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Atrial Fibrillation-Linked Germline GJA5/Connexin40 Mutants Showed an Increased Hemichannel Function

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ATRIAL FIBRILLATION-LINKED GERMLINE *GJA5*/CONNEXIN40 MUTANTS
SHOWED AN INCREASED HEMICHANNEL FUNCTION

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by

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Abstract

Mutations in *GJA5* encoding the gap junction protein connexin40 (Cx40) have been linked to lone atrial fibrillation (AF). Some of these mutants result in impaired gap junction function due to either abnormal connexin localization or impaired gap junction channels, which may play a role in promoting AF. However, the effects of the AF-linked Cx40 mutants on hemichannel function have not been studied. Here we investigated two AF-linked germline Cx40 mutants, V85I and L221I. These two mutants formed putative gap junction plaques at cell-cell interfaces, with similar gap junction coupling conductance as that of wild-type Cx40. Connexin deficient HeLa cells expressing either of these two mutants displayed prominent propidium iodide (PI)-uptake distinct from cells expressing wild-type Cx40 or other AF-linked Cx40 mutants, I75F, L229M and Q49X. The PI-uptake was sensitive to $[Ca^{2+}]_o$ and carbenoxolone, but was not affected by probenecid, indicating that uptake is mediated via connexin hemichannels. A gain-of-hemichannel function in these two AF-linked Cx40 mutants may provide a novel mechanism underlying the etiology of AF.

Statement of Co-Authorship

This entire set of work was completed by Matthew Hills with the following exceptions: all electrophysiology, as well as any work done on IRES-tagged constructs, was completed by Dr. Yiguo Sun.

Dedication

      I would like to dedicate my thesis to my mom and dad who have supported me throughout this entire process in every possible way. Without you this would not have been possible.

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I would like to thank a number of people, starting with my supervisor Dr. Donglin Bai. He gave me a great opportunity by allowing me to work in his lab and gain hands on laboratory experience as well as greatly expand my knowledge. His constant guidance and help was instrumental in allowing me to complete this thesis. I would also like to thank my committee members, Dr. Stan Leung and Dr. Mike Jackson, for all of their support and advice. I would like to thank the members of the Bai lab, both past and present, who assisted me with this project, as well as members of the Laird lab who were great both in and outside of the lab. Finally, I would like to thank all of my friends and family who have supported me throughout this process.

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List of Abbreviations and Nomenclature

$[Ca^{2+}]_i$ intracellular calcium concentration

$[Ca^{2+}]_o$ extracellular calcium concentration

AF   atrial fibrillation

CBX   carbenoxolone

CL   cytoplasmic loop

CMTX   Charcot-Marie-Tooth syndrome

CT   carboxyl-terminus

Cx   connexin

DAD   delayed afterdepolarization

DCC   divalent cation containing

DCF   divalent cation free

ddH_2O double distilled water

DMEM   Dulbecco's modified Eagle's medium

E1   first extracellular loop

E2   second extracellular loop

EAD   early afterdepolarization

ECS   extracellular solution

EDTA   ethylenediaminetetraacetic acid

EGTA   ethylene glycol tetraacetic acid

ER   endoplasmic reticulum

F   fluorescence intensity at time t

F/F0   ratio of fluorescent intensity at time t over baseline intensity

F0	baseline fluorescence intensity
GFP	green fluorescent protein
GJ	gap junction
G$_j$	macroscopic junctional conductance
HeLa	cervical cancer cell line
I$_j$	junctional current
IP3	inositol trisphosphate
IP3R	inositol trisphosphate receptor
IRES	internal ribosome entry site
kDa	kilo-dalton
N2A	neuroblastoma cells
NT	amino terminus
PBS	phosphate buffered saline
PI	propidium iodide
RFP	red fluorescent protein
RMP	resting membrane potential
ROI	regions of interest
SEM	standard error of the mean
SR	sarcoplasmic reticulum
TM	transmembrane domain
V$_j$	transjunctional voltage
V$_j$-gating	transjunctional voltage-dependent gating
YFP	yellow fluorescent protein

1. Introduction

1.1 - Gap Junctions

Gap junctions (GJ) are intercellular channels that allow for the direct exchange of ions and small molecules less than 1 kDa in size between neighbouring cells. GJs are found in almost all tissues in the body and play key roles in a number of different physiological processes including development, differentiation, growth, immune response, neural activity and synchronous heart contraction (Maeda and Tsukihara 2011). Hexamers of connexin (Cx) proteins form a connexon, also known as a hemichannel. A connexon from one cell can then dock end-to-end with a connexon from a neighbouring cell to create a GJ channel (Fig. 1.1) (Goodenough, Goliger et al. 1996).

GJs are crucially important in electrically excitable cells including neurons, cardiomyocytes and smooth muscle cells, because of their ability to provide a direct, low-resistance, high speed passageway for ions to travel between cells, leading to synchronous electrical as well as mechanical activities (Goodenough and Paul 2009). In addition to their roles for coupling cells electrically, GJs are also able to provide a direct conduit for metabolites, nutrients and second messengers for virtually all tissues in the body.

GJs can be regulated in a number of different ways. A rapid regulation comes from their sensitivity to transjunctional voltage, known as transjunctional voltage-dependent gating (V_j-gating). Vj-gating of gap junction channels varies depending on the component Cxs (Kumar and Gilula 1996). GJs can also be regulated by other relatively slower mechanisms, which result in changes in the abundance of the GJ channels at the cell-cell interface at one time. This is achieved by a number of different processes including changing the rates of biosynthesis/degradation as well as different post-

translational modifications, such as phosphorylation, hydroxylation (Locke, Koreen et al. 2006) and acetylation (Shearer, Ens et al. 2008). Other factors including, changes in intracellular concentrations of Ca^{2+} and pH, can also modulate the chemical gating of GJs, which cause slow, stepwise transitions from fully open to fully closed states. Increases in $[Ca^{2+}]_i$ have been shown to close GJ channels and it is believed that this gating is mediated by Ca^{2+} interacting with calmodulin, which then acts directly on Cxs to close GJs (Peracchia 2004). Low intracellular pH has been shown to close most GJ channels and is assumed to be a mechanism that acts to prevent the spread of injury from damaged to healthy cells (Bukauskas and Verselis 2004). The mechanisms of pH-mediated GJ gating are not fully understood and have been shown to act both directly and indirectly to cause GJ closure. Slow GJ gating also occurs with increases in intracellular CO_2, as well as after the addition of long chain n-alkanols or arachidonic acid (Peracchia 2004).

Mutations in Cxs have been linked to a number of human diseases including hearing loss (Cx26 and Cx30) (Al-Achkar, Moassass et al. 2011; Wang, Liu et al. 2011), hearing loss with associated skin disease (Cx26) (Laird 2008), X-linked Charcot-Marie-Tooth syndrome (CMTX) (Cx32) (Hanemann, Bergmann et al. 2003), lone atrial fibrillation (Cx40) (Gollob, Jones et al. 2006; Yang, Liu et al. 2010; Yang, Zhang et al. 2010; Sun, Yang et al. 2013), oculodentodigital dysplasia (Cx43) (Paznekas, Boyadjiev et al. 2003), Pelizaeus Merzbacher-like disease (Cx47) (Henneke, Combes et al. 2008) and cataracts (Cx50 and Cx46) (Arora, Minogue et al. 2008; Wang and Zhu 2012).
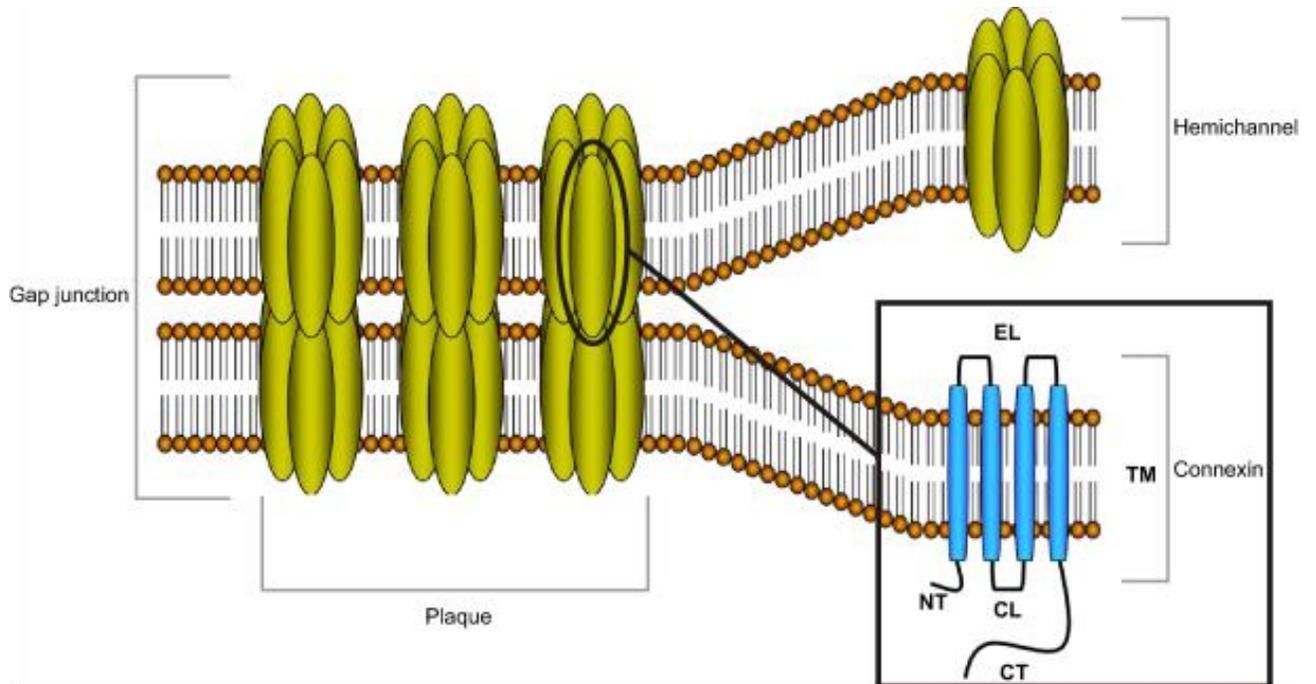
Figure 1.1 - Schematic diagram of gap junction channels and connexin topology. Gap junction channels are formed by the head-to-head docking of connexons, or hemichannels, which are each made of six Cx proteins. All Cxs share a common structural topology with an intracellular amino-terminus (NT), four transmembrane spanning domains (TM1-TM4), two extracellular loops (E1 and E2), a cytoplasmic loop (CL) and an intracellular carboxyl-terminus (CT) (Vinken 2012).

1.2 - Connexins

Connexins are a highly conserved family of integral membrane proteins that are the building blocks for GJs. There are 21 different Cx isoforms found in the human genome and they are named for their calculated molecular weight in kDa. In addition, all Cx proteins share a common structural topology. They all have an intracellular amino-terminus (NT), four transmembrane spanning domains (TM1-TM4), two extracellular loops (E1 and E2), a cytoplasmic loop (CL) and an intracellular carboxyl-terminus (CT) (Fig. 1.1) (Sohl and Willecke 2004). The amino-terminus, four transmembrane domains and two extracellular loops are the most conserved sequences within the Cx protein family. The differences in Cxs stem mostly from the varying length and sequence of the cytoplasmic loop and carboxyl-terminus (Kovacs, Baker et al. 2007). Usually one or more Cxs are expressed in a given cell type, leading to the formation of various types of gap junctions that might have specific functions (Sohl and Willecke 2004).

The subcellular organelles responsible for oligomerization of Cxs into connexons can be different depending on the Cx type. For example, Cx32 subunits oligomerize in the endoplasmic reticulum (ER) and Cx43 subunits oligomerize in the trans-Golgi network (Saez, Berthoud et al. 2003). Although the location of Cx40 oligomerization has not been fully described, it is believed that most Cxs begin to oligomerize in the ER, as well as ER-Golgi intermediate compartments. From there, assembly progresses to form stable oligomers in the trans-Golgi network (Laird 2006). Currently, it is not completely clear where oligomerization takes place for each Cx subtype and further studies are needed to resolve this. Once oligomerization is complete, a number of different transport intermediates, with the help of microtubules to increase efficiency, deliver the connexons

to the cell surface. From here, they can dock with connexons from apposing cells to create gap junction channels (Laird 2006).

1.3 - Hemichannels

In addition to forming gap junction channels, it has also been shown that once inserted into the plasma membrane, connexons can remain unapposed and function as hemichannels. These hemichannels provide a direct passage between the intracellular environment and the extracellular space, which allows for the exchange of ions as well as the release of small signalling molecules such as ATP (Dale 2008), glutamate (Parpura, Scemes et al. 2004), NAD+ (Bruzzone, Guida et al. 2001) and prostaglandin E2 (Cherian, Siller-Jackson et al. 2005). These signalling molecules can then act in an autocrine or paracrine fashion on their respective receptors, to cause a downstream cellular response.

Hemichannels provide a large aqueous pore for ions and small signalling molecules to travel through, which is why the opening of hemichannels must be tightly regulated. The large pore size means that even a small number of open hemichannels can have a large impact on both a cell's electrical properties as well as its integrity (Verselis and Srinivas 2008). As a result of this, a common feature of all hemichannels is that at physiological conditions they have a low open probability, but they can be opened by a number of different stimuli including reduced concentrations of extracellular divalent cations, such as Ca^{2+} and Mg^{2+}, large and prolonged membrane depolarization, mechanical membrane stress and metabolic inhibition (Evans, De Vuyst et al. 2006; Wang, De Bock et al. 2013). Furthermore, hemichannel opening can also be influenced by pH, phosphorylation and redox status. Hemichannel opening under at least one of these conditions has already been described for the following Cxs: Cx26, Cx30, Cx32,

Cx35, Cx37, Cx38, Cx43, Cx44, Cx46, Cx50 and Cx56 (Evans, De Vuyst et al. 2006).

While hemichannel gating is not yet fully understood, there are currently two voltage -dependent mechanisms which can regulate hemichannel gating. One mechanism, known as fast or V_j-gating, has been previously described as the gating mechanism for gap junctions. This V_j-gating causes transitions from the fully open state to a subconductance state through either large negative or positive depolarizations depending on the Cx type (Bukauskas and Verselis 2004). The second gating mechanism, known as slow or loop-gating, causes transitions between the fully open state and the fully closed state of hemichannels in response to membrane depolarization and hyperpolarization, but is also controlled by extracellular divalent cations, especially Ca^{2+} and Mg^{2+} (Fasciani, Temperan et al. 2013). Extracellular divalent cations act on loop-gating by binding to a common extracellular site, which promotes a stable, long-lived closure configuration (Verselis and Srinivas 2008). In addition, it has also been demonstrated that $[Ca^{2+}]_o$ could act to modify loop-gating independently from Mg^{2+} by binding to a site located on the channel pore (Fasciani, Temperan et al. 2013). Although the basic mechanisms behind V_j and loop-gating are assumed to be a common property of all hemichannels (Bukauskas and Verselis 2004), Cx-specific differences exist in divalent cation regulation. For example, Cx46 hemichannels have a higher affinity for external Ca^{2+} than Cx32 hemichannels, but they have similar affinity for external Mg^{2+} (Verselis and Srinivas 2008). The gating mechanisms described above are not yet fully understood and for many Cx isoforms, including Cx40, it is unknown whether they exhibit any hemichannel function.

1.4 - Gap Junctions in the Heart

In the heart, gap junctions are critical for the direct electrical coupling of cardiomyocytes, facilitating the rapid conduction of action potentials throughout the myocardia, which is essential for synchronous heart contraction (Alex, Cale et al. 2005). Gap junctions are primarily located at the intercalated disks and are found in very low numbers at the lateral membrane (Jansen, van Veen et al. 2010). The heart expresses three main Cx isoforms: Cx40, Cx43 and Cx45 (Fig. 1.2). Cx40 is one of the main Cxs found in the atria, but it is also expressed in the His-bundle, the upper and lower bundle branches and the Purkinje fibres in the ventricular conduction system (Jansen, van Veen et al. 2010). Cx40 forms very high conductance homotypic channels that are believed to allow rapid propagation of the action potential in the conduction system (Alex, Cale et al. 2005). Cx43, which is the most abundant Cx found in the heart, is located throughout the atria, except the nodal area, and is also found in the ventricles and parts of the ventricular conduction system (Manias, Plante et al. 2008). Cx45 is mainly found in the sinoatrial and atrioventricular nodes, but also in the His-bundle and the upper and lower bundle branches (Jansen, van Veen et al. 2010).
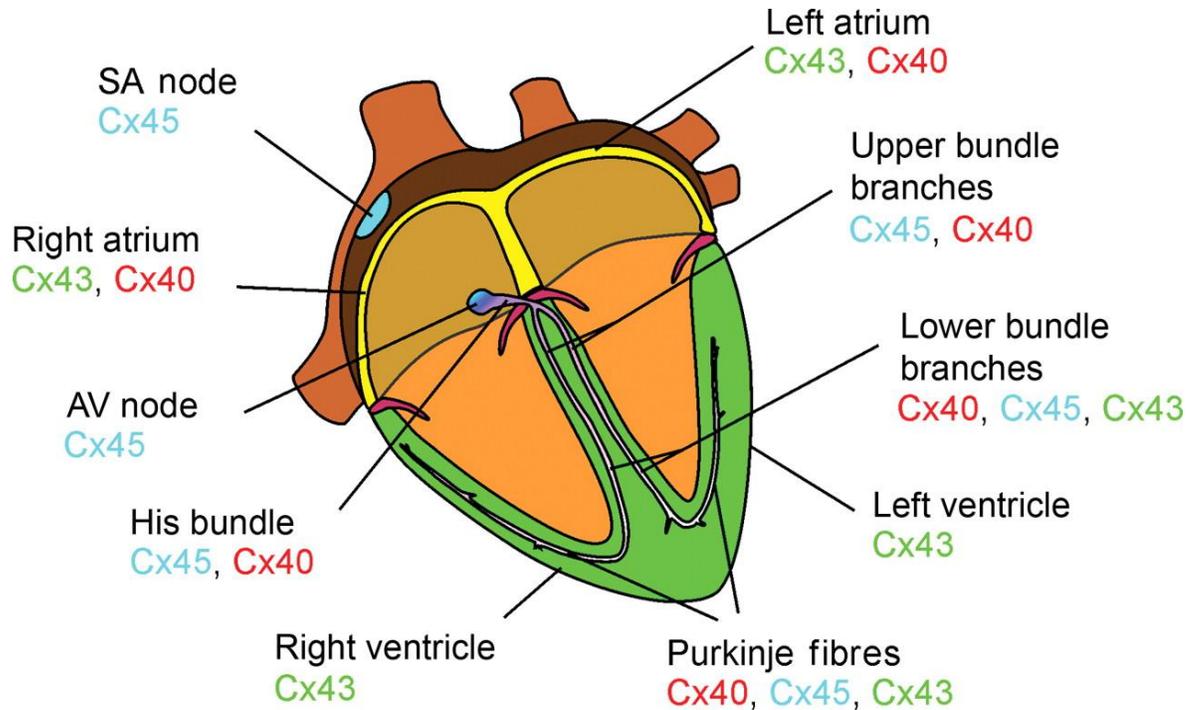
Figure 1.2 - Expression of gap junctions in the heart. Cx40 is highly expressed in the atria and can also be found in parts of the ventricular conduction system. Cx43 is also highly expressed in the atria as well as the ventricles. Cx45 is mainly found in the sinoatrial and atrioventricular nodes (Severs, Bruce et al. 2008).

1.5 - Atrial Fibrillation

Atrial fibrillation (AF) is the most common sustained cardiac arrhythmia and the prevalence of AF is increasing due to the aging of our population (Chaldoupi, Loh et al. 2009). Normal sinus rhythm is initiated in the sinoatrial node, propagated to the atria and then passed on to the ventricles. Sinus rhythm shows regular activation and contraction of the atria, followed by the regular and rhythmic activation and contraction of the ventricles. AF however, is characterized by rapid and irregular atrial activation, which is followed by uncoordinated and ineffective atrial contraction as well as rapid and irregular ventricular activity, which weakens ventricular contractions. This can lead to stagnant blood pooling in the atria and lead to thrombosis formation, which is why AF is a leading cause of embolic stroke (Wakili, Voigt et al. 2011).

AF is a very heterogeneous disease with a pathophysiology that is still not fully understood. Focal ectopic activity, which is described as automatic action potential firing that occurs out of sync with sinus rhythm, can act as a driving force to create and maintain AF, or it can act as a trigger in conjunction with a vulnerable substrate to cause a single or multiple re-entry circuit (Wakili, Voigt et al. 2011). There are two main mechanisms that contribute to focal ectopic activity. The first is known as an early afterdepolarization (EAD). If an action potential has a prolonged duration, the inactivated Ca^{2+} channels recover, which allows for Ca^{2+} to move inward leading to an EAD. EADs can raise nearby normally repolarizing cells to threshold and cause focal ectopic activity. However, the more significant contribution to focal atrial activity comes from delayed afterdepolarizations (DADs). DADs result from abnormal diastolic Ca^{2+} leak from the sarcoplasmic reticulum (SR) due to a malfunction of the ryanodine receptor, which is

caused by either overload of SR Ca^{2+} or hyperphosphorylation of the ryanodine receptor (Dobrev, Voigt et al. 2011). If a DAD is large enough, it can cause focal ectopic firing and if they are continual, they can lead to atrial tachycardia (Wakili, Voigt et al. 2011).

Re-entry circuits are created by the presence of both a trigger, such as ectopic firing, and a substrate, which is due to either structural or electrical changes in the heart. Under normal conditions where the re-entry substrate is absent, an ectopic beat will have a conduction time that is shorter than the refractory period of the cell it originated from, rendering the cell unexcitable. If however, the re-entry substrate is present, it can lead to beat re-entry in two ways. If the substrate causes a decreased action potential duration such that the refractory period is sufficiently reduced, the re-entering impulse is able to maintain itself in the circuit. Additionally, the substrate could cause slowed conduction of the ectopic beat, allowing for the refractoriness of the cell to dissipate, which will allow the ectopic beat to re-enter (Wakili, Voigt et al. 2011).

Approximately 70% of AF cases are secondary to pre-existing cardiovascular problems including hypertension, heart failure, valvular dysfunction and myocardial infarction. The rest of patients (~30%) have AF that is not due to other cardiovascular problems and this is known as idiopathic or lone atrial fibrillation (Saffitz 2006; Chaldoupi, Loh et al. 2009). Increasing evidence suggests that lone AF has a genetic basis and both somatic and germline mutations have been found in the gene encoding Cx40 in patients suffering from AF (Fig. 1.3) (Gollob, Jones et al. 2006; Yang, Liu et al. 2010; Yang, Zhang et al. 2010).
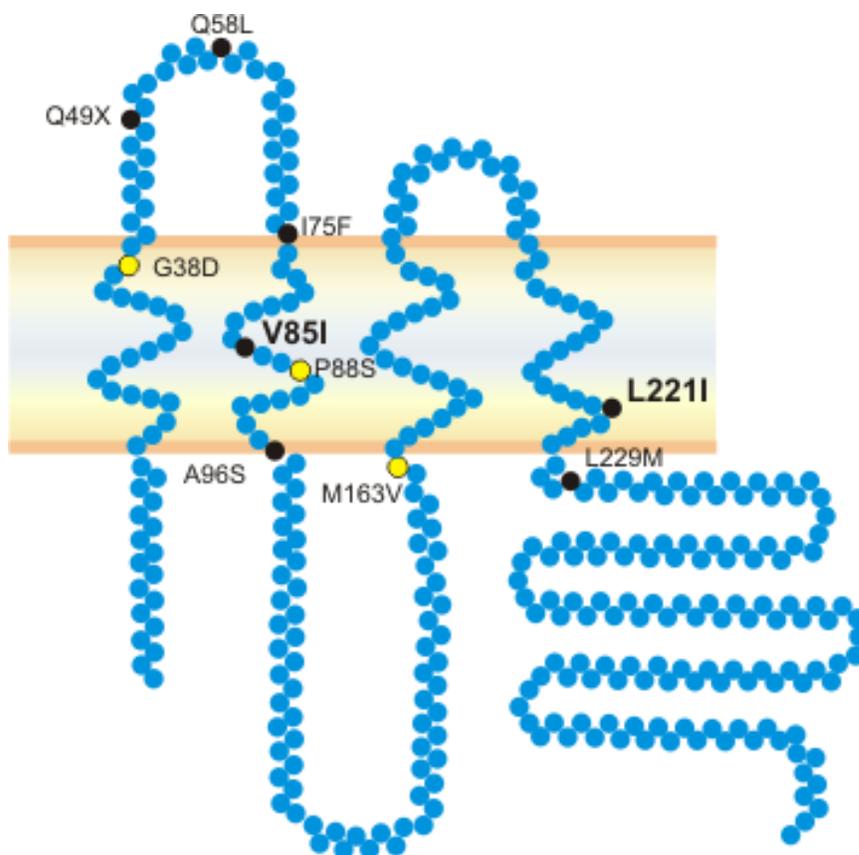
Figure 1.3 - Cx40 topology and mutant map. Three somatic Cx40 mutations (yellow) and seven germline Cx40 mutations (black) have been identified and linked to lone AF. The two bolded germline mutants (V85I and L221I) were chosen for this study.

1.6 - Cx40 and Atrial Fibrillation

The genetic factors determining lone AF are still unclear, however, there are a number of gene polymorphisms found in Cx40 that are linked to lone AF. In addition, studies have shown that Cx40 acts as the dominant Cx for conduction through the atria, and therefore changes to Cx40 can have a significant effect on atrial conduction (Chaldoupi, Loh et al. 2009). In mouse models, knockout of the Cx40 gene has shown decreased atrial conduction velocities, which increases their susceptibility to inducible AF (Hagendorff, Schumacher et al. 1999). In addition, in a goat model where AF was induced, the expression patterns of Cx40 became abnormal, although the protein levels remained unchanged. It was observed that there were areas of cells showing almost no Cx40 expression neighbouring cells that showed normal Cx40 expression. These changes in Cx40 expression eventually lead to a sustained version of AF (van der Velden, Ausma et al. 2000). In humans, abnormal Cx40 expression is also seen, but it presents in a different fashion. AF remodelling in humans is characterized by lower Cx40 expression at the intercalated disks and increased expression of Cx40 at the lateral membrane, leading to conduction heterogeneity and maintenance of re-entrant circuits (Severs, Coppen et al. 2004). Although there are many reports stating that AF leads to a number of different and sometimes conflicting changes in Cx40, most of these can be attributed to either species specific differences or the difference between the manner in which AF was induced; that is, pacing induced AF compared to naturally occurring AF (Severs, Coppen et al. 2004). Even with these differences, it is clear that Cx40 plays a major role in the pathogenesis of AF and it needs to be studied more thoroughly to give us a better understanding of the molecular mechanisms that contribute to AF.

A number of the previously identified Cx40 germline mutants have not yet been studied and at this point, none of them have been investigated for a possible change in hemichannel function. Here we have selected two novel Cx40 mutations, V85I and L221I, which were identified by Yang et al. (2010) from patients suffering from lone AF. The V85I mutation signifies a valine to isoleucine substitution at amino acid 85 and is found in the second transmembrane domain of Cx40. The L221I mutation indicates a leucine to isoleucine substitution found at amino acid 221 in the fourth transmembrane domain of Cx40. These mutations were found only in patients who suffered from lone AF, as well as all family members who suffered from lone AF. The mutations were not found in 200 unrelated, healthy, ethnically, age-matched control subjects, indicating that these are germline mutations (Fig. 1.4) (Yang, Liu et al. 2010).
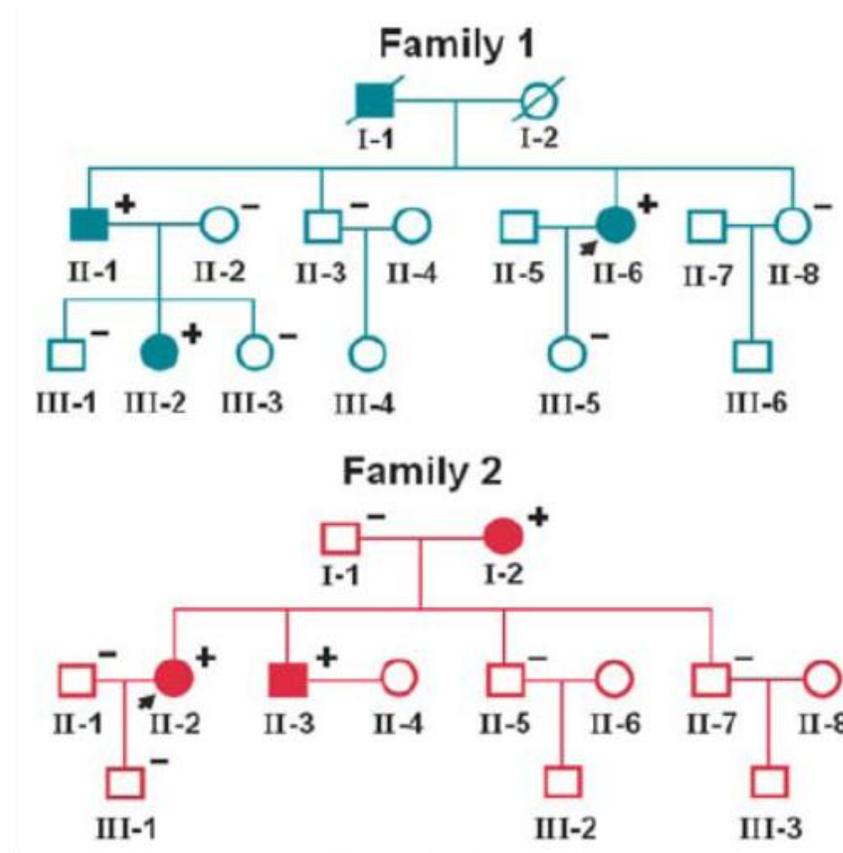
Figure 1.4 - Pedigrees representing the heritability of autosomal dominant atrial fibrillation-linked Cx40 mutants. Family 1 is affected by the V85I mutation. Family 2 is affected by the L221I mutation. Squares indicate males; circles, females; symbols with a slash, deceased; closed symbols, affected members; open symbols, unaffected members; arrow, proband; +, carriers of heterozygous mutation; - non-carriers (Yang, Liu et al. 2010).

1.7 - Hypothesis and Objectives

1) Hypothesis

The AF-linked Cx40 mutants, V85I and L221I, alter the gap junction channel, or hemichannel, distribution and/or function.

2) Objectives

I. To determine whether the Cx40 mutants are able to traffic to the cell-cell junction and form gap junction plaque-like structures in model cell systems.

II. To determine whether the Cx40 mutants can form functional homotypic gap junction channels in gap junction deficient N2A cells.

III. To determine if Cx40 and Cx40 mutants can form functional, unapposed hemichannels at the cell membrane of connexin-deficient HeLa cells.

1.8 - References

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2. Atrial fibrillation-linked germline *GJA5*/connexin40 mutants showed an increased hemichannel function

Mutations in *GJA5* encoding the gap junction protein connexin40 (Cx40) have been linked to lone atrial fibrillation (AF). Some of these mutants result in impaired gap junction function due to either abnormal connexin localization or impaired gap junction channels, which may play a role in promoting AF. However, the effects of the AF-linked Cx40 mutants on hemichannel function have not been studied. Here we investigated two AF-linked germline Cx40 mutants, V85I and L221I. These two mutants formed putative gap junction plaques at cell-cell interfaces, with similar gap junction coupling conductance as that of wild-type Cx40. Connexin deficient HeLa cells expressing either of these two mutants displayed prominent propidium iodide (PI)-uptake distinct from cells expressing wild-type Cx40 or other AF-linked Cx40 mutants, I75F, L229M and Q49X. The PI-uptake was sensitive to $[Ca^{2+}]_o$ and carbenoxolone, but was not affected by probenecid, indicating that uptake is mediated via connexin hemichannels. A gain-of-hemichannel function in these two AF-linked Cx40 mutants may provide a novel mechanism underlying the etiology of AF.

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2.1 - Introduction

Gap junctions are intercellular channels formed by dodecamers of integral membrane protein subunits known as connexins (Cxs). Gap junctions allow for the direct exchange of ions and small molecules between opposing cells (Goodenough, Goliger et al. 1996). The Cx family of proteins all share a common structural topology, which consists of an intracellular amino-terminus, four transmembrane domains, two extracellular loops, a cytoplasmic loop and an intracellular carboxyl-terminus (Kumar and Gilula 1996). The oligomerization of six Cxs forms a hemichannel (also known as connexon) and two hemichannels on the plasma membrane of neighbouring cells can dock end-to-end to form a gap junction channel.

In addition to forming gap junction channels, Cxs are also able to form undocked hemichannels on the plasma membrane. These hemichannels provide a direct passage between the intracellular environment and the extracellular space, which allows for the release of small intracellular molecules such as ATP (Dale 2008), glutamate (Parpura, Scemes et al. 2004), NAD+ (Bruzzone, Guida et al. 2001) and prostaglandin E2 (Cherian, Siller-Jackson et al. 2005). These signaling molecules can then act on their respective receptors located on the same cell (autocrine) or its neighbour cells (paracrine). A common feature of all hemichannels is that under physiological conditions they have a low open probability, but can be opened by a number of different stimuli including reduced concentrations of extracellular divalent cations, such as Ca^{2+} and Mg^{2+}, large and prolonged membrane depolarization, mechanical membrane stress and/or metabolic inhibition (Evans, De Vuyst et al. 2006; Wang, De Bock et al. 2013).

In the heart, gap junctions facilitate direct electrical coupling between cardiomyocytes, allowing for rapid propagation of action potentials in the atria and ventricles, which is essential for synchronous contractions (Davis, Rodefeld et al. 1995). The human heart expresses three main Cx isoforms: Cx40, Cx43 and Cx45. Both Cx40 and Cx43 are highly expressed in the atria and Cx43 is also abundant in the ventricles. In contrast, Cx45 is mainly found in the sinoatrial and atrioventricular nodes (Jansen, van Veen et al. 2010). In addition to its extensive expression in the atria, Cx40 is also found in parts of the ventricular conduction system, such as the His-bundle, the upper and lower bundle branches and the Purkinje fibres. Several recent studies indicate somatic and germline mutations in the Cx40 gene (*GJA5*) are associated with lone atrial fibrillation (AF) (Gollob, Jones et al. 2006; Yang, Liu et al. 2010; Yang, Zhang et al. 2010; Sun, Yang et al. 2013). Studies by us and others on these AF-linked Cx40 mutants revealed various changes in cellular distribution and gap junction function (Gollob, Jones et al. 2006; Thibodeau, Xu et al. 2010; Sun, Yang et al. 2013). However, it is not known if there are any changes in the hemichannel function for any of the AF-linked Cx40 mutants. Here we investigated two novel germline Cx40 mutations, V85I and L221I, identified by Yang et al. (2010) from two Chinese families with inherited lone AF. These mutations were autosomal dominantly inherited and mutant carriers in the family showed early onset of AF. These mutations were not found in other members in the family or in 200 unrelated, healthy, ethnic- and age-matched control subjects (Yang, Liu et al. 2010). We observed little change in the gap junction distribution and function of these two Cx40 mutants in our model cells. However, these two AF-linked Cx40 mutants showed an increase in propidium iodide (PI) uptake under conditions favoring hemichannel opening.

Interestingly, under the same conditions, we did not observe PI-uptake in wild-type Cx40 expressing cells, indicating that the mutants showed a gain of hemichannel function, which may play a role in the pathogenesis of AF.

2.2 - Methods

2.2.1 - Plasmid Construction

The human Cx40-YFP, Cx40-IRES-GFP, Cx43-IRES-GFP and Cx26-GFP constructs were created as previously described (Thomas, Telford et al. 2004; Sun, Yang et al. 2013). The C-terminal fusion YFP-tagged (V85I-YFP and L221I-YFP) and the non-fusion GFP-tagged (V85I-IRES-GFP and L221I-IRES-GFP) constructs were generated by the Quick-Change site directed mutagenesis kit (Stratagene, La Jolla, CA) on the respective template with the following primers: the forward 5'-CAGATCATCTTCATCTCCACGCCCT-3' and the reverse 5'-AGGGCGTGGAGATGAAGATGATCTG-3' for V85I and the forward 5'-CTGTCCCTCCTCATTAGCCTGGCTG-3' and the reverse 5'-CAGCCAGGCTAATGAGGAGGGACAG-3' for L221I. All connexin clones were sequenced to confirm the accuracy of the nucleotide sequence and no additional variations were introduced.

2.2.2 - Cell Culture and Transfection

HeLa (human cervical carcinoma) cells were grown in Dulbecco's modified Eagle's medium (DMEM, Invitrogen, Burlington, ON) containing 4.5 g/L D-glucose, 584 mg/L L-glutamine, 110 mg/L sodium pyruvate, 10% fetal bovine serum and 1% penicillin and streptomycin, in an incubator with 5% CO_2 at 37°C. HeLa cells were plated at 60-80% confluence on 35 mm Petri dishes 12-24 hours before transfection. For each transfection, HeLa cells were incubated with 1.5 µg of a cDNA construct and 3 µl of X-tremeGENE HP DNA transfection reagent (Roche, Mississauga, ON) in Opti-MEM I + GlutaMAX-I medium supplemented with HEPES and 2.4 g/L sodium bicarbonate (Invitrogen) for 4 hours. Medium was then changed back to the modified DMEM and cells were used for either localization studies or dye uptake assays approximately 18-24 hours after transfection.

2.2.3 - Localization Study

To observe the localization of Cx40-YFP, V85I-YFP and L221I-YFP, HeLa cells were cultured on glass bottom dishes and were transfected individually with the respective cDNA constructs. After culturing for 24 hours, the cells were fixed with a solution of 80% methanol and 20% acetone for 20 minutes at -20°C. Wild-type Cx40-YFP and YFP-tagged mutants were imaged using a Zeiss LSM 510-META confocal microscope as described earlier (Sun, Yang et al. 2013). To quantify the percentage of gap junction plaque-like structures at the cell-to-cell interfaces of successfully transfected cells, approximately 20-30 cells were counted for each transfection.

To observe the localization of untagged Cx40 and mutants, HeLa and N2A cells were transfected with Cx40-IRES-GFP, V85I-IRES-GFP or L221I-IRES-GFP. After culturing for 24h, cells were rinsed with PBS and fixed for 10 minutes in a 1:1 solution of acetone and methanol at -20°C. Cells were then blocked for 1 hour with 5% BSA in PBS. Anti-Cx40 antibody (Millipore, Billerica, MA) was incubated for 1h at room temperature. The cells were washed and subsequently stained for 30 min with the secondary Alexa 594–conjugated antibody (Invitrogen) prior to confocal microscopy.

2.2.4 - Dye Uptake Assay

Propidium iodide (PI)-uptake assay was used to assess the hemichannel function of YFP-tagged Cx40 and mutants. HeLa cells were plated at a low density to allow for isolated, single cells to be transiently transfected as described above. The cells were washed with regular extracellular solution (ECS) (also known as divalent cation-containing ECS, DCC-ECS) containing 142 mM NaCl, 5.4 mM KCl, 1.4 mM $MgCl_2$, 2 mM $CaCl_2$, 10 mM HEPES and 25 mM D-Glucose. The pH of ECS was adjusted to 7.35 and the osmolarity was adjusted to 298 mOsm. The cells were then washed with divalent cation free-ECS (DCF-ECS), which contains no Ca^{2+} or Mg^{2+}. The addition of EGTA (2 mM) to this solution chelated the remaining ambient divalent cations. The cells were incubated in DCF-ECS-containing PI (150 μM) at 37°C for 15 minutes to assess PI-uptake. After incubation, the cells were washed three times with regular ECS and the percentage of transfected cells showing PI-uptake was measured under a fluorescent microscope (DMIRE2, Leica). Cells in pairs and clusters were excluded from measurement to avoid errors produced by gap junctions. Negative controls (untransfected

cells and YFP-transfected cells) and positive control (Cx26-GFP transfected cells) and the various incubation conditions (e.g. with hemichannel blocker carbenoxolone or different $[Ca^{2+}]_o$) were indicated in each experiment. For each experiment approximately 30-40 cells were counted to obtain a percentage of PI-uptake. The bar graphs were generated with 5-15 transfections. Similar PI-uptake experiments were performed on HeLa cells transfected with the mutant-IRES-GFP constructs with a slightly longer PI-incubation time (20 minutes).

For the experiment with time lapsed measurement of PI-uptake, HeLa cells were cultured in glass bottom dishes. Fluorescence measurements of PI were performed with a confocal microscope (LSM 510 Meta, Zeiss, Germany). Baseline fluorescence (F0) in regular ECS and the increase of PI-uptake during the incubation of DCF-ECS (F) were collected at 1 minute intervals for 20 minutes. The obtained images were quantitatively analyzed using ZEN software for changes in fluorescence intensities within regions of interest (ROIs) of isolated GFP-positive cells, which expressed mutant-IRES-GFP. The intracellular fluorescence changes during PI incubation are expressed as the ratio of current fluorescence intensity over that of the baseline (F/F_0).

2.2.5 - Electrophysiological Studies

Electrophysiological recordings for measuring gap junction coupling were carried out in connexin-deficient N2A cells. N2A cells were grown at 37°C in 35-mm culture dishes to 70% confluence in Dulbecco modified Eagle medium containing 10% FBS. Cells were transiently transfected with mutant or wild-type connexin DNA by X-tremeGENE HP reagent. The dual whole-cell patch clamp technique was performed to

assess the gap junctional conductance (G_j) between cell pairs 24 hours after transfection (Roscoe, Veitch et al. 2005; Sun, Yang et al. 2013). The junctional current (I_j) was amplified via a MultiClamp 700A amplifier (Molecular Devices, Sunnyvale, CA) and was digitized at a sampling rate of 10 kHz with a Digidata 1322A (Molecular Devices, Sunnyvale, CA). Data were analyzed with pClamp9 software. Each cell of a pair was initially held at a common holding potential of 0 mV. To evaluate junctional coupling, 20 mV pulses for 7 seconds were applied to one cell to establish a transjunctional voltage (V_j), while the junctional currents (I_j) were measured in the other cell. Macroscopic junctional conductance (G_j) was calculated as follows: $G_j = I_j/V_j$. In all cases, cells were studied after multiple independent transfections and only cell pairs with fluorescent protein signals were selected for double patch clamp recording.

2.2.6 - Statistical Analysis

One-way ANOVA followed by Newman-Keuls test was used to compare the multiple groups of data on G_j and PI-uptake percentage. Statistical significance is denoted with asterisks (*, $P < 0.05$ or ***, $P < 0.001$) on the graphs. The data presented on the graphs are expressed as mean ± standard error of the mean (SEM). Unless specified, all experiments were performed at least three times.

2.3 - Results

2.3.1 - Localization of YFP-tagged Cx40 mutants

The localization of wild-type Cx40-YFP and the AF-linked Cx40 mutants, V85I-YFP or L221I-YFP, were examined in connexin-deficient HeLa cells expressing the respective constructs. As shown in Fig. 2.1A, Cx40-YFP, V85I-YFP and L221I-YFP were all able to traffic to the plasma membrane and form gap junction plaque-like structures at cell-cell interfaces. Free YFP did not form gap junction plaque-like structures at cell-cell interfaces (data not shown). To further quantify the probability of gap junction plaque formation, we calculated the percentage of the cell pairs/clusters displaying putative gap junction plaques at cell-cell interfaces. The percentage of successful formation of gap junction plaques of V85I-YFP- and L221I-YFP-expressing cells were $65 \pm 2\%$ (n = 7) and $46 \pm 2\%$ (n = 7), respectively and were found to be statistically lower than that of the cells expressing Cx40-YFP ($89 \pm 2\%$, n = 7; P < 0.001 for both mutants), indicating that these two AF-linked mutants showed a modest but statistically significant decrease in the formation of gap junction plaque-like structures at cell-cell interfaces.

2.3.2 - G_js of the V85I- and L221I-expressing cell pairs were the same as that of cell pairs expressing wild-type Cx40

Dual patch clamp technique was used to measure the coupling conductance (G_j) of N2A cell pairs expressing Cx40-YFP, V85I-YFP or L221I-YFP. Transjunctional currents (I_js) in response to a 20 mV transjunctional voltage pulse (V_j) are shown in Fig.

2.1B. Our results indicate that the averaged coupling conductance (G_js) for each mutant was not statistically different from the control (Cx40), demonstrating that the gap junction function of these two mutants was not impaired in the N2A cells.
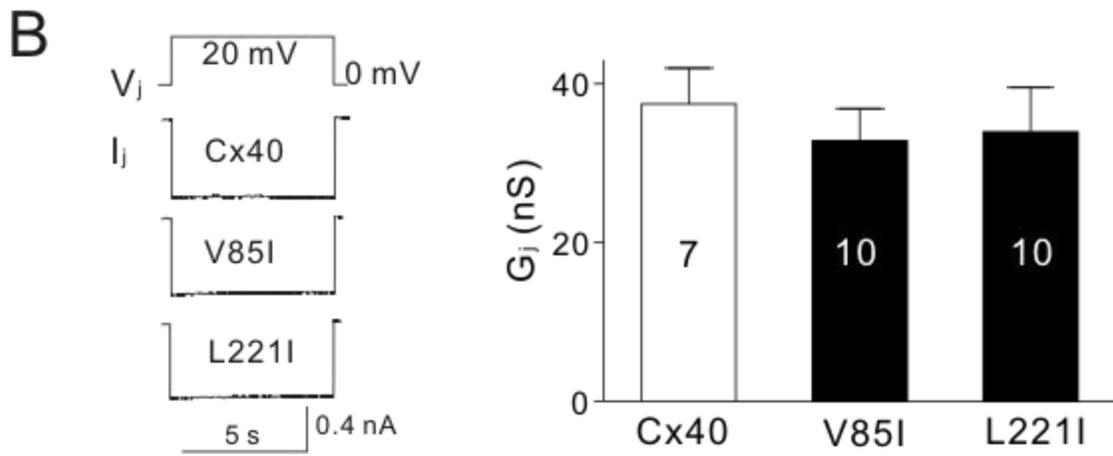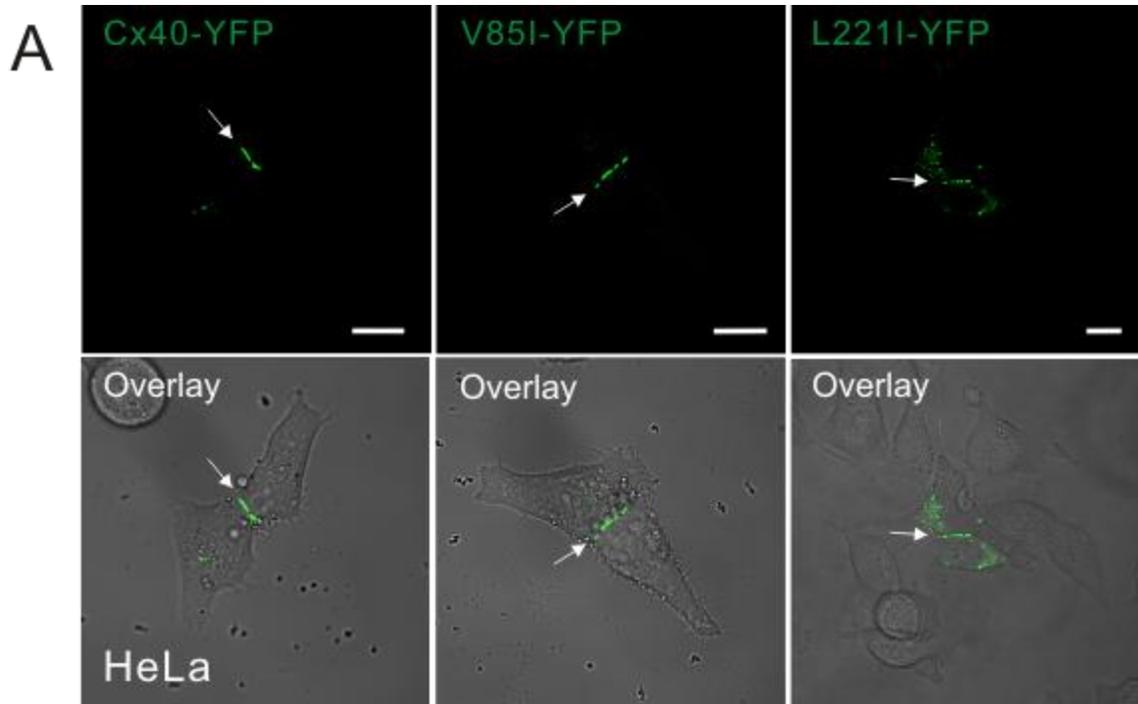
Figure 2.1 - The localization and macroscopic dual whole-cell patch clamp recordings of YFP-tagged homotypic Cx40 and Cx40-mutant gap junctions. (A) Representative fluorescent confocal images of HeLa cells transfected with Cx40-YFP, V85I-YFP and L221I-YFP (top panels). The overlaid fluorescent images on top of phase contrast images are also shown (bottom panels). All three constructs were able to form gap junction plaque-like structures at the cell-cell junction. Scale bar = 10 μm. **(B)** Voltage steps of 20 mV were applied to one cell of a transfected N2A cell pair and the junctional current (I_j) was recorded in the second cell. There was no significant difference between the I_j of Cx40-YFP, V85I-YFP or L221I-YFP. The junctional conductance (G_j) was calculated and there was no significant difference between the G_j of Cx40-YFP, V85I-YFP or L221I-YFP.

2.3.3 - V85I- and L221I-expressing cells showed increased propidium iodide-uptake

Since we observed no apparent gap junction function defects of these two Cx40 mutants in our model cells, this prompted us to look into possible changes in non-gap junction linked functions, including hemichannel function. To facilitate the opening of undocked Cx40 gap junction hemichannels in HeLa cells, we removed both Ca^{2+} and Mg^{2+} and added EGTA (2 mM) to chelate the ambient low level of divalent cations. This solution was defined as divalent cation free-extracellular solution (DCF-ECS). HeLa cells expressing Cx26-GFP, incubated DCF-ECS and PI, showed a prominent PI-uptake in 86% of cells (Fig. 2.2A, B), while the majority of untransfected HeLa cells or YFP-expressing HeLa cells failed to show PI-uptake (Fig. 2.2A, B), suggesting that undocked Cx26 hemichannels may be responsible for the PI-uptake. Interestingly, positive PI-uptake was identified in the majority of HeLa cells expressing YFP-tagged Cx40 mutants, V85I ($67.6 \pm 6.6\%$, n = 10) and L221I ($83.2 \pm 2.8\%$, n = 10). In contrast to these findings, Cx40-YFP-expressing cells failed to show a significant PI-uptake under the same conditions (4.6 ± 1.2 %, n = 14). This was significantly different from the PI-uptake observed for the two mutants ($P < 0.001$), but was similar to that of the negative control, YFP-expressing cells (4.0 ± 1.6 %, $P > 0.05$).

Our previous studies showed that AF-linked Cx40 mutants, I75F and Q49X, impaired homotypic gap junction function, while L229M did not impair homotypic gap junction function, but specifically impaired the gap junction function when co-expressed with Cx43 (Sun, Yang et al. 2013). Here we tested PI-uptake of HeLa cells expressing these Cx40 mutants individually. As shown in Fig. 2.2B, these mutants all failed to show

any substantial PI-uptake, indicating that their undocked hemichannels are unlikely to be

in the open state during the incubation with DCF-ECS.
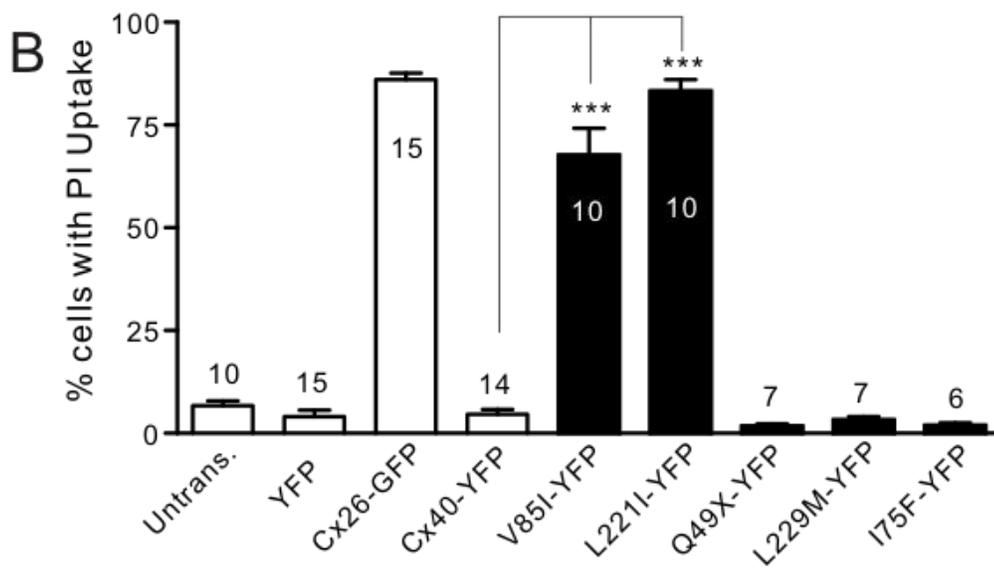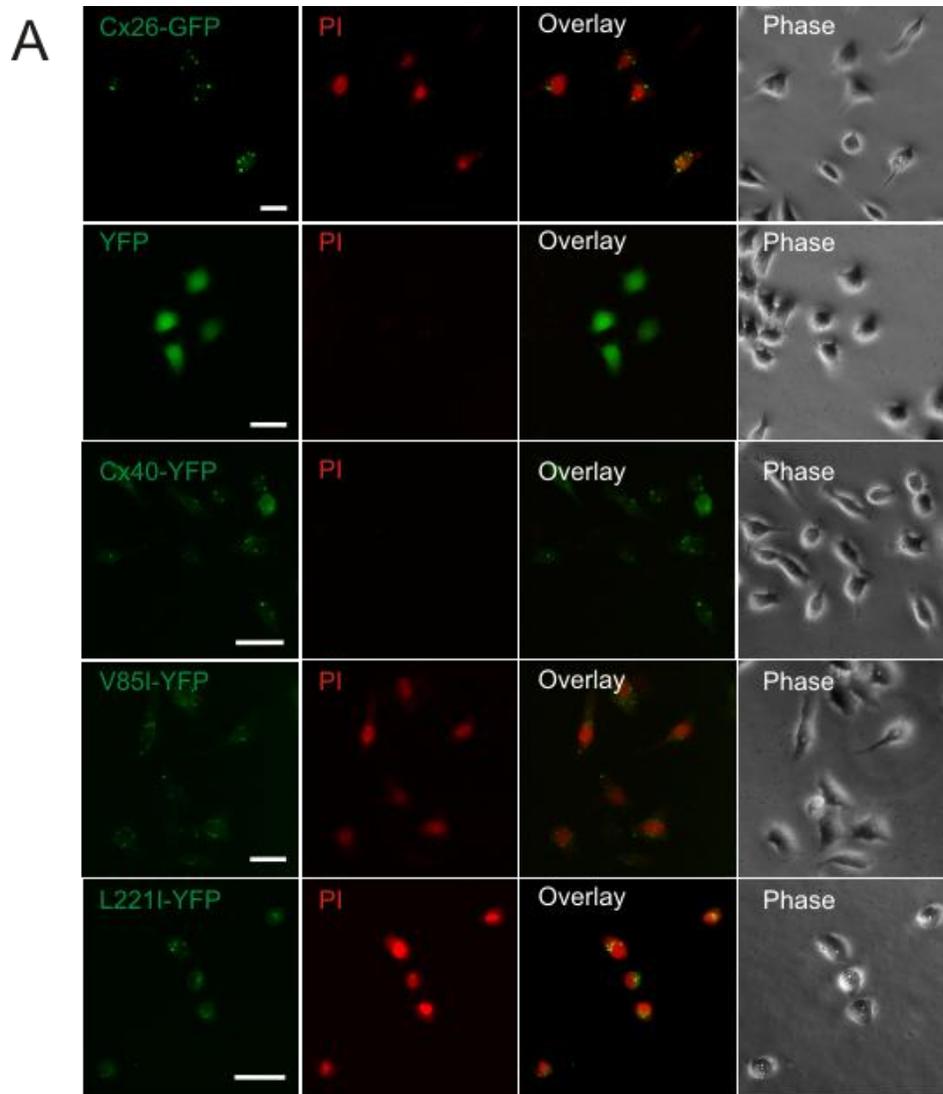
Figure 2.2 - PI-uptake under DCF+EGTA conditions. (A) Representative images of PI-uptake under DCF+EGTA conditions for isolated, individual, transfected HeLa cells. Successful transfection can be identified by their tagged green/yellow fluorescent proteins (green colour in the first column images). PI-uptake (red colour in the second column images) can be seen in cells expressing Cx26-GFP, V85I-YFP and L221I-YFP, but no uptake was seen in cells expressing YFP alone or Cx40-YFP. Scale bar = 20 μm. **(B)** Quantification of PI-uptake under DCF+EGTA conditions. V85I-YFP (67.6%, n = 10) and L221I-YFP (83.2%, n = 10) showed a significant increase in PI-uptake compared to Cx40-YFP (4.6%, n = 14) at P < 0.001. Other AF-linked Cx40 mutants, Q49X, L229M and I75F, were also studied and did not show any PI-uptake.

2.3.4 - The role of extracellular divalent cations and hemichannel blocker carbenoxolone on PI-uptake

Previous studies indicate that several gap junction hemichannel-mediated dye-uptake can be blocked by the elevation of extracellular calcium concentration or addition of the hemichannel blocker CBX (Wang, De Bock et al. 2013). We hypothesized that the PI-uptake was due to the undocked connexin hemichannels on the plasma membrane. To test this, transfected HeLa cells were incubated with PI in the presence of divalent cation containing solution (DCC-ECS) or CBX (100 μM). Both DCC-ECS and CBX effectively eliminated PI-uptake in cells expressing Cx26-GFP and Cx40 mutants, V85I and L221I (Fig. 2.3A).

To quantitatively assess $[Ca^{2+}]_o$ dependence of the PI-uptake, several $[Ca^{2+}]_o$ concentrations from nominal Ca^{2+}-free to 2 mM were tested. Our data indicated that cells expressing Cx26 or the Cx40 mutants, V85I and L221I, displayed $[Ca^{2+}]_o$ concentration-dependent PI-uptake (Fig. 2.3B). In the range of 0.2 and 0.02 mM $[Ca^{2+}]_o$, Cx26-expressing cells showed a higher level, L221I-expressing cells showed an intermediate level and V85I-expressing cells showed a lower level PI-uptake (Fig. 2.3B), suggesting that these hemichannels may have different sensitivities to $[Ca^{2+}]_o$. For Cx40-expressing cells, no PI-uptake was observed for any calcium concentrations tested (Fig. 2.3B).
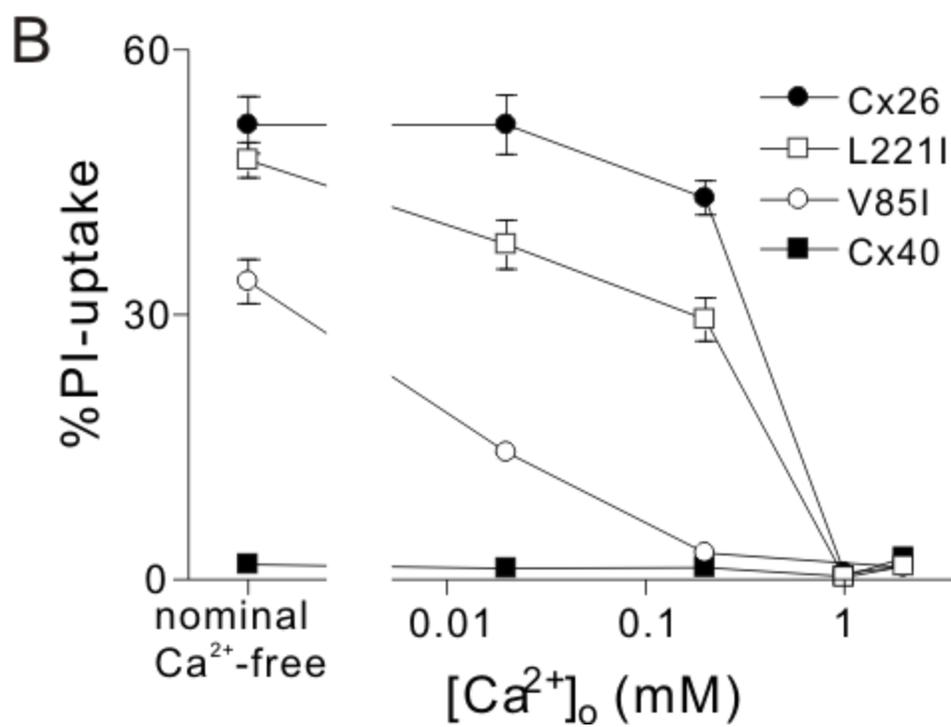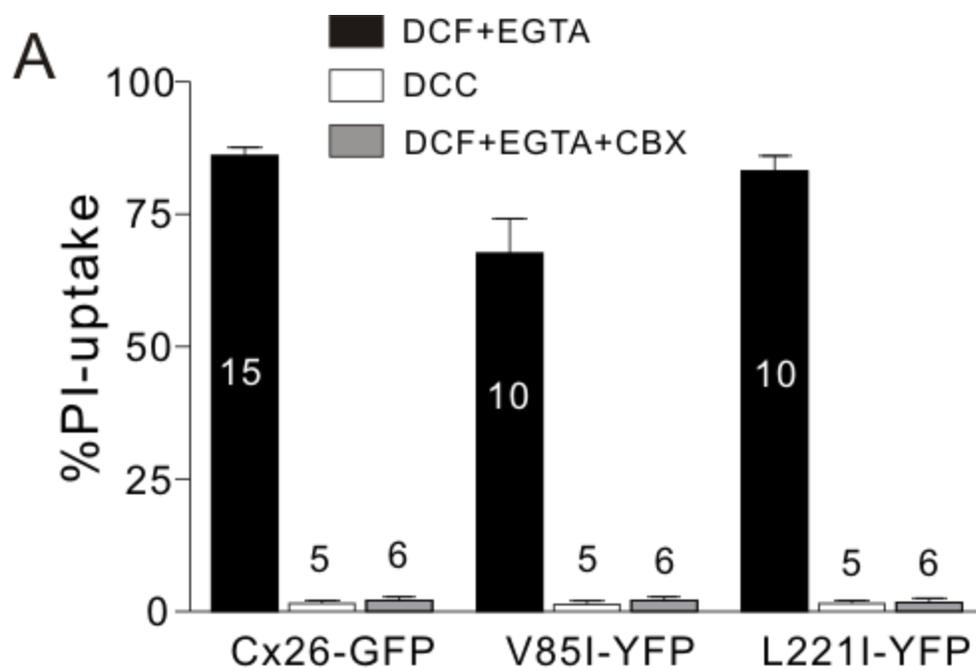
Figure 2.3 - The effect of external Ca^{2+} concentration on PI-uptake. (A) Comparison of PI-uptake for divalent cation containing (DCC) and divalent cation-free (DCF) conditions. The PI-uptake for cells expressing Cx26-GFP, V85I-YFP and L221I-YFP was significantly increased under DCF conditions compared to DCC conditions. Also the addition of the hemichannel blocker CBX (100 µM) under DCF+EGTA conditions significantly decreased PI-uptake. **(B)** [Ca^{2+}]$_o$ dose dependent PI-uptake. Cx26-GFP, V85I-YFP and L221I-YFP all showed [Ca^{2+}]$_o$ dependent PI-uptake. Comparatively speaking, Cx26-GFP showed the highest amount of PI-uptake under all concentrations. L221I-YFP showed intermediate PI-uptake and V85I-YFP showed low amounts of PI-uptake. Cx40-YFP did not show PI-uptake for any concentrations tested.

2.3.5 - Untagged Cx40 mutants

Fusion of fluorescent proteins (GFP, YFP or RFP) at the carboxyl terminus of connexins is very useful in determining the distribution and function of the connexin in live cells. However, to verify our results obtained from using YFP-tagged Cx40, we also studied untagged Cx40 mutants using mutant-IRES-GFP constructs. We expressed V85I-IRES-GFP and L221I-IRES-GFP in HeLa and N2A cells. Anti-Cx40 antibody was used to reveal the localization of expressed Cx40 mutants. As shown in Fig. 2.4A, V85I and L221I showed a similar intracellular distribution pattern and both of them were able to reach cell-cell interfaces to form gap junction plaque-like structures, similar to that observed for wild-type Cx40, in both HeLa and N2A cells.

The coupling conductance (G_j) of N2A cell pairs expressing either of these mutants showed a similar level as that of Cx40-expressing cells. All of these G_js were much higher than cell pairs expressing GFP (Fig. 2.4B).

PI-uptake was assessed the same way as described earlier by incubating HeLa cells in DCF-ECS. Both V85I and L221I showed a significantly higher level of PI-uptake than that of Cx40 (Fig. 2.5A). We also found that cells expressing these two mutants displayed higher levels of PI-uptake than that of wild-type Cx43 (Fig. 2.5A). Adding divalent cations or CBX (100 μM) virtually eliminated PI-uptake, while the pannexin 1 channel blocker, probenecid (200 μM), failed to decrease the PI-uptake (Fig. 2.5B). These results indicated that the PI-uptake was likely via undocked hemichannels and not pannexin 1 channels.

To evaluate the time course of PI-uptake during the incubation with DCF medium, we monitored the cellular PI-fluorescent level changes. L221I- and V85I-expressing cells

showed a time-dependent increase in PI-uptake and saturated near the end of the 20 minute incubation (Fig. 2.6). V85I-expressing cells showed a slightly slower rate of PI-uptake within the first 10 minutes than that of L221I-expressing cells. However, they both reach a similar level of PI-uptake near the end of 20 minute-incubation. Again, addition of CBX (100 μM) abolished the PI-uptake of mutant-expressing cells.
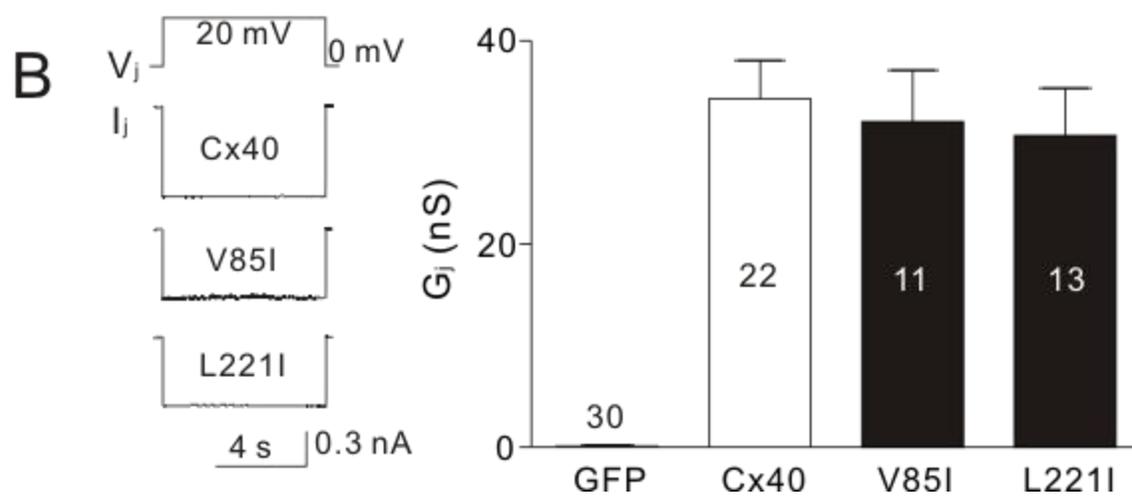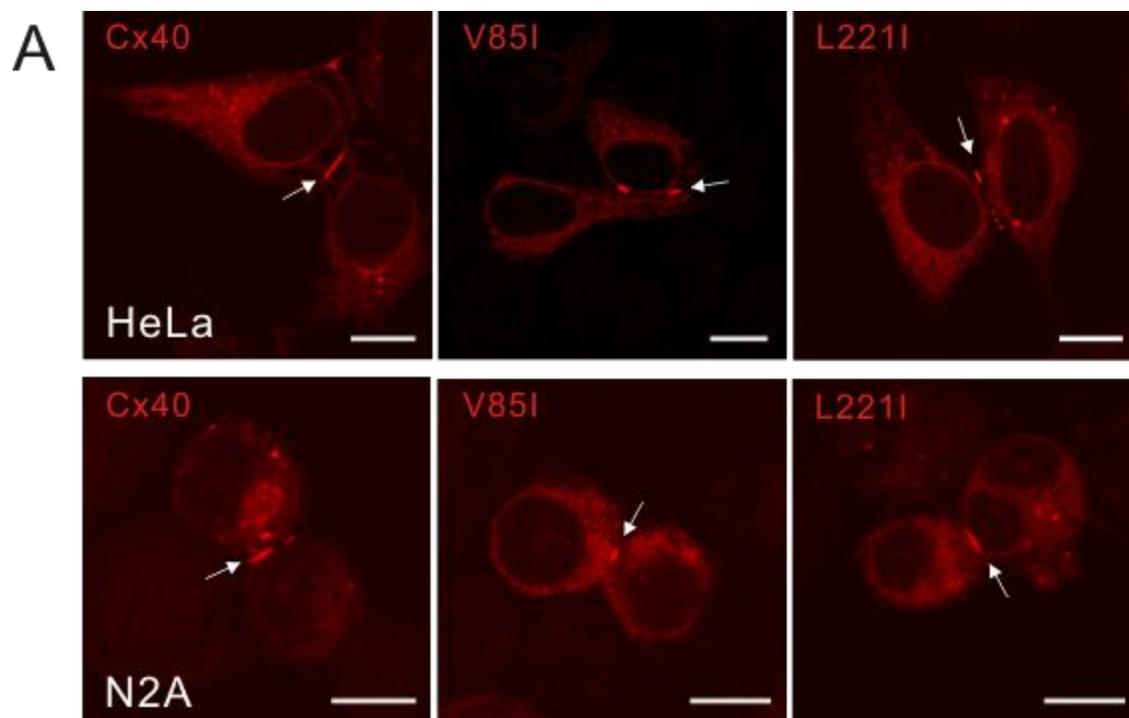
Figure 2.4 - The localization and function of untagged homotypic Cx40 and Cx40-mutant gap junctions. (A) Representative confocal images showing the antibody localizations of untagged Cx40, V85I and L221I expressed in HeLa (top panels) and N2A (bottom panels) cells. In both cell lines, Cx40, V85I and L221I were all able to traffic to the cell-cell interface and form gap junction plaque-like structures. Scale bar = 10 μm. **(B)** Voltage steps of 20 mV were applied to one cell of a transfected N2A cell pair and the junctional current (I_j) was recorded in the second cell. There was no significant difference between the I_j or G_j of Cx40, V85I or L221I.
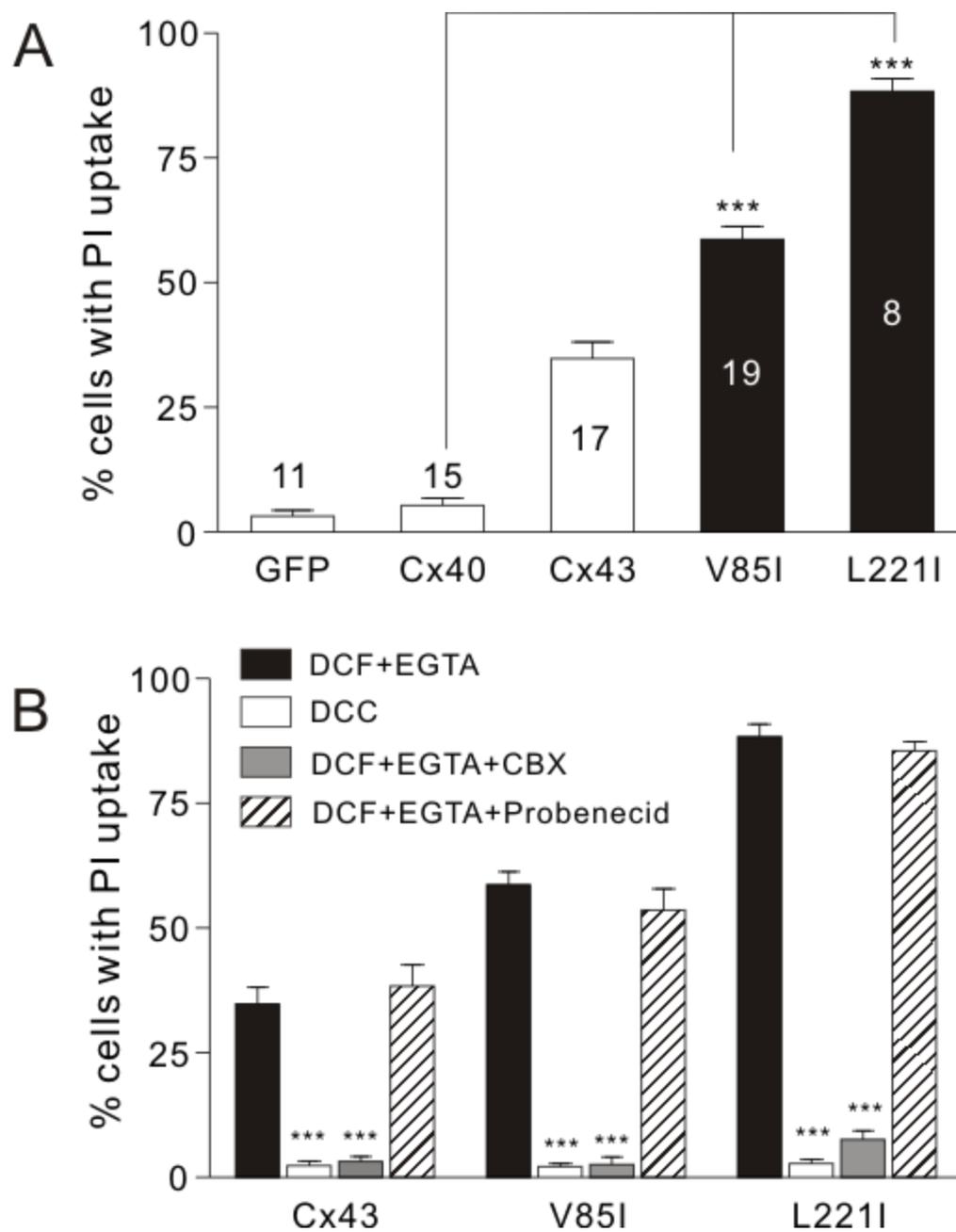
Figure 2.5 - PI-uptake of untagged Cx40 and Cx40 mutants. (A) Untagged V85I and L221I showed a significant increase in PI-uptake compared to both wild-type Cx40 and Cx43 under DCF+EGTA conditions. **(B)** The addition of divalent cations (DCC, open bars) or CBX (100 µM, gray bars) blocked the PI-uptake for Cx43, V85I and L221I, however, the addition of the pannexin 1 channel blocker probenecid (200 µM, hatched bars) did not affect PI-uptake.
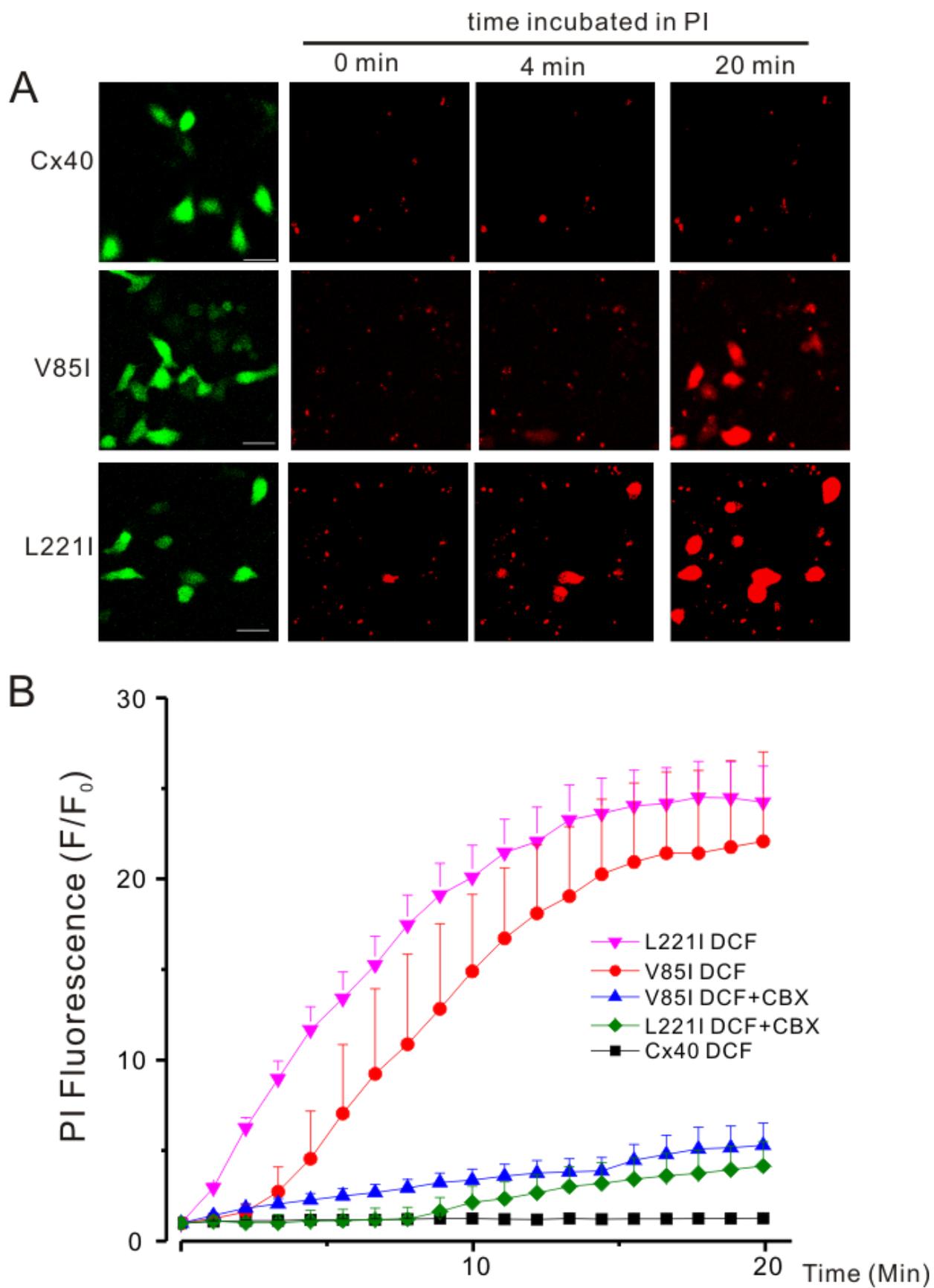
Figure 2.6 - Time course of PI-uptake under DCF+EGTA conditions. (A) Representative confocal images of PI-uptake for HeLa cells transfected with Cx40, V85I and L221I, showing time points of 0, 4 and 20 minutes of incubation with PI. During 20 minutes of incubation with PI, only cells expressing the V85I and L221I mutants showed PI uptake. Scale bar = 50 μm. **(B)** Ratio of current PI fluorescence intensity over the initial baseline fluorescence over a 20 minute incubation. L221I showed the fastest rate of PI-uptake, with V85I having a slightly slower rate of uptake, however, both L221I and V85I had similar levels of PI-uptake after the 20 minute incubation. The addition of 100 μM CBX blocked PI-uptake. Cx40 expressing cells failed to show any PI-uptake.

2.4 - Discussion

Here we studied two previously identified AF-linked germline mutations in the Cx40 gene. Our data indicate that V85I and L221I showed a statistically significant reduction in gap junction plaque formation at cell-cell interfaces. However, the functional inspection of the coupling conductance in N2A cell pairs expressing any of the mutants did not show a change from that of the wild-type Cx40, indicating that these mutants were unlikely to impair gap junction function. To further evaluate if there were any changes in the hemichannel function, we performed PI-uptake assays in divalent cation-free medium. To our surprise, cells expressing wild-type Cx40 did not show any PI-uptake, but V85I- and L221I-expressing cells showed a pronounced PI-uptake at a level similar to Cx26-expressing cells. $[Ca^{2+}]_o$ and carbenoxolone sensitivities, as well as not being blocked by a pannexin 1 channel blocker, argue that the PI-uptake is due to the opening of connexin hemichannels on the plasma membrane. A gain of hemichannel function for these two AF-linked Cx40 mutants provide a completely novel mechanism for possible AF-pathogenesis. Our tests on several previously studied AF-linked Cx40 mutants suggest that a number of different mechanisms can link these mutants to AF. This suggests that mutations of the same gene (*GJA5*) can result in a variety of impairments, including impaired trafficking to the gap junction site, reduced/eliminated gap junction function or increased hemichannel function. A detailed understanding of the AF-linked Cx40 mutants will be crucial in developing proper, effective strategies to treat AF.

Atrial fibrillation (AF) is the most common sustained cardiac arrhythmia and the prevalence is predicted to increase due to the aging of our population (Chaldoupi, Loh et

al. 2009). AF is characterized by rapid and irregular atrial activation, which is followed by uncoordinated and ineffective atrial contraction. This can lead to stagnant blood pooling in the atria and lead to thrombosis formation and is therefore a leading cause of embolic stroke (Wakili, Voigt et al. 2011). Approximately 30% of AF patients have a form of AF that is not secondary to other cardiovascular problems, such as hypertension, heart failure or myocardial infarction, and this is known as lone or idiopathic AF (Saffitz 2006; Chaldoupi, Loh et al. 2009). Although most of the Cx40 mutants show some sort of impairment in terms of forming functional gap junction channels, V85I and L221I show normal channel function, but are gain of hemichannel function mutants. With this result comes the question of how this gain of hemichannel function can contribute to AF. Several possibilities associated with the opening of hemichannels may directly or indirectly change electrical properties of the cardiomyocytes, which could promote atrial arrhythmias. 1) Open hemichannels would allow for the inward and outward fluxes of Na^+ and K^+ ions according to their electrochemical gradient, the result of which is membrane depolarization. Transient membrane depolarization brings the cell closer to/over the threshold of firing an action potential, while sustained depolarization may lead to substantial Na^+ channel inactivation, which can reduce the excitability of cardiomyocytes and lead to a slower conduction velocity. Both actions could increase the heterogeneity of cardiomyocyte excitability, increasing the susceptibility to arrhythmias (Rudy 2008). 2) Extracellular calcium could enter cardiomyocytes through open hemichannels and elevate $[Ca^{2+}]_i$. This may cause calcium-induced calcium release from the sarcoplasmic reticulum, altering calcium homeostasis, which may promote arrhythmias. Overloading cardiomyocytes with calcium may also lead to cell death (John,

Kondo et al. 1999; Li, Sugishita et al. 2001; Shintani-Ishida, Uemura et al. 2007). 3) Open hemichannels may lead to the release of ATP to the extracellular space. The released ATP can act in an autocrine and paracrine manner to cause Ca^{2+}_i wave propagation via purinergic receptors (Dale 2008). 4) From the well studied gap junction hemichannels, we learned that the channel is usually large enough to pass small signaling and nutrient molecules. The mutant Cx40 hemichannels may provide a passage to lose some of these important molecules, which may be critical to normal cardiomyocyte function. All of the above possibilities have the potential to lead to abnormal activities of the cardiomyocytes and may play a role in generating arrhythmias in patients carrying these mutations.

Previous studies on AF-linked gap junction mutants are focused on the localization and function of gap junction channels. Cx40 mutants, P88S and Q49X, as well as the Cx43 mutant, G60S, showed impaired trafficking to the cell surface (Gollob, Jones et al. 2006; Manias, Plante et al. 2008), while Cx40 mutants, I75F, A96S, L229M and G38D did not show any alterations in their localization, but displayed various degrees of reduction of the GJ coupling conductance (Gollob, Jones et al. 2006; Sun, Yang et al. 2013). In any case, the GJ function is impaired via different underlying mechanisms. It is predicted that the connexin mutants with impaired localization would also likely eliminate/reduce their hemichannel function. However, it is not known whether hemichannel function is reduced, increased or unchanged in those mutants with impaired gap junction function because other disease-linked Cx43 and Cx30 mutants with GJ impairment can increase (Dobrowolski, Sasse et al. 2008) or decrease hemichannel function (Lai, Le et al. 2006). Here we tested AF-linked Cx40 mutants,

Q49X, I75F and L229M, PI-uptake in DCF conditions and found no increase in PI-uptake in our experimental conditions. At this time we are unable to identify if there is a decrease in the hemichannel function for these mutants due to the fact that we did not observe any substantial PI-uptake in wild-type Cx40 hemichannels. This is in contrast to the results seen with V85I and L221I, which show no obvious defect in gap junctional conductance when expressed as homotypic cell pairs; however they showed prominent PI-uptake under DCF conditions, indicating a gain of hemichannel function.

Although many Cxs have had their hemichannel function investigated, a number of them have not yet been reported, including Cx40. Our current study is the first reported functional study of wild-type Cx40 hemichannels. One study by Allen et al. (2011), used atomic force microscopy to evaluate the three-dimensional molecular topology and calcium-dependent conformational changes of Cx40 hemichannels. They demonstrated that at low $[Ca^{2+}]_o$ (<10 μM), Cx40 hemichannels showed surface openings. The increase of external Ca^{2+} concentration closed most of the Cx40 hemichannels, suggesting that Ca^{2+} ions cause conformational rotation of Cx40 subunits, which act to close the pore. They also noted that the addition of EDTA, which acts as a Ca^{2+} chelator in a similar way to EGTA, also led to Cx40 hemichannel opening (Allen, Gemel et al. 2011). Although this study reported structural hemichannel opening of Cx40, they were not able to characterize the functions of Cx40 hemichannels due to limitations with atomic force microscopy. Allen et al. (2011) showed that Cx40 hemichannels were Ca^{2+} sensitive, however we established that the conformational changes of Cx40 hemichannels under low Ca^{2+} conditions did not cause functional hemichannel opening, as demonstrated by a lack of PI-uptake. At present, we could not rule out the possibility that

low extracellular Ca^{2+} may lead to the opening of Cx40 hemichannels, however, this open state may be too small to allow PI to pass through.

Specific increases in connexin hemichannel function have been associated with human disease-linked mutations in several connexin genes. For example, two Clouston syndrome-linked Cx30 mutants, G11R and A88V, have been previously reported to increase hemichannel function while maintaining normal gap junction function. Under physiological conditions, both G11R and A88V show increased ATP release via hemichannels compared to wild-type Cx30 (Essenfelder, Bruzzone et al. 2004). The Cx32 mutation S85C, which is associated with X-linked Charcot-Marie-Tooth (CMTX) disease, has shown large hemichannel-mediated voltage-dependent currents that are not seen with wild-type Cx32 (Abrams, Bennett et al. 2002). Another CMTX Cx32 mutation, F235C, also forms leaky hemichannels that contribute to a very severe neuropathy (Liang, de Miguel et al. 2005). Leaky hemichannels have also been reported in Cx26 mutations linked to keratitis-ichthyosis-deafness syndrome, G45E (Stong, Chang et al. 2006) and A40V (Gerido, DeRosa et al. 2007). Both mutants exhibit normal gap junction function, but A40V has altered extracellular Ca^{2+} regulation leading to a higher hemichannel open probability and G45E shows increased permeability to Ca^{2+} (Sanchez, Mese et al. 2010). Finally, three oculodentodigital dysplasia-linked Cx43 mutants, I31M, G138R and G143S, all showed greater than a 2-fold increase in hemichannel activity compared to wild-type Cx43 as demonstrated by an increase in ATP release (Dobrowolski, Sommershof et al. 2007). It should be noted that the G138R mutant also impaired GJ function in addition to increasing hemichannel activity. Our current study provides the

first demonstration of Cx40 and Cx40 mutant hemichannel function, giving us new insights into the etiology of lone AF.

2.5 - Conclusion

Two germline Cx40 mutations, V85I and L221I, were identified and linked to a Chinese family suffering from lone AF. These mutations showed impaired trafficking to the cell surface, but did not significantly impair gap junction function. Wild-type Cx40 did not show significant PI-uptake, signifying a closed hemichannel under our experimental conditions. Both Cx40 mutants showed significantly increased PI-uptake compared to wild-type Cx40, suggesting a gain of hemichannel function. These findings demonstrate the first functional study of Cx40 hemichannels and also describe a novel mechanism by which Cx40 mutants can contribute to the pathogenesis of lone AF.

Disclosures

The authors have no financial conflicts to disclose.

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3. Discussion

3.1 - Overall Study

In this study, we examined two lone AF-linked Cx40 germline mutations, V85I and L221I. We investigated their localization at the cell-cell interface using confocal microscopy, gap junction function by the dual whole-cell patch clamp technique and hemichannel function by PI-uptake assays. Results showed that both Cx40 mutants had a statistically significant reduction in gap junction plaque formation, although there was no change in gap junctional conductance, suggesting no functional change compared to wild-type Cx40. Interestingly, under DCF-ECS conditions, wild-type Cx40 did not show significant amounts of PI-uptake compared to negative controls, suggesting that the Cx40 hemichannel either remains closed or has a pore diameter too small to allow for PI passage under low $[Ca^{2+}]_o$ conditions. However, under the same conditions, V85I and L221I showed prominent PI-uptake, which was statistically similar to the positive control of Cx26. This PI-uptake could be blocked by the addition of external Ca^{2+}, as well as 100 μM CBX, both of which have previously been reported to close unapposed hemichannels (Wang, De Bock et al. 2013). In addition, the pannexin 1 channel blocker probenecid, did not affect the PI-uptake of these mutant hemichannels. These results suggest that the PI-uptake observed was hemichannel-mediated and the Cx40 V85I and L221I mutants cause a gain of hemichannel function.
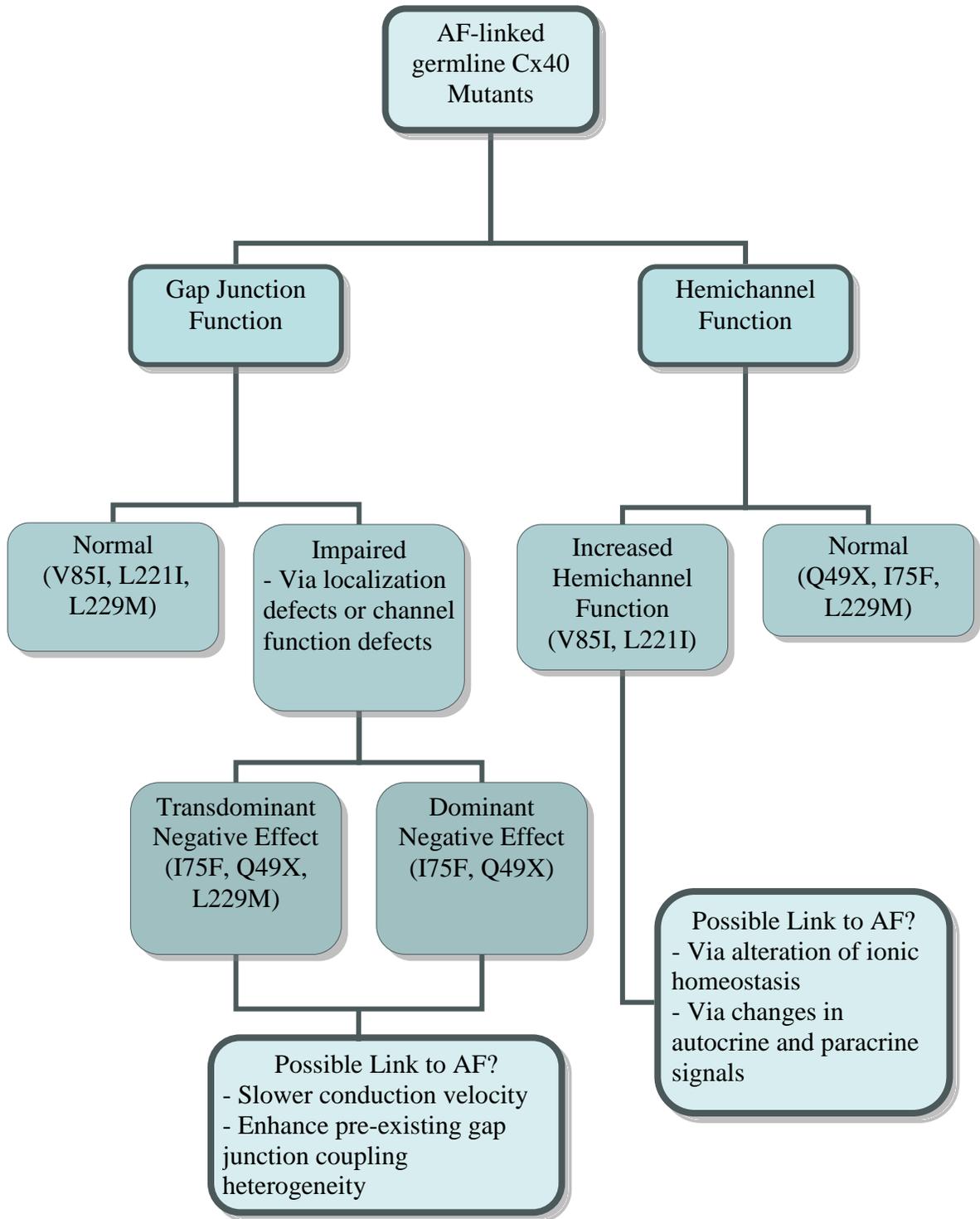
Figure 3.1 - Overall summary of AF-linked germline Cx40 mutants. A number of AF-linked germline Cx40 mutants have shown changes in their gap junction function (Q49X, I75F, L229M) (Sun, Yang et al. 2013, Sun et al. submitted). V85I and L221I have shown increased hemichannel function.

3.2 - Localization of V85I-YFP and L221I-YFP

In order to visualize the localization of Cx40 mutants, HeLa cells were transiently transfected with either V85I or L221I tagged with YFP at the C-terminus. The observation that both Cx40 mutants showed reduced plaque formation at the cell-cell interface could have several explanations. One possible explanation is that V85I and L221I somehow impair the trafficking of the Cx proteins to the plasma membrane, although this seems unlikely because a majority of transfected cell pairs showed gap junction plaque-like structures for both mutants. Another explanation is that the Cx40 mutant proteins are able to traffic to and be inserted into the plasma membrane, but do not always incorporate themselves into gap junction plaques and prefer to remain as unapposed hemichannels. It has been previously demonstrated that Cx hemichannels are located within lipid rafts of the plasma membrane and gap junction plaques do not co-localize with these lipid rafts (Locke, Liu et al. 2005). These lipid rafts are involved in the trafficking of undocked hemichannels to gap junction plaques. The Cx40 mutants may change the interactions between hemichannels and lipid rafts, thereby decreasing their trafficking to the cell-cell junction and their subsequent incorporation into gap junction plaques. We observed reduced plaque formation of homotypic Cx40 mutant gap junctions, but it would be very interesting to see the result when wild type and mutant Cx40 are both used to create either heteromeric or heterotypic gap junction channels. This would give us a more physiologically relevant viewpoint about protein trafficking and would allow us to see if the Cx40 mutants have a dominant negative effect on the trafficking of wild type Cx40.

3.3 - Gap Junction Function of V85I and L221I

Dual whole-cell patch clamp was used to examine the gap junction function of homotypic channels formed of either V85I or L221I in N2A cells. The results showed that there was no change in the gap junctional conductance of both V85I and L221I, compared to that of wild-type Cx40, indicating that these mutants were able to form functional gap junction channels.

A number of other previously studied AF-linked germline Cx40 mutants (Q49X, L229M and I75F) have specific defects in their gap junction function. The Q49X mutant, when expressed alone, did not form gap junction plaque-like structures and was not functionally coupled. In addition, when co-expressed with either Cx40 or Cx43, Q49X had a dominant negative effect on both plaque formation and conductance levels (Sun et al. submitted). The L229M mutant showed normal plaque formation, as well as normal conductance levels when expressed alone or when co-expressed with Cx40. When the L229M mutation was co-expressed with Cx43, there was normal plaque formation, but a decreased conductance level was observed (Sun, Yang et al. 2013). The I75F mutation formed gap junction plaque-like structures when expressed alone or when co-expressed with either Cx40 or Cx43. However, when expressed alone, I75F was not functionally coupled and when co-expressed with either Cx40 or Cx43, there was a decreased gap junctional conductance (Sun, Yang et al. 2013). In contrast to these mutations, since V85I and L221I did not impair homotypic gap junction channel function, we investigated a possible change in their hemichannel function.

3.4 - Hemichannel Function of V85I and L221I

When subjected to DCF+EGTA conditions, cells expressing wild-type Cx40 did not show significant amounts of PI-uptake and in fact, the level of PI-uptake observed was statistically similar to the negative controls (YFP-expressing or non-transfected cells). In contrast, when exposed to the same conditions, cells expressing V85I or L221I showed statistically significant increases in PI-uptake compared to that of Cx40 and this uptake was statistically similar to the uptake seen with our positive control (Cx26-expressing cells). This PI-uptake decreased with increases in $[Ca^{2+}]_o$, eventually leading to no significant uptake at 2 mM $[Ca^{2+}]_o$, and was also blocked with the addition of 100 μM CBX, suggesting that this PI-uptake was mediated through open hemichannels. A number of other AF-linked germline Cx40 mutations (Q49X, L229M and I75F) were also tested under DCF+EGTA conditions to investigate their PI-uptake properties. None of these Cx40 mutants showed significant PI-uptake, suggesting that under DCF+EGTA conditions, they are unlikely to have open hemichannels. However, as previously stated, these mutations have been shown to cause specific defects in their gap junction function.

This is not the first case of a mutant Cx showing a gain of hemichannel function. As described previously, a number of human disease-linked Cx mutants, including Cx26, Cx30, Cx32 and Cx43 mutants, have shown an increase in hemichannel function (Abrams, Bennett et al. 2002; Essenfelder, Bruzzone et al. 2004; Liang, de Miguel et al. 2005; Stong, Chang et al. 2006; Dobrowolski, Sommershof et al. 2007; Gerido, DeRosa et al. 2007; Sanchez, Mese et al. 2010). However, this is the first time that Cx40 hemichannel function has been investigated and also represents the first demonstration of a gain of hemichannel function for Cx40 mutants.

The sensitivity of Cx hemichannels to $[Ca^{2+}]_o$ has been demonstrated to be a common feature of all studied Cx hemichannels. Lowering $[Ca^{2+}]_o$ to zero, or near zero, as was done in this project, has become a common method to open hemichannels so their function can be studied (Quist, Rhee et al. 2000; Contreras, Saez et al. 2003; Srinivas, Calderon et al. 2006). The majority of studies on hemichannel function have been done *in vitro* under low $[Ca^{2+}]_o$ conditions. It is not known whether the $[Ca^{2+}]_o$ is low enough under physiological conditions to cause hemichannel opening. In certain pathological conditions such as ischemia, the $[Ca^{2+}]_o$ are predicted to be low enough to open Cx hemichannels, including those formed by V85I and L221I.

In addition to being sensitive to $[Ca^{2+}]_o$, hemichannels are also sensitive to changes in $[Ca^{2+}]_i$. It was previously demonstrated that significant ATP release, as well as significant dye uptake, was observed in Cx32-expressing cells when exposed to an increase in $[Ca^{2+}]_i$ above 200 nM, but below 1000 nM, signifying hemichannel opening under these conditions (De Vuyst, Decrock et al. 2006). Similar results of PI-uptake and ATP release were also seen when Cx43-expressing cells were exposed to similar changes in $[Ca^{2+}]_i$ (Evans, De Vuyst et al. 2006). Intracellular Ca^{2+} concentrations are constantly changing, especially in contractile cells such as cardiomyocytes, and have a normal, physiological range of 100 nM to 1 μM (Evans, De Vuyst et al. 2006), leading us to believe that Cx40 mutant hemichannels could also open in response to $[Ca^{2+}]_i$ changes within this physiological range. As a result, for future experiments, it would be interesting to test the role of $[Ca^{2+}]_i$ on the PI-uptake for Cx40 V85I and L221I-expressing cells.

Another mechanism that causes hemichannel opening is mechanical membrane stress. This was first discovered in Cx46-expressing *Xenopus* oocytes which, when exposed to a hypotonic solution and membrane depolarization, showed hemichannel opening. In contrast, exposing the oocytes to a hypotonic solution and membrane hyperpolarization resulted in closed hemichannels, leading us to believe that, at least for Cx46 hemichannels, the mechanosensitive properties are closely tied to the membrane potential (Bao, Sachs et al. 2004). Other studies expanded on this by showing that a number of other Cxs, including Cx26, Cx32 and Cx43, also show hemichannel opening in response to mechanical stimulation (Schlosser, Burgstahler et al. 1996; Stout, Costantin et al. 2002; Gossman and Zhao 2008). It has also been stated that mechanical stimulation may occur in any experiment where the medium needs to be changed, for example in this project where there is a change from regular to low Ca^{2+} medium (Evans, De Vuyst et al. 2006). This mechanical strain could cause further hemichannel opening in addition to that caused by low $[Ca^{2+}]_o$. Notably, the cells of the atria are constantly undergoing mechanical stress and strain via the normal beating the heart. As a result, this constant mechanical membrane stress could facilitate the opening of unapposed Cx40 mutant hemichannels. Although the mechanisms behind mechanical stress-induced hemichannel opening are not yet fully understood, mechanical strain remains an important mechanism to promote hemichannel opening.

3.5 - Cx40 Mutants and Lone AF

Although most Cx40 mutants show some sort of impairment in terms of forming functional gap junction channels, V85I and L221I showed normal gap junction channel function, but exhibited a gain of hemichannel function. These results raise the question of how this gain of function contributes to the pathogenesis of lone AF. While the answer is still up for debate, it is possible that open hemichannels, which should normally be closed, could drastically affect the resting membrane potential (RMP) of the cardiomyocytes. Open hemichannels would allow for the exchange of Na^+ and K^+ ions according to their own electrochemical gradients, which results in membrane depolarization. If this depolarization is transient, the cell will be closer to, or if the depolarization is large enough, over, the threshold level for action potential firing, causing significant changes in cardiomyocyte excitability, leading to increased vulnerability to arrhythmias. On the other hand, a sustained depolarization will inactivate a large number of Na^+ channels, abolishing their ability to open (Rudy 2008). This means that the cardiomyocyte will be less excitable, causing a slowing of conduction through the heart, increasing the susceptibility to arrhythmias.

In addition to the exchange of Na^+ and K^+, other ions, especially Ca^{2+}, can pass through open hemichannels as well. This permeability to Ca^{2+} will act to increase $[Ca^{2+}]_i$ and lead to the phenomenon known as calcium-induced calcium release. This occurs when $[Ca^{2+}]_i$ is elevated and more Ca^{2+} is available to bind to ryanodine receptors on the sarcoplasmic reticulum (SR), leading to further Ca^{2+} release and further elevation of $[Ca^{2+}]_i$ (Berridge 1997). This change in intracellular Ca^{2+} homeostasis can contribute significantly to arrhythmias and has also been shown to increase both cellular injury and

cell death (John, Kondo et al. 1999; Li, Sugishita et al. 2001; Shintani-Ishida, Uemura et al. 2007). Calcium-induced calcium release can also be triggered by the binding of inositol trisphosphate (IP3) to IP3 receptors (IP3R). IP3 has previously been shown to pass through open hemichannels (Gossman and Zhao 2008). IP3 entering cardiomyocytes binds to IP3R, which are found in specific cytoplasmic compartments as well as the nucleus, but are not co-localized with ryanodine receptors on the SR (Kockskamper, Zima et al. 2008). Binding of IP3 to IP3R causes Ca^{2+} release from endogenous Ca^{2+} stores, independent from those in the SR, leading to elevated $[Ca^{2+}]_i$ and pro-arrhythmogenic effects (Zima and Blatter 2004).

Another possible mechanism linking these Cx40 mutants to lone AF could be via the release of ATP through open hemichannels. ATP can be released from open hemichannels and act in a paracrine manner to cause $[Ca^{2+}]_i$ wave propagation by binding to purinergic receptors (Dale 2008). Purinergic receptors are found in almost all mammalian tissues, including the heart, and are classified as either P1 or P2. P1 receptors have a higher affinity for adenosine and AMP, which is why they are sub-classified as adenosine receptors, where as P2 receptors preferentially bind with ATP and ADP (Vassort 2001). In the heart, a number of P2 receptor subtypes are present. In particular, the $P2X_1$, $P2X_2$ and $P2X_4$ receptors are found in the atria, while only $P2X_4$ is found in the ventricles (Nori, Fumagalli et al. 1998). ATP released from open hemichannels is able to bind to $P2X_2$ receptors on the cell membrane, leading to Ca^{2+} release from the sarcoplasmic reticulum and an increase in $[Ca^{2+}]_i$ at either a single focus or at multiple foci (Capogrossi, Houser et al. 1987). The $[Ca^{2+}]_i$ increase causes a transient inward current that is assumed to be arrhythmogenic (Kass, Lederer et al. 1978). The $[Ca^{2+}]_i$ can

then propagate to the rest of the cytosol in a wave-like pattern, causing membrane depolarization and has been shown to induce an action potential (Capogrossi, Houser et al. 1987).

In addition, a number of P1 subtypes, A_1, A_{2A}, A_{2B} and A_3, are found in the heart. A_1 receptors are found in both the atria and ventricles, A_{2A} and A_{2B} receptors are mainly located in the coronary vessels, and it is currently unknown if A_3 receptors are present in adult cardiomyocytes, although it is assumed they are present in the ventricles (Auchampach and Bolli 1999; Mubagwa and Flameng 2001). As stated above, P1 receptors preferentially bind to adenosine as well as AMP. When ATP is released from open hemichannels into the extracellular space, it can be rapidly hydrolysed into either adenosine or AMP, which can then bind to A_1 receptors (Vetri, Xu et al. 2011). This activation of A1 receptors leads to downstream activation of G protein-coupled receptors, which causes what is known as an adenosine-induced inwardly rectifying K^+ current (Martynyuk, Kane et al. 1995). This inward current has been shown to cause a slowing of heart rate and atrioventricular conduction, as well as a reduction of atrial contractility (Srinivas, Shryock et al. 1997).

3.6 - Limitations and Future Studies

Although this study provides a novel mechanism by which Cx40 mutants can contribute to lone AF, a number of limitations have to be considered before making any strong conclusions. This starts with the fact that all results were obtained through the use of *in vitro* methods using the model cell systems of HeLa and N2A cells. HeLa and N2A cells were chosen because they are immortal cell lines that do not contain endogenous Cxs, but express transfected Cxs well. As a result, this makes them ideal for studying

localization, as well as gap junction and hemichannel function. In addition, HeLa cells have a flat morphology, which is better for imaging and N2A cells have a rounder morphology, which makes them ideal for patch clamp. In addition, using transient transfection of HeLa and N2A cells over expresses the Cx proteins, which may differ from the endogenous protein levels found in cardiomyocytes. As a result, the observations made may not accurately portray what occurs *in vivo*. The fluorescent dye propidium iodide is a biologically irrelevant molecule that does not necessarily mimic the exchange of endogenous nutrients and metabolites through open hemichannels. Also, the gap junction hemichannel blocker carbenoxolone is widely used in research, however, it has been shown to block more than just hemichannels and all of its effects are yet to be elucidated (Wang, De Bock et al. 2013). Of course, *in vitro* model systems are just a stepping stone in the research process and do not necessarily correlate with what would be observed *in situ* or even *in vivo*, however they still represent an important research tool.

With all this in mind it is important to remember that our study is the first report of Cx40 and Cx40 mutant hemichannel function and should open the door to more in-depth research. Future studies should look at single channel recordings of mutant Cx40 gap junction channels to further evaluate any changes in channel biophysics. In addition, heteromeric and/or heterotypic gap junctions should be investigated, where Cx40 mutants are co-expressed with either Cx40 or Cx43, to see if these arrangements cause any change in trafficking or junctional conductance levels.

Hemichannel dye uptake assays could be expanded to include fluorescently labelled metabolic derivatives that are more biologically relevant than PI, although these

molecules may be too large to permeate open hemichannels. Hemichannel function could also be examined further with the use of ATP release and mechanical stimulation assays. ATP release assays were attempted for this project, although they were very difficult to optimize and the results obtained were not consistent. Mechanical stimulation assays were also carried out, however they were unsuccessful. HeLa cells were not robust enough to survive the strong mechanical force applied by dropping fluorescent dye directly onto them. In addition, HeLa cells were subjected to hypo-osmotic stress conditions as a method of mechanical stimulation, which induces cell swelling. The mechanical strain of cell swelling was not enough to cause increased PI-uptake and more details from this experiment are described Appendix A.

In addition, this work should be repeated in more physiologically relevant cells. This could include the use of atrial cell lines, for example HL-1 cells, which are derived from mouse atrial cells and have the ability to contract in culture and maintain consistent atrial morphological, biochemical and electrophysiological properties, or even the use of primary atrial cells. However, primary cell lines are difficult to transfect and may necessitate the use of viral transformation, which can be more toxic to cells and lead to virus-related side effects.

3.7 - Conclusion

AF-linked Cx40 mutants, V85I and L221I, were able to traffic to the cell-cell junction and form gap junction plaque-like structures, although in reduced amounts compared to wild-type Cx40. This reduction of plaque formation did not however, change the homotypic gap junctional conductance of these mutant-expressing cell pairs,

implying no functional impairment of gap junction channels. Although wild-type Cx40 did not show significant PI-uptake under DCF+EGTA conditions, the Cx40 mutants showed significant uptake. PI-uptake was blocked by the addition of external Ca^{2+}, as well as the presence of the hemichannel blocker CBX. This was the first time that Cx40 hemichannel function was investigated and under our experimental conditions, Cx40 hemichannels did not show any PI-uptake. We also demonstrated for the first time, a gain of hemichannel function for two AF-linked germline Cx40 mutants, V85I and L221I. Although this project necessitates future work, here we provided the first functional study of Cx40 hemichannels as well as identified a novel mechanism with which these Cx40 mutants could contribute to the development of lone AF.

3.8 - References

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Appendix A

Methods

Dye uptake was performed as described above. HeLa cells were treated with a solution containing 0.02 mM Ca^{2+} ECS, but were also exposed to minor mechanical strain in the form of hypo-osmotic stimulation. This was achieved by adding 20% more double distilled water (ddH_2O) to the solution of 0.02 mM Ca^{2+} ECS and incubating at $37°C$ for 15 minutes. PI-uptake was assessed from single, isolated, transfected cells using a Leica DMIRE2 fluorescent microscope. Approximately 30-40 cells were counted for each transfection.

Results

PI-uptake was compared between cells treated with 0.02 mM Ca^{2+} ECS and 0.02 mM Ca^{2+} + 20% ddH_2O (hypo-osmotic conditions). When treated with 0.02 mM Ca^{2+} ECS, Cx26-GFP showed 51.5 ± 3.3% (n=6), V85I-YFP showed 14.4 ± 0.6% (n=6) and L221I-YFP showed 38 ± 2.8% (n=6) uptake. Under hypo-osmotic conditions Cx26-GFP showed 55.1 ± 2.9% (n=4), V85I-YFP showed 18.8 ± 2.2% (n=4) and L221I-YFP showed 42.2 ± 3.4% (n=4) uptake (Fig. A1). Cx26-GFP, V85I-YFP- and L221I-YFP-expressing cells failed to show any increase in PI-uptake under hypo-osmotic conditions ($P > 0.05$ for each of these conditions).
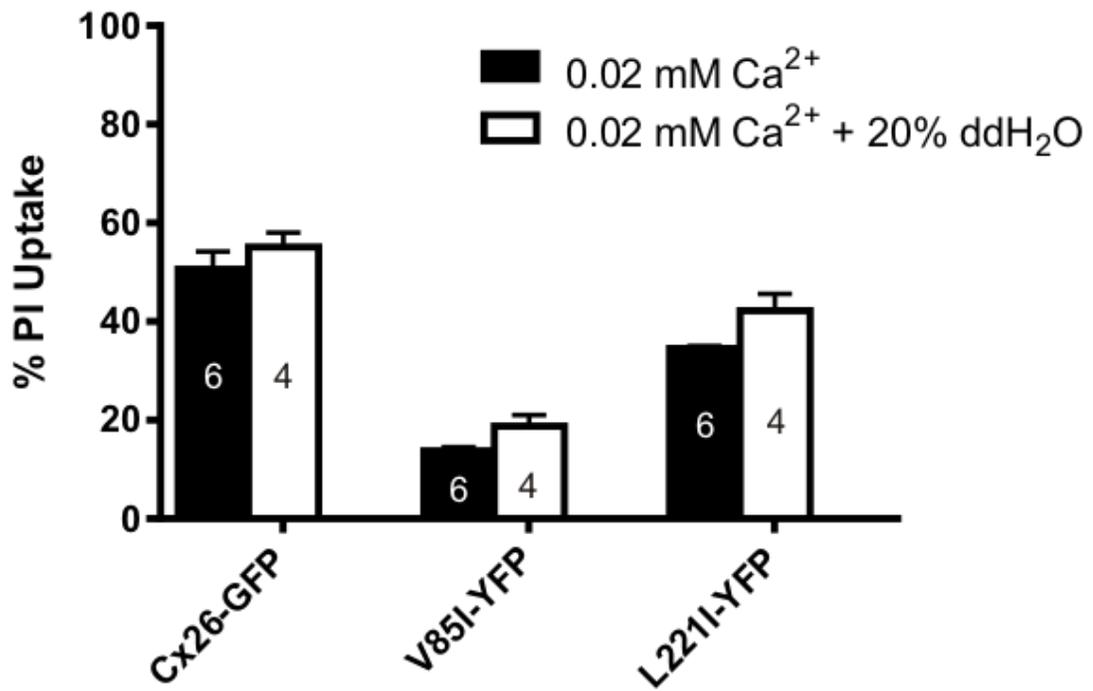
Figure A1 - PI-uptake under hypo-osmotic conditions. Comparison of cells treated with 0.02 mM Ca^{2+} and 0.02 mM Ca^{2+} + 20% ddH_2O (hypo-osmotic conditions) to cause mechanical stress-induced hemichannel opening. Cells exposed to hypo-osmotic conditions did not show a statistically significant increase in PI uptake.

Discussion

Previous experiments have shown that Cx hemichannels open in response to a number of different types of mechanical membrane stress including shear stress (Cherian, Siller-Jackson et al. 2005), point stimulation of a single cell (Romanello, Veronesi et al. 2003; Gomes, Srinivas et al. 2005) and changes in osmolarity (John, Cesario et al. 2003; Lu, Soleymani et al. 2012). Atrial cells are constantly being subjected to mechanical stress as a result of the natural beating of the heart. As a result, we wanted to assess whether mechanical stimulation could open the mutant Cx40 hemichannels and cause PI-uptake.

As stated previously, point stimulation of a single cell was attempted on transfected HeLa cells by applying Rhodamine dye in a drop-wise fashion. This method proved unsuccessful because HeLa cells were not robust enough to survive this strong mechanical force. As a result, we attempted mechanical stimulation via changes in osmolarity, in the form of hypo-osmotic stimulation. Dye uptake was performed under hypo-osmotic conditions to cause a form of mechanical membrane stress, known as cell swelling, which has been shown to open a number of connexin hemichannels including those composed of Cx26, Cx32, Cx43, Cx45 and Cx46 (Stout, Costantin et al. 2002; Saez, Retamal et al. 2005; Lu, Soleymani et al. 2012). This minor mechanical strain was not enough to cause a statistically significant increase in PI-uptake under our experimental conditions. A previous study on Cx46 hemichannels in *Xenopus* oocytes demonstrated that when exposed to a hypotonic solution and membrane depolarization, the Cx46 hemichannels would open (Bao, Sachs et al. 2004). It is possible that if we had added membrane depolarization in addition to hypo-osmotic stress, the Cx40 mutants

would have shown increased PI-uptake. It is also possible however, that the 20% change in osmolarity was not enough to cause mechanical swelling that was forceful enough to lead to hemichannel opening. A previous study on ATP release through open Cx43 and Cx45 hemichannels from rat cardiac fibroblasts created a hypo-osmotic solution with an osmolarity of 150-160 mOsm, a 50% reduction in osmolarity (Lu, Soleymani et al. 2012), whereas our experiment used a hypo-osmotic solution with an osmolarity of 230-240 mOsm, a 20% reduction. It is possible that this larger reduction in osmolarity lead to a more forceful mechanical stress, which caused increased hemichannel opening and perhaps this technique should be used on Cx40 mutant hemichannels.

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