present in both fractions (C).

4.3.3. Immunofluorescent analysis of connexin expression/co-expression patterns

The localisation of Cx45 (Figure 4.3) and co-localisation of Cx40 and Cx43 (Figure 4.4) was examined by immunofluorescent labelling and confocal microscopy. In all three clones immunostaining for Cx40, Cx43 or Cx45 gave labelling at the cell interface between neighbouring cells representing gap junctions. There was also some perinuclear distribution within the cytoplasm likely representing connexins within the biosynthetic pathway.

Cells from clone 2 appeared to form gap junctions with predominantly Cx45 (Figure 4.4 (A)). Labelling for Cx45 was homogenous but at low levels across the cell monolayer in all the clones with the exception of clone 3.

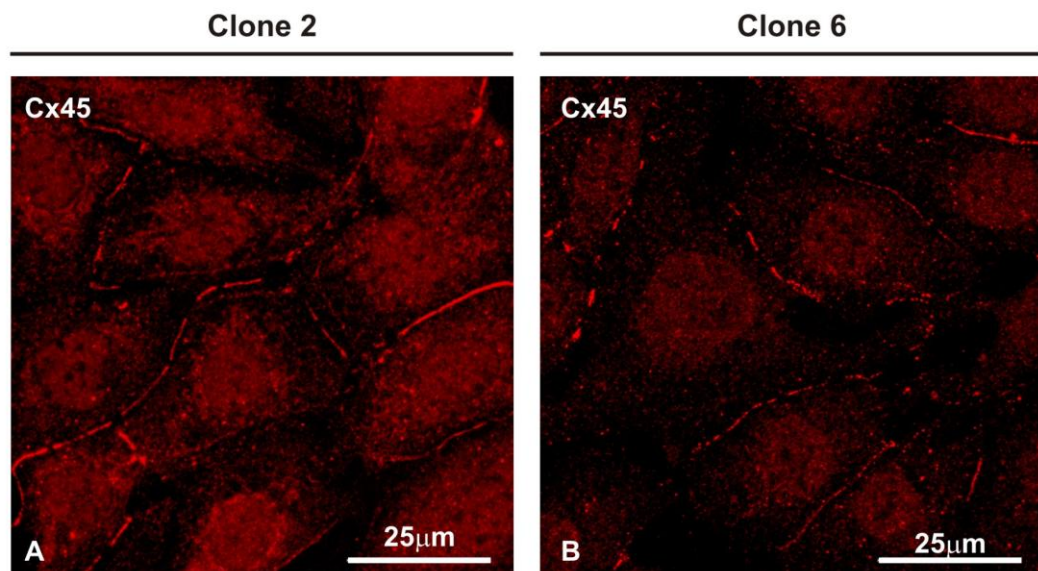


Figure 4.3: Immunofluorescent labelling of Cx45 in clones 2 and 6

In both clones 2 (A) and 6 (B), Cx45 was expressed homogeneously at low levels at the cell interface between neighbouring cells representing gap junctions.

The co-localisation of Cx40 and Cx43 was examined by double immunofluorescent labelling. Clone 2 expressed Cx40 and Cx43 at low levels with large areas of the culture giving no positive staining (Figure 4.4 (A)), confirming the data obtained by northern and western blot. In clones 3 (Figure 4.4 (B)) and 6 (Figure 4.4 (C)) expression for both Cx40 and Cx43 was heterogeneous in an interface-to-interface basis indicated by the different colours. For example the gap junctions of one interface were prominently labelled for Cx40 (red) while at a different interface of the very same cell, Cx43 (green)

predominated or a mixture of the two connexins (yellow). Although we can observe these distinctive patterns of different interfaces of the same cell, there was little variation within an individual interface where one cell abuts to another. Labelling was red, green or yellow but seldom more than one colour.

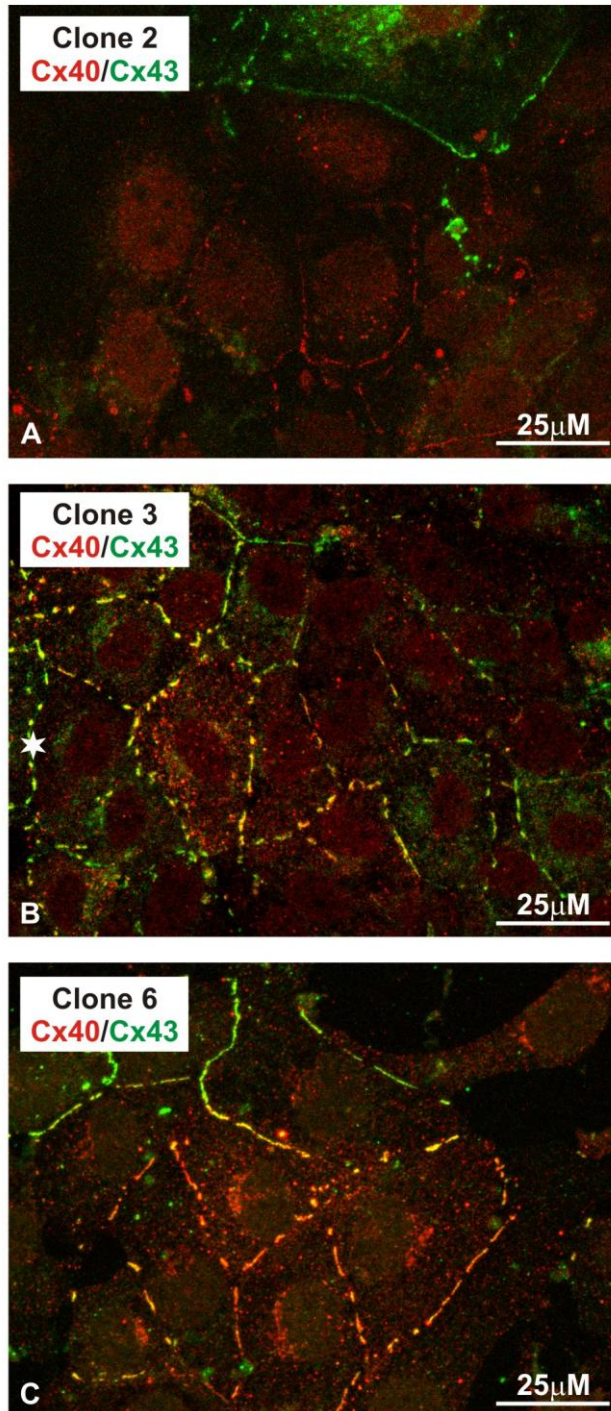


Figure 4.4: Immunofluorescence analysis of Cx40/Cx43 co-expression in the HL-1 clones

Images show double labelling for Cx40 (red) and Cx43 (green) in clones 2, 3 and 6. Labelling was weak in clone 2 since it expressed low levels of Cx40 and Cx43 (A). In clones 3 (B) and 6 (C) expression for both Cx40 and Cx43 was heterogeneous. A single cell may display very different distribution patterns on opposite interfaces. This is highlighted by the star in (B) where one interface displays a predominance for Cx43 (green) while at a different interface of the same cell there was a mixture of the two signals (yellow).

Triple labelling of Cx40, Cx43 and Cx45 in clone 6 was used to examine their relative co-localisation. The three connexins when visualised separately within the same area typically revealed similar labelling patterns (Figure 4.5 and Figure 4.6). The triple labelling image was generated by superimposing the image for each connexin subtype indicating that at specific cell interfaces all three connexins co-localise to form gap junctions, but the relative amount of signal for each connexin varied substantially from one cell interface to the next (Figure 4.5 and Figure 4.6, panel (A)).

To demonstrate the varying degrees of co-localisation three different interfaces from the triple labelling were separated to allow simultaneous visualisation of Cx40 and Cx43, Cx40 and Cx45 and Cx43 and Cx45 (Figure 4.5 and Figure 4.6, panel (B)). Labelling for Cx40 and Cx43 gave a single colour per interface within a given cell with some interfaces predominantly showing Cx40 label while others largely showed labelling for Cx43 or a mixture of the two confirming the co-localisation pattern obtained earlier. Labelling for Cx40 and Cx45 showed isolated spots of Cx40 only (blue), Cx45 only (red) and a mixture of the two (purple) at different cell interfaces and within an individual interface. A similar pattern was also observed for Cx43 (green) and Cx45 (red) with some areas of overlap of the two signals (yellow). In contrast to the Cx40 and Cx43 labelling no major differences in the labelling patterns at the different cellular interfaces were apparent. In certain areas of the cell monolayer all three connexins co-localised within the same gap junction plaque to generate a white colour.

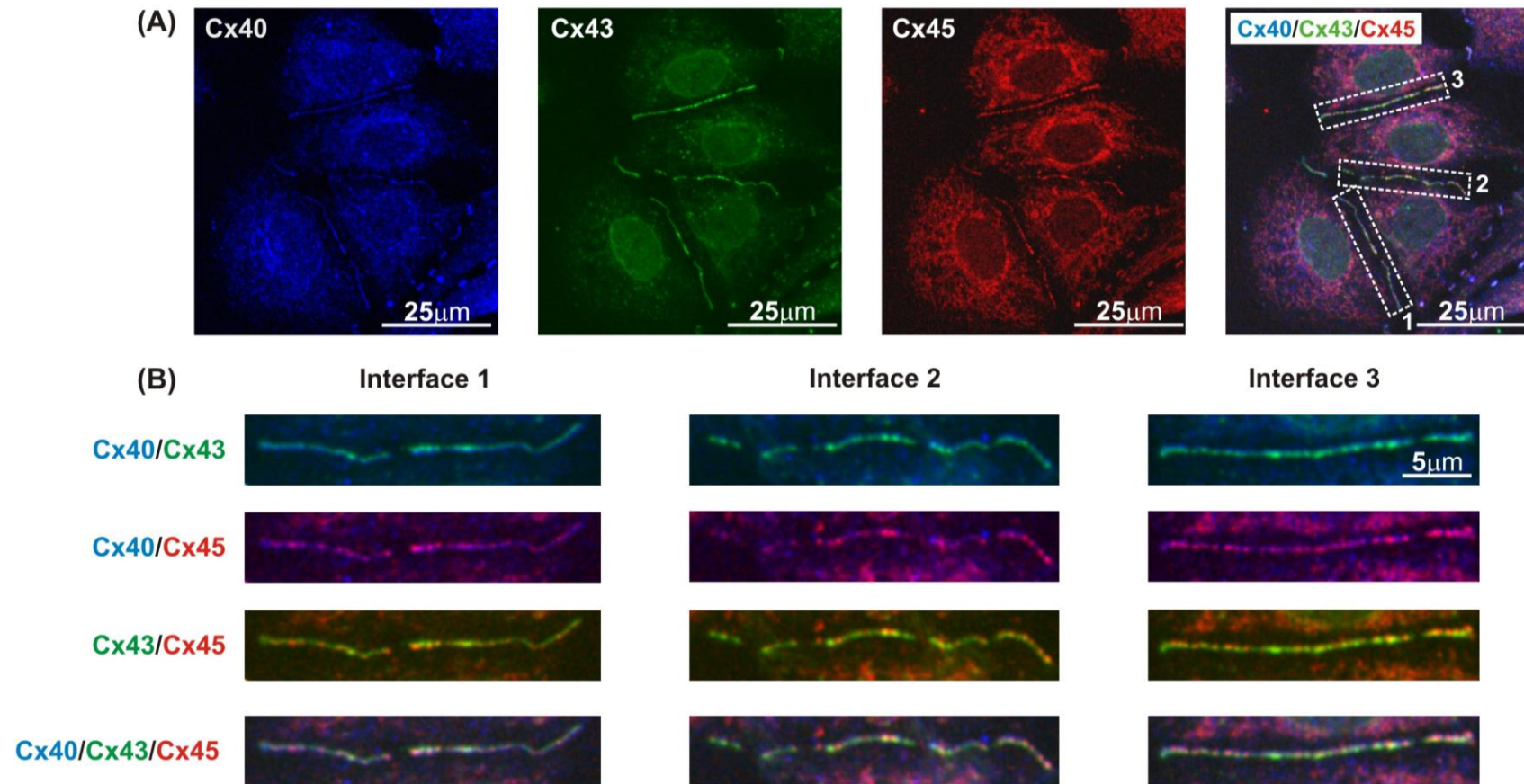


Figure 4.5: Co-localisation of Cx40, Cx43 and Cx45 in clone 6 to illustrate the spatial distribution patterns of all three connexins (Example 1)

Each connexin can be seen in the individual channels within the same area and the triple labelling image was generated by superimposing the image for each connexin subtype (A). To demonstrate the varying degrees of co-localisation at each cell interface, sections 1, 2 and 3 highlighted from triple labelling were separated into dual images of Cx40/Cx43, Cx40/Cx45, Cx43/Cx45 and triple image of Cx40/Cx43/Cx45 as indicated on the left of the image (B).

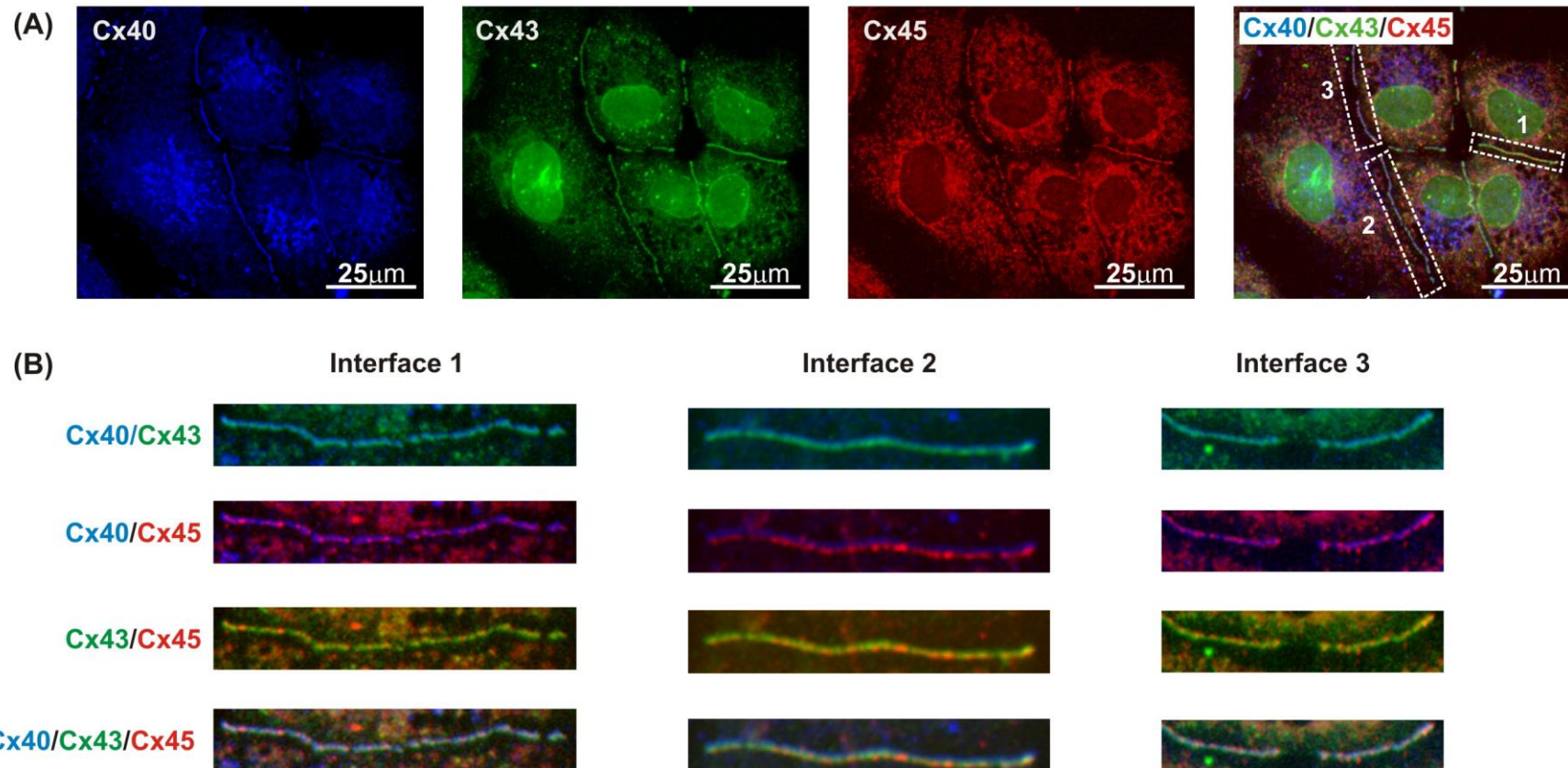


Figure 4.6: Co-localisation of Cx40, Cx43 and Cx45 in clone 6 to illustrate the spatial distribution patterns of all three connexins (Example 2)

Another example of triple labelling from a different group of cells to confirm the various colours per cell interface observed when signals for Cx40, Cx43 and Cx45 are superimposed (A). Sections 1, 2 and 3 indicated by the white boxes from triple labelling were separated into Cx40/Cx43, Cx40/Cx45, Cx43/Cx45 and Cx40/Cx43/Cx45 images indicated on the left of the image (B).

4.3.4. Cx40 and Cx43 form heteromers in clone 6

To assess the levels of heteromers formed, cells from clone 6 were extracted into their junctional and non-junctional fractions. The fractions were subjected to immunoprecipitation with a Cx40 antibody (Figure 4.7). Samples containing the total amount of junctional and non-junctional fractions together with their respective bound (sample bound to the connexin antibody that is specifically adsorbed on the beads) and unbound (sample not bound to the connexin antibody) fractions were loaded for western blot analysis. When the Cx40 antibody was immobilised on the beads we found that approximately $19 \pm 1.0\%$ of the junctional fraction and $7 \pm 1.0\%$ of the non-junctional fraction formed heteromers with Cx43 Figure 4.7 (A) and (B)). These differences were statistically different ($n = 3$; $p < 0.001$).

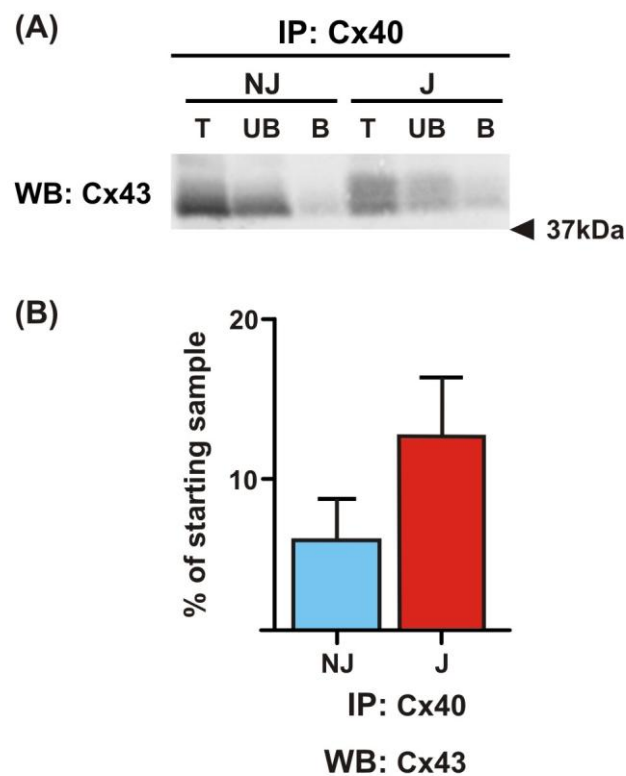


Figure 4.7: Cx40 and Cx43 form heteromers in clone 6

The total (T), junctional (J) and non-junctional (NJ) fractions were subjected to immunoprecipitation (IP) with a Cx40 antibody (A). The bound (B) and unbound (UB) samples from the non-junctional and junctional fraction were loaded for western blot (WB) analysis. Detectable levels of Cx40 interacting with Cx43 were observed in both the non-junctional and junctional fractions. Bar chart depicting the level of Cx40 and Cx43 interaction in the junction ($19\% \pm 1.0$) and non-junction ($7\% \pm 1.0$) is illustrated in (B) ($n = 3$; $p < 0.001$).

4.3.5. Connexin interaction with ZO-1 protein

Co-localisation of ZO-1 with Cx40, Cx43 and Cx45 can be visualised using immunoconfocal microscopy in clone 6 as shown in Figure 4.8. ZO-1 was expressed at high levels at the plasma membrane and was localised exclusively at inter-cellular contacts. ZO-1 showed varying degrees of co-localisation with Cx40 (A), Cx43 (B) and Cx45 (C) to give a yellow colour when the two signals overlapped. Isolated spots of ZO-1, Cx40, Cx43 or Cx45 signals were observed highlighted by the enlarged images in Figure 4.8 (D), (E) and (F).

In certain areas, closer inspection of the ZO-1 co-labelling with connexin indicated that ZO-1 was located at the edge of the gap junction plaque forming a 'belt' along the gap junctions (Figure 4.9). This observation is in keeping with the proposed role of this protein in accretion of connexons to the plaque thereby regulating gap junction size (Hunter et al. 2005). 'Holes' in the labelled junction may represent areas in which patches of gap junction membrane have been internalised for degradation (Falk et al. 2009).

Although the immunofluorescence images demonstrated varying degrees of co-localisation of ZO-1 with Cx40 and Cx43, this did not demonstrate an interaction. To determine the extent of any interaction between the proteins co-immunoprecipitation experiments were carried out in clone 4 (Figure 4.10). Clone 4 was used in this figure to illustrate that although all the clones have different expression profiles regarding the ion channels and connexins, they still retain the characteristics of a myocyte cell line. A ZO-1 antibody was used to precipitate ZO-1 from the junctional and non-junctional fractions and any interaction with Cx40 and Cx43 were detected by western blot analysis (Figure 4.10). Cx40 and Cx43 were also detected in the bound and unbound samples. In the bound fraction, Cx40 and Cx43 interacted with ZO-1 in both the junctional and non-junctional fractions. However this interaction was significantly lower in the junction in comparison to the non-junctional sample. In general there also appears to be a greater level of interaction of ZO-1 with Cx40 in contrast to Cx43.

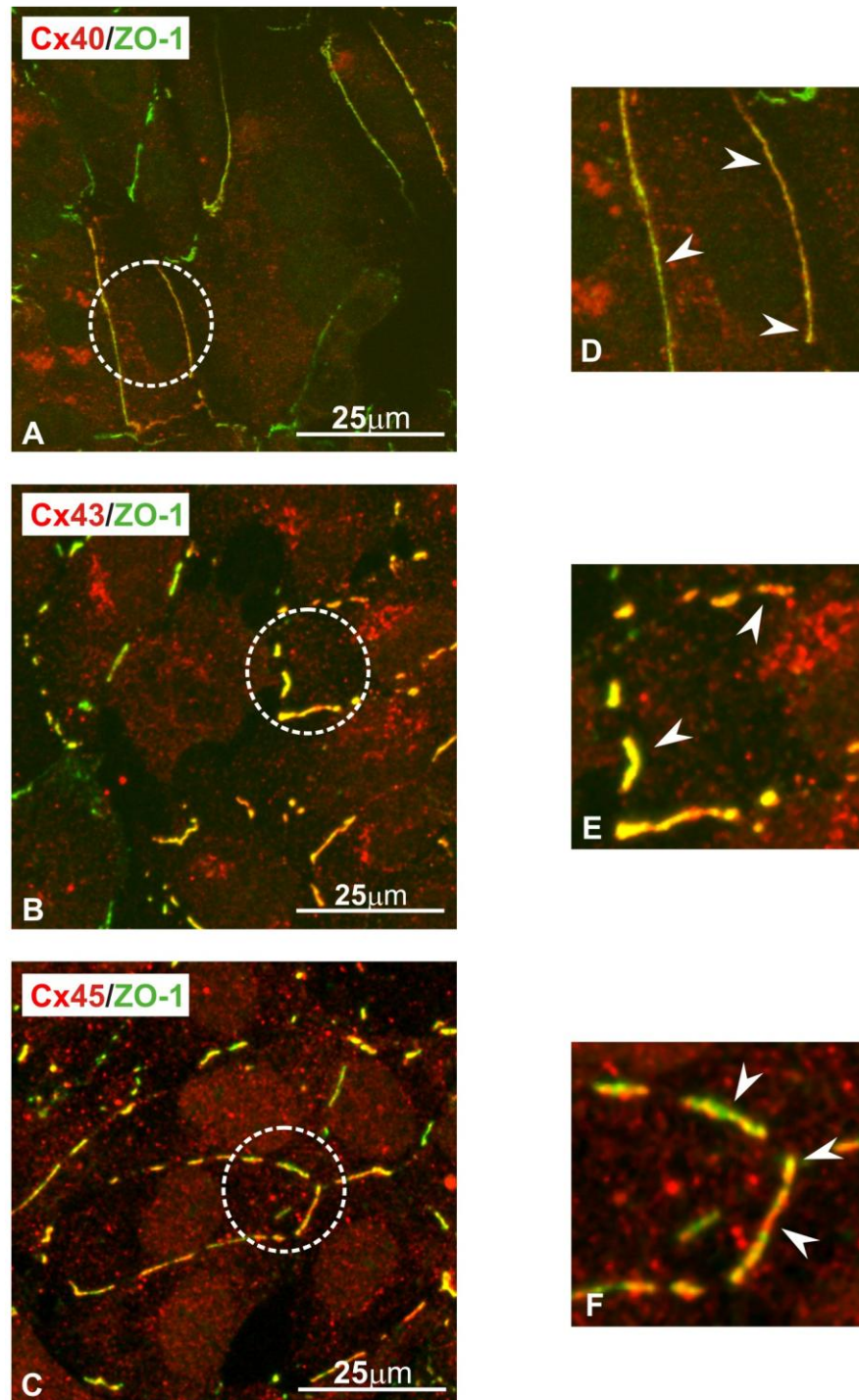


Figure 4.8: Co-localisation of ZO-1 with Cx40, Cx43 and Cx45 in clone 6

ZO-1 is expressed exclusively at the cell interface and only when there is cell-to-cell contact. Double labelling of ZO-1 with Cx40 (A) and Cx43 (B) and Cx45 (C) showed varying degrees of co-localisation. Images (D), (E) and (F) represent enlarged views of ZO-1 interaction with Cx40, Cx43 and Cx45 respectively. At a specific cell interface it can be observed that there are certain areas of connexin (red) or ZO-1 only (green) and fraction where each signal overlaps (yellow) indicating co-localisation. These areas as highlighted by the arrows.

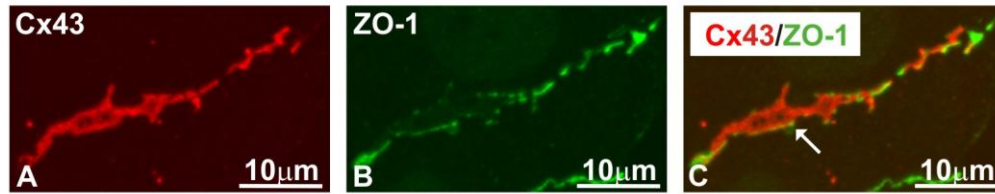


Figure 4.9: Localisation of ZO-1 at the edges of the plaque

Cells were co-labelled with Cx43 (A) and ZO-1 (B). When the individual signals were superimposed (C) ZO-1 appeared to be restricted to the edges of the plaque (indicated by the arrow) with Cx43 expressed in the centre of the plaque.

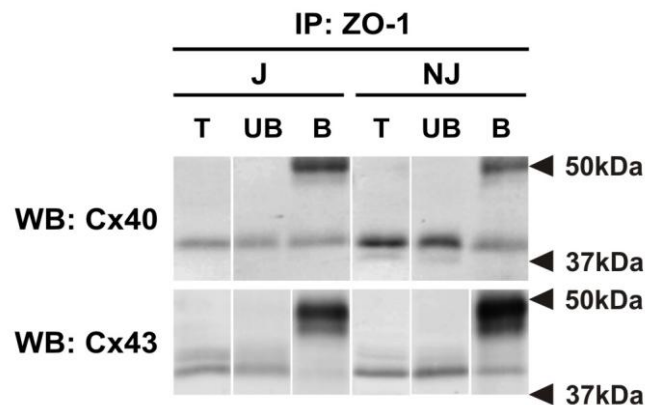


Figure 4.10: Co-immunoprecipitation of ZO-1 with Cx40 and Cx43 in clone 4

Proteins were extracted into their junctional (J) and non-junctional (NJ) fractions in clone 6. Both the fractions were subjected to co-immunoprecipitation with the ZO-1 antibody and detected for Cx40 (top immunoreplica) and Cx43 (bottom immunoreplica). Cx40 and Cx43 were tested in the total (T), unbound (UB) and bound (B) samples in both the junctional and non-junctional fractions. There were detectable levels of Cx40 interacting with ZO-1 in both fractions. However for Cx43, interaction with ZO-1 was greater in the non-junctional sample compared with the junctional fraction. Remnants of the IgG heavy chain from the antibody used for co-immunoprecipitation are visible in the bound fractions, but are readily distinguishable from Cx43 signal, which is the smaller of the two bands. Image was kindly gifted by Dr K. Grikscheit.

4.4. Discussion

Differential patterns of connexin expression is thought to confer distinctive electrical properties and play a fundamental role in the orderly spread of current flow (Severs et al. 2008). Thus it was important to distinguish the connexin expression/co-expression patterns in the HL-1 clones before their role in action potential propagation could be studied. The data presented in this chapter characterised the HL-1 clones in terms of: (1)

connexin expression (mRNA and protein levels); (2) connexin co-expression patterns; (3) connexin heteromerisation and (4) connexin interaction with ZO-1. This information can then be utilised to exploit each clone; their individual properties making them suitable for different experiments.

Northern and western analysis confirmed that the HL-1 clones expressed a combination of Cx40 and Cx43 with the exception of clone 2. Only cells from clone 2 displayed major differences with the other clones and adult mouse heart with considerably lower levels of Cx40 and Cx43 mRNA. The differences in Cx40 expression between the HL-1 clones and the mouse heart can be accounted for the fact that Cx40 only represents a small proportion of the total connexin expression *in vivo* where Cx43 is the predominant connexin. Cx40 expression is restricted to the atria, bundles within the conduction system and endothelial cells whereas in the working ventricular muscle it is almost completely absent (Duffy et al. 2006; Severs et al. 2008). The connexin expression pattern was very similar to that observed in normal mouse atria indicating a relatively normal connexin expression in HL-1 derived atrial myocytes (Coppen et al. 1998a). Cx45 could only be detected by northern blot. Based on the exposure times required to obtain signal intensities similar to that of Cx40 and Cx43, levels of Cx45 messenger were considerably lower in all the HL-1 clones as reported *in vivo* (Vozzi et al. 1999). From the quantification system developed in our laboratory that uses tagged HeLa transfectants with known amounts of connexin (Severs et al. 2006; Thomas 2007) we estimated that Cx45 protein was expressed at least twenty times lower than Cx40 and Cx43. Quantitative immunofluorescence could be performed to measure Cx45 expression in each of the clones. In this study there were an insufficient number of images recorded for a complete quantification and statistical analysis.

We found a positive correlation between the level of mRNA and the corresponding protein levels suggesting that connexin expression was predominantly regulated at the transcriptional level similarly to what has been reported in the human myocardium (Vozzi et al. 1999; Dupont et al. 2001a). Connexin gene expression is regulated by a variety of factors. These include (1) transcription factors TATA box-binding protein, specificity protein 1/3 (Sp1/Sp3) and activator protein-1 (AP-1) expressed to regulate basal expression and possibly modulate large changes in expression; (2) chemical and biological substances such as cAMP, phorbol esters and retinoids and (3) cell-type

specific transcription factors (e.g. Nkx2.5) which bind to connexin gene promoters (Beyer and Berthoud 2009; Oyamada et al. 2005). However further experiments will be required to deduce the exact mechanism involved in connexin synthesis.

Further western blot analysis of the junctional and non-junctional connexins provided data on the relative amounts of Cx40 and Cx43 docked as channels compared with those in intracellular trafficking pathways and as single connexons present in the plasma membrane. For Cx40 there was a higher proportion present in the junctional fraction than the non-junctional fraction. By contrast Cx43 was expressed at an equal amount in both fractions. There also appears to be some phosphorylation of Cx43 at the junctional fraction. However to substantiate the amount of phosphorylation, phosphatase inhibitors will need to be used and the samples run on higher resolute western blot gels containing 8-10% acrylamide (Musil et al. 1990). There are also a range of antibodies that are available targeting specific residues only when phosphorylated (e.g. S368) (Solan et al. 2003).

Immunoconfocal visualisation of Cx40, Cx43 and Cx45 provided us with an overall distribution pattern. Single labelling demonstrated that Cx40 and Cx43 were heterogeneously expressed at high levels within the gap junction plaque (data not shown) with Cx45 homogeneously present at low levels throughout the monolayer. Multiple labelling was performed to study the spatial relationship between the distributions of connexins relative to each other. From the double and triple imaging, a clear difference was observed in the distribution of each connexin at individual cell-to-cell interfaces. The labelling pattern for Cx40 with Cx43 showed that a large proportion of cells displayed an individual colour per interface: either high Cx40, high Cx43 or a mixture of the two. This suggests that in those areas the amount of connexin present in the junctions and/or the make-up of connexons are near identical between the two cells. Similar observations were found in the RLE cell line transfected to co-express Cx40 (Halliday et al. 2003; Thomas 2007) (Figure 4.11 (A)), N2a cells transfected with Cx40 and Cx43 (Lin et al. 2010) and A7r5 which naturally co-express Cx40 and Cx43 (Cottrell et al. 2002). This suggests that irrelevant of the cell type these labelling patterns observed are dependent on the connexins present at the membrane. Cx40 and Cx43 are frequently co-localised in cells of the cardiovascular system (Gros et al. 1994; Vozzi et al. 1999; Yeh et al. 2000) and are present within the same gap junction plaque

(Kanagaratnam et al. 2002; Severs et al. 2005). Inspection of the human Purkinje fibres, which co-express CX40 with CX43 at the intercalated disk commonly demonstrated a predominance of label for one connexin subtype at one interface and for the other connexin subtype at another interface confirming the labelling pattern observed in the HL-1 clones was a true representation of cardiac tissue *in vivo* (Figure 4.11 (B)).

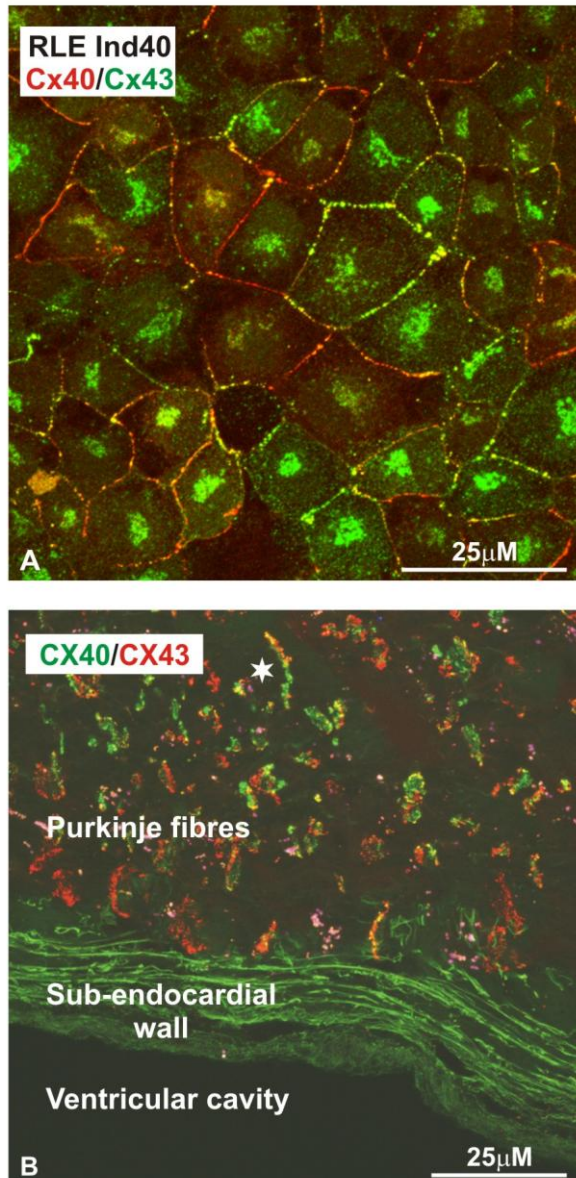


Figure 4.11: Immunofluorescence analysis of Cx40/ Cx43 co-expression in two different cell models

Double labelling with anti-Cx40 and anti-Cx43 in the RLE cell line that endogenously express Cx43 transfected to co-express Cx40 (RLE Ind40) (A) and human Purkinje fibres (B) that co-express Cx40 with Cx43. Similar to the labelling pattern observed in clones 3 and 6, connexin localisation was heterogeneous. In both images an individual cell interface may demonstrate different labelling patterns indicating a predominance for one connexin subtype at one interface and for the other connexin subtype at another interface. Images were kindly gifted by Dr N. Thomas (A) and Dr R. Kaba (B).

The single colour per interface was not observed for Cx40 with Cx45. We found isolated spots of Cx40 only, Cx45 only or an overlap of the two connexins throughout the monolayer. Studies on Cx40 and Cx45 mRNA levels showed that levels of both

Cx40 and Cx45 messenger followed a similar trend and variation between the four chambers indicating the two connexins may be spatially associated at the protein level (Vozzi et al. 1999) . In the rat conduction system the common bundle was shown to express Cx40 and Cx45 in a similar pattern to clone 6 (Severs et al. 2001). Labelling for Cx43 with Cx45 also revealed pattern of co-localisation comparable to that of Cx40 and Cx45. Studies using the RLE Ind45 cell line (Thomas 2007) and rabbit sinoatrial nodal cells (Honjo et al. 2002) reported a mixture of labelling with signals for Cx43 only, Cx45 only and an overlapping of the two connexins corroborating our observations in the HL-1 clones. When the three connexins were superimposed a clear difference was observed in the distribution of each connexin at the individual gap junction and cell-to-cell interface indicated by the resulting colours. There were specific areas where all three connexins were present in the same gap junction producing a white signal similar to what has been observed at the intercalated disks in the human atrial myocardium and Purkinje fibres (Vozzi et al. 1999; van Veen et al. 2001).

The varying degrees of co-localisation between the three connexins indicate a variety of structural arrangements of connexins may be present at the individual gap junction channel (e.g. heterotypic and heteromeric) although not all connexin combinations are compatible. Numerous investigations on *Xenopus* oocytes and HeLa transfectants have reported an absence of measurable gap junction currents between cells expressing Cx40 and Cx43 in a heterotypic fashion (Bruzzone et al. 1993; White et al. 1995; Haubrich et al. 1996; Gu et al. 2000). This conclusion was supported by Lucifer yellow dye transfer studies showing there was no dye transfer between channels formed by Cx40 and Cx43 (Elfgang et al. 1995) and in co-cultures in which no immunofluorescent signals were detected on apposed membranes (Haubrich et al. 1996; Rackauskas et al. 2007a). Haubrich et al (1996) further demonstrated an inherent structural basis to connexin incompatibility that implicated both extracellular loops, preventing the docking of mismatched connexons. Work in our laboratory also demonstrated that cells co-cultured with the RLE wild type (Cx43 homomeric connexons) and RLE Ind40 (Cx40 homomeric, Cx43 homomeric and Cx40/Cx43 heteromeric connexons), no label for Cx40 was detected at any interface between the two cell types indicating an absence of heterotypic channels (Thomas 2007). However studies performed in HeLa and RIN cell transfectants reported the presence of heterotypic channels on cell pairs with a significantly reduced gap-junctional conductance which could be an indication of

reduced channel function and the inability of Cx40 and Cx43 to dock (Valiunas et al. 2000; 2001; Cottrell and Burt 2001). It is also possible that the low levels of coupling could be accounted for by the presence of endogenous Cx45 interacting with Cx40 and Cx43 as found by Rackauskas et al. (2007a), which would also explain the differences of asymmetry in voltage dependence (Moreno 2004; Elenes et al. 2001). Since these studies by Valiunas et al. (2000) and Cottrell et al. (2001) were performed on cell pairs, and our experimental model was based on a cell monolayer, the assembly of Cx40 with Cx43 homomeric-heterotypic channels may be rarer in multicellular cultures because highly compatible connexons have an increased chance to form gap junctions and are less likely to form low compatibility channels such as Cx40 with Cx43.

Connexons made from Cx45 are able to dock with connexons made up of either Cx43 or Cx40 to form functional channels (Elfgang et al. 1995; Desplantez et al. 2004; Rackauskas et al. 2007a; Thomas 2007). Thus the presence of Cx45 makes it possible for a variety of different channel configurations to be formed. White et al. (1994) demonstrated that heterotypic channel formation is a selective property dependent on the expression of compatible connexins in the adjacent cells. In the lens Cx50 does not form heterotypic channels with Cx43, while Cx46 is compatible with both Cx43 and Cx50. In our cell line Cx45 could play a similar role in compatibility facilitating the functional linkage of gap junction channels in zones that express otherwise incompatible connexins. As a result if a large proportion of homomers are formed, then the known low compatibility between Cx40 and Cx43 (Elfgang et al. 1995; White and Bruzzone 1996) would also produce this specific connexin distribution. The connexon content of each interface would depend on the level of expression of individual connexins by adjacent cells modified by the docking of compatible connexons at other cell-to-cell interfaces.

Alternatively results from the triple labelling could be due to a large proportion of heteromers produced containing channels composed of different ratios of Cx40, Cx43 and Cx45. Co-immunoprecipitation experiments in clone 6 showed that the level of heteromers produced were considerably lower than theoretical models predicted assuming the connexon subtypes are free to associate in a random fashion (Brink et al. 1997). Heteromerisation between Cx40 and Cx43 have been reported in several studies using affinity chromatography on a Nickel-nitrolacetate matrix (Ni^{2+} -NTA) (He et al.

1999; Beyer et al. 2001; Valiunas et al. 2001). However one caveat of these studies is that analysis was only performed in the Triton X-100 fraction, which contains the non-junctional connexins only. There is not only an assumption that the level of heteromers formed in the synthetic pathways is similar to that in the plasma membrane but this procedure is not quantitative both of which we were able to overcome in our laboratory. We found that Cx40 and Cx43 heteromerise in both the junctional (19%) and non-junctional (6%) fractions indicating that hetero-oligomerisation begins during the assembly of connexons in the *trans*-Golgi network before its incorporation into the gap junction plaque (Musil and Goodenough 1993; Laird 2006). The lower levels of Cx40 and Cx43 interaction in the non-junctional fraction could be explained by presence of connexin monomers prior to oligomerisation in the ER-Golgi compartments.

Due to the low levels of Cx45 expressed in the HL-1 clones we were unable to detect any interaction with Cx40 or Cx43. However using the RLE Ind45 cell line no heteromers were observed between Cx45 and Cx43 indicating that detection of an interaction in cells where only low amounts are expressed is unlikely to be feasible (Grikscheit 2010). This data is quite different from studies performed by Martinez et al. (2002a) who showed co-purification of Cx45 with V5-(His)6-tagged Cx43 on a Ni²⁺-NTA matrix. Differences in these results and could be due to technical factors or to cell type specific heteromerisation

Work on connexin hetero-oligomerisation report that this process may be an intrinsic process specific to each connexon type. For example the ‘selectivity’ signal that regulates connexin compatibility might be located in the amino terminal portion and differ between the connexin subtypes thereby enabling or preventing hetero-oligomerisation (Lagree et al. 2003; Segretain and Falk 2004; Koval 2006). The segregation of connexins within a gap junction channel could also be dependent on the different affinities each connexin has for surrounding lipids or connexin-binding proteins (e.g. ZO-1) (Segretain and Falk 2004). Work by Das et al. (2001) demonstrated that the ability to form heteromeric channels is also dependent on the context of cell expression. Alveolar epithelial cells express Cx43 and Cx46 however only type I cells are able to form heteromers compared with type II in which Cx46 is retained in the *trans*-Golgi network preventing interactions with Cx43. Quantification of the level of heteromerisation in both the junctional and non-junctional fractions in clone 6 was also

consistent with what has been found in the human atria and the RLE Ind40 cell line (Grikscheit 2010). This suggests that heteromereisation is an intrinsic property of the connexins expressed and not the cell or tissue type.

In recent years the major focus regarding connexin interacting proteins has been on ZO-1 and its involvement in regulating the size and cellular distribution of gap junctions in the healthy and diseased myocardium (Hunter et al. 2005; Laing et al. 2007; Bruce et al. 2008). In the HL-1 clones ZO-1 expression was confined to areas of inter-cellular contact only. A similar expression pattern was found *in vivo* where the distribution of ZO-1 was only present at the intercalated disk and preferentially associated at the edges of gap junctions (Barker et al. 2002; Bruce et al. 2008). Using double labelling we observed some degree of co-localisation between ZO-1 with either Cx40, Cx43 and Cx45 similar to what has been reported in the human ventricle (Bruce et al. 2008) and rat myocytes (Barker et al. 2002). In specific regions we found that ZO-1 did not directly overlap with the plaque interior but was associated at the perimeter in keeping with studies by Hunter et al. (2005) on HeLa and neonatal cardiac myocytes. These specialised microdomains within the plaque are likely to represent gap junction recruitment sites since work by Gaietta et al. (2002) and Lauf et al. (2002) demonstrated that Cx43 insertion into the plasma membrane occurred at the periphery of gap junction plaques. We also found areas of ZO-1 not co-localised with Cx40, Cx43 or Cx45. In these areas ZO-1 is most likely to be complexed with another type of cell junction such as adhesion junctions; e.g. N-cadherin (Barker et al. 2002). Molecular ‘cross-talk’ between different junction types has been proposed between mechanical and electrical junctions at the intercalated disk in the formation and stabilisation of gap junctions (Rohr 2007). For example the deletion of N-cadherin resulted in the destabilisation of adhesion junctions at the intercalated disk and a decrease in the levels of Cx43 (Hertig et al. 1996; Kostetskii et al. 2005).

Co-immunoprecipitation studies confirmed the interaction of ZO-1 with Cx40 and Cx43 in both the junctional and non-junctional fraction as found in the healthy and diseased heart (Bruce et al. 2008). These interactions are most likely through the C-terminus of the connexin motif and the PDZ domain of ZO-1 (Laing et al. 2005; Herve et al. 2007). ZO-1 interaction with Cx40, Cx43 and Cx45 through the PDZ binding domain has been demonstrated in a variety of cell models including porcine vascular endothelial cells

(Nagasawa et al. 2006), osteoblastic cells (Laing et al. 2001) and adult rat ventricular myocytes (Barker et al. 2002). Interactions in the non-junctional fraction include single connexons at the plasma membrane and may reflect a possible role of ZO-1 in gap junction turnover and compartmentalisation of connexins within the plasma membrane (Laing et al. 2007). For example the expression of ZO-1 at the edge of gap junction could promote the accretion of non-junctional connexons into the plaques (Hunter et al. 2005; Laing et al. 2005) and the internalisation of connexons from the plaque via the formation of annular gap junctions (Bruce et al. 2008). Although we have not confirmed the exact role of ZO-1 in the HL-1 clones we can speculate that by controlling the rate of turnover, ZO-1 could potentially influence the level of cell-to-cell communication in these cells.

In summary, the HL-1 clones expressed Cx40, Cx43 with very low levels of Cx45. All three connexins co-localised at varying levels resulting in the different colours produced by immunofluorescence. This heterogeneity is possibly due to (1) incompatibility of connexons composed Cx40 and Cx43 and (2) ability of Cx45 to freely dock with Cx40 or Cx43 to form functional channels. Co-immunoprecipitation experiments showed that levels of heteromerisation were much lower than would be predicted from random association. ZO-1 interaction with Cx40 and Cx43 was detected by immunoprecipitation indicating a potential role for this protein in gap junction assembly and function in the HL-1 cells. Collectively these are all influential factors in impulse propagation, which will be studied, in the next chapter.

CHAPTER 5.

The study of action potential propagation in the HL-1 clones

5.1. Introduction

Cardiac excitation involves action potential generation by individual cells and its conduction from cell-to-cell through gap junctions (Kleber and Rudy 2004). For a single cell to reach the threshold of activation and serve as a source the liminal length requirements must be fulfilled resulting in a re-generative propagating action potential; otherwise only local electrotonic effects occur. The liminal length is defined as the length of tissue required to be raised above threshold so that the inward depolarising current from that region is greater than the repolarising influences of adjacent tissue (Fozzard and Arnsdorf 1991).

Cellular parameters that govern action potential propagation are: (1) *excitable membrane properties* defined by distribution, density and type of voltage-gated ion channels participating in the action potential; (2) *intracellular longitudinal resistance* that affects the spread and charge of depolarising current in tissue is determined by the cytoplasmic and gap junction resistance; (3) *cell membrane capacitance* and (4) *extracellular longitudinal resistance* (Kleber and Rudy 2004; Spach et al. 2004; Desplantez et al. 2007). A modification of any of these elements is predicted to alter conduction velocity (CV) in the heart. For example reducing the availability of sodium (Na^+) channels is accompanied by a decrease in the safety factor (SF) and drop in CV by a third followed by conduction block (Shaw and Rudy 1997; Wang and Rudy 2000; Kucera et al. 2001). In order to study action potential propagation, microelectrode arrays (MEA) in combination with optical mapping will be used to study the HL-1 clones.

A MEA consists of a matrix arrangement of 60 electrodes that allows the recordings of extracellular field potentials across a cell monolayer. The signal recorded by the MEA reflects changes of membrane potential caused by the flow of ions through the voltage-gated ion channels crossing the cell membrane by local depolarisation (Yamamoto et al. 1998; Stett et al. 2003; Hescheler et al. 2004). Thus any difference in transmembrane potential between cells would cause a current to flow through the cell interior across the membrane giving rise to a voltage gradient in the extracellular environment. The size of the voltage gradient is influenced by the amount of current produced by the neighbouring cells and the distance the signal is from the electrode. This technique

enables non-invasive recordings of field potential in any electrogenic tissue including neuronal (Baruchi et al. 2008; Brewer et al. 2009) and cardiac (Halbach et al. 2006; Fahrenbach et al. 2007) cell preparations.

In contrast to MEA, optical mapping measures the differences in transmembrane potentials in excitable cells. The changes in membrane potential are detected using voltage-sensitive dyes (e.g. di-8- butyl-amino-naphthyl-ethenyl-pyridinium-propyl-sulfonate (ANNEPS)) that insert into the external lipid bilayer of the cell. At the cell membrane the dye reacts to local changes in transmembrane potential with a shift in their optical properties. These changes are then recorded using appropriate detectors. The technique has a wide range of applications particularly in studying source-and-sink mismatches (Rohr et al. 1997) and the dependence of impulse propagation on tissue geometry (Fast and Kléber 1993; Rohr and Salzberg 1994; Fast et al. 1996). It not only provides a high temporal resolution necessary to detect the rapid change in membrane potential during the action potential upstroke but it is also able to provide better spatial resolution enabling the investigation of finer details of wave propagation (Morley and Vaidya 2001). This part of the study was done in collaboration with Dr M. Miragoli (Cardiovascular Science, NHLI).

MEA and optical mapping provide efficient tools to study the propagation of the wavefront giving detailed descriptions of: (1) origin and spread of electrical activity; (2) CV; (3) curvature and homogeneity of the propagated wavefront and (4) electrical activity across the entire cell monolayer, an advantage over electrophysiological techniques such as patch-clamp which only work on a single cell at a time. In addition there are advantages unique to each technique; MEA enables the recordings of electrical activity on the same preparation over a length of time whereas optical mapping allows the action potential morphology and duration to be monitored from several sites at a high resolution across the monolayer. All these aspects on impulse are presented and discussed in detail in this chapter.

5.2. Materials and methods

HL-1 clones were routinely cultured as described in Chapter 2, section 2. Effects of cell density on connexin protein expression were detected by western blot technique (Chapter 2, section 5).

5.2.1. Measurement of impulse propagation

Cell preparation

The seeding procedure used was the same for MEA and optical mapping. Once cells reached full confluency, they were trypsinised according to methods described in Chapter 2, section 2. After centrifugation the cell pellet was re-suspended in Claycomb medium using a volume equivalent to 10-20 μ l per 1cm² of cells. The MEA and 35mm glass bottom dishes were coated with fibronectin (10mg/ml) in water. 10-20 μ l of the cell suspension was seeded as a drop directly on the electrodes of the MEA. For optical mapping 10-20 μ l of the cell suspension was seeded as a drop on 35mm glass bottom dishes (MatTek Corporation). Cells were left to attach for 20-30 minutes with incubation in an atmosphere of 5% CO₂ at 37°C. After cell seeding the chambers were flooded with Claycomb medium (~800 μ l). The next day the medium was changed and viewed under a microscope to ensure cells had attached over the electrodes and the 35mm glass bottom dishes. Recordings were performed 24-48 hours after seeding.

MEA apparatus

The equipment used in this study was composed of glass MEA plates, the MEA 1060 amplifier, the MC_Stimulus generator 2.1.5, the TC01/02 temperature controllers and the MC_Rack data acquisition and analysis software (Multichannel systems).

MEA plates

The standard MEA plate consist of 60 titanium nitride (TiN) electrodes in an 8x8 grid fabricated on a glass substrate (Figure 5.1 (A)). The electrodes have a diameter of 30 μ m and the spatial resolution is 200 μ m. The total distance across an MEA plate is 1400 μ m and this value is used to calculate CV across a cell preparation. This will be explained in greater detail later in this section. Cell preparations can be cultivated directly on the

MEA plate and extracellular action potentials can be recorded over a length of time (up to several weeks).

After each experiment the MEA plate was cleaned for re-use. The plate was thoroughly rinsed with tap water and filled with a 1% sodium dodecyl sulphate (SDS) solution in distilled water (dH₂O) for a minimum of 3 hours to remove cellular debris from the surface of the electrodes. The plate was rinsed under a continuous stream of tap water between 2-24 hours followed by dH₂O for a few minutes to ensure complete removal of the detergent. The MEA plate was sterilised with 75% ethanol and baked at 120°C for a minimum of 2 hours before dry storage in a clean 100mm petri dish until ready for use.

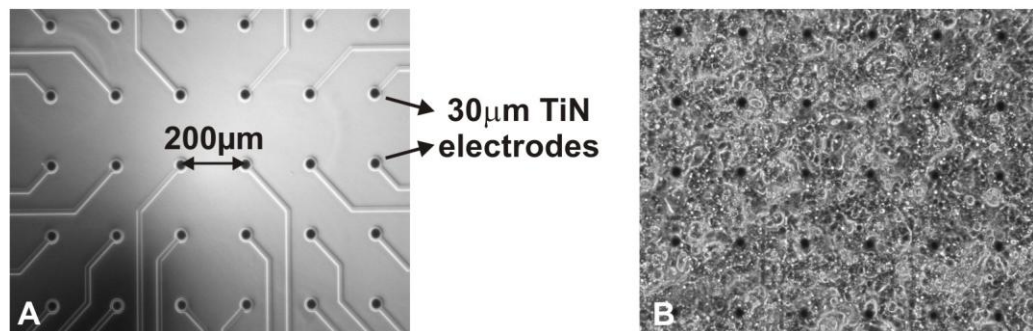


Figure 5.1: MEA plate

The MEA plate consists of 60 titanium nitride (TiN) electrodes (A). Each electrode has a diameter of 30µm and the space between each electrode is 200µm from centre to centre. Cells are seeded as a confluent drop directly on the TiN electrodes (B) and extracellular recordings performed 24-48 hours post-seeding.

MEA recordings

All the electrodes were in contact with the cell monolayer as shown in Figure 5.1 (B). This was essential to ensure a sufficient amount of electrical activity was produced by the cells for the signal to be detected by the electrodes.

Prior to mounting the MEA onto the amplifier, the contact pads on the MEA were thoroughly cleaned with 75% ethanol. This was to remove any contaminants, for example grease, from touching the pads with bare fingers and to prevent poor contact between the pads and the amplifier pins. A bad contact results in increased noise levels on the affected channel. The medium was grounded either via: (1) an internal reference

electrode connected to the ground with the provided connector or (2) a silver wire connected to the amplifier's ground and placed into the chamber.

Once connected to the amplifier the MC_Card pre-installed on the data acquisition computer acquired the analog input signals at a sampling rate of 25kHz and digitized the raw data (Figure 5.2 (A)). Extracellular recordings can be visualized using MC_Rack. All recordings were carried out at 37°C using the temperature controller (TC01/02) connected to the base of the amplifier. Usually signals were visually observed for 1-2 minutes to determine if the MEA was suitable for experimental use. The selection criteria were based on the size and the frequency of the cellular signals.

For data acquisition, intrinsic beats were recorded prior to stimulation for 30-60 seconds using the MC_Rack software. Cells were then stimulated (MC_Stimulus 2.1.5) with a 10ms bipolar pulse of 0.3-1V for 1 minute at either 1 or 2Hz (Figure 5.2 (B)). The frequency used was directly dependent on the intrinsic frequency generated by the cells. MC_Rack allowed the experimenter to select the location and number of electrodes required to stimulate the cells. Analysis of extracellular recordings and excitations maps were performed using MC_Rack, Microsoft Excel and GraphPad prism.

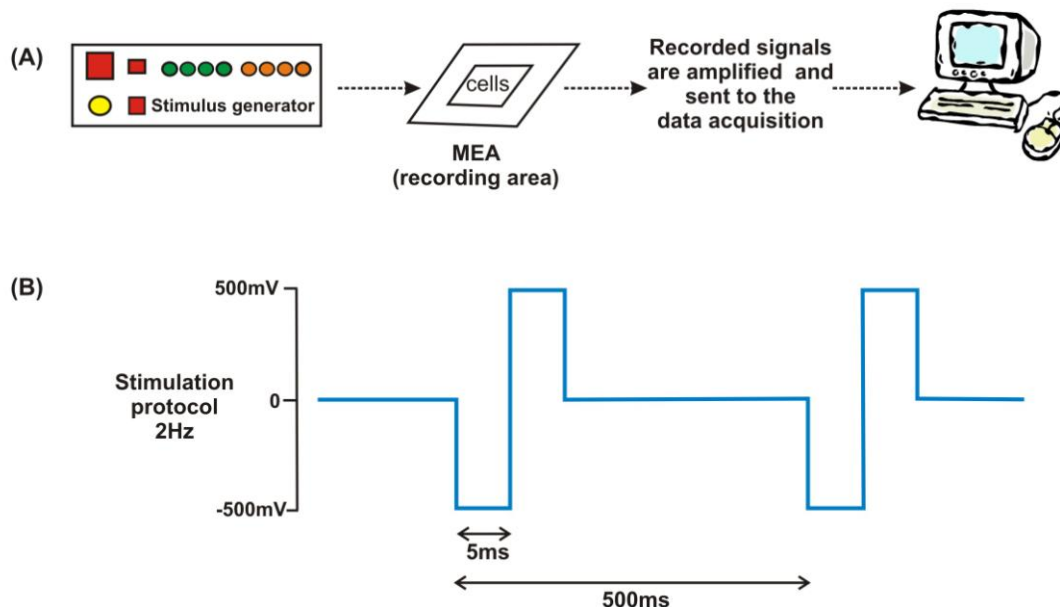


Figure 5.2: Set-up of the stimulus generator to the MEA amplifier

Cells were stimulated using a stimulus generator directly transmitting the stimulation to any of the TiN electrodes present on the MEA plate. Action potentials recorded extracellularly were amplified and sent to the data acquisition system to be recorded and analysed on the computer (A). (B) is an example of a bipolar protocol used to stimulate the cellular preparation. Cells were normally stimulated at a frequency of 1-2Hz.

Measurement of CV

Spontaneous preparations

Extracellular field potentials produced by the HL-1 clones are shown in Figure 5.3 (A). The field potential has several characteristic components that can be distinguished which include: (1) the first negative peak of the field potential (FP_{Min}); (2) the last positive peak of the field potential (FP_{Max}) and (3) the duration of the field potential (FP_{dur}) defined as the time between (FP_{Min}) and (FP_{Max}) (Halbach et al. 2003) (Figure 5.3 (A), enlarged electrode (E86)). Studies of simultaneous recordings of field potentials and intracellular action potentials have established a close correlation between: the time of upstroke and repolarising phases of the action potential to the FP_{Min} and FP_{Max} respectively (Halbach et al. 2003; Reppel et al. 2004).

For extracting CV from the field potentials, a trigger detector was set-up in a single electrode (can be any electrode) using MC_Rack. The purpose of the trigger detector was to detect electrical activity above a threshold value of the field potential thereby

identifying the spontaneous activity (e.g. the dotted line on the enlarged electrode (E86) in Figure 5.3 (A)). This in turn generated a data stream (Figure 5.3 (B)) in the remaining electrodes relative to the trigger, which was always set at 0ms. Depending on the origin of activation the field potentials in the remaining electrodes occurred before or after the trigger electrode. This data stream was then used to set-up an analyser through MC_Rack defining the region of interest (ROI) representing the time range in which the field potentials on all 60 electrodes were analysed (Figure 5.3 (B), E85). Data within the ROI gave trace plots of the time taken for the action potential to reach each electrode from the origin of wavefront activation (Figure 5.3 (C)). CV was calculated using Equation 5.1 using Figure 5.3 (C) as an example.

Equation 5.1: Measurement of CV from spontaneous preparations (mm/sec)

$$\frac{\Delta D_{(A-B)} \times 1000}{\Delta T_{(A-B)}}$$

D = distance; T = time delay (ms); A = origin of activation in Figure 5.3 (C)); B = point furthest away from the origin of activation in Figure 5.3 (C))

The trace plot showed the consistency of each beat in each of the electrodes across the length of the recording (x-axis) highlighted by the enlarged image of the electrode (E75) in Figure 5.3 (C).

To illustrate the spread of activation and electrical activity in different areas within a monolayer we were able to create two-dimensional false colour maps (Figure 5.3 (D)). Each colour map consisted of 64 colours representing time intervals determined by the ROI. For example in Figure 5.3 (D) the darkest colour denoted by the asterisk represented the fastest time delay and the lightest colour represented the slowest time delay in the ROI from the origin of activation.

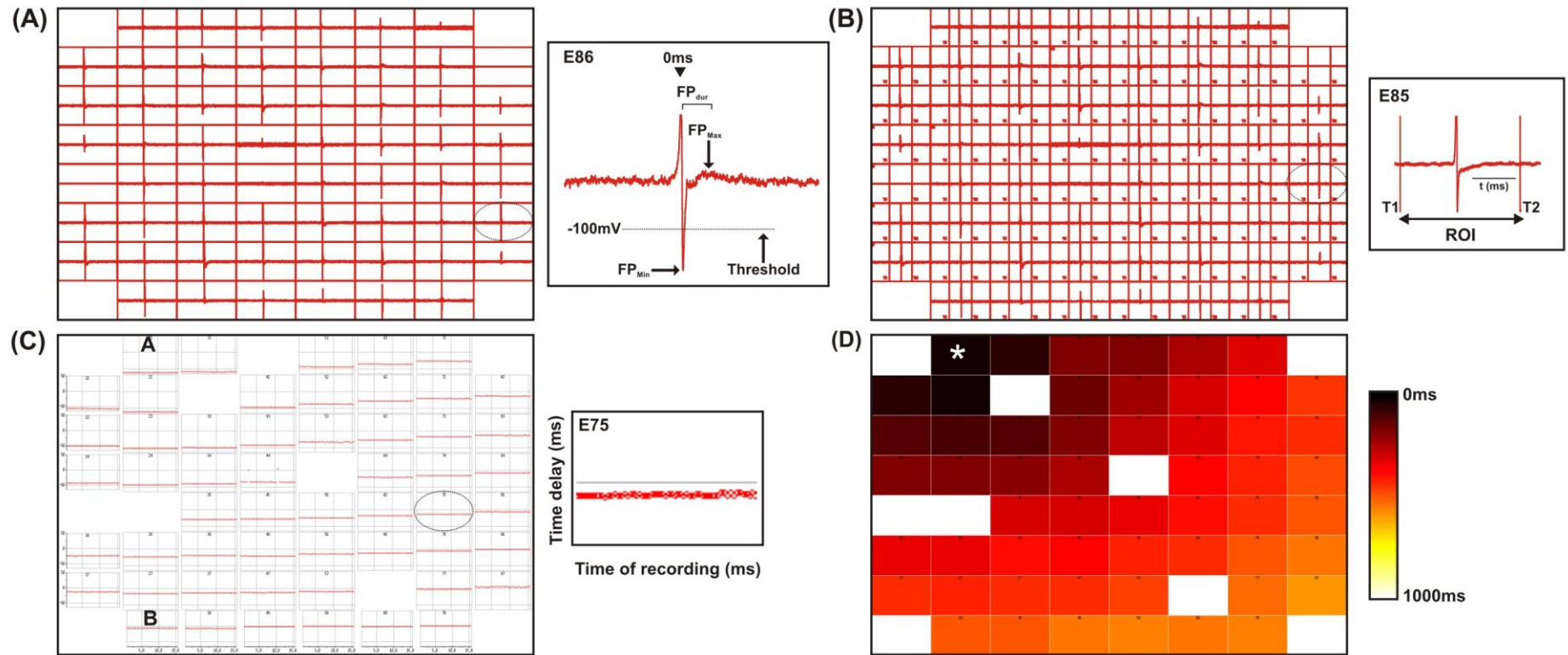


Figure 5.3: Measuring CV in spontaneously contracting preparations

(A) is a typical example of extracellular recordings produced by spontaneously contracting cell preparations. The key parameters of the field potential are indicated in the enlarged electrode 86: F_{Min} is the size of the largest negative peak; F_{Max} is the last positive peak and F_{Dur} is the duration of the field potential. To calculate CV using recording (A) as an example, a trigger detector was set-up in one electrode (e.g. E86), which detects any electrical activity above a certain threshold (-100mV) of the field potential generating a data stream (B). This data stream was used to set-up an analyser enabling to define a region of interest (ROI) and measure CV from trace plots (C). Two-dimensional false colour maps illustrate the origin of activation (D). Blank electrodes in the trace plots and false colour maps represent electrodes that were excluded because of electrical noise.

Stimulated preparations

When cell preparations were electrically stimulated (e.g. top row of electrodes) the time delay between the artefact of stimulation and the field potential can be measured in any of the electrodes (e.g. Figure 5.4 (A), E28). An enlarged image of the time delay in action potential propagation along a row of electrodes from the point of stimulation (e.g. E71) to the remaining electrodes down the array (e.g. E72-78) is shown in Figure 5.4 (B). CV was calculated using Equation 5.2.

Equation 5.2: Measurement of CV from stimulated preparations (mm/sec)

$$\frac{\Delta D_{(\text{point of stimulation} - \text{electrode } X)} \times 1000}{\Delta T_{(\text{field potential} - \text{artefact of stimulation})}}$$

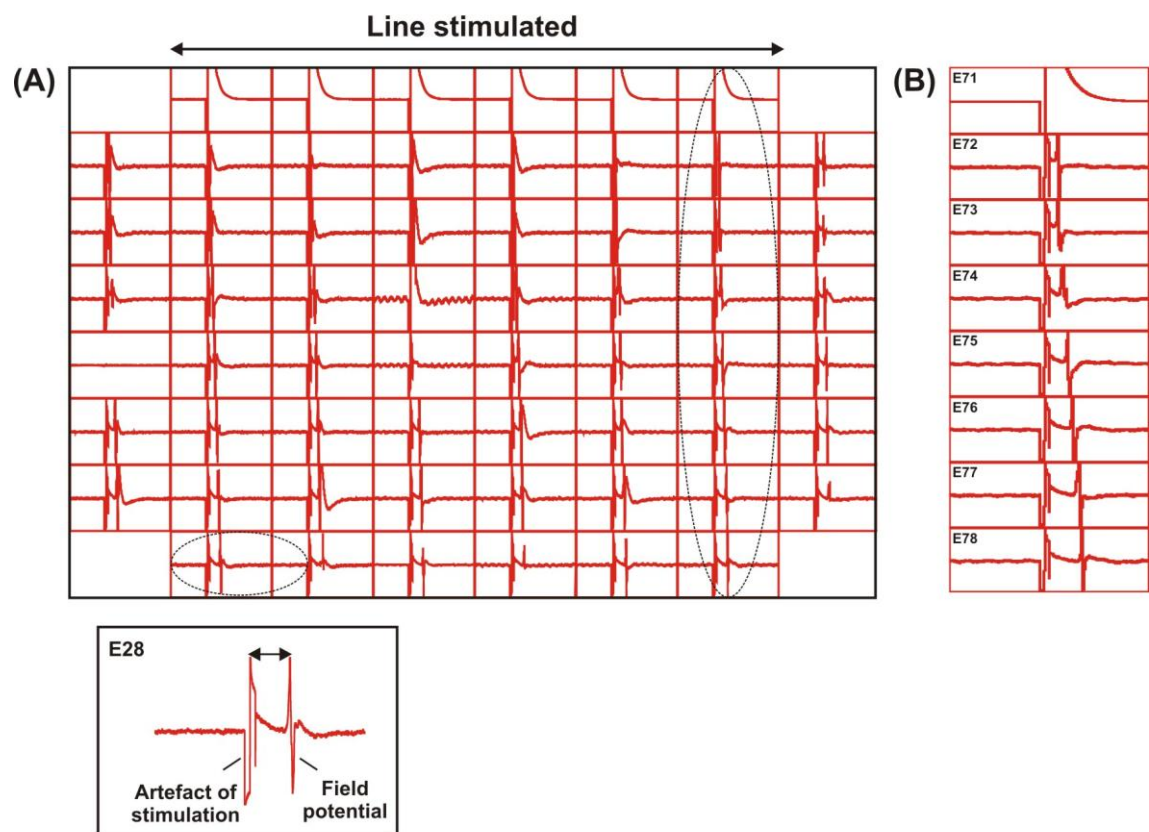


Figure 5.4: Measurement of CV in stimulated cellular preparations

Cells were field stimulated with a 10ms bipolar pulse between 0.3-1V along a row of electrodes (e.g. top row). The time taken between the artefact of stimulation and the resulting field potential in any electrode (e.g. E28) was measured (A). The delay of excitation spread in a single row of electrodes is plotted against the respective distance of the electrodes from the origin of excitation (B).

Optical mapping

The characteristics of impulse propagation were assessed optically with the voltage sensitive dye di-8-ANNEPS (Biotium). The dye was prepared in a stock solution of 1ml 75% dimethyl sulphoxide (DMSO) and 25% pluronic acid. 10 μ l of the stock solution was added to the cell preparation containing 1ml of normal Tyrode to give a final concentration of 135 μ M and incubated for 2 minutes at room temperature. The cell preparations were washed in normal Tyrode to remove any unbound dye. During the experiments, preparations were constantly superfused with normal Tyrode at 37°C. Tyrode was necessary for the optical mapping recording because the autofluorescence of the medium would interfere with the actual signal. The emitted fluorescence corresponding to transmembrane voltage changes were measured at an excitation wavelength of 470 $\pm$ 30nm and a recording wavelength of 550 $\pm$ 40nm using a CMOS fast-resolution camera (Ultima, SciMedia). Spontaneous activity in a given preparation was limited to recordings lasting 4 seconds each in order to avoid phototoxic effects of the dyes. Optical raw data was analyzed using software provided by camera manufacturer (BV_Analyze V9.09, SciMedia). After offset correction and digital filtering of the raw data, the frequency of spontaneous activations and the spatial activation patterns were determined separately for each preparation.

5.3. Results

5.3.1. Optimisation of seeding parameters

From preliminary experiments we found that field potentials most suitable for measuring CV were obtained from cells covering the electrodes only compared to cells plated over the entire MEA plate. In both techniques cells were seeded as a monolayer with the only difference being in the surface area covered by the cells. In preparations covering the entire MEA plate, cells spontaneously contracted at a higher frequency of 4-5Hz with poor signal to noise ratio (data not shown) when compared with cells covering only the electrodes (1-2Hz). Thus cells were always seeded as a drop covering only the electrodes. Despite this seeding optimisation, CV varied considerably between each experiment depending on the volume and size of the seeding drop.

To provide more consistency in CV, a range of cell densities ($0.5\text{-}2\text{cm}^2$ of cells) and seeding volumes ($5\text{-}40\mu\text{l}$) were tested using clone 6. Figure 5.5 (A) displays a chart with the key volumes and cell densities tested with their corresponding CV (mm/sec). From these experiments we established that 0.5 and 1cm^2 of cells in a volume of $10\mu\text{l}$ was optimum in providing the highest CV and most consistent data between experiments ($1\text{cm}^2/10\mu\text{l}$: $44 \pm 1\text{mm/sec}$; $0.5\text{cm}^2/10\mu\text{l}$: $38 \pm 1\text{mm/sec}$) that peaked within 3-4 days post-seeding. Seeding cells below or above these values lead to lower CV ($0.5\text{cm}^2/5\mu\text{l}$: $27 \pm 1\text{mm/sec}$; $2\text{cm}^2/30\mu\text{l}$: $22 \pm 1\text{mm/sec}$) and on the occasions maximum velocities were reached on days 5-6 post-seeding. Seeding volumes of $10\mu\text{l}$ were optimum as smaller volumes (e.g. $5\mu\text{l}$) were insufficient for the cells to be seeded in and larger volumes (e.g. $30\text{-}40\mu\text{l}$) resulted in the spreading of the drop and cells not forming a confluent monolayer.

To explain these differences in CV western blots measuring connexin40 (Cx40) and Cx43 expression were performed on cells cultured at different densities (50%-300%). Cells were harvested on day 3 which was typically the time point when CV peaked (Figure 5.5 (B)). We found that Cx40 and Cx43 expression reduced considerably when cells were seeded at 250% confluency (Cx40: $\sim 75\%$ drop; Cx43: $\sim 50\%$ drop) compared with when cells were at 100-200% confluency. This indicates that there is a range in which cells need to be seeded where connexin expression is optimum and the maximum CV is recorded.

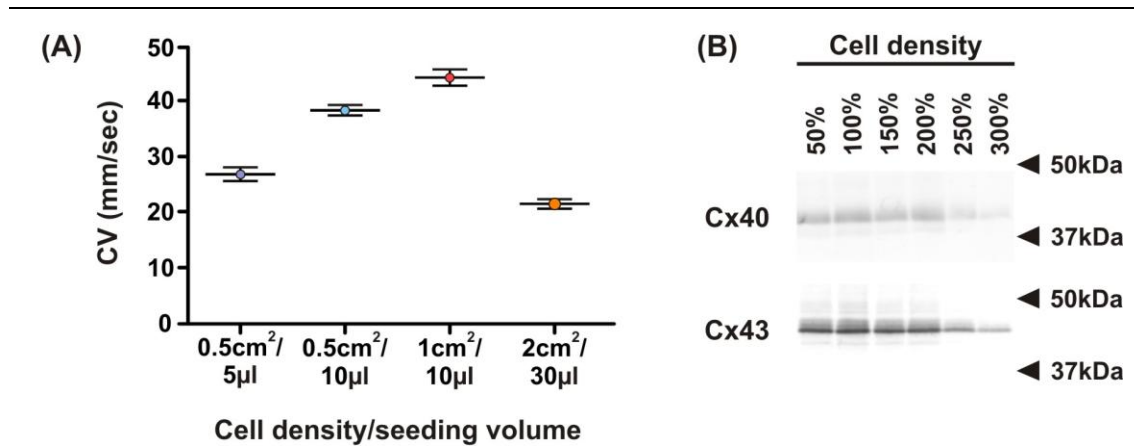


Figure 5.5: Optimisation of seeding parameters

Cells were seeded at different densities (0.5-2cm² of cells) and seeding volumes (5-30μl) on the MEA and the resulting CV measured (A). The exact cell density and seeding volume is listed below the chart. (B) are immunoblots of cells seeded at varying densities and detected for Cx40 and Cx43. The initial seeding density is indicated above the immunoblots.

5.3.2. Action potential propagation in spontaneously contracting cultures using MEA and optical mapping

Using the seeding protocol described in the section above, action potential propagation was measured using MEA and optical mapping in spontaneously contracting cultures in clone 6. Figure 5.6 (A) and (B) show typical examples of two-dimensional false colour maps generated from MEA and optical mapping respectively. In both examples the origin and the direction of the propagating wavefront is highlighted by the asterisk and arrows. In the majority of the preparations the electrical activity originated from the edge of the monolayer formed by the initial seeding drop and the spread of activation appeared relatively homogenous. CV were measured using MEA and optical mapping and no significant difference ($p = 0.2684$) was observed in the two techniques (MEA: 41 ± 2 mm/sec; optical mapping: 37 ± 2 mm/sec; $n = 8$ and 18 respectively) (Figure 5.6 (C)).

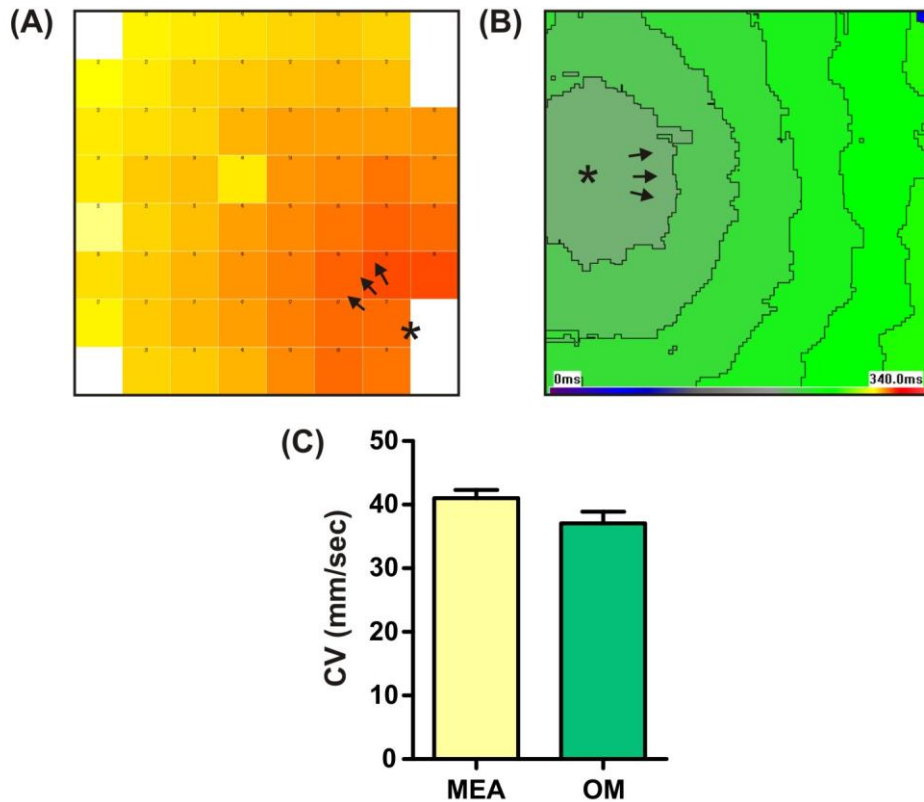


Figure 5.6: CV in spontaneously contracting cultures using MEA and optical mapping

Typical examples of two-dimensional false colour maps from MEA (A) and optical mapping (OM) (B) in spontaneously contracting cultures of clone 6. In images (A) and (B) origin and spread of activation is denoted by the asterisk and arrows mark the direction of the propagating wavefront. CV was measured using both techniques and there was no significant difference ($p = 0.2684$) in the values (MEA: 41 ± 2 mm/sec; optical mapping: 37 ± 2 mm/sec; $n = 8$ and 18 respectively) (C).

In many of the recordings analysed the natural pacemaker was always present at the edge of the preparation. We also observed that the origin of activation was not always at the same location in the preparation, indicating that there was more than one pacemaker. Optical mapping was used to illustrate the switch in the natural pacemaker within the same recording (Figure 5.7). At the beginning of the recording (Figure 5.7 (A), top left panel) electrical activity spread from the top right corner across the preparation. However ~ 300 ms later during the same recording there were two pacemakers within the same preparation (Figure 5.7 (A), bottom panel) which collided in the centre.

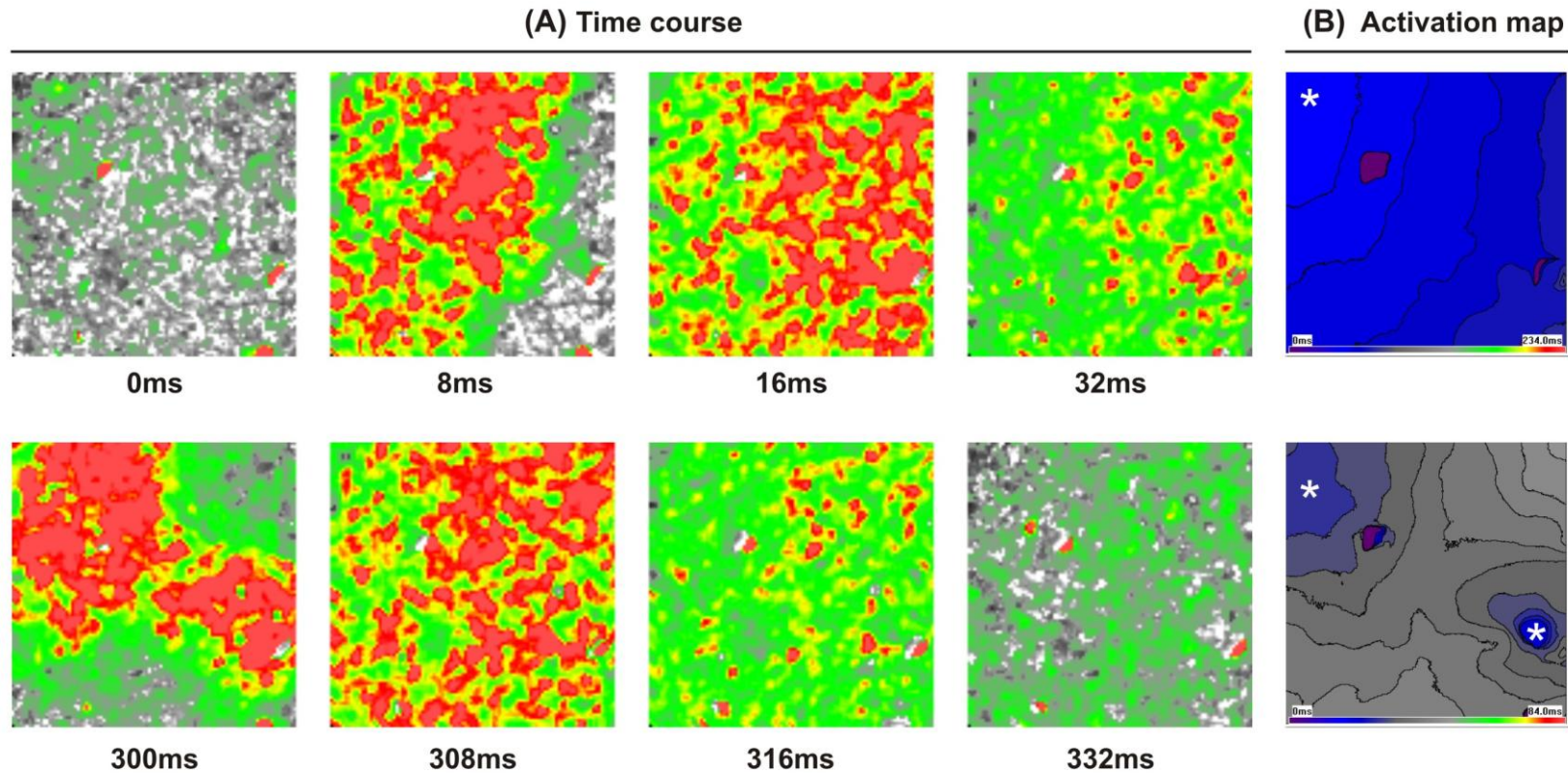


Figure 5.7: Origin of natural pacemaker using optical mapping

(A) shows a time course of wavefront propagation within the same cell preparation. The time difference in milliseconds (ms) is indicated below each of the images. In the top left panel of the recording, spread of activation is initiated from the top left corner. Approximately 300ms later during the same recording there appears to be two pacemakers at the top left and bottom right corner (bottom left panel). The red colour represents full-sized action potentials; green means there is an absence of depolarisation. (B) illustrates the corresponding activation maps for each of the panels. Asterisks mark the pacemaker location in each of the activation maps.

The overall spread of activation in the two panels are summarised by their corresponding activation maps and are marked by the asterisks indicating the pacemaker locations (Figure 5.7 (B)).

To test the homogeneity of wavefront propagation, optical mapping was used to record action potential size, shape and duration from multiple sites within a monolayer. Figure 5.8 (A) is an example of an activation map illustrating impulse propagation across the entire cell monolayer with examples of two optically recorded action potentials from the origin of activation (point 1) and at a location (point 2) further away from the origin. The action potential shape was homogenous across the monolayer and showed a good correlation to the action potentials recorded by patch-clamp (Figure 5.8 (B)). On average cells had an action potential duration at 50% repolarisation (APD_{50}) of 46 ± 2 ms ($n = 32$) which was significantly longer ($p > 0.05$) compared to values calculated using patch-clamp (17 ± 6 ms, $n = 4$). The differences in APD_{50} are possibly a result of motion artefacts due to cell or muscle contraction that distorts the optical action potential signal preventing accurate recordings of the repolarisation phase (Morley and Vaidya 2001; Efimov et al. 2004). To avoid motion artefacts and for accurate recordings of action potential durations, cell preparations will need to be supplemented with electromechanical uncoupling agents such as diacetyl monoxime and 2,3-butanedione monoxime (Morley et al. 1999; Sharifov and Fast 2003). Nevertheless optical mapping can still give an approximate representation of the action potentials generated by cells across a monolayer.

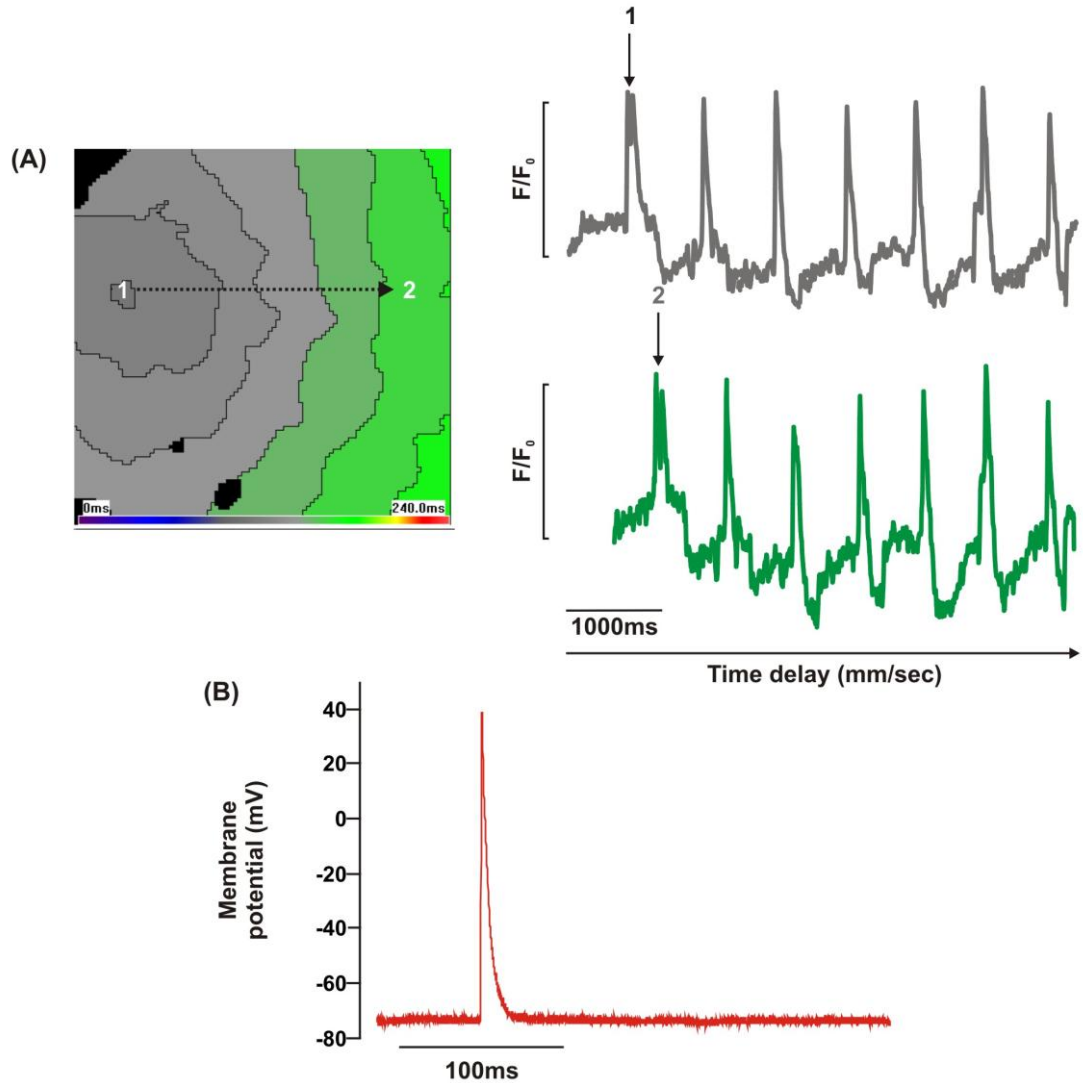


Figure 5.8: Action potential recordings by optical mapping

A typical example of impulse propagation across the entire cell monolayer. In many of the preparations the natural pacemaker was always present at the edge of the monolayer as shown in this example (A). Analysis of action potential size and shape at multiple locations within a monolayer indicated a homogenous morphology (A). Points 1 and 2 on the activation map correspond to the grey and green traces of the optically recorded action potentials respectively. The black dotted line on the activation map denotes the spread of impulse propagation, initiated from the edge (point 1) and spreading across the preparation (point 2). This in turn corresponds to the difference in time delay observed in action potential activation between the two points. Comparing optically recorded action potentials to those recorded by patch-clamp (B) revealed a similar morphology.

5.3.3. Frequency of intrinsic spontaneous rate in the HL-1 clones

Extracellular field potentials were typically observed 1-2 days post-seeding. All the HL-1 clones had an intrinsic spontaneous rate ranging from 1.3-1.5Hz with the exception of

clone 1 which had a higher intrinsic rate of 2.5Hz. The only significant difference in intrinsic frequency was between clones 1 and 6 ($p < 0.05$). It should be noted that after sub-cloning clone 1 had drifted and cells were unable to contract and generate action potentials spontaneously or after electrical stimulation. The data presented in Table 5.1 and in Figure 5.12 were calculated when the cells were still contracting and able to generate action potentials. Table 5.1 is a summary of the spontaneous frequencies obtained in the HL-1 clones two days post-seeding.

Table 5.1: Intrinsic rate of frequency in the HL-1 clones

HL-1 clones	Intrinsic frequency (Hz)
1	2.5 ± 0.31 (n = 10)
2	1.5 ± 0.22 (n = 6)
3	1.4 ± 0.24 (n = 5)
4	1.4 ± 0.24 (n = 5)
6	1.3 ± 0.16 (n = 8)

5.3.4. Action potential propagation under electrical stimulation in the HL-1 clones

After days 2 and 3 post-seeding, some preparations did not always display spontaneous electrical activity but extracellular recordings could be elicited by electrical stimulation. Furthermore only certain preparations of spontaneously contracting cultures could be used to calculate CV accurately depending on location of the pacemaker. Thus for consistency, all the data from this stage of the study will be presented on electrically stimulated cultures.

Cells could either be stimulated using point or line stimulation from the edge of the array (top, bottom, left and right rows). Line stimulations were preferred over point stimulations to produce a flat activation front. Electrical stimulation using a single electrode, produced a convex wavefront (i.e. excitation front is curving outwards) (Figure 5.9) comparable to studies by Knisley and Hill (1995) on the effects of stimulation on excitation wavefront.

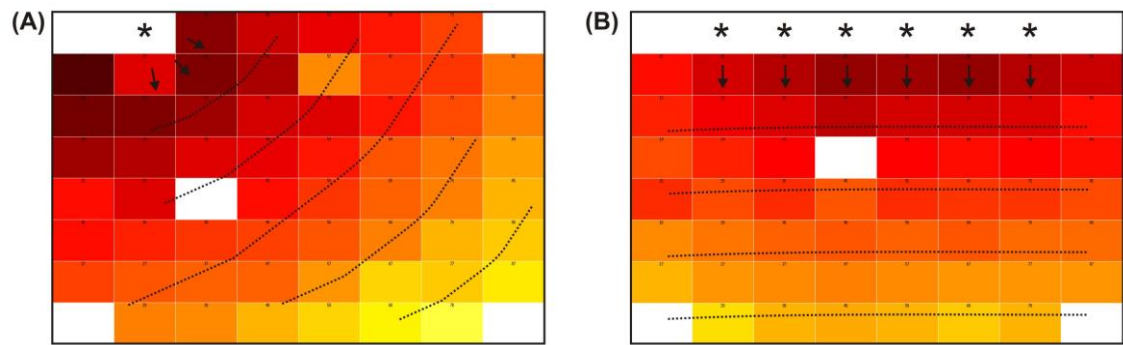


Figure 5.9: Effects of point stimulation versus linear stimulations on activation spread

Cells were stimulated using a point (A) or line (B) stimulation. Stimulation using a single electrode produced a convex wavefront whereas stimulation with a row of electrodes produced an almost flat excitation. The arrows mark the direction of wavefront propagation and the curvature of the wavefront is indicated by the black dotted lines. The stimulating electrodes are denoted by asterisks. White squares in the false colour maps are electrodes excluded due to electrical noise.

When cells were stimulated in different directions no significant difference ($p = 0.0682$) in CV was observed (Table 5.2). No anisotropic propagation was detected, thus any of the rows at the edge of the MEA array could be used to electrically stimulate the cells.

Table 5.2: CV when stimulated in different directions

Stimulation	CV (mm/sec)
Top	36 ± 2 (n = 3)
Bottom	35 ± 1 (n = 4)
Left	40 ± 2 (n = 5)
Right	38 ± 1 (n = 6)

The electrical stimulation parameters used to dominate the natural pacemaker were dependent on the cells intrinsic rate of frequency. The HL-1 clones had an intrinsic rate between 1-1.5Hz with the exception of clone 1 (Table 5.1) and the rate of electrical stimulation sufficient to override this was 1-2Hz. However in certain occasions a higher rate of stimulation was required. For example in clone 1 which needed a higher frequency at 2.5Hz. To test if the frequency of stimulation was a determining factor in CV cells were paced at a range of frequencies from 1-5Hz. We observed that an increase in the rate of stimulation was accompanied by a decrease in CV, a pattern found in all the HL-1 clones. Figure 5.10 shows CV recorded from clones 4 (n = 3) and

6 (n = 4) at different rates of stimulation. CV was only significantly different in clone 4 when stimulated at a frequency above 2Hz (3Hz: $p < 0.01$; 5Hz: $p < 0.001$).

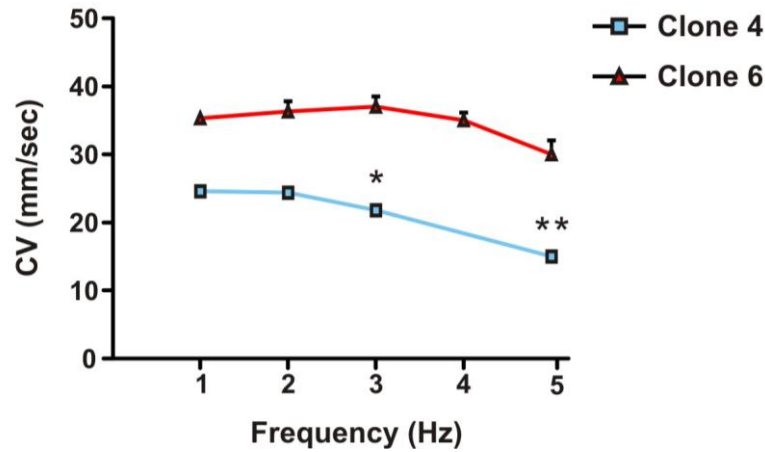


Figure 5.10: Effects of pacing frequency on CV

Cells from clones 4 and 6 were electrically stimulated at different frequencies (1-5Hz) and the CV measured. No significant difference in CV was observed at 1-2Hz in both clones. At frequencies above 2Hz a drop in CV was observed but the values were not statistically different ($p > 0.05$) in cells from clone 6 (n = 4). However in clone 4 the reduction in CV upon stimulation above 2Hz was significantly different compared with 1Hz (n = 3; * $p < 0.01$; ** $p < 0.001$). This pattern in conduction drop upon an increase in frequency was observed in all the HL-1 clones.

Recordings of action potential propagation monitored over time (days 1-5) indicated that CV gradually increased over days 1-2 and typically peaked on days 3-4 and dropped thereafter. This pattern was followed by all the HL-1 clones but data from clones 3 (n = 3) and 6 (n = 4) are shown as examples in Figure 5.11. As a general rule for all MEA experiments CV were recorded between days 2-5 when possible.

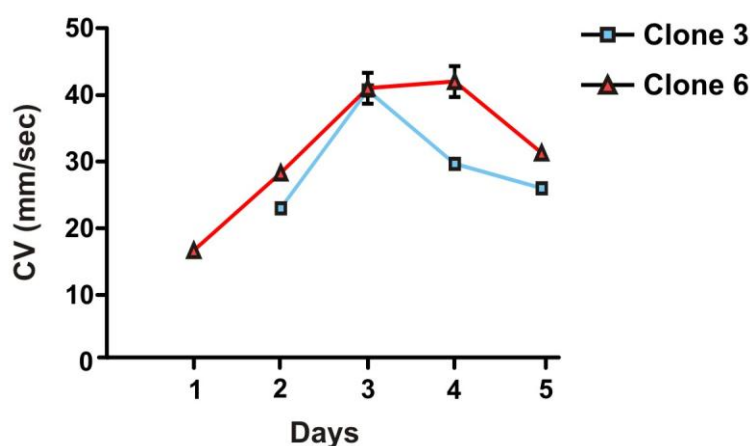


Figure 5.11: CV measured over days

CV was recorded over days 1-5. Conduction gradually increased over days 1-2 and typically peaked at days 3-4. This was a pattern followed by all the HL-1 clones but a time course for only clones 3 (n = 3) and 6 (n = 4) is shown. At some of the points the error bars are too small to be seen.

Using the stimulation parameters described in Figure 5.10 action potential propagation was measured in the HL-1 clones when paced at 1-2Hz. The maximum CV recorded between days 3-4 is shown in Figure 5.12. We were unable to electrically stimulate clone 2 although they displayed spontaneous field potentials. Thus the maximum CV in clone 2 was calculated from spontaneous electrical activity. CV in clone 2 was significantly different ($p < 0.001$) compared to the rest of the HL-1 clones.

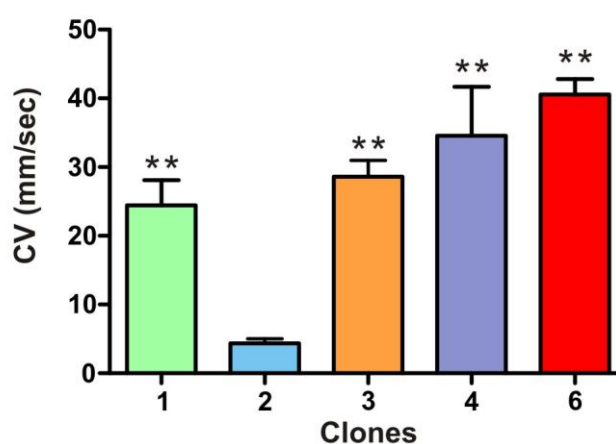


Figure 5.12: CV in the HL-1 clones

For each of the HL-1 clones, cells were paced at 1-2Hz and maximum CV (mm/sec) was recorded over days 3-4. Clone 6 was the fastest propagating clone (41 ± 2 mm/sec; n = 13) followed by clone 4 (35 ± 7 ; n = 7), clone 3 (29 ± 3 ; n = 10), clone 1 (24 ± 2 ; n = 4) and 2 (4 ± 1 ; n = 6). CV from clones 1, 3, 4 and 6 were compared only with clone 2 and velocities were significantly different (** $p < 0.001$).

5.4. Discussion

The key findings in this chapter were: (1) MEA can be used to measure impulse propagation from spontaneously contracting and electrically stimulated cultures of the HL-1 clones; (2) optical mapping not only confirmed the CV measured by MEA but gave valuable information on the homogeneity of the propagated wavefront using optically recorded action potentials from multiple sites across the cell monolayer; (3) the natural pacemaker normally originated from the edge of the cell monolayer with sometimes more than one pacemaker present; (4) the HL-1 clones had an intrinsic frequency rate of 1-2.5Hz; (5) CV was dependent on the time in culture and the frequency at which cells were electrically stimulated and (6) the HL-1 clones displayed differences in CV particularly in clone 2.

We started by optimising the cell seeding protocol for MEA analysis to ensure cell preparations not only produced a sufficient amount of electrical activity to be detected by the electrodes but also with a high signal to noise ratio. It was important to seed the cells as a confluent monolayer ensuring cells were well interconnected with no gaps within the preparation. Recordings of field potentials most suitable for measuring CV were obtained from cells covering only the electrodes compared to cells seeded over the entire MEA plate. Although in both approaches cells were seeded as a monolayer, it appeared that the larger the surface area the more likely it was for the cells to intrinsically pace at a higher frequency. This made it difficult to accurately measure CV. Studies exploring the relationship between frequency and the size of cell aggregates demonstrated that the rhythm and strength of beating is not only influenced by the initial beat rate but also by the size of the network (i.e. an increase in rate is observed as the cell aggregates increase) (Kojima et al. 2006). Hachiro et al. (2007) also demonstrated that beating rhythm in an aggregate of cells is dependent on the strength of coupling through gap junctions. It is also possible the larger the cell aggregate the more likely it is for the monolayer to accommodate more pacemakers thereby increasing the strength of beat rate (Kojima et al. 2006). Thus the larger the cell aggregate, the greater the cell-to-cell interaction, which could explain the higher frequencies, observed in the larger monolayer.

Cells seeded on top of the electrodes, had an intrinsic rate of electrical activity of 1-2Hz. However CV varied depending on the number and the volume in which cells were seeded. For example the same number of cells was seeded in a volume of 5 μ l and 10 μ l but CV were slower in the smaller volume despite no difference in connexin expression. It is possible that the 5 μ l was insufficient to maintain the cells in a healthy state particularly as they were left to adhere to the MEA for a minimum of 20 minutes. The smaller volume could have also prevented the cells from spreading so forcing a more compact aggregate to be formed and reducing the cell size. Mathematical studies comparing adult and neonatal cells with their respective gap junction distribution demonstrated that cell size is a greater determining factor in CV than differences in gap junction distribution. Thus the larger the cell the lower the internal resistance (determined by cell size, cytoplasmic resistivity, gap junctions and their conductance) and the faster the conduction (Spach et al. 2001; 2004).

Increasing the number of cells seeded also resulted in lower CV. This was supported by western blot data showing that at high densities connexin expression is reduced. Changes in connexin expression due to cell density have also been reported in endothelial cells with a decrease in both Cx43 mRNA and protein levels at high confluency (Larson et al. 1997). Since gap junctions are key determinants in controlling the passage of current between adjacent cells, changes in intercellular coupling will alter the velocity of the propagating action potential (Shaw and Rudy 1997; Rudy 2005). Computer modelling demonstrated that reducing the gap-junctional coupling decreases CV but also increases the SF by almost three times before a conduction block (Shaw and Rudy 1997; Rudy 2005). From this data correlating different seeding densities and CV there is a 'threshold' of the number of cells seeded whereby: (1) the cell aggregate is large enough to contract; (2) cells within the aggregate are of the right size to electrically communicate and (3) connexin expression is optimal. For future the exact number of cells required for seeding an MEA plate and for protein measurements would need to be calculated rather than expressing the values as a percentage of the cell density.

Using the optimised seeding parameters CV were measured in spontaneously contracting cultures of clone 6 using MEA and optical mapping. No difference in

velocities were observed since both techniques are similar in function with the key difference being MEA records extracellular field potentials from a cell monolayer (Halbach et al. 2006) whereas optical mapping detects local changes in membrane potential (Rohr and Salzberg 1994). In the majority of recordings the natural pacemaker was observed at the edge of the monolayer formed by the initial seeding. Using MEA we were only able to approximate the origin of activation since the natural pacemaker was not always observed within the array but just outside the recording area. Optical mapping overcame this problem, as we were able to always visualise the entire monolayer and pin point the exact location of the pacemaker confirming that the origin of propagation was mainly from the edge of the monolayer. This is likely since the sink at the edge of the preparation is smaller for the source to serve as a charge for the depolarising neighbouring cells. Therefore the source is better matched to the load permitting a successful propagation (Rohr et al. 1997).

Further analysis of the natural pacemaker location indicated there was sometimes more than one pacemaker present within the monolayer with even two pacemakers colliding. Groups of cells intrinsically contracting at different rates are expected to synchronise their pacing, forming a focal pacemaker providing a better current source for activation of the surrounding quiescent tissue (Kucera et al. 2000; Joyner et al. 2007). This process of synchronisation in which cells with greater intrinsic automaticity dominate the overall rate of contraction is determined by intercellular coupling (gap junctions) (Hachiro et al. 2007; Joyner et al. 2007). In cell aggregates with multiple pacemakers a single group may not be dominant enough to drive the preparation, which could explain a switch in pacemaker midway through a recording or even pacemakers colliding.

The HL-1 clones had similar rates of intrinsic frequency (1-1.5Hz) with the exception of clone 1 (2.5Hz). Comparisons in the amount of mRNA for Ca^{2+} handling proteins (e.g. sodium-calcium exchanger (NCX) and L- and T-type Ca^{2+} channel) showed that only clone 2 displayed major differences with considerably reduced levels of messenger expression compared with the other clones. There also appears to be no direct correlation between the generation and frequency of spontaneous activity and the absolute steady state levels of transcript coding for these proteins in the HL-1 clones. It is likely that the proportion of protein expression for each ion channel or Ca^{2+} handling protein (e.g. spontaneous contracting rate dependent on ryanodine receptor function and

NCX activation) is likely to determine the frequency of spontaneous action potentials. Further experiments will need to be performed to study the exact mechanisms that determine the intrinsic frequency.

In spontaneously contracting cultures the propagating action potential had a convex wavefront indicating a source-and-sink mismatch whereby the local excitatory current supplied by the source is distributed over a larger area (sink) (Fast and Kleber 1997). In tissue, the geometry of the wavefront (i.e. flat, convex or concave) is a determinant of CV and a mismatch between the current source and the current required for the cells to be activated can cause delay in propagation (Fast and Kleber 1997; de Bakker and van Rijen 2006). Any potential problems caused by the wavefront curvature were circumvented by linearly stimulating the preparation ensuring a near flat activation. We found that CV did not differ significantly when stimulated in different directions. In the heart anisotropy arises from the spatial alignment of elongated cardiac myocytes and the preferential location of gap junctions at the end of the cells producing a higher resistivity in the transverse direction compared with the longitudinal direction. As a result propagation is faster in the longitudinal direction than in the transverse direction (Fast et al. 1996). Other factors governing anisotropy include cell size and shape and the spatial distribution of neighbouring cells (Bursac et al. 2002). In our model anisotropy was eliminated since the cells do not have a structured shape and gap junctions were distributed along the cell perimeter of the cells rather than at the cell ends similar to neonatal ventricular myocytes (Bursac et al. 2002).

The frequency of stimulation was a key factor when measuring CV whereby an increase in pacing negatively correlated with a drop in CV. After action potential generation the voltage-gated ion channels that are activated enter an inactivated state and to regain their ability to open, the channels must undergo a recovery process at hyperpolarised potentials (Grant 2009). Keeping this in mind increasing the frequency shortens the time intervals between action potentials. Shortening the action potential duration could potentially interfere with the relative refractory period, an interval during which a second action potential can be elicited if the stimulus strength is greater than usual (Widmaier et al. 2006). In this period not all the sodium (Na^+) channels would have returned to their resting state and some of the potassium channels that repolarised the membrane are still open. As a result if stimulation does take place during the relative

refractory period the action potential upstroke may not be as fast as when all the sodium channels were to be open thereby decreasing membrane excitability.

CV increased with time in culture with maximum velocities recorded on days 3-4 post-seeding. The gradual increase in CV over time could be due to cells needing time to recover from the process of trypsinisation. Contraction of the HL-1 cells depends very much on their developmental stage. Over time cells become more mature and the localised activation sites recruit more neighbouring cells with more cell-to-cell contact, which could affect CV. Usually after day 4, a decrease in CV was observed. This could be attributed by cell growth and cultures becoming too dense. We know that connexin expression levels are reduced by at least 50% when cells are overgrown thereby reducing the intercellular coupling between cells. It is also possible that cells become damaged over time. Studies on the original HL-1 cell line also reported an increase in cell degeneration with myolysis and membrane blebbing that steadily increased with time in culture (Hong et al. 2008). Effects of cell density on ion channel expression are not known and will also need to be tested in high and low density cultures to further explain these differences in CV over time.

When comparing the maximum CV in the HL-1 clones, clone 2 displayed the largest difference with significantly lower CV with the other clones. To confirm the role gap junctions play in conduction, uncoupling agents such as palmitoic acid and carbenoxolone should be used and the CV measured (Rohr et al. 1997; Spray et al. 2002). In this study only heptanol was applied to the cell preparations which resulted in a complete but reversible block in conduction (data not shown). It would also be useful to use the antiarrhythmic peptides AAP10 and ZPP123 (for further details see Chapter 1, section 5.5) on clone 2 to enhance gap junctional communication and measure its effects on CV (Clarke et al. 2006; Easton et al. 2009).

In Chapter 3 we reported no difference in current density of the voltage-gated ion channels (Na^+ , L- and T-type Ca^{2+}) and intracellular Ca^{2+} release in both clones 2 and 6 although we did find differences in resting membrane potential with clone 2 exhibiting a more depolarised potential. The lack of action potential recordings from clone 2 could be attributed to cell density problems since the cells clearly displayed spontaneous field potentials from a confluent monolayer. In Chapter 4 differences in connexin expression

were observed with clone 2 expressing significant lower amounts of Cx40 and Cx43 compared with clone 6. Since gap junctions are key contributors to the spread of charge in action potential propagation, RNA interference (RNAi) was used to study the role of connexin expression in clone 6. The next chapter describes the optimisation of the RNAi technique using short interfering RNA to knock-down connexin expression.

CHAPTER 6.
**Applying RNA interference to knockdown connexin
expression**

6.1. Introduction

RNA interference (RNAi) was employed to knockdown specifically the expression of connexins and establish their respective importance in action potential propagation. RNAi is a naturally occurring post-transcriptional gene silencing mechanism employed by the cell to mediate gene regulation. The pathway is triggered by small RNA molecules that cause either a translational block or degradation of mRNA homologous in sequence to the RNA molecules. Post-transcriptional gene silencing was originally observed in plants where it was termed ‘co-suppression’ (Napoli et al. 1990) and later correctly described by Fire et al. in 1998 in *C. Elegans* as homology-dependent gene silencing. Since then similar findings have been described in several organisms including fruit flies (Kennerdell and Carthew 1998), mice (Lewis et al. 2002) and human cells (Elbashir et al. 2001a).

In animals and plants the two major classes of RNA molecules that regulate gene expression are: (1) short interfering RNA (siRNA) exogenously excised from long double-stranded (ds) RNA derived from foreign nucleic acids such as viruses and transposons functioning as part of the innate cellular defence mechanism (Carthew and Sontheimer 2009) and (2) microRNA (miRNA) which is non-coding RNA processed from stem-loop precursors transcribed in the nucleus to regulate endogenous gene expression (Filipowicz et al. 2008; Bartel 2009). Despite these differences in origin both processes require the cleavage of dsRNA by the ribonuclease III protein Dicer (Bernstein et al. 2001) producing RNA duplexes 21-23 nucleotides in length with dinucleotide overhangs at their 3’ end and monophosphate groups at their 5’ end (Zamore et al. 2000; Elbashir et al. 2001b). The 3’ overhangs in the RNA duplex provides a recognition site for the nuclease-containing multi-protein structure called the RNA induced silencing complex (RISC) (Hammond et al. 2000; Martinez and Tuschl 2004). The RNA duplex enters the RISC assembly pathway through the unwinding of its ds by RNA helicase in an ATP-dependent step culminating in the stable association of one of the two strands with the Argonaute effector protein (Zamore et al. 2000; Nykanen et al. 2001). Strand selection is dictated by the relative thermodynamic stabilities of the two duplex ends (Carthew and Sontheimer 2009). This guide strand directs mRNA target recognition by Watson-Crick base pairing, whereas the other strand of the original small RNA duplex is discarded. In the final step siRNA guide

strands direct the RISC to (1) perfectly complementary RNA targets, which are then degraded by the RISC-associated endoribonuclease (Martinez et al. 2002b; Schwarz et al. 2004) or (2) mismatches between the siRNA/target duplex leading to the suppression of mRNA translation or exonucleolytic degradation.

In mammalian cells, RNAi can be induced artificially by the introduction of synthetic ds siRNA molecules (21-23 nucleotides) the preferred approach for inducing RNAi in this study, long dsRNA (>30 nucleotides), or by transduction of plasmid or viral vector systems that express short hairpin RNA (shRNA) which are subsequently processed to siRNA by the cellular machinery. A schematic representation of the RNAi mechanism is illustrated in Figure 6.1.

The rapid development of the RNAi technique has been enhanced by a more complete knowledge of gene sequences. However its application in mammalian cells has been hindered by an anti-viral interferon response triggered in the presence of long dsRNA molecules resulting in the cessation of all protein synthesis (Clemens and Elia 1997; Player and Torrence 1998). However Elbashir et al. demonstrated that the interferon response and the subsequent non-specific degradation of mRNA, was only triggered by dsRNA longer than 30 nucleotides and dsRNA of 19-21 nucleotides in length were sufficient for RNAi activity (Bass 2001; Elbashir et al. 2001a; Caplen 2002). Collectively these findings have enabled the application of RNAi in mammalian cells and its potential use in therapeutic treatments including malignant, infectious and autoimmune diseases (McManus and Sharp 2002; Lieberman et al. 2003; Sioud 2004).

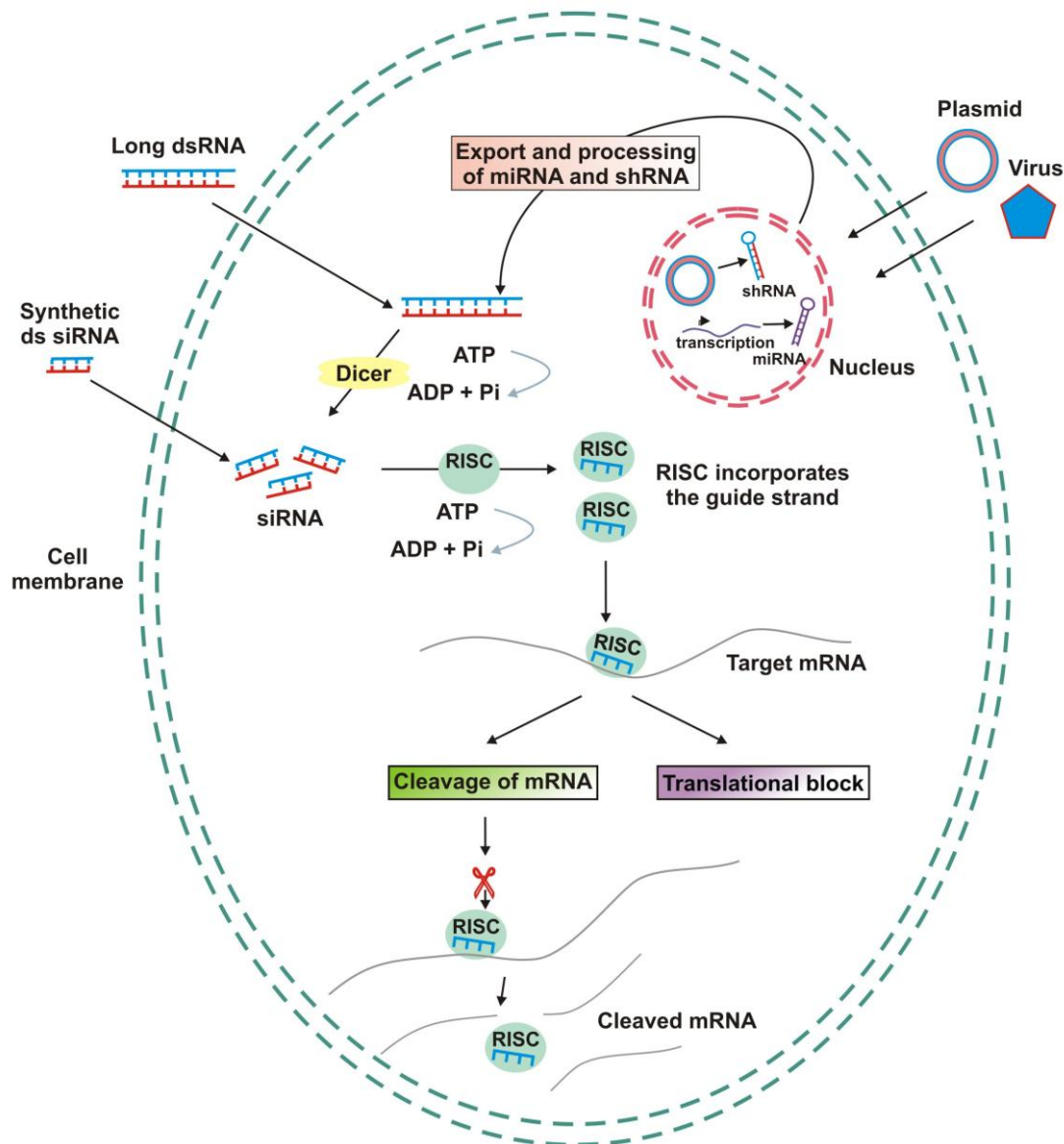


Figure 6.1 Schematic representation of the RNAi pathway

RNAi is a naturally occurring pathway that can be activated by: (1) siRNA produced in response to a virus; (2) miRNA to regulate gene expression and (3) artificially either by synthetic double-stranded (ds) siRNA molecules (19-21 nucleotides in length), long dsRNA molecules or through short hairpin RNA (shRNA) expressed in plasmid or viral vector systems. miRNA and shRNA are produced in the nucleus as hairpin structures which are then exported and processed into long dsRNA molecules. dsRNA are cleaved into 21-23 nucleotide long RNA molecules by the enzyme Dicer in an ATP-dependent step. The processed siRNA is then incorporated into the RISC multi-protein complex which contains a helicase that unwinds the duplex. The single strands of the RNA the duplex are then able to guide the RISC complex to the target mRNA. In the final step selective gene silencing is achieved by one of two ways (1) the target mRNA is either degraded by the RISC-associated endoribonuclease or (2) repression of mRNA translation.

One of the main challenges in using RNAi as a tool to study gene function is the successful delivery of siRNA into cells. A variety of strategies have been developed for *in vitro* delivery of dsRNA either by transfection (e.g. electroporation and lipid-mediated reagents) or through plasmid and viral vectors expressing shRNA (e.g. adenovirus and lentivirus). Each of the techniques has its benefits and drawbacks. Electroporation was the transfection method used in this study because it is a convenient and non-toxic delivery strategy of siRNA molecules into cells. It is also the method of choice for *in vitro* delivery of a large variety of molecules from ions to drugs, antibodies, cytokines and plasmid DNA and *in vivo* drug delivery to malignant tumours (Gehl 2003; Mir 2009). siRNA are small (21 nucleotides) and the molecules only need to be delivered into the cytoplasm to be active. Thus electroporation conditions can be less extreme, requiring lower voltages and shorter pulse durations. An alternative strategy is using lentiviral particles expressing shRNA that can now be purchased as transduction-ready viruses for a target gene in any mammalian cell type including primary and non-dividing cells. The benefits of using shRNA lentiviral particles include high transduction efficiency and the possibility of developing a stable expression of the shRNA while retaining high cell survival. Stable gene silencing for longer periods of time may also be advantageous if the target protein has a slow rate of turnover. A problem of this method is that integration of the transgene is random, so the silencing efficiency can vary considerably depending on the integration site. However this can be overcome by selection of the clones using an antibiotic resistance.

Another important aspect for induction of successful RNAi is the effectiveness of the siRNA sequence against the mRNA target. With siRNA molecules being commercially available, some empirical rules for the basic structure of effective siRNA sequences have emerged (Reynolds et al. 2004; Luo and Chang 2004; Elbashir et al. 2002). Such guidelines have been taken into account in the development of siRNA design tools (*i.e.*, algorithms) that are able to predict possible siRNA candidates for a target gene. Some of the rules include: (1) avoiding target regions where the mRNA can form a hairpin structure affecting siRNA accessibility; (2) selecting a target site in a gene that is only specific to its sequence; (3) low guanine-cytosine content (less than 50%) to reduce thermal stability and facilitate the unwinding and incorporation of the strands into RISC (Khvorova et al. 2003; Schwarz et al. 2004) and (4) keeping the length of dsRNA to 21-23 nucleotides preventing an interferon response.

In this chapter we only discuss the optimisation for the connexin40 (Cx40) and Cx43 siRNA sequences. As mentioned in Chapter 4 we were unable to detect Cx45 by western blot, therefore the effectiveness of Cx45 siRNA sequences were characterised functionally using microelectrode arrays (MEA). This will be discussed in greater detail in Chapter 7. Several preliminary steps were required before RNAi could be used reliably to specifically knockdown connexin expression. The first stage of my investigation was to optimise: (1) the siRNA transfection efficiency of the different siRNA sequences available; (2) electroporation parameters to ensure high transfection efficiency with minimal cell death; (3) the concentration of siRNA required to maintain gene silencing without causing cell toxicity and off-target effects and (4) the duration of the silencing effect.

6.2. Materials and methods

The HL-1 clones were cultured using the methods described in Chapter 2, section 2. Effects of RNAi on connexin protein expression were detected by western blot, as described in Chapter 2, section 5. Immunofluorescent labelling and confocal microscopy was used to assess the specificity of connexin down-regulation according to the methods described in Chapter 2, section 5.

6.2.1. siRNA sequences

Pre-designed siRNA sequences (*Silencer*[®] Select Pre-designed & Validated siRNA) were purchased from Ambion. siRNA sequences were designed by applying an algorithm optimized to produce highly specific and efficient siRNA molecules that cause minimal off-target effects. The sequences used were chemically synthesised, high-performance liquid chromatography-purified (>95% purity) in the form of annealed ds molecules ready for transfection. For Cx40 and Cx43 three different molecules targeting different locations of the respective mRNA sequence were tested for the best knockdown efficiency by western blot analysis. A range of siRNA concentrations (0.25-2 μ M) was also tested for the best down-regulation and for any non-specific effects on gene expression. A summary of the siRNA sequences tested in this study are listed in Table 6.1.

Table 6.1: List of Cx40 and Cx43 siRNA sequences tested

Name	Abbreviation	Sequence	Homology (%)
Mouse Cx40-siRNA	A	Cx40mRNA 1034 1052 5'-GGCUUCAUCCACAUGCACUtt-3' 3'-ctCCGAAGUAGGUGUACGUGA-5'	100
	B	Cx40mRNA 3059 3077 5'-GGAAAUACUAUGUUUGACUtt-3' 3'-ctCCUUUAUGAUACAAACUGA-5'	100
	C	Cx40mRNA 1108 1126 5'-GGGCUACCAUAGUGACAAAAtt-3' 3'-tcCCCGAUGGUUAUCACUGUUU-5'	100
Mouse Cx43-siRNA	A	Cx43mRNA 1126 1144 5'-GCGCAGAGCAAAAU CGAAUtt-3' 3'-gtCGCGUCUCGUUUUAGCUUA-5'	100
	B	Cx43mRNA 1579 1597 5'-GCUUAAAUUCUAGUAAGAGtt-3' 3'-ctCGAAUUUAAGAUCAUUCUC-5'	100
	C	Cx43mRNA 1106 1124 5'-GCAAAACUGGGCGAAUUACtt-3' 3'-tgCGUUUUUGACCCGCUUAUG-5'	100
Rat Cx43-siRNA		Cx43mRNA 1046 1064 5'-GCUGGUUACUGGUGACAGAtt-3' 3'-ttCGACCAAUGACCACUGUCU-5'	94

The siRNA used in this study were pre-designed to be 21 nucleotides in length with the dinucleotide 3' overhangs that can be incorporated directly into the RISC complex by-passing the initial processing step involving the Dicer. Either of the two strands from the ds siRNA were able to guide the RISC to the target mRNA in a complementary driven fashion. Homology searches were performed with the basic local alignment search tool, BLAST (<http://www.ncbi.nlm.nih.gov/blast/Blast.cgi>). The location where each of the above siRNA sequences binds Cx40 mRNA and Cx43 mRNA is shown by the highlighted numbers.

6.2.2. siRNA delivery

Electroporation requires the application of a pulsed electrical field to induce the reversible breakdown of the lipid bilayer creating temporary pores (40-120nm in diameter) in the plasma membrane that re-seal within seconds (Neumann et al. 1982). This process permeabilises the host cells to a wide variety of molecules such as siRNA. For small molecules, diffusion alone may be sufficient for intracellular uptake (Neumann et al. 1998). In our study we used a capacitance to increase the pulse duration and provide an electrophoretic drive for the negatively charged siRNA to enter the cells. Although electroporation is a convenient and efficient method of delivery, cell viability is a crucial aspect and is dependent on the experimental conditions used (e.g. voltage parameter) and the parameters are specific for each cell type. Thus a range of electroporation conditions including the field strength (voltage) and pulse lengths (capacitance) were tested to ensure maximum transfection efficiency and cell recovery.

Cells were cultured to sub-confluency in a 75cm² flask (~8.5 x 10⁶ cells at confluency) and were no more than 4-5 days old. The cells were treated with trypsin, collected by centrifugation (130gmax, 5 minutes) and re-suspended in a volume of 1.2ml in serum free Claycomb medium (SFM) to give an approximate density of 2x10⁶ cells per 400µl SFM. siRNA molecules were purchased as a powder form of the dried oligonucleotides which were then re-suspended in nuclease-free water (in accordance to manufacturer's instructions) to give a final concentration of 100µM. siRNA was gently mixed with the ~2 x 10⁶ cells in a volume of 400µl (to give a final concentration of 0.25-2µM depending on the experiment) in a sterile electroporation cuvette, where the two metallic plates are separated by 0.4cm and pulsed using an Electro Cell Manipulator 630 (PrecisionPulse, Harvard Apparatus). During a single experiment all siRNA treatments were pulsed using the same electroporation cuvette to minimise any variability of the electrical field. Between siRNA treatments the cuvette was thoroughly rinsed with sterile distilled water to remove any remnant of siRNA and transfected cells from the previous electroporation. Although parameters for electroporation had been developed previously in our laboratory for smooth muscle cells, re-optimisation was necessary to maximise siRNA delivery and minimise cell death. Thus cells were subjected to a range of voltages and capacitance and the time constant (τ) recorded after electroporation. The time constant is the length of time required for the voltage in the

circuit to fall exponentially ($1/e$) by approximately 63% of its initial value. The time constant is dependent on the volume in the electroporation cuvette and the conductivity of the medium.

Following transfection, cells were removed from the cuvette by gentle pipetting and seeded in a 6-well plate. Cells were seeded at confluency since these cells are mildly 'contact-inhibited' making them less likely to divide and prevent any dilution of the siRNA. The medium was changed 3-4 hours post-seeding to remove any dead cells and cellular debris caused by the electroporation and incubated in an atmosphere of 5% CO₂ at 37°C for a minimum of 48 hours to recover from electroporation injury. The effects of electroporation on cell morphology and survival rate were examined visually using a phase contrast microscope 24-48 hours post-seeding. siRNA knockdown was assessed by western blot procedure in cells harvested 48-72 hours post-transfection.

6.3. Results

6.3.1. Identification of siRNA sequences that effectively knockdown connexin expression

Clone 6 was transfected with three different siRNA molecules (2 μ M) for Cx40 and Cx43 and the best down-regulatory effects on connexin expression examined. The electroporation parameters (250V and a 25ms time constant) and the siRNA concentration used had been previously established in our laboratory (2 μ M). A typical example of a western blot obtained from the same batch of cells exposed to different siRNA sequences is shown in Figure 6.2 (A).

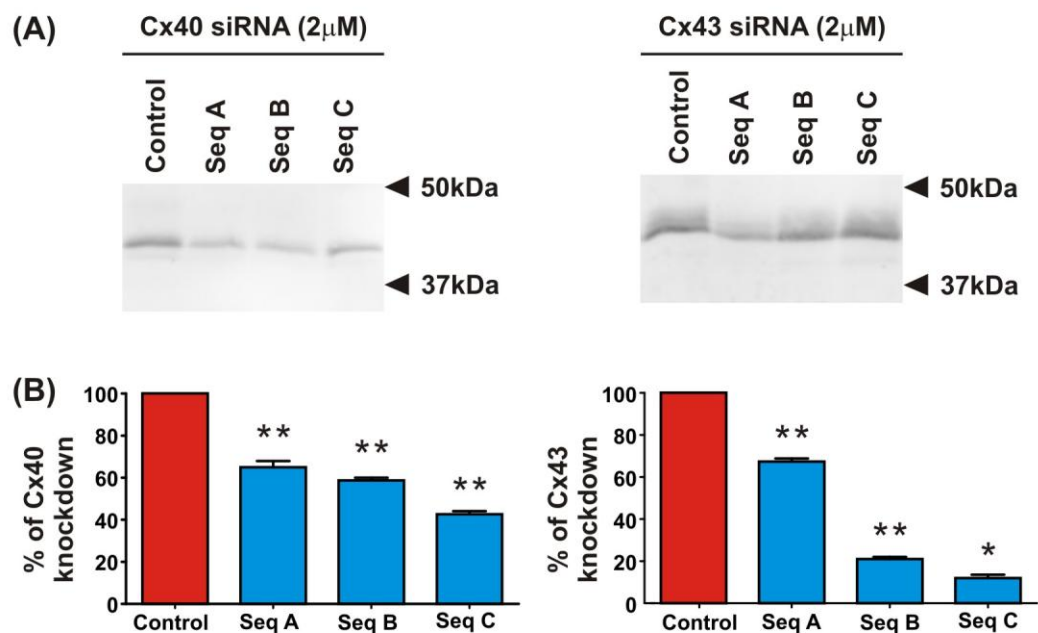


Figure 6.2: Western blot analysis of the ability of different siRNA sequences to knockdown Cx40 and Cx43 expression

Cells were treated with three different siRNA sequences, A (Seq A), B and C (2 μ M) for both Cx40 and Cx43 (A). Knockdown efficiency was compared to the expression levels in the control sample (cells electroporated without siRNA) which was regarded as 100%. For Cx40, all three sequences used produced significant down-regulatory effects (n = 3; ** p < 0.001) in comparison to the control. Similar to Cx40, the three sequences used for Cx43 also resulted in significant protein knock down (n = 3; * p < 0.05; ** p < 0.001).

All down-regulatory effects through siRNA were compared to connexin expression levels in the control sample, which were cells electroporated without siRNA. The immunoblots were quantified by densitometric scanning and a summary of the results obtained from the different siRNA sequences for Cx40 (n = 3) and Cx43 (n = 3) is shown in Figure 6.2 (B). Although all the siRNA sequences used produced significant down-regulatory effects (p < 0.05), for the remainder of the investigation only sequences A were used for both Cx40 and Cx43 siRNA.

6.3.2. Optimisation of siRNA down-regulation for Cx40 and Cx43

The electroporation parameters used lead to approximately 50% cell death. To account for this, twice the number of cells normally required to seed a 6-well plate, were seeded to produce comparable densities to the seeding control. To optimise parameters for siRNA transfection and reduce cell death, a range of different voltages 200V-280V and

capacitances 100-1000 μ F were tested. Table 6.2 lists a summary of the key voltages and capacitances tested with their corresponding effects on cell death.

Table 6.2: Range of pulse durations obtained with the varying capacitances

Volume of cells used (μl)	Voltage (V)	Capacitance (μF)	Time constant (ms)	Cell death (%)
400	250	1000	~25	75
400	200	1000	~25	50
400	280	400	~10	50

The images recorded 24-48 hours post-seeding demonstrated that voltage appeared to be a greater determinant on cell survival than the time constant, which is controlled by the size of the capacitance. We also found that cell death was exclusively caused by the electroporation procedure and not from the siRNA. As a result the voltage was reduced to 200V with the same time constant of 25ms. This not only reduced cell death (Figure 6.3 (A)) but also the transfection efficiency of the siRNA (Figure 6.3 (B)). Thus voltage was a greater determinant of siRNA delivery than the capacitance but the disadvantage being more cell death (30-40% cell survival). Previous experiments have shown that reducing the time constant can also decrease cell death (data not shown). Therefore we chose to increase the voltage to 280V, to improve siRNA efficiency and decrease the time constant to ~10ms. When these values were used, maximum siRNA efficiency and minimum cell death were achieved (Figure 6.3 (B)).

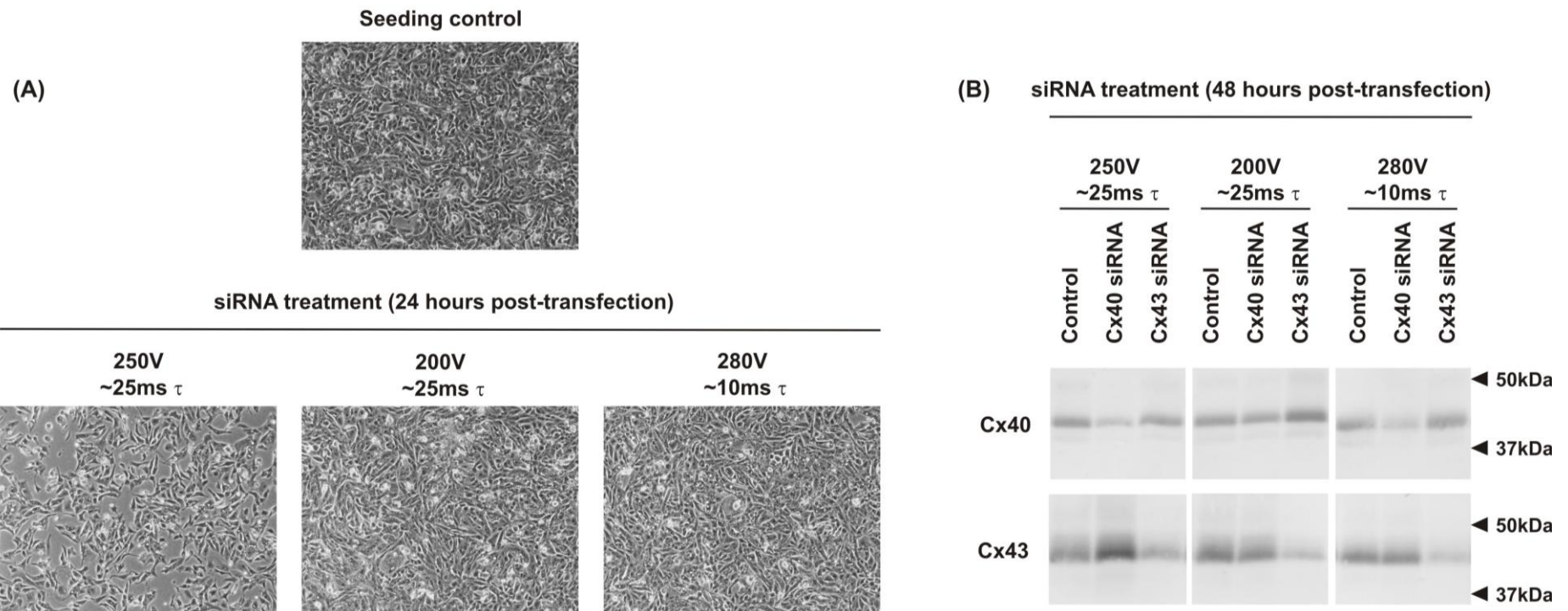


Figure 6.3: Optimisation of electroporation parameters

Cells were electroporated at three different voltages (200V/250V/280V) and two different capacitances (1000 or 400 μ F per 400 μ l of cells). The phase contrast micrographs show the effects of the different electroporation parameters on cell death in comparison to the seeding control 24 hours post-transfection (A). The transfection efficiency of the different parameters for both Cx40 and Cx43 siRNA delivery was assessed by western blot technique 48 hours post-transfection (B). The electroporation parameters used and the time constant (τ) obtained are indicated above the images for (A) and (B). The different siRNA treatments are also indicated above the immunoblots for (B). Results indicate that siRNA delivery was greatest and cell death was minimal at 280V and 400 μ F per 400 μ l of cells.

These results were not only reproducible within the same clone but the electroporation parameters could also be applied to a different clone such as clone 3 and achieve similar results in knockdown efficiency as shown in Figure 6.4.

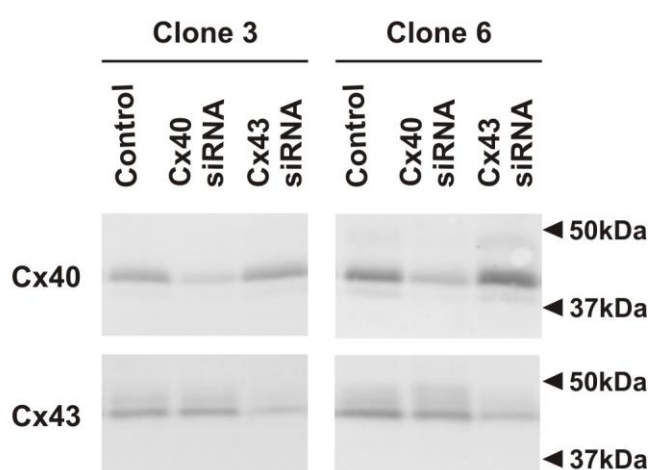


Figure 6.4: Cx40 and Cx43 knockdown in HL-1 clones

Two different HL-1 clones were treated with Cx40 and Cx43 siRNA (2 μ M) using the same optimised electroporation parameters. Clone numbers and the different treatments are indicated above the immunoblots. Controls were cells electroporated without siRNA. Efficiency of connexin knockdown was similar in both clones.

To minimise any off-target effects and/or an interferon response from the siRNA it was important to use the lowest and most effective siRNA concentration. A range of concentrations from 0.25-2 μ M were tested for Cx40 and Cx43 down-regulation in clone 6 (Figure 6.5). Levels of Cx40 expression were reduced significantly at 2 μ M (~60%). This knockdown effect gradually declined as the siRNA concentration decreased. A similar pattern was also observed for Cx43 siRNA with maximal effect shown at 1-2 μ M (60-70%). Knockdown of either connexin did not appear to affect the expression of the co-expressed connexin.

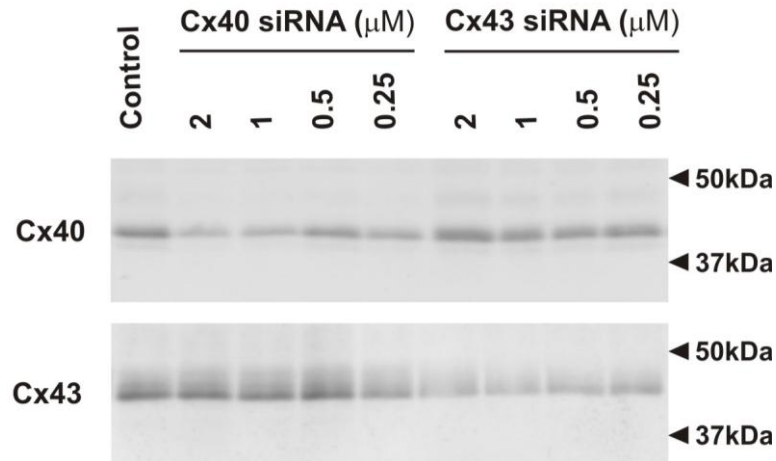


Figure 6.5: Western blot of siRNA dose response for Cx40 and Cx43 siRNA

Cells were treated with a range of siRNA concentrations for both Cx40 and Cx43 (0.25-2μM). siRNA treatments were compared to the control of cells electroporated without siRNA. The specific siRNA treatment and the concentrations used are indicated above the immunoblots. Maximal effects of siRNA knockdown for both Cx40 and Cx43 were obtained at 2μM. Knockdown of either connexin had no effect on the co-expressing connexin.

To visualise the effects of Cx40 and Cx43 siRNA and verify that knockdown of either connexin did not affect Cx45 expression, clone 6 was transfected simultaneously with Cx40 and Cx43 siRNA and analysed by immunofluorescence. Double labelling of Cx40 and Cx43 showed that after siRNA treatment many of the cells lack Cx40 and Cx43 compared to electroporated control (Figure 6.6 (A) and (B)). However a small proportion of the cells expressed low levels of Cx40 and Cx43 within the cytoplasm and junction indicating that the connexins had not yet been degraded or that some cells were not efficiently transfected. This also further demonstrates that the siRNA technique is a form of protein knockdown rather than a knockout. Figure 6.6 (C) demonstrated that in the presence of Cx40 and Cx43 siRNA, Cx45 was still detected at the protein level indicating the specificity of the siRNA technique. To show species specificity, cells (mouse cell line) were transfected with a rat Cx43 siRNA (one base mismatch with Cx40 mouse mRNA) that was effective in the rat epithelial cell line (RLE) (personal communication with Dr K. Grikscheit). The rat Cx43 siRNA had no effect on Cx43 expression levels (data not shown).

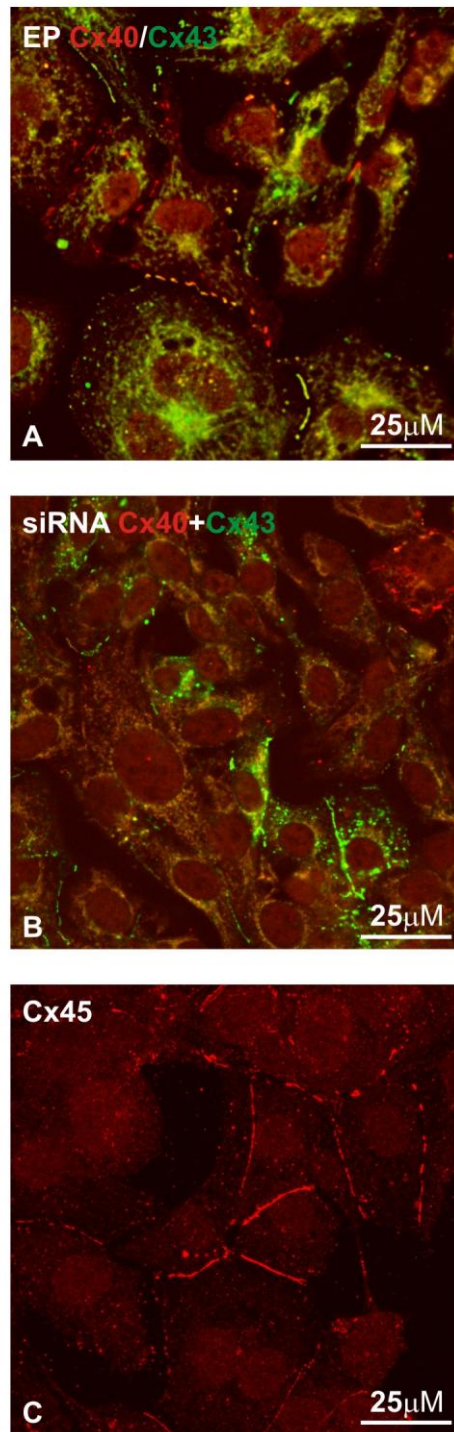


Figure 6.6: Immunofluorescence analysis of Cx40 and Cx43 knockdown in clone 6

Double labelling of Cx40 (red) and Cx43 (green) after electroporation (EP) (A) and treatment with Cx40 and Cx43 siRNA (2µm) (B). After siRNA treatment many of the cells lack Cx40 and Cx43 at the junction. Knockdown of either connexin did not affect Cx45 expression (C).

To assess the duration of connexin knockdown cells were treated with Cx40 and Cx43 siRNA (2 μ M) and evaluated at days 2 and 3. Western blot analysis for the time course of connexin suppression is presented in Figure 6.7 (A). Quantification of Cx40 and Cx43 siRNA over the two days (Figure 6.7 (B)) indicated that the siRNA mediated effects was most prominent on day 2 in contrast to day 3 but connexin suppression was still maintained at over 50%.

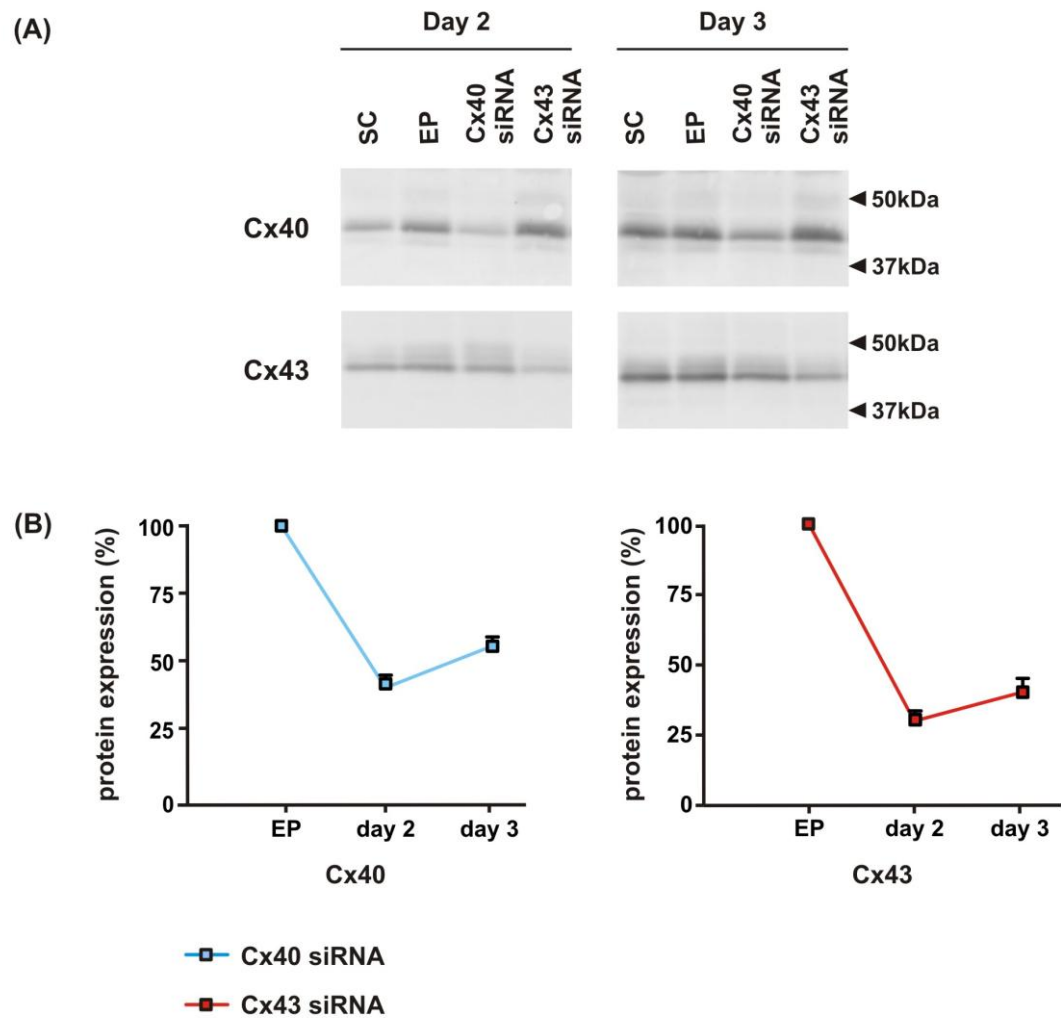


Figure 6.7: Western blot analysis of Cx40 and Cx43 suppression over time

Cells were electroporated with Cx40 and Cx43 siRNA (2 μ M) and detected for both connexins at days 2 and 3 post-transfection (n = 3) (A). siRNA was able to down-regulate both Cx40 and Cx43 by over 60% in comparison to the seeding and electroporated controls at day 2 (B). Although connexin expression levels increased at day 3, knockdown of either connexin was still maintained at over 50% (B).

6.4. Discussion

There are currently a range of techniques available to study connexin gene function including the transfection of cell lines (Halliday et al. 2003), the use of mutant constructs through molecular cloning (Joshi-Mukherjee et al. 2008) and cells from animal knockout studies (Beauchamp et al. 2004). In this investigation we have used RNAi to knockdown and study connexin(s) expression because of its specificity against a target gene. Before connexin function could be assessed, siRNA delivery and transfection efficiency needed to be optimised to give maximum protein suppression and minimum cell death.

An important aspect for the activation of RNAi is the effectiveness of the siRNA sequence used against the target mRNA. Although Ambion guarantee that at least one of the three siRNA sequences purchased should knockdown either Cx40 or Cx43, their effectiveness in reducing protein expression still needed to be tested. We found that sequence B was most effective in reducing Cx40 expression and sequence A for Cx43 and the electroporation procedure itself did not affect connexin expression.

Previous studies in our laboratory have shown that siRNA delivery by chemical transfection caused a wide range of side effects depending on the cell type (Charolidi 2008). This led us to explore the physical delivery method of electroporation. A key advantage of electroporation over other methods is that electropermeabilization is independent of the cell-cycle stage making it possible to deliver siRNA to non-dividing cells or slowly dividing cells (Ovcharenko et al. 2005). It also requires fewer pipetting steps thereby reducing variability of procedures and is more cost effective when compared with chemical transfection.

The optimum electroporation parameters are generally specific for each cell type. The key parameters that influence electroporation efficiency are the voltage and the pulse-length. From the range of voltages and capacitances tested, the optimum electroporation parameters for siRNA delivery in the HL-1 clones were a high voltage and low capacitance. We found that the voltage, which presumably determines the pore size and number of pores formed in the plasma membrane, was the more important parameter in determining the effectiveness of transfection. However an increase in voltage caused a significant decrease in cell survival. This negative effect on cell viability was minimised

by decreasing the pulse lengths (capacitance) of the electrical field. Both the size of the electrical field and the pulse length are also critical in ensuring cell recovery and membrane pore re-sealing. When the pores are open for a prolonged amount of time cellular toxicity may be increased by the influx of external medium (Bonnafeous et al. 1999). In extreme conditions, intracellular content may leak out due to osmotic swelling, leading to cell death (Gehl 2003). It is also important to ensure the parameters are not too extreme on the cellular structure since intact function of the cytoskeleton is important for pore closure (Teissie and Rols 1994).

Reducing cell death was crucial to enable the transfected cells to be studied on MEA as part of the functionality study and allow a direct comparison with the siRNA treated cells prepared for western blot analysis. This aspect of the study will be discussed in greater detail in Chapter 7. To verify the voltage and time constant were optimal in siRNA delivery, two of the HL-1 clones were transfected with Cx40 and Cx43 siRNA and comparable results were obtained. These parameters were also used on the RLE cell line by a colleague in the laboratory (Grikscheit 2010) to reduce Cx43 expression.

siRNA is highly specific, but its use at high concentrations may cause non-specific target effects (e.g. interferon response) or off-target effects that include any alterations of non-target genes (Shrey et al. 2009). It was also important to check for siRNA mediated toxicity since a potential disadvantage of electroporation is that it requires higher amounts of siRNA for successful gene silencing in comparison to chemical transfection (Ovcharenko et al. 2005). At all the siRNA concentrations tested (0.25-2 μ M) knockdown of either Cx40 or Cx43 did not affect cell survival and the protein expression of the co-expressed connexin. Species specificity was demonstrated using a rat Cx43 siRNA that contained a single mismatch with the mouse Cx43 mRNA sequence demonstrating that 100% homology of the siRNA-target mRNA duplex is required to activate RNAi (Tuschl et al. 1999; Elbashir et al. 2001c).

The effectiveness of siRNA is transient and is also dependent on the half-life of the target protein (Kurreck 2009). A pronounced inhibition of protein expression was observed within 48 hours post-transfection. The level of inhibition decreased at day 3 but a significant level of down-regulation was still maintained. This may be expected since the half-life of connexins is short ranging from 1-5 hours (Laird 2006). A

reduction in the effectiveness of siRNA may also be caused by cell division. It was also important the duration of gene silencing was sustained over days 2 and 3 for MEA recordings, which was the primary technique used to study connexin function.

In conclusion siRNA parameters were optimised in terms of: (1) siRNA sequences used for their best down-regulatory effects; (2) electroporation parameters to maximise siRNA transfection efficiency and minimise cell death and (3) siRNA concentration for best knockdown without causing any off-target effects. The next step was to apply siRNA in the clone 6 to study the role(s) of the individual connexins in relation to action potential propagation, which will be discussed in the following chapter.

CHAPTER 7.

Functional effects of connexin suppression on action potential propagation

7.1. Introduction

In Chapter 5, it was shown using microelectrode arrays (MEA) that the HL-1 clones displayed different conduction velocities (CV) particularly clones 2 and 6, which also displayed significant differences in connexin expression. To examine the role played by each connexin and their various combinations in action potential propagation, we used RNA interference (RNAi) optimised and detailed in Chapter 6 to knockdown connexin(s) expression. Changes in CV caused by altered connexin expression levels were assessed by MEA.

Gap junctions play a key role in mediating electrical coupling in the heart by permitting low resistance pathways for action potential propagation (Rohr 2004). Connexin43 (Cx43) is the principal cardiac connexin expressed throughout the atrial and ventricular myocardium; Cx40 is typically expressed in atrial tissue and in the His bundle and Purkinje fibres; Cx45 is present in the sinoatrial and atrioventricular nodes and at very low levels in the ventricular myocardium where it co-localises with Cx43 (van Veen et al. 2001; Severs et al. 2008). The biochemical and electrical characteristics of gap junction channels depend on their constituent connexin subtypes. Cx40 forms channels with the highest conductance of 150-200pS followed by Cx43 with a conductance of 60-120pS and Cx45 that forms low conductance channels of 22-39pS depending on the species and experimental conditions (van Veen et al. 2001). These properties confer distinctive passive electrical properties to specific regions of the heart (Vozzi et al. 1999).

Changes in cell-to-cell coupling through alterations in gap junction organisation and connexin expression contribute to abnormal impulse propagation as shown by theoretical modelling (Shaw and Rudy 1997; Spach et al. 2001) and in both human (Dupont et al. 2001b; Kanagaratnam et al. 2002) and animal knockout models (Gutstein et al. 2001a; Leaf et al. 2008; Bagwe et al. 2005). For example, patients with congestive heart failure have reduced levels of CX43 and increased heterogeneity of CX43 expression. These changes in connexin expression would result in slow macroscopic conduction and inhomogeneous wavefront propagation increasing the susceptibility to arrhythmias (Dupont et al. 2001b; Yamada et al. 2003). Thus the precise relationship

between Cx40, Cx43 and Cx45 in action potential propagation is critical for understanding the role of connexin expression in arrhythmogenesis.

Our goals in this study were to: (1) identify the most effective siRNA sequence in Cx45 knockdown; (2) assess the role of Cx45, Cx40 and Cx43 in impulse propagation; (3) assess the effects of knocking down more than one connexin subtype (Cx40+Cx43; Cx40+45; Cx40+43+45; Cx40+Cx43+Cx45) and (4) quantify the relative amounts of Cx40 and Cx43 co-expressed at the junctional and non-junctional fractions after siRNA treatment.

7.2. Methods

The HL-1 clones were cultured using the methods described in Chapter 2, section 2. Small interfering RNA (siRNA) was delivered into cells by electroporation as described in Chapter 6, section 2. The functional consequences after siRNA treatment with Cx45, Cx40 and Cx43 were determined by MEA (Chapter 5, section 2). The siRNA data in this chapter were from electrically stimulated preparations of clone 6 at a frequency of 1-2Hz. MEA recordings were performed 2-5 days post-transfection and only the maximum down-regulatory effects on CV were presented. A list of the Cx45 siRNA sequences used in this study is listed in Table 7.1. Effects of siRNA on the total, junctional and non-junctional connexin expression were detected by western blot (Chapter 2, section 5).

Table 7.1: List of Cx45 siRNA sequences used in this study

Abbreviation	Cx45 sequences	Homology (%)
A 157777	Cx45mRNA 1341 1359 5'-CCAGAUCAGAUCCAGUACAtt-3' 3'-ctGGUCUAGUCUAGGUCAUGU-5'	100
B 157775	Cx45mRNA 922 941 5'-GCCAACC AAAACCUAAGCAtt-3' 3'-ctCGGUUGGUUUUGGAUUCGU-5'	100
C 61750	Cx45mRNA 851 869 5'-GGACCAUGAAGAGGAUCCUtt-3' 3'-tcCCUGGUACUUCUCCUAGGA-5'	100
D 61834	Cx45mRNA 863 881 5'-GGAUCCUAUGAUGUAUCCAAtt-3' 3'-gtCCUAGGAUACUACAUAGGA-5'	100
E 61662	Cx45mRNA 573 591 5'-GGAGAGUCCAUCUACUAUGtt-3' 3'-ctCCUCUCAGGUAGAUGAUAC-5'	100

Homology searches were performed with the basic local alignment search tool, BLAST (<http://www.ncbi.nlm.nih.gov/blast/Blast.cgi>). The location where each of the above siRNA sequences binds to Cx45 mRNA is shown by the highlighted numbers. siRNA sequences C and D overlap part of the mouse mRNA sequence by 6 base-pairs.

7.3. Results

7.3.1. Effects of Cx45 knockdown on Cx40 and Cx43 protein expression

As described in Chapter 4, Cx45 was expressed at levels at least twenty times lower levels compared to Cx40 and Cx43 rendering it difficult to detect the protein by western blot. Because of this, five siRNA sequences targeting different regions of the Cx45 mouse mRNA were used as there was no alternative way of checking and confirming that what we observed by MEA was only caused by Cx45 knockdown. Selection criteria for Cx45 siRNA were based on sequences that caused minimal interference with Cx40 and Cx43 expression levels. Cells were electroporated with Cx45 siRNA using the

optimised transfection parameters of 280V with a time constant of 10ms and scraped on day 2 post-transfection. The total expression levels for Cx40 and Cx43 were detected after Cx45 siRNA by western blot (Figure 7.1 (A)) and quantified using densitometric scanning (Figure 7.2 (B) and (C)). Expression levels of Cx40 and Cx43 in the control, which were cells electroporated without siRNA, were taken as 100%. Thus any effect observed after siRNA treatment was normalised to that level. After Cx45 siRNA, Cx40 expression levels were either comparable to the control or greater, with a maximum increase of 30% observed with sequence A. Similarly following Cx43 siRNA treatment, protein levels were either similar to the control or slightly increased again with the maximum increase of 30% observed with sequence A. Although the total Cx40 and Cx43 expression was not greatly affected after Cx45 siRNA treatment we also tested for any alterations in the junctional and non-junctional fractions for Cx40 and Cx43 using two of the sequences (B and C) and no change was observed (data not shown).

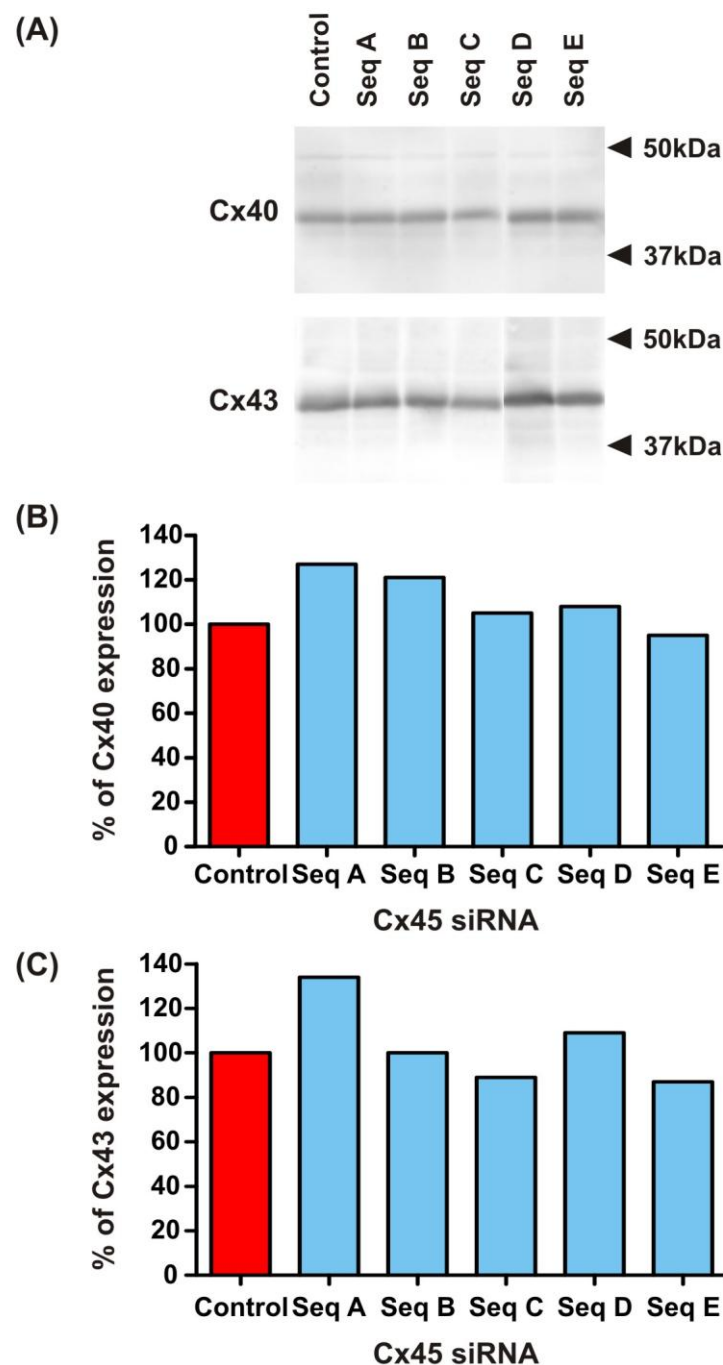


Figure 7.1: Effects of Cx45 knockdown on Cx40 and Cx43 total protein expression

Cx40 and Cx43 total protein levels were measured after treatment with Cx45 siRNA using five different sequences. Cx40 and Cx43 expression after siRNA treatment was normalised to the control sample (cells electroporated without siRNA) and was expressed as a percentage. Overall levels of Cx40 and Cx43 were either comparable to the control or increased by approximately 10-30% depending on the siRNA sequence used. Cx45 siRNA with sequences D and E were done separately to sequences A, B and C with their own controls to which levels of expression were compared to. For the purposes of illustration the immunoblots were cropped with sequences D and E added at the end.

7.3.2. Functional assessment of Cx45 knockdown using MEA

Effects of Cx45 knockdown on action potential propagation were examined by MEA as shown in Figure 7.2. No significant difference ($p = 0.31$) was observed between the seeding (no electroporation) and electroporated (cells electroporated without siRNA) controls indicating that the electroporation procedure itself did not affect action potential propagation (seeding control: 41 ± 3 mm/sec; electroporated only control: 37 ± 2 mm/sec; $n = 8$ and 6 respectively). All five sequences of Cx45 siRNA reduced CV significantly ($p < 0.001$) when compared to the electroporated control. On average propagation velocities decreased from 37 ± 2 mm/sec; $n = 6$ in the electroporated control to 14 ± 6 mm/sec; $n = 3$ in sequence A; 19 ± 2 mm/sec; $n = 3$ in sequence B; 16 ± 5 mm/sec; $n = 3$ in sequence C; 12 ± 2 mm/sec; $n = 3$ in sequence D and 14 ± 1 mm/sec; $n = 3$ in sequence E. This significant reduction in CV ($p < 0.001$) by at least 50% was unexpected considering the low levels of Cx45 expression in clone 6. Although a strong reduction in CV was recorded with all five Cx45 siRNA sequences, only sequences B and C were used for the remainder of this chapter.

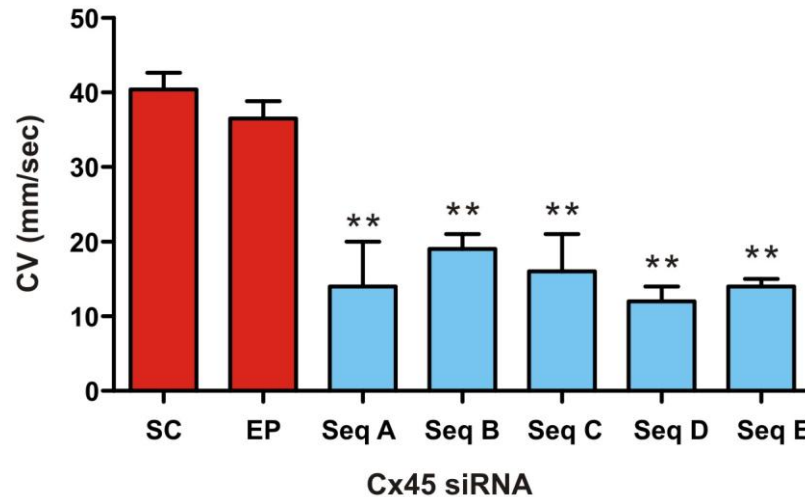


Figure 7.2: Effects of Cx45 knockdown on action potential propagation

Cells from clone 6 were electroporated with five different siRNA molecules of Cx45 and effects on CV were recorded using MEA. CV were comparable between the seeding control (SC) and electroporated (EP) control that were cells electroporated without siRNA (SC: 41 ± 3 mm/sec; EP: 37 ± 2 mm/sec; $p = 0.31$). After siRNA treatment all five Cx45 siRNA sequences (Seq) significantly reduced CV (** $p < 0.001$) by at least 50% compared to both the electroporated control (sequence A: 14 ± 6 mm/sec; $n = 3$; sequence B: 19 ± 2 mm/sec; $n = 3$; sequence C: 16 ± 5 mm/sec; $n = 3$; sequence D: 12 ± 2 mm/sec; $n = 3$; sequence E: 14 ± 1 mm/sec; $n = 3$).

7.3.3. Effects of Cx40, Cx43 and Cx45 knockdown on action potential propagation

The most effective siRNA sequences used for Cx40 and Cx43 knockdown were established in Chapter 6. Using these sequences and those for Cx45 described in the section above CV were recorded over days 2-5 post-transfection. Results show an overall decrease in CV regardless of which connexin or combination of connexins was being used (Figure 7.3). When expression of either Cx40 or Cx43 was down-regulated, an approximately 30% reduction ($p < 0.01$) in CV was observed from the electroporated control (control: 37 ± 2 mm/sec $n = 6$; Cx40 siRNA 24 ± 2 mm/sec; $n = 10$; Cx43 siRNA: 28 ± 2 mm/sec; $n = 8$) (Figure 7.3 (A)). For Cx45 knockdown the average CV recorded from the five siRNA sequences tested showed a 50% reduction in CV (Cx45 siRNA: 16 ± 2 mm/sec; $n = 3$; $p < 0.001$).

After assessing the respective importance of each connexin in impulse propagation, the next step was to use a combination of two or more connexin siRNA (Cx40+43; Cx40+45; Cx43+45 and Cx40+43+45) and measure CV following treatment (Figure 7.3 (B)). Simultaneous knockdown of two connexins irrespective of the connexin subtype

resulted in CV being halved (control: 37 ± 2 mm/sec; $n = 6$; Cx40+43 siRNA 21 ± 2 mm/sec; $n = 3$; Cx40+45 siRNA: 22 ± 1 mm/sec; $n = 3$; Cx43+45 siRNA: 23 ± 1 mm/sec; $n = 3$; $p < 0.001$ using a one-way analysis of variance with Tukey's multiple comparison test). Furthermore a reduction of Cx40, Cx43 and Cx45 at the same time also resulted in CV being halved with no add-on effect of all three connexins being suppressed (Cx40+43+45 siRNA: 23 ± 2 mm/sec; $n = 3$; $p < 0.001$). When using Cx45 siRNA, two different molecules (sequences B and C) were used to study the effects observed by MEA and an average of both these values are presented.

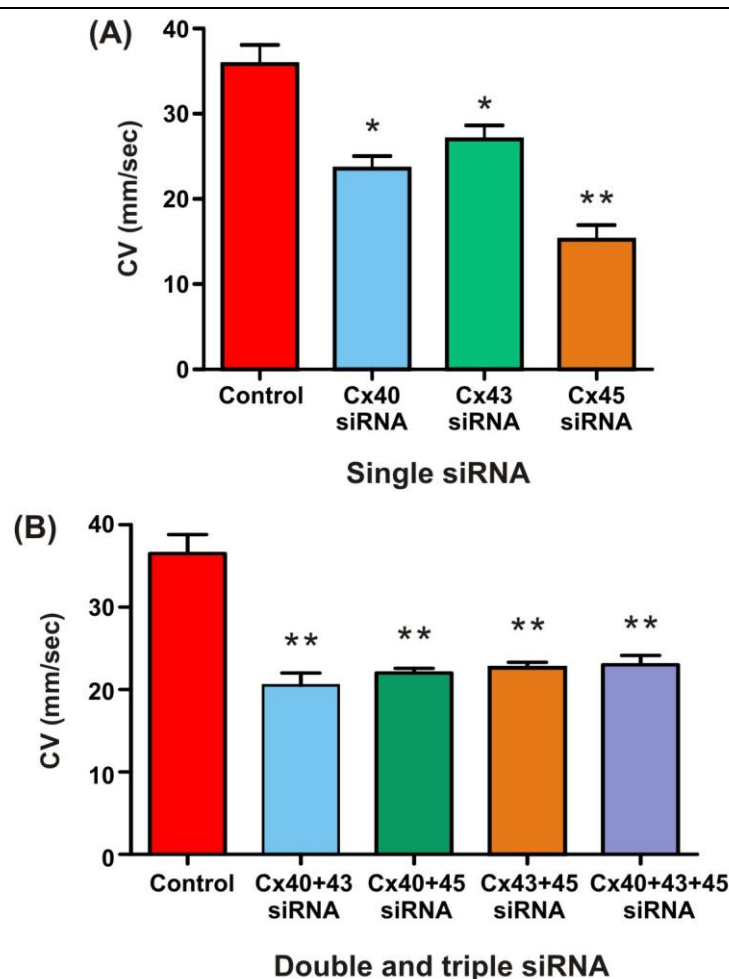


Figure 7.3: Effects of Cx40, Cx43 and Cx45 knockdown on action potential propagation

Cells were transfected with siRNA for Cx40, Cx43, Cx45, Cx40+43, Cx40+45, Cx43+45 and simultaneous knockdown of the three connexins. Effects of siRNA treatment on CV were compared to the electroporated control. Knockdown of Cx40 or Cx43 resulted in a 30% drop in CV (control: 37 ± 2 mm/sec; $n = 6$; Cx40 siRNA 24 ± 2 mm/sec; $n = 10$; Cx43 siRNA: 28 ± 2 mm/sec; $n = 8$) and suppression of Cx45 reduced CV by approximately half (Cx45 siRNA: 16 ± 2 mm/sec; $n = 8$) (A). Comparable results were obtained by simultaneous knockdown of Cx40+43, Cx40+45, Cx43+45 and Cx40+43+45 (Cx40+43 siRNA 21 ± 2 mm/sec; $n = 3$; Cx40+45 siRNA: 22 ± 1 mm/sec; $n = 3$; Cx43+45 siRNA: 23 ± 1 mm/sec; $n = 3$; Cx40+43+45 siRNA: 23 ± 2 mm/sec; $n = 3$) (B). The levels of significance are denoted above each sample (* $p < 0.01$; ** $p < 0.001$).

7.3.4. Quantification of Cx40/Cx43 co-expression ratio after siRNA treatment

To correlate the functional effects of CV with the levels of respective connexin expressed after siRNA treatment it was important to determine the levels of connexin expressed and to quantify the exact ratios of each co-expressed connexin. Effects of

altered Cx40/Cx43 co-expression ratios were examined only because Cx45 could not be detected by western blot.

Figure 7.4 (A) shows western blot analysis of clone 6 after Cx40 and Cx43 siRNA treatment. Clone 6 was electroporated with siRNA and cells were extracted into their junctional and non-junctional fractions on day 2 post-transfection. Using the Cx40- and Cx43-V5tag described in Chapter 2 we were able to quantify the relative amount of Cx40 and Cx43 co-expressed by the cells. Effects of siRNA on protein levels were compared to both the seeding and electroporated controls. Blots show that in the control samples, Cx40 and Cx43 were expressed at comparable levels in the total, junctional and non-junctional fractions. After treatment with the respective siRNA, the total cellular expression (junctional + non-junctional) for Cx40 and Cx43 was significantly reduced by at least 50% ($p < 0.05$). No significant effect was observed in protein expression of the co-expressed connexin ($p > 0.05$).

The relative amounts of Cx40 and Cx43 in the control and siRNA treated samples are shown in Figure 7.4 (B) and (C). Although the bands for total connexins were quantified, only data for the junctional and non-junctional connexins are shown since the signal intensities were equal to the sum of both the fractions. In the electroporated only sample, levels of Cx40 were 25% higher at the junction in comparison to the seeding control but was not considered to be significant ($p = 0.07$). All siRNA effects were compared with the electroporated control. After Cx40 siRNA treatment, the junctional and non-junctional levels of Cx40 were reduced significantly with a more pronounced knockdown in the junctional sample ($p = 0.0011$) demonstrating that knockdown of Cx40 is in fact greater than the values calculated for the cells as a whole. A similar pattern was also observed after Cx43 siRNA with a greater down-regulatory effect at the junction ($p = 0.0019$). Knockdown of either connexin had minimal effects on both the junctional and non-junctional expression levels of the co-expressed connexin ($p > 0.05$). A summary of the quantification data in Figure 7.3 (B) and (C) is presented in Table 7.2.

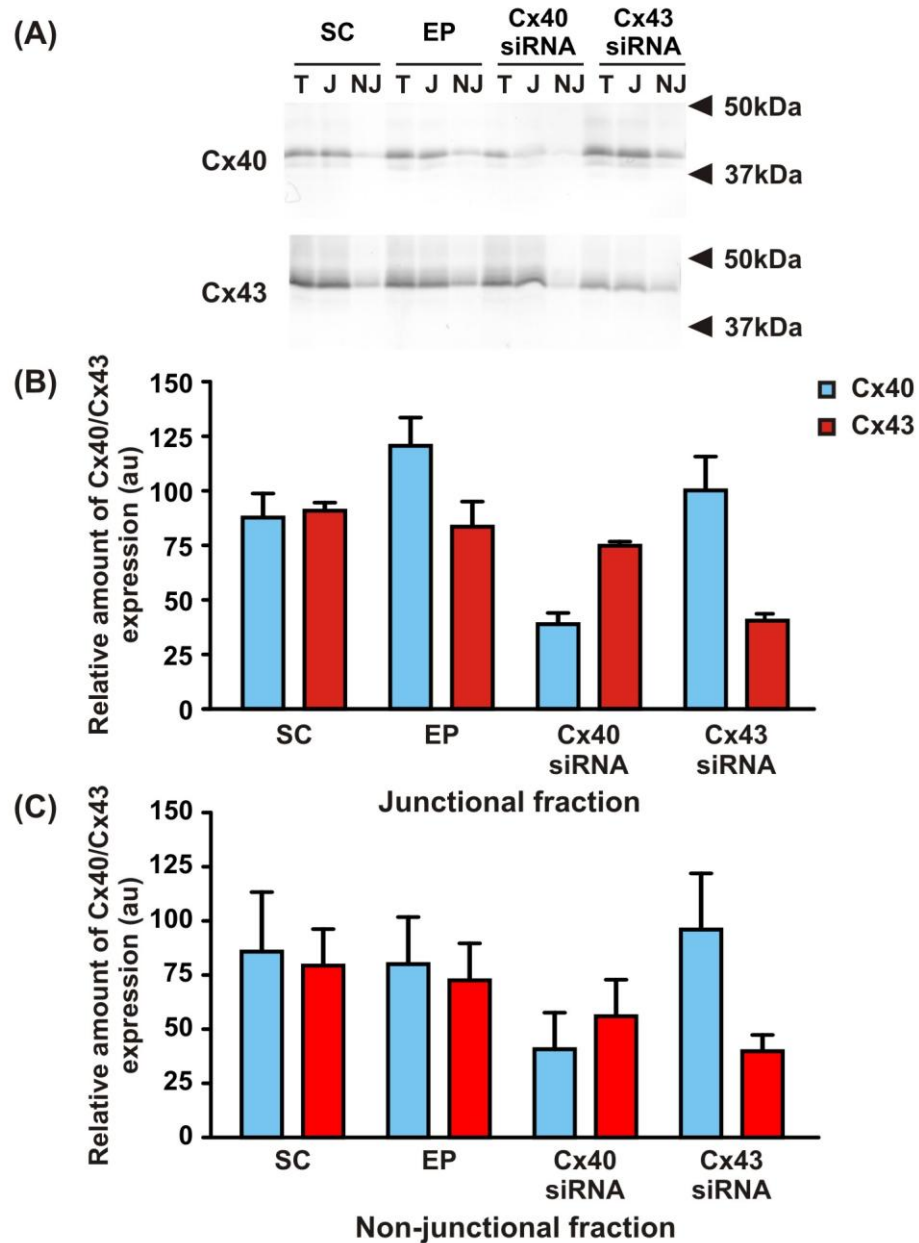


Figure 7.4: Quantification of Cx40/Cx43 co-expression after siRNA treatment

Cells from clone 6 were treated with Cx40 and Cx43 siRNA (2 μ M) and extracted into their junctional (J) and non-junctional fractions (NJ) on day 2 post-transfection and analysed by western blot (A). Total (T) samples were prepared by mixing equal volumes of the junctional and non-junctional fractions. Control samples included the seeding control (SC) and cells electroporated (EP) without siRNA. Treatment with Cx40 and Cx43 siRNA resulted in reduced levels of expression of their respective protein. Effects of siRNA were a significantly greater at the junctional fraction compared to the non-junctional fraction (Cx40 siRNA: $p = 0.0011$; Cx43 siRNA: $p = 0.0019$). Knockdown on either connexin did not affect the expression of the co-expressed connexin ($p > 0.05$ using an unpaired t test).

Table 7.2: Cx40 and Cx43 expression levels in the junctional and non-junctional fractions after siRNA treatment

Samples	Cx40 arbitrary units; n = 4		Cx43 arbitrary units; n = 4	
	Junctional	Non-junctional	Junctional	Non-junctional
Seeding control	88 ± 11	86 ± 27	91 ± 4	80 ± 17
Electroporated control	120 ± 13	80 ± 21	84 ± 11	73 ± 17
Cx40 siRNA	39 ± 5	40 ± 16	75 ± 2	60 ± 17
Cx43 siRNA	100 ± 15	96 ± 25	30 ± 3	39 ± 7
Cx40 + 43 siRNA	34 ± 1	57 ± 19	27 ± 3	44 ± 10

Similar analysis by western blot was performed on cells treated with the simultaneous knockdown of Cx40 and Cx43 as shown in Figure 7.5 (A). Effects of Cx40 and Cx43 knockdown in the total, junctional and non-junctional fractions were compared with similar fractions from the electroporated cells. Reduction of both connexins was significantly greater ($p < 0.005$) at the junctional membrane similar to that observed after Cx40 and Cx43 siRNA treatment. These data for simultaneous knockdown are presented in Table 7.2.

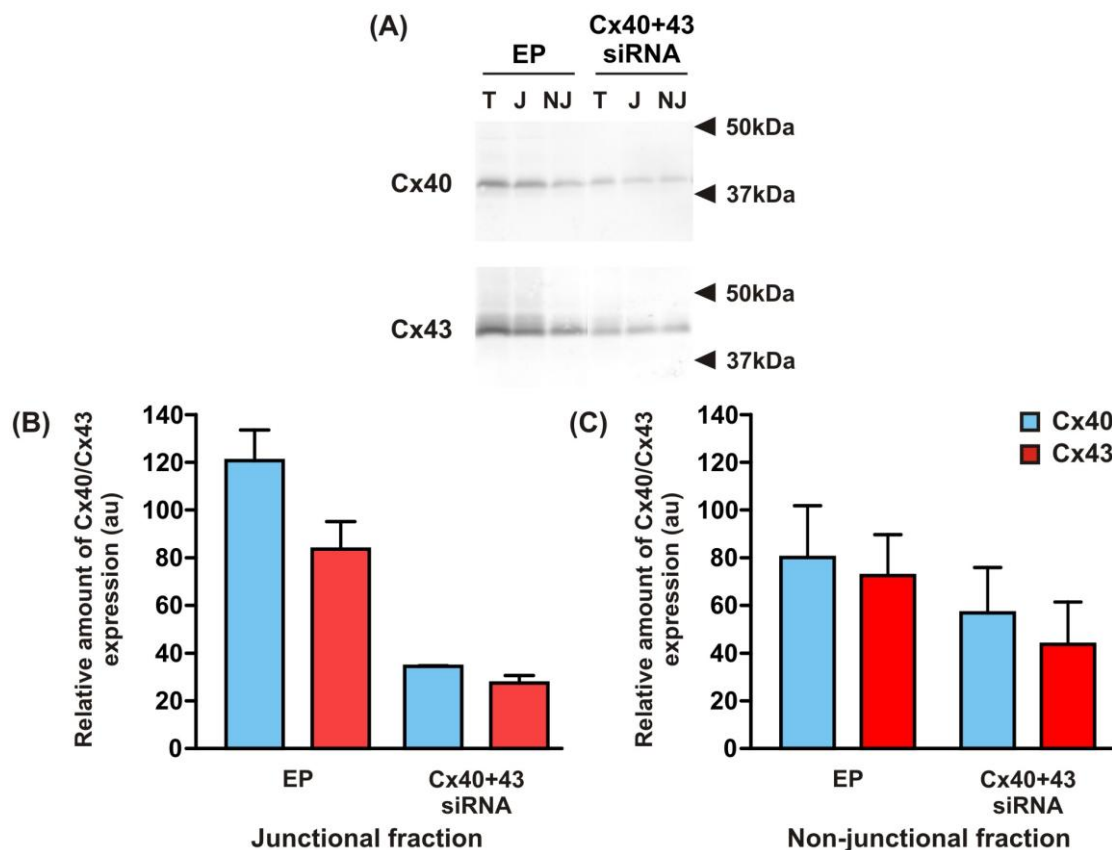


Figure 7.5: Western blot analysis data of simultaneous knockdown of Cx40 and Cx43

Clone 6 was electroporated with Cx40 and Cx43 simultaneously and scraped on day 2 post-transfection. The total (T), junctional (J) and non-junctional (NJ) fractions were analysed and siRNA effects were compared to electroporated (EP) control (A). Knockdown of both connexins resulted in approximately 60-70% reduction in Cx40 and Cx43 expression. It should be noted that reduction in connexin expression was significantly greater in the junctional fraction (B) than the non-junctional fraction ($p < 0.005$) (B).

After establishing the relative amounts of connexin expressed in the control samples (seeding and electroporated only) and after siRNA treatment the Cx40/Cx43 co-expression ratios was calculated based on the quantification values presented in Table 7.2. In the control samples the Cx40/Cx43 co-expression ratio was approximately 1:1 at the junctional and non-junctional fractions (Figure 7.5). After Cx40 siRNA the ratio decreased due to the lower levels of Cx40 expressed although the ratio was slightly higher in the non-junctional fraction reflecting the greater proportion of Cx40 knockdown in the junctional fraction. The opposite effect was seen after Cx43 siRNA where Cx43 levels were considerably reduced. No change was observed in the ratio of Cx40/Cx43 when Cx40 and Cx43 were simultaneously reduced (Figure 7.6). A

summary of the co-expression ratios in the control and siRNA treated samples are presented in Table 7.3.

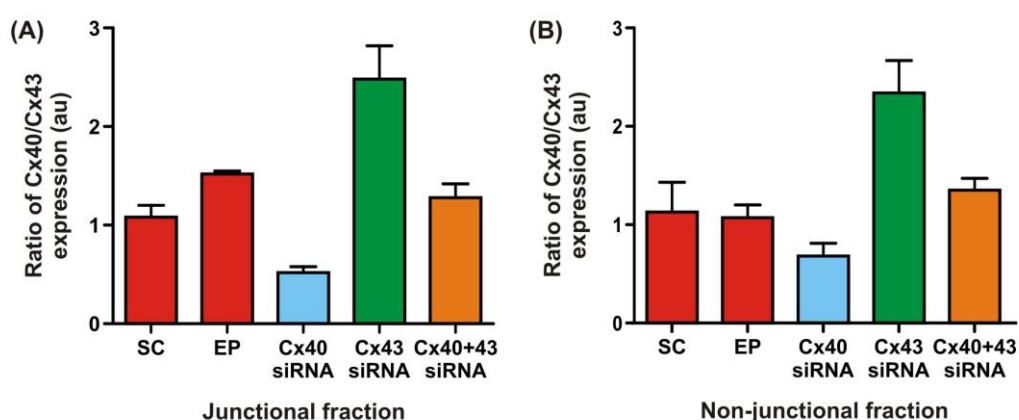


Figure 7.6: Co-expression ratios of Cx40/Cx43 after Cx40 and Cx43 siRNA treatment

Cx40/Cx43 co-expression ratios were calculated in the control samples and after siRNA treatment in the junctional (A) and non-junctional (B) fractions. The electroporated (EP) sample had a slightly higher ratio of Cx40 and Cx43 compared with the seeding control. Co-expression ratios decreased when Cx40 was reduced and vice versa when Cx43 was decreased. Simultaneous knockdown of both connexins did not alter the co-expression ratio. All data were expressed as arbitrary units (au).

Table 7.3: Cx40/Cx43 co-expression ratio in the junctional and non-junctional fractions

Samples	Cx40/Cx43 co-expression ratio arbitrary units; n = 4	
	Junctional	Non-junctional
Seeding control	1.08 ± 0.03	1.13 ± 0.12
Electroporation only	1.52 ± 0.03	1.07 ± 0.03
Cx40 siRNA	0.52 ± 0.06	0.68 ± 0.13
Cx43 siRNA	2.4 ± 0.34	2.4 ± 0.33
Cx40 + 43 siRNA	1.28 ± 0.14	1.35 ± 0.12

7.4. Discussion

Changes in gap junction organisation and connexin expression can result in disturbances of the normal cardiac rhythm. In this study we have assessed the effects of changing Cx40, Cx43 and Cx45 expression levels on the CV in cardiac myocytes using RNAi to knockdown connexin(s) expression. The major findings were: (1) knockdown of Cx45 produced a decrease in CV by approximately 50% with little or no effect on Cx40 and Cx43 protein expression; (2) knockdown of Cx40 or Cx43 resulted in an approximately

30% reduction in CV; (3) knockdown of two or all three connexins reduced CV by 40-50% and (4) depending on the siRNA treatment the reduced CV approximately corresponded to alterations in Cx40 and Cx43 protein levels at the membrane.

We began our investigation by identifying the most effective siRNA sequence for Cx45 since this had already been established for Cx40 and Cx43 in Chapter 6. As described in Chapter 4, Cx45 expression levels were too low to be detected by western blot. Thus to select the most appropriate siRNA for Cx45 knockdown we chose sequences that showed no interference with Cx40 and Cx43 total expression levels indicating specificity. Only one of the five siRNA used resulted in an increase of Cx40 and Cx43 expression with minimal effects caused by other sequences. This led us to functionally assess the effects of Cx45 suppression by MEA. All five sequences tested resulted in a 50% reduction of CV. From the BLAST homology searches (Table 7.2) we knew that these effects were not caused by siRNA sequences targeting the same region of the mouse Cx45 mRNA and are a result of true Cx45 knockdown. The findings of decreased CV caused by Cx45 siRNA were unexpected considering its mRNA was detected in low abundance comparable to studies in the human (Vozzi et al. 1999), neonatal mouse hearts (Lin et al. 2010) and the northern blot data presented in Chapter 4. Cx45 also has the lowest current-carrying capacity compared with Cx40 or Cx43 with a single channel conductance of approximately 33pS (Moreno et al. 1995; Elenes et al. 2001; Beauchamp et al. 2004). However when Cx45 was detected by immunofluorescence expression levels was homogenous across the cell monolayer albeit at low levels.

The majority of experimental data investigating the role Cx45 in cardiac development and conduction comes from transgenic animal models. Cx45 knockout studies resulted in death at embryonic day 10 from endocardial cushion defects and contractile failure (Alcolea et al. 1999; Kruger et al. 2000; Kumai et al. 2000) indicating the critical role of Cx45 in cardiac development and differentiation. Cx45 deletion could potentially have a variety of down-stream targets, which in turn could affect impulse propagation. For example immunofluorescence of cryosections through the atrioventricular canal revealed that the sub-cellular translocalisation from the cytoplasm to the nucleus of Ca^{2+} dependent transcription factor NF-ATc1 was significantly reduced in Cx45 deleted mice at embryonic day 10. This translocation process of NF-ATc1 requires a rise in

intracellular Ca^{2+} for phosphatase activity of calcineurin. The dephosphorylation of NF-ATc1 by calcineurin in the cytoplasm enables it to enter the nucleus and bind to DNA and other factors (Kumai et al. 2000). Accordingly it can be assumed that Cx45 plays a crucial role in the activation of Ca^{2+} dependent transcription factors in early cardiogenesis (Kumai et al. 2000). Although the precise mechanism of Cx45 function is unclear, these studies have highlighted the importance of Cx45 in cardiac morphogenesis and function.

Due to the embryonic lethality of Cx45 deletion, studies on mice that were deficient in Cx40 and lacked one allele of Cx45 were used to further deduce the functions of Cx45 beyond embryonic day 10 although only 15% of the mice survived to adulthood (Kruger et al. 2006). Subsequent electrocardiograms (ECG) of these mice revealed a significant prolongation of the PQ interval and QRS duration compared to mice with the Cx40 deletion only in the presence of normal Cx45 levels (Kruger et al. 2006). This supports the present findings that even the loss of one allele for Cx45 can slow conduction considerably and aggravate the cardiovascular abnormalities of Cx40 deficient mice. Despite its low levels of expression, Cx45 appears critical in impulse propagation.

Suppression of Cx45 had very little effect on Cx40 and Cx43 expression levels particularly at the junction so effects on CV could not be readily explained by this mechanism. However immunofluorescence data from the triple labelling of Cx40, Cx43 and Cx45 (Chapter 4, section 3) illustrated that all three connexins co-localise at the same cell-to-cell interface. There is also substantial evidence from biochemical and electrophysiological data that Cx45 can freely dock with Cx40 or Cx43 to form heterotypic channels with unique biophysical properties (Koval et al. 1995; Desplantez et al. 2004; Rackauskas et al. 2007a). In our laboratory co-culture studies of the rat liver epithelial cell model induced to express Cx45 revealed strong immunolabelling between cells expressing Cx43 only and those expressing Cx43 and Cx45 indicating compatibility (Thomas 2007). From this information we could speculate that the loss of Cx45 could have a modulatory effect on the formation of gap junction channels composed of Cx40 and Cx43 as these connexins do not appear to be functionally compatible with each other (White et al. 1995; Haubrich et al. 1996; Rackauskas et al. 2007a). Cx45 could play a similar role to Cx46 in the lens which is required if heterotypic channels are to be formed between Cx50 and Cx43 (White et al. 1994).

Through this mechanism the loss of Cx45 could alter the type of connexins constituting the gap junction channels and thereby intercellular coupling between cells. As a result, effects of Cx45 may be considerably greater than its low level of expression would suggest. Further experiments by dual voltage patch-clamp measuring the macroscopic and single channel conductance will be required to determine the exact properties of gap junction channels that remain after Cx45 siRNA.

Suppression of Cx40 resulted in a 30% reduction in CV. Quantification after Cx40 siRNA revealed that Cx40 was reduced by approximately 70% and this knockdown was more pronounced at the junction with a lesser effect in the non-junctional fraction. After Cx40 siRNA there were minimal effects on Cx43 expression in both the junctional and non-junctional fractions. The Cx40/Cx43 co-expression ratio also decreased by approximately 50%. We were unable to quantify Cx45 levels but protein expression was still detected by immunofluorescence after Cx40 siRNA (Chapter 6, section 3).

The decrease in CV after Cx40 suppression is consistent with findings from Cx40 homozygous knockout mice (Kirchhoff et al. 1998). ECG studies on these mice revealed prolonged P wave duration, PQ interval and QRS duration (Kirchhoff et al. 1998; Simon et al. 1998; Hagendorff et al. 1999; Schrickel et al. 2009). Impulse propagation studied by transesophageal atrial stimulation in Cx40 knockout studies also revealed a number of conduction abnormalities in sinoatrial, intra-atrial, atrioventricular and His-Purkinje conduction including an increased susceptibility for re-entrant arrhythmias (Hagendorff et al. 1999; Tamaddon et al. 2000). Verheule et al. (1999) showed that in Cx40 knockout mice a 30% decrease in CV in the right atrium produced no significant effect on ventricular conduction highlighting the importance of Cx40 in atrial conduction. Supportive results were also obtained by Bagwe et al. (2005). The ablation of Cx40 can also lead to developmental malformations which need to be taken into account as inclusion of these animals could influence analysis of conduction (Gu et al. 2003; Leaf et al. 2008).

Beauchamp et al. (2006) reported an increase in CV in Cx40 ablated atrial cell strands from neonatal cardiac myocytes. The group also observed a 2.4 fold increase in the Cx43 signal at the junctional membrane although there was no change in the total Cx43 expression. This property of increase in Cx43 at the junctional membrane may decline

during growth and development as it was absent in adult Cx40 knockout atria in the same study (Beauchamp et al. 2006). Results from patients undergoing coronary artery surgery also showed a strong correlation between high levels of CX40 immunosignal and decreased CV during sinus rhythm (Kanagaratnam et al. 2002). Differences in results could be due to the experimental methods used. Connexin quantification by Kanagaratnam et al. (2002) was based on the antibody specificity which can be affected by the topological arrangement and steric hindrance of co-expressed connexins. Problems with steric hindrance were also reported by Grikscheit et al. (2008) where measurements of gap junction size differed with the antibody used. In this study it is clearly demonstrated that knockdown of Cx40 has little or no effect on Cx43 expression, particularly at the junctional membrane. This indicated that the decrease in CV is a true effect of reduced Cx40 levels.

Cx43 siRNA resulted in an approximate 70% knockdown of Cx43 protein expression with a 30% reduction in CV. Similar to Cx40, Cx43 suppression was more pronounced at the gap junction plaque and no effect on Cx40 expression was observed in both the junctional and non-junctional fractions. These findings of higher Cx40 and Cx43 levels in the non-junctional fraction may suggest that connexin trafficking and degradation are regulated by the amount of the non-junctional connexins. Studies by VanSlyke et al. (2009) hypothesised that connexin trafficking is a concentration-driven process in which a certain non-junctional level is necessary to obtain successful oligomerisation and transport to the plasma membrane.

The significance of reduced levels of Cx43 has been well illustrated in transgenic models. Knockout mice of Cx43 resulted in mice surviving to term but dying shortly after birth due to obstruction of the right ventricular outflow tract (Reaume et al. 1995). The development of cardiac-restricted conditional knockout Cx43 circumvented these problems enabling the role of Cx43 in cardiac conduction to be assessed (Gutstein et al. 2001a). Despite a normally formed heart, conditional knockout mice demonstrated marked reduction of ventricular CV with the development of spontaneous ventricular arrhythmias and sudden cardiac death by two months of age (Gutstein et al. 2001a). These studies demonstrated that there is no essential requirement for Cx43 during cardiac development but it is required for maintaining cardiac electrical stability in the more mature ventricle (Gutstein et al. 2001a). In mice where cardiac specific Cx43 is

ablated progressively during postnatal development, Cx43 expression levels were reduced finally to 20% whereupon there was a significant slowing of CV to half of that observed in control hearts (Danik et al. 2004). There are conflicting data on conduction properties in heterozygous Cx43 mice. Some studies report conduction slowing (Guerrero et al. 1997; Thomas et al. 1998) whereas others find no electrophysiological differences compared with wild type hearts (Morley et al. 1999). Discrepancies in results could be due to different methodologies or other factors such as phosphorylation which plays a key role in regulating intercellular communication (Kwak and Jongsma 1996; Ai et al. 2010).

In cultured myocytes isolated from rabbit left ventricles, Cx43 levels were reduced to those observed in heart failure (up to 70% knockdown) using siRNA (Ai et al. 2010). This reduction produced a significant decrease in intercellular dye coupling. Correspondingly, in parallel studies overexpression of these connexins improved dye coupling. This work by Ai et al. (2010) most importantly illustrated that even moderate changes in Cx43 protein (~40%) can contribute to impaired intercellular coupling in the failing heart contradictory to previous studies suggesting that at least 80-90% of uncoupling had to occur to affect CV (Danik et al. 2004; Peters 2006). Studies on Cx43 deleted atrial strands reported a 40% drop in CV comparable to our results obtained by siRNA (Beauchamp et al. 2006).

The expression levels of a large number of genes have been shown to be modified in Cx43 heterozygous and null mice (Iacobas et al. 2005). The loss of Cx43 in both homozygous and heterozygous knockout mice lead to a variety of electrophysiological alterations including a higher potassium (K^+) current density, shorter action potential (Danik et al. 2008; Kontogeorgis et al. 2008) and faster upstroke velocity (dV/dt_{max}) (Beauchamp et al. 2004). This electrical remodelling may be a compensatory response of the myocytes, which could subsequently contribute to the conduction delays observed. In this work we could exclude effects of compensation of other connexins particularly at the junctional membrane. No difference in the shape and size of the extracellular field potentials were observed with or without siRNA indicating that the changes in CV are a gap-junction-mediated effect. However measurements of the dV/dt_{max} with and without siRNA will need to be performed to confirm this.

The mechanism underlying the decreased CV following Cx40 and Cx43 siRNA treatment is most likely due to reduced intercellular coupling and gap-junctional conductance rather than through the formation of heterotypic-heteromeric channels. Evidence for decreased gap-junctional conductance in hearts lacking Cx43 has come from numerous studies (Beauchamp et al. 2004; Vink et al. 2004; Yao et al. 2003). For example Beauchamp et al. (2004) reported a 96% reduction in conductance in ventricular strands from Cx43 knockout mice which corresponded to a 97% drop in CV. Further experiments on clone 6 will need to be performed by dual cell patch-clamp to assess the total gap-junctional conductance after siRNA and if coupling is influenced by the formation of Cx40-Cx43 heterotypic channels.

After Cx40 and Cx43 siRNA treatment CV decreased by approximately 30% but this effect was slightly greater following the reduction of Cx40 compared with Cx43. Channels composed of Cx40 have a higher unitary conductance ranging from 150-200pS in comparison to those composed of Cx43 which ranges from 60-120pS (Gros and Jongsma 1996). These differences in unitary conductance could explain why Cx40 channels may conduct action potentials more rapidly than cells connected by Cx43 channels (Kirchhoff et al. 1998).

Simultaneous knockdown of two and all three connexins resulted in CV being halved. We were only able to quantify protein expression after the simultaneous knockdown of Cx40 and Cx43. Reduction of Cx40 and Cx43 was achieved to similar levels with no difference in the Cx40/Cx43 co-expression ratio. No additive effect on CV was observed after Cx45 and Cx40, Cx45 and Cx43 and all three connexins. Results also indicate that the loss of Cx45 was equal to that of both Cx40 and Cx43 knockdown. When levels of all three connexins were reduced, action potential propagation was still maintained albeit at considerably reduced levels compared to the controls.

The lack of additive effects on propagation following the loss of two or more connexins could be explained by the siRNA not completely suppressing the expression of the connexin gene. The residual 20-30% remaining after knockdown is still functional. Theoretical models have demonstrated that reduced coupling can cause large reductions in velocity before conduction block is observed. Shaw and Rudy (1997) have shown that markedly slowed CV can still be maintained with reduced gap junction coupling

through an increase in the safety factor greater than during normal conduction. This suggests that after siRNA treatment cells must remain coupled though low-level expression of gap junction channels composed of Cx40, Cx43 and Cx45 thereby maintaining propagation. siRNA could also be utilised to assess the individual roles of each connexin in the various diseased states. For instance reduced levels of CX43 expression have been consistently reported in patients with heart failure which are thought to increase the susceptibility to arrhythmias as shown in the transgenic animal models ((Chapter 1, section 5.4). In addition it would be useful to study the effects of siRNA with optical mapping which provides a higher spatial resolution compared with the MEA to analyse differences in the pattern of excitation and propagation.

CHAPTER 8.
General discussion

8.1. Introduction

In the concluding chapter of my thesis I would like to summarise the principal findings of my project, discuss ideas emerging from them and suggest future developments.

8.2. Overview of key findings

The aim of my study was to investigate the influence of connexin expression/co-expression levels on action potential propagation using the HL-1 cell line. Because the cells were tumour-derived, they were heterogeneous in population containing cells blocked at various developmental stages (Trosko et al. 1993). To obtain more homogenous cell lines, clones were selected from low density HL-1 cultures on their ability to contract and therefore, possess some functional excitation-contraction coupling. Homogeneity was essential in correlating a change in function with molecular intervention and exclude differences due to a heterogeneous cell line. To study action potential propagation the clones had to fulfil two main criteria: (1) the expression of ion channels necessary to generate action potentials and (2) the expression of gap junctions that form cell-to-cell pathways for the propagation of current flow in the heart.

We demonstrated that the HL-1 clones expressed the major Ca^{2+} handling proteins and displayed spontaneous intracellular Ca^{2+} transients $[\text{Ca}^{2+}]_i$. Functional expression of the major cardiac ion channels involved in excitation (sodium (Na^+), L and T-type Ca^{2+}) were studied in clones 2 and 6. No difference in current densities were observed despite major differences in mRNA channel expression indicating differences in post-transcriptional regulation between the two clones. Cells in clone 2 displayed a more depolarised membrane potential than clone 6. Action potentials were always recorded from clone 6 but were rare in clone 2. This was most likely due to differences in cell culture density since patch-clamp experiments required the cells to be isolated and the development of spontaneous contraction in the HL-1 cells is dependent on the development stage of the culture (Claycomb et al. 1998). Spontaneous electrical activity and contraction suggested the existence of some form of pacemaking in the cells. Hyperpolarisation-activated ‘funny’ current (I_f) was present in clone 6 but not in clone 2 suggesting that in this clone other currents could contribute to action potential generation such as $[\text{Ca}^{2+}]_i$ (Lakatta et al. 2003).

All the HL-1 clones expressed connexin40 (Cx40), Cx43 and Cx45 at varying levels with significantly lower amounts present in clone 2. There was a good correlation between the level of mRNA and the corresponding connexin expression indicating that regulation was predominantly at the transcriptional level. Using the Cx40- and Cx43-V5tag and -tag developed in our laboratory, Cx40/Cx43 co-expression was quantified with a ratio of approximately 1:1. Despite Cx45 being expressed at a level that was least twenty times lower than Cx40 and Cx43, immunofocal analysis revealed that expression was relatively homogenous. After triple labelling, a clear difference was observed in the distribution of each connexin at each cell-to-cell interface. Differences in connexin co-localisation were attributed to incompatibilities between connexons particularly between Cx40 and Cx43 to form heterotypic channels (Bruzzone et al. 1993; Haubrich et al. 1996). Evidence for a substantial amount of interaction between Cx40 and Cx43 was found in similar amounts in both clone 6 and human atrial tissue but the levels of heteromerisation were a lot lower than theoretically calculated (Brink et al. 1997). In the healthy and diseased heart connexins also interact with various partner proteins that influence the size and organisation of gap junctions (Hunter et al. 2005; Bruce et al. 2008). Zonula occludens-1 (ZO-1) was shown to co-localise with all three connexins by immunofluorescence and co-immunoprecipitation experiments provided further evidence of interactions with Cx40 and Cx43 as expected for a cell of myocyte origin.

Action potential propagation was recorded in the HL-1 clones including clone 2 using microelectrode arrays (MEA). The clones displayed differences in conduction velocities (CV) with clone 6 producing the fastest propagation across a monolayer. The natural pacemaker came from the edge of the preparation most likely due to source-and-sink factors (Rohr et al. 1997). Further analysis indicated there was sometimes more than one pacemaker present within the monolayer. Optical mapping confirmed the observations made with MEA and gave further details on action potential amplitude and duration at multiple sites on a monolayer revealing homogenous impulse propagation.

To deduce the role of gap junction expression on impulse propagation, RNA interference (RNAi) with small interfering RNA (siRNA) was used in clone 6 to knockdown connexin expression. Cells were electroporated with siRNA for Cx40, Cx43 and Cx45 and expression was down-regulated by approximately 60-70%. Cx45 could

not be detected by western blot so siRNA effects were assessed using MEA. Knockdown of Cx40 and Cx43 resulted in an approximately 30% reduction in CV. All the siRNA sequences used to reduce Cx45 levels resulted in a 50% reduction in CV. Double (Cx40 + Cx43, Cx40 + Cx45, Cx43 + Cx45) and triple (Cx40 + Cx43 + Cx45) siRNA also resulted in CV being halved. These results indicate that although gap junctions play a key role in conduction, synergism between membrane excitability (e.g. Na⁺ channels) and intercellular coupling are likely to govern CV (Shaw and Rudy 1997; van Veen et al. 2005).

8.3. Relevance of connexin co-expression in the heart

As discussed in the introduction Cx40, Cx43 and Cx45 are co-expressed in the heart in characteristic combinations in a chamber related, myocyte type-specific and developmentally-regulated manner (Vozzi et al. 1999; Severs et al. 2004a). This opens the possibility for a wide range of different molecular stoichiometries (Cottrell and Burt 2005). However it is still unclear how the different stoichiometries translate into different electrophysiological properties *in vivo*.

So far the functional properties of connexin co-expression in the intact heart have been extrapolated from *in vitro* cell models and transgenic animals. For example pairing of a Cx40 connexon with a Cx45 connexon showed asymmetrical voltage dependence, a property associated with rectifying channels. However whether such unidirectional channels play a role in current spread *in vivo* in the heart is unknown but may be functionally relevant in the central nervous system where signal propagation in one direction is preferred (Rackauskas et al. 2007a). Co-expression of incompatible connexons (e.g. Cx40 and Cx43) could lead to communication barriers. In the rat heart Cx40 is preferentially located in the His bundle and the proximal region of the bundle branches compared with myocytes of the surrounding tissue expressing mainly Cx43 (Gros et al. 1994). If these two connexons are incompatible, then the conduction system could be electrically isolated from the surrounding tissue and the amount of current flowing through Cx40 channels will be greater than that flowing through Cx43 channels providing a more efficient conduction (Bastide et al. 1993).

Knockdown of each of the three connexins affected impulse propagation with the largest effect produced by Cx45 indicating that, despite low levels of expression, it is crucial in regulating intercellular communication in the HL-1 clones. Collectively these approaches have enabled CV to be assigned to each combination of co-expressed connexins that occurs *in vivo* and to predict the electrophysiological consequences of disease-related alteration in connexin levels.

8.4. Strengths and limitations

The use of the HL1- cell line was highly advantageous due to its ability to be serially passaged without the loss of contraction and differentiation. As demonstrated throughout my thesis the HL-1 clones exhibited a gene expression profile similar to that of primary cardiac myocytes. The connexin expression profiles in the HL-1 clones, which resemble those of atrial myocytes, present an advantage over existing *in vitro* cell models designed to investigate cell-to-cell communication.

Majority of the studies investigating the biophysical properties of the different gap junction channel types formed are based on communication-deficient cell lines engineered to express or co-express exogenous connexins such as *Xenopus* oocytes (Bruzzzone et al. 1993; 1994) and HeLa cells (Haubrich et al. 1996; Gemel et al. 2006; Rackauskas et al. 2007a). Although these cellular models have provided valuable information, they are unlikely to represent the actual ratios of connexins co-expressed in cardiac tissue and the expression levels of the transfected connexins are generally static. Selected data comparing the HL-1 clones and human tissue indicated similarities in connexin co-localisation patterns (Cx40 and Cx43), co-expression ratios of Cx40/Cx43 and levels of heteromerisation (Cx40 and Cx43), a unique advantage over transfected cell models. In addition results from the HL-1 clones were reproducible, time and cost effective and amenable to transfection using RNAi or plasmid DNA constructs followed by selection with antibiotics to modulate a specific protein.

One of the limitations in our study was the inability to study the biological significance of Cx45. The low levels of Cx45 expressed in the HL-1 clones made detecting this protein by western blot difficult and we were unable to correlate the amount of Cx45 reduced and the decrease in CV after siRNA. In our cell model we were also not able to

study anisotropy by the lack of a structured cell shape and the distribution of gap junctions along the cell perimeter compared with cardiac myocytes in which the gap junctions are located at cell-to-cell ends at the intercalated disk. When relating CV to connexin expression and its potential role in arrhythmias *in vivo* additional factors are likely to be involved. Arrhythmias are multifactorial in origin involving an interplay between gap-junctional coupling, membrane excitability and cell and tissue architecture (Severs et al. 2008). For example atrial fibrillation leads to electrical and structural remodelling including fibroblast proliferation, fibrosis and cell death (de Bakker and van Rijen 2006). Fibrosis involves a large accumulation of collagen sheets, which act as electrical insulators so impairing electrical coupling between myocytes. Moreover fibroblasts could modulate impulse propagation by forming gap junctions with cardiac myocytes (Rohr 2004; Miragoli et al. 2007).

In summary findings *in vitro* particularly between connexin co-expression could be extrapolated to *in vivo* and patient samples to further our knowledge on the physiological consequences of altered connexin expression in modulating impulse propagation in both the healthy and diseased heart.

8.5. Future directions

mRNA quantification

In this study mRNA transcribed by the HL-1 clones was quantified by Northern blot. Although this technique provides a direct relative comparison of mRNA abundance between samples and additional information on the transcript size and alternative splicing there are limitations associated with northern analysis (Bustin 2000). For example quantification relies on the integrity of the RNA samples and how efficiently it has transferred onto the membrane. As a result, if the samples are degraded or RNA transfer is incomplete, the ability to quantify is severely compromised. For future experiments a more sensitive approach such as real-time polymerase chain reaction (PCR) could be employed to study gene expression. real-time PCR relies on the specific amplification of target RNA and its detection through a fluorescence tag at the end of every cycle rather than after a fixed number of cycles. Thus the higher the initial starting copy number of the mRNA target, the quicker the fluorescence is observed. In addition

compared with northern blot, real-time PCR can be used to quantify mRNA levels from small samples, is less time consuming and above all accurate (Wang and Brown 1999).

Action potential recordings in clone 2

Action potentials were recorded from clones 2 and 6 using patch electrodes. However we were only able to record a single action potential from clone 2 despite the clone producing spontaneous $[Ca^{2+}]_i$ and extracellular field potentials. Both these experiments indicate that cells in clone 2 are able to produce action potentials once confluence is achieved. This is an important factor in HL-1 cells as their differentiation is very much dependent on cell density and time spent in culture (Claycomb et al. 1998). To test the hypothesis that action potential production in clone 2 is truly density dependent, the cell monolayer needs to be accessible to micro electrodes, already used in a variety of studies (Jongsma and van Rijn 1972; Bursac et al. 2003). A future protocol would involve the seeding of a confluent cell monolayer and sharp micropipettes fabricated with a tip resistance of 50 M Ω used to impale the cell monolayer. Recordings would be made in cells superfused with normal Tyrode. Using this protocol should give us further information on action potential shape and duration particularly in clone 2 and if any differences are observed in action potential morphology between single cells and a cell monolayer.

Pacemaker generation

The HL-1 clones provide an ideal cell model in which pacemaker automaticity could be generated. Although this was not extensively studied we were able to record I_f but only in clone 6. This suggests there are other mechanisms likely to be involved: (1) cyclic release of $[Ca^{2+}]_i$ from the sarcoplasmic reticulum (SR) through the ryanodine receptor (RyR) and (2) sodium-calcium exchanger (NCX) that provides a transient inward current associated with SR Ca^{2+} release and an increase in $[Ca^{2+}]_i$ (Lakatta et al. 2003). RNAi could be used to knockdown these key proteins (e.g. RyR2 and NCX 1.1) seeing as there are a range of siRNA commercially available. Alternatively pharmacological agents could be used to block ion channels. RNAi is preferable because of the specificity of the technique. This protocol could also be employed to study the role of membrane excitability (e.g. Na^+ and Ca^{2+}) in action potential propagation since suppression of all three connexins only resulted in a slowing of CV rather than

conduction block. The functional consequences could be analysed not only by patch-clamp but also through MEA which will potentially provide a bridge between single cells and the whole tissue. Figure 8.1 is a schematic view of the ion channels that could affect automaticity and membrane excitability.

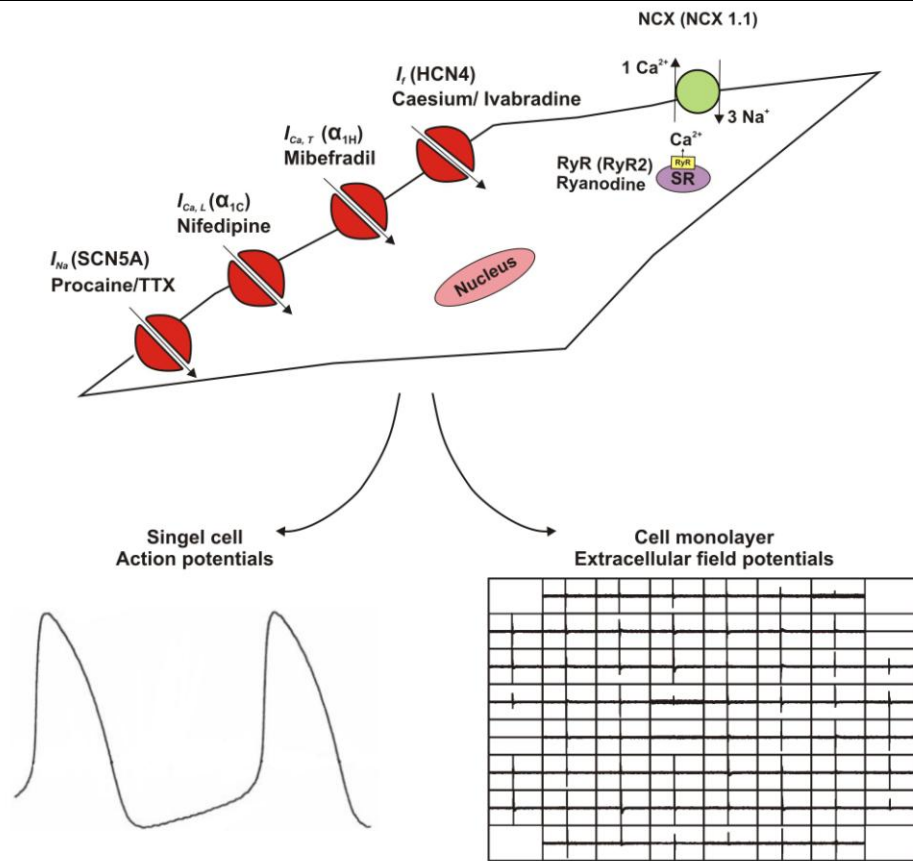


Figure 8.1: Schematic diagram of the ion channels responsible for pacemaker generation and membrane excitability

RNAi or pharmacological agents could be used to test the role of each ion channel responsible for action potential generation and membrane excitability (i.e. the amount of charge produced by the inward currents). Ion channels are listed with the corresponding gene name and pharmacological agent. Functionality could be assessed on: (1) action potentials recorded from single cells or (2) extracellular field potentials from a cell monolayer using MEA. Image was modified from Dobrzynski et al. (2007).

Connexin compatibility

To test the hypothesis that cells composed of Cx40 and Cx43 are not capable of forming heterotypic channels, co-culture studies using siRNA could be performed. We know that cells in clone 6 expressed Cx40 and Cx43 at a ratio of 1:1 and siRNA suppressed connexin expression by approximately 60-70%. To investigate this hypothesis cells

treated with Cx40 siRNA can be co-cultured with cells treated with Cx43 siRNA. One group of cells will be stained with membrane permeant fluorescent probes such as CMFDA (5-chloromethylfluorescein diacetate) or CMHC (4-chloromethyl-7-hydroxycoumarin) to help discriminate between Cx40 and Cx43 expressing cells. Compatibility could be verified by immunoconfocal analysis. Electrophysiological studies on cell pairs from the co-culture would help gain further information regarding the precise molecular make-up of the type of channels formed when Cx40 and Cx43 are co-expressed. We were unable to study cell-to-cell communication using scrape-loading in the HL-1 clones as no dye transfer was observed with Lucifer yellow (data not shown). However there are a variety of different molecules that could be tested (e.g. neurobiotin, ethidium bromide) to characterise the permeability-selectivity of the channels formed (Elfgang et al. 1995).

Assessing the role of connexin interacting proteins

In this study we did not examine the functional role of ZO-1 in the HL-1 clones. Previous studies have demonstrated that ZO-1 plays an important role in modulating gap junction size and the recruitment of connexons to the gap junction periphery. To assess of role of ZO-1 in regulating cell-to-cell communication, siRNA could be applied and the effects assessed by MEA. It is also likely that ZO-1 functions in parallel with other connexin interacting partners such as N-cadherin and β -catenin that are known to interact with Cx43 (Hertig et al. 1996; Kostin et al. 1999). Thus the HL-1 clones could be used to study connexin protein interactions and further our understanding in their role in formation and stability of gap junctions particularly as mutations or suppression of these proteins can: (1) lead to slow conduction and arrhythmias and (2) reductions in Cx43 expression (Li et al. 2006).

8.6. Concluding comment

In summary we have demonstrated the HL-1 clones provide a useful model in studying varying aspects of cardiac biology and electrophysiology including cellular signalling, signal transduction and Ca^{2+} handling. The work on gap junction expression using RNAi has clearly identified that each of the connexins plays a specific role in conduction. This data should aid our understanding of gap junction function in the

healthy and diseased heart by showing how alterations of connexin co-expression can affect the communication behaviour of cells.

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Abstracts

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