CHAPTER 1: EFFECTS OF COMPLETE REPLACEMENT OF TRANSMEMBRANE α HELICES OF CONNEXIN 43 ON GAP-JUNCTIONAL HEMICHANNEL FUNCTION

INTRODUCTION

Approximately 25% of all genome coding sequences correspond to membrane proteins, which perform varied and essential functions in cells. Eukaryotic integral membrane proteins are predominantly α-helical proteins that span the membrane several times. Recent years have seen significant progress in understanding the structure-function of complex membrane proteins (Watkins *et al*., 2001). However, our current knowledge of these important molecules is still very limited. Membrane embedded transmembrane helices, like M1 through M4 in Cx43, are well known for their tolerance for mutations, although the evolutionarily-conserved residues play an important role of protein-packing interactions (Baldwin *et al*., 1993 and Frillingos *et al*., 1998). If some α-helices in membrane proteins do not contain residues required for proper helix packing, then it may be possible to replace them completely while preserving function. Because of the poor resolution of the hemichannel structure available (see general Introduction), there are currently several models of connexin hemichannel folding, which are not consistent with each other. Some are based on experimental results using SCAM, connexin chimeras of different isoforms and mutagenesis and are discussed in subsequent chapters (Zhou *et al.*, 1997; Pfahnl *et al.*, 1998; Skerrett *et al.*, 2002; Kronengold *et al.*, 2003). The other model, currently most widely accepted, is based mostly on the comparison of connexin sequences and the notion that the most conserved transmembrane residues correspond to amino acids in pore-lining helices. This contention seems true in the case of channels

with pores of small diameter, but may not be true for large-diameter pores, such as that of hemichannels, where the mechanisms of permeation are different (Hille, 2001).

The most frequent approach to identify transmembrane-helix amino acids essential for function is to substitute native residues, one at a time, with Cys or Ala (Cys- and Ala-scanning mutagenesis), and then assay the function of each mutant. This approach is extremely labor intensive, and has been carried out only for a few membrane proteins (e.g., Lac Permease, Frillingos *et al.*, 1998.). Here, I present a new approach where complete transmembrane-helix native sequences are substituted with poly-Ala sequences. According to the notion used to develop the hemichannel model in Fig. 1.1 (Fleishman *et al*., 2004), pore-forming helices contain one or more conserved residues essential for function, and therefore the mutants in which one or several of these helices are replaced with a poly-Ala sequence will not be functional. The mutants of helices that do not contain essential residues will be functional, and will not likely form the pore. It was reasoned that this approach ("helix scanning mutagenesis") would allow for rapid identification of helices containing pore-lining residues essential for function, and can be used as a primary helix-screening tool, to be followed by individual amino-acid substitutions when specific-helix poly-Ala replacements cause functional changes in the protein.

The hydrophobic amino acid residues Leu and Ala are the most frequently found in transmembrane helices (Ulmschneider *et al*., 2001). Poly-Ala sequences capped with sequences such as those regularly found in inter-helical loops of membrane proteins form α-helices (Miller *et al*., 2001). The methyl side-chain group of Ala lacks unusual dihedral angle preference and does not contribute to hydrogen bonds. Ala-scanning mutagenesis takes advantage of these properties of Ala, and has been used to identify the so-called functional epitopes, i.e., "hot spots" in the structural epitope identified in high-resolution

structures, which contribute most binding energy in protein-ligand interactions (Cunningham *et al*., 1989).

In this chapter, the primary sequence requirements for Cx43 hemichannel function were studied by multiple simultaneous mutagenesis, replacing the Cx43 transmembrane helices, one at a time, with poly-Ala sequences (helix-scanning mutagenesis).

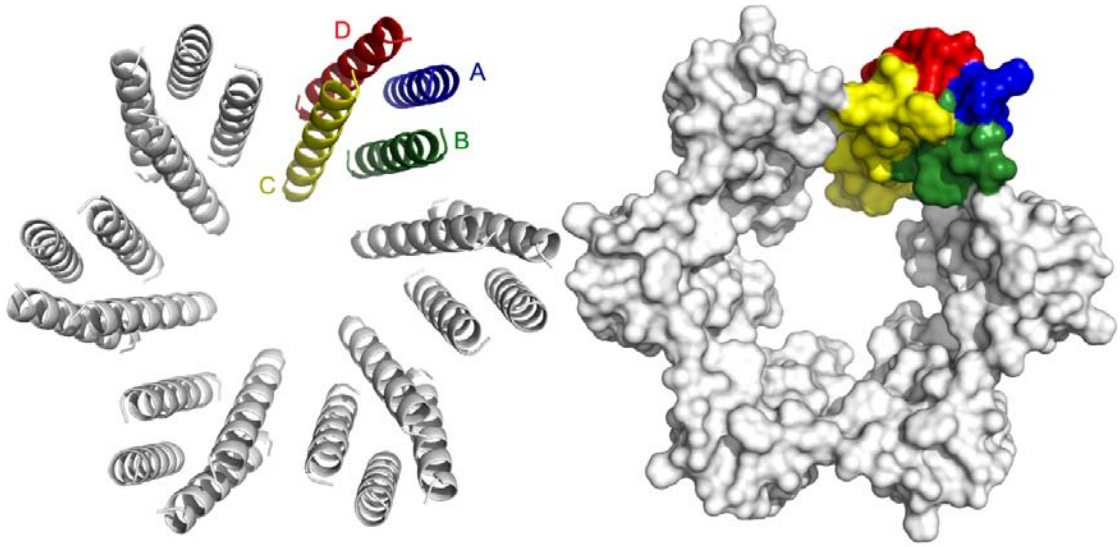

Figure 1.1: Structural model of the transmembrane hemichannel pore.

Transmembrane hemichannel pore viewed from the cytoplasmic side. The representation is based on the coordinates provided by Dr. M. Yeager, see Fleishman *et al*., 2004), and the helices are labeled A-D, as done in the original cryoelectron microscopy work from Yeager and collaborators. The helices from one monomer are labeled with different colors (green, blue, yellow and red represent helices 1, 2, 3, and 4, respectively, according to the Fleishman *et al*. model). The coordinates were provided by Dr. Mark Yeager from The Scripps Institute in La Jolla, CA.

MATERIALS AND METHODS

Plasmid engineering

To generate the poly-Ala mutants, unique restriction sites were introduced flanking the transmembrane helices of interest to rat Cx43 cloned into pOcyt7 (Bao *et al*., 2004a; Bao *et al*., 2004b; Bao *et al*., 2004c; Bao *et al*., 2005). In the resulting plasmid, pOcyte-Cx43 NRS, the DNA sequences coding for a specific transmembrane helix were replaced with a poly-Ala coding set of oligonucleotide adaptors ready for ligation to the cut sites of the plasmids. The stretches of amino acids replaced with Ala included Lys23 to Glu42 (transmembrane segment 1), Arg76-Val96 (transmembrane segment 2), Gly150-Phe169 (transmembrane segment 3) and Thr207-Ile226 (transmembrane segment 4). The unique sites flanking the transmembrane helices 1 to 4 were *SacII*/*BamHI*, *PmlI*/*SphI*, *NcoI*/*SpeI* and *AgeI*/*NsiI*, respectively.

The restriction sites were introduced by site-directed mutagenesis (Quick Change Multisite Site-Directed Mutagenesis kit, Stratagene, La Jolla, CA) using the following mutagenic primers (sites underlined): 5'-GCCTACTCCACCGCGGGAGGGAAGG-3' (*SacII*); 5'-GTCAGCTTGGGGTGATGAAGGATCCGCCTTTCGCGCTAACAC-3' (*BamHI*); 5'-CCACCCTCCTGTACTTGGCGCATGCGTTCTATGTGATGAGGAAGG -3' (*SphI*); 5'-GTACGGGATTGAAGACCATGGCAAGGTGAAAATGAGGGGC-3' (NcoI); 5'-CGAGGTGGCCTTCCTACTAGTCCAGTGGTACATCTATGGG-3' (*SpeI*); 5'-GCCTTCCTCTCACGACCGGTGGAGAAAACCATCTTC-3' (*AgeI*); 5'-CATCATT GAGCTCTTCTATGCATTCTTCAAAGGCGTTAAGG-3' (*NsiI*). The *PmlI* site was endogenous and an endogenous *BamHI* site (upstream of the Cx43 coding sequence) was removed by site-directed mutagenesis. Because of the addition of the unique restriction sites, some amino-acid substitutions were introduced (Q49G, V96A, E141D, F169A,

I172V, T204V and V231A), which had no effect on carboxyfluorescein uptake (data not shown).

The adaptors used to replace the wild-type transmembrane-helix sequences were: 5'-GGGAGGGGCCGCTGCTGCTGCGGCTGCGGCGGCCGCTGCGGCTGCTGCGG CTGCGGCGGCCGCTGCGTCAGCTTGGGGTGATGAAG-3' (transmembrane helix 1 sense); 5'-GATCCTTCATCACCCCAAGCTGACGCAGCGGCCGCCGCAGCCGCAG CAGCCGCAGCGGCCGCCGCAGCCGCAGCAGCAGCGGCCCCTCCCGC-3' (transmembrane helix 1 antisense); 5'-GTGGCCGCTGCTGCCGCGGCTGCCGCGGCCGCT GCGGCTGCCGCGGCTGCCGCGGCCGCGCATG-3' (transmembrane helix 2 sense); 5'-CGCGGCCGCGGCAGCCGCGGCAGCCGCAGCGGCCGCGGCAGCCGCGGCA GCAGCGGCCAC-3' (transmembrane helix 2 antisense), 5'-CATGGCAAGGTGAAAA TGAGGGGCGCCGCTGCTGCCGCGGCTGCCGCGGCCGCTGCGGCTGCCGCGGC TGCCGCGGCCGCTGCGCTA-3' (transmembrane helix 3 sense); 5'-CTAGTAGCGC AGCGGCCGCGGCAGCCGCGGCAGCCGCAGCGGCCGCGGCAGCCGCGGCAGC AGCGGCGCCCCTCATTTTCACCTTGC-3' (transmembrane helix 3 antisense); 5'-CC GGTGGAGAAAGCCGCGGCCGCTGCTGCCGCGGCTGCCGCGGCCGCTGCGGCT GCCGCGGCTGCCGCGGCGGAGCTCTTCTATGCA-3' (transmembrane helix 4 sense); 5'-TAGAAGAGCTCCGCCGCGGCAGCCGCGGCAGCCGCAGCGGCCGCG GCAGCCGCGGCAGCAGCGGCCGCGGCTTTCTCCA-3' (transmembrane helix 4 antisense). DNA sequencing of all constructs was performed at the Protein Chemistry Core Laboratory of the University of Texas Medical Branch.

Oocyte preparation and cRNA injection

For T7-directed capped cRNA synthesis (mMessage machine, Ambion, Austin, TX), the Cx43 plasmids in pOcyt7 were linearized with *SalI* and used them as templates.

Xenopus laevis oocytes were isolated and prepared for cRNA injection as described (Sharon *et al*., 1997). In brief, ovarian lobes were surgically removed from frogs anesthetized by tricaine methanesulfonate (1g/L). Stage V and VI oocytes were then isolated by manual dissection and defolliculated with 1mg/ml type I collagenase in Barth's solution (in mM, 88 NaCl, 1.0 KCl, 1.0 $CaCl_2$, 1.0 $MgCl_2$, and 10 HEPES/ NaOH, pH 7.4) containing 10 μg/ml penicillin and streptomycin, and 100 μg/ml gentamycin, for 16 h. After this procedure, the cells were incubated in calcium-free Barth's solution at 16°C for cRNA injection. Oocytes were injected with 7.4 ng of antisense Cx38 oligonucleotide (to reduce endogenous expression of Cx38, the native connexin) alone or together with 10 to 28 ng of wild-type or mutant Cx43 cRNA.

Western blots of biotinylated plasma-membranes

Two days after injection, the oocytes were subjected to membrane surface biotinylation with a cell-membrane impermeable reagent. Details on the methodology to assess plasma-membrane expression in oocytes have been previously published (Button *et al*., 1999). In brief, membrane proteins were labeled by incubating the oocytes with 0.5 mg/ml biotin (EZ-Link Sulfo-NHS-Biotin; Pierce Chemical Co.) for 30 min at 4°C in Barth's solution. Oocytes were washed three times with 4 ml of ice-cold phosphate-buffered saline solution to remove unbound biotin, resuspended in Barth's solution, and lysed at 4°C by sonication with an ultrasonic processor (model GE501; Sonics and Materials). Yolk and debris were collected by centrifugation at 1,000 *g* for 5 min at 4°C. The supernatant was gently mixed for 1 h at 4°C with 50 μl of streptavidin attached to beads (ImmunoPure immobilized streptavidin; Pierce Chemical Co.). Bound membranes were collected by centrifugation at 1,000 *g* for 2 min at 4°C. The pellet was washed twice with PBS, resuspended in electrophoresis sample buffer, and subjected to 10-20% SDS-

PAGE electrophoresis. The biotinylated Cx43 was detected by chemiluminescence on a blot probed with Cx43 and streptavidin-horseradish peroxidase.

Uptake of 5(6)-carboxyfluorescein

Two days after cRNA injection, the oocytes were incubated for 40 min at 16°C in ND96 solution (in mM, 96 NaCl, 2 KCl, 1 $MgCl_2$, 1.8 $CaCl_2$, and 5 HEPES/NaOH, pH 7.4) containing 2 mM carboxyfluorescein. The low-Ca solution was nominally Ca^{2+}- and Mg^{2+}-free ND96 containing 1 mM EDTA. After the 40-min carboxyfluorescein-uptake period, extracellular carboxyfluorescein was removed by washing with ice-cold ND96 solution containing 10 μM Gd^{3+}, to minimize leakage of intracellular carboxyfluorescein *via* hemichannels during washing. Individual oocytes were lysed in 5 mM Tris/HCl, pH 9, and carboxyfluorescein was measured by spectrofluorometry, as described (Bao *et al*., 2004a; Bao *et al*., 2004b; Bao *et al*., 2004c).

Measurements of gap-junctional currents

Transjunctional currents were measured in paired oocytes. The vitelline membrane was manually removed 1 day after cRNA injection (Methfessel *et al*., 1986), before pairing the cells with the vegetal poles facing each other. The oocyte pairs were incubated in Barth's solution prior to the electrophysiological measurements using the dual two-microelectrode voltage clamp technique (Bao *et al*., 2004a and Spray *et al*., 1981). The paired cells were clamped at -60 mV, and a transjunctional potential was generated by stepping the voltage of one oocyte from -60 mV, while holding constant the voltage of the other cell (used as reference). The current supplied to the cell clamped at -60 mV is equal in amplitude, but opposite in sign, to the transjunctional current. The

transjunctional voltage was stepped from -120 to 120 mV for 10 s, at 20-mV intervals, with 15-s intervals between pulses.

Statistics

Data are expressed as means ± SEM of 20-30 oocytes/condition.. Statistical differences were assessed by one-way ANOVA.

RESULTS

Poly-Ala Cx43 mutants form functional gap-junctional hemichannels

Individual replacement of each of the Cx43 transmembrane segments with poly-Ala sequences yielded mutant proteins that were expressed at the plasma membrane (Fig. 1.2)

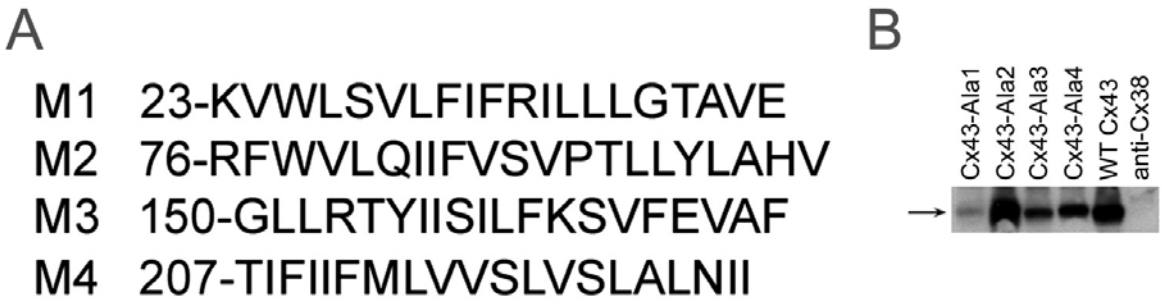

Figure 1.2: Cell surface expression of Cx43 hemichannels.

A. Wild-type sequences of the Cx43 transmembrane segments. Each residue in these sequences was replaced with Ala in the poly-Ala mutants. The position of the first residue of each sequence is indicated. B. Immunoblots of plasma membranes from frog oocytes injected with anti-Cx38 antisense oligonucleotide alone (anti-Cx38) or in combination with cRNA coding for wild-type rat Cx43 (WT Cx43) or the Cx43 poly-Ala mutants (Cx43-Ala1 to Cx43-Ala4). From Bao *et al*., 2005.

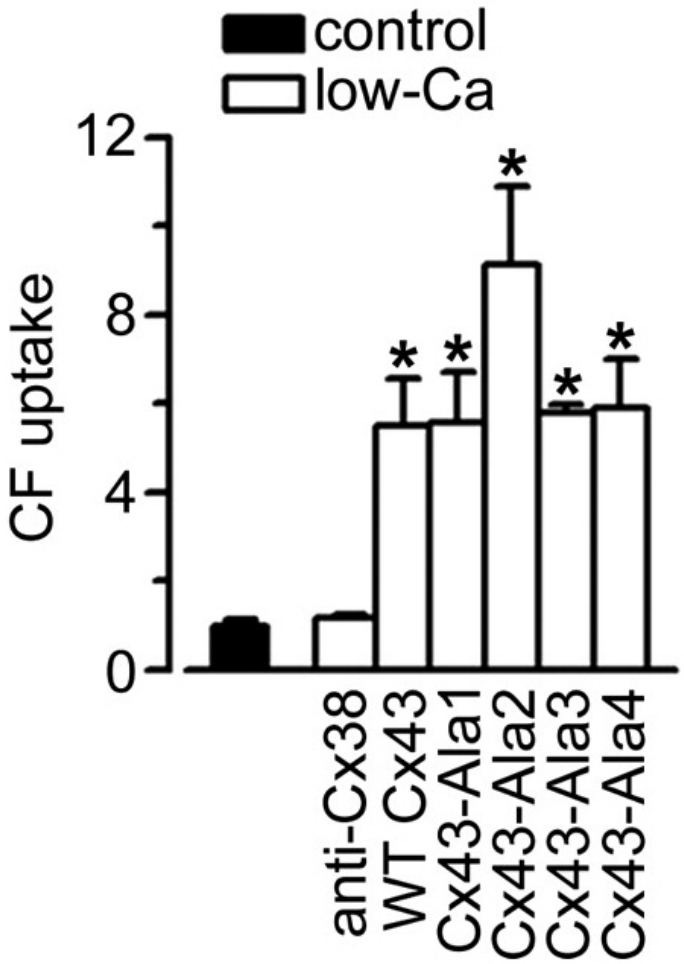

Figure 1.3: Uptake of carboxyfluorescein *via* hemichannels formed by Cx43 poly-Ala mutants.

Data were normalized to the carboxyfluorescein (CF) uptake in normal [Ca^{2+}] solution in oocytes expressing WT Cx43. The filled bar corresponds to the mean ± SEM of the data from all groups in normal [Ca^{2+}]. Data are means ± SEM of 30-40 oocytes *per* condition. * $P < 0.05$ compared to the control value, in the presence of 1.8 mM Ca^{2+}. From Bao *et al*., 2005. See Fig. 1.2 for abbreviations.

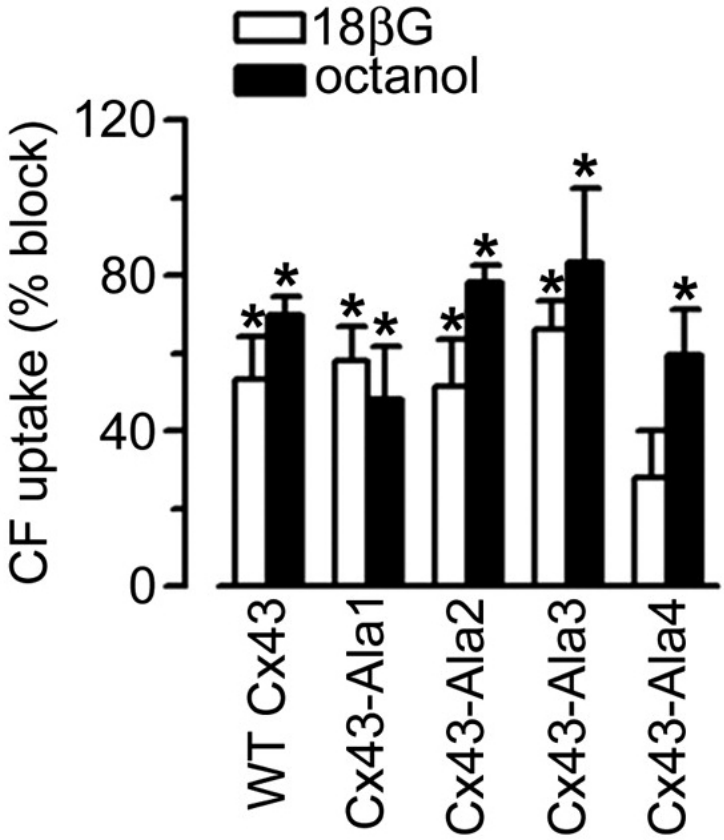

Figure 1.4: Inhibition of carboxyfluorescein uptake by blockers of gap-junctional communication.

The uptake of carboxyfluorescein (CF) *via* hemichannels was measured in low-Ca^{2+} solution, in the absence and presence of 18β-glycyrrhetinic acid (18βG, 20 μM) or octanol (1 mM). Data are means ± SEM of 20-30 oocytes *per* condition, and were normalized to the uptake in the absence of blockers. * $P < 0.05$ compared to the value in the absence of blockers. From Bao *et al.*, 2005.

and were functional (Fig. 1.3 and Fig 1.4). Functional Cx43 mutant proteins were defined as those capable of forming a plasma-membrane gated pore permeable to large hydrophilic solutes.

We have recently shown that heterologous expression of recombinant Cx43 in single frog oocytes results in uptake of the M_r 376 hydrophilic probe carboxyfluorescein *via* Cx43 hemichannels (Bao *et al*., 2004a and Bao *et al*., 2004b). Fig. 1.3 shows that carboxyfluorescein uptake is very low with normal extracellular [Ca^{2+}], due to hemichannel block (Harris, 2001 and Pfahnl *et al*., 1999), and that exposure to low-[Ca^{2+}] medium resulted in a significant increase in carboxyfluorescein uptake in the poly-Ala mutants. This carboxyfluorescein uptake is blocked by 18β-glycyrrhetinic acid and octanol (Fig. 1.3 and Fig. 1.4), two blockers of gap-junctional channels and hemichannels (Bao *et al*., 2005 and Harris, 2001). We have also demonstrated that carboxyfluorescein uptake in normal [Ca^{2+}] is increased by the PKC inhibitor calphostin C, because of dephosphorylation of Ser368 of the C-terminal domain (Bao *et al*., 2004a, and Bao *et al*., 2004b). Calphostin C increased carboxyfluorescein uptake in oocytes expressing Cx43 with transmembrane helix 4 replaced with a poly-Ala sequence (Cx43 Ala4, 7.9 ± 1.4-fold increase, n = 10, $P < 0.0005$), indicating that the permeability of mutant hemichannels is increased by lowering [Ca^{2+}] and blocking PKC, both known regulatory mechanisms of WT Cx43 gap-junctional channels and hemichannels (Lee *et al*., 2003; Harris, 2001; Pfahnl *et al*., 2003; Lampe *et al*., 2000b).

Formation of functional gap-junctional channels by a Cx43 poly-Ala mutant

Large gap-junctional currents, such as those shown in Fig. 1.5A (top right), were easily measured in essentially all pairs of WT Cx43-expressing oocytes. Paired oocytes expressing poly-Ala mutants (n = 6-7 pairs *per* mutant) had no detectable gap-junctional

currents (not shown), but Cx43-Ala4 formed gap-junctional channels with WT Cx43 (Fig. 1.5A, bottom right).

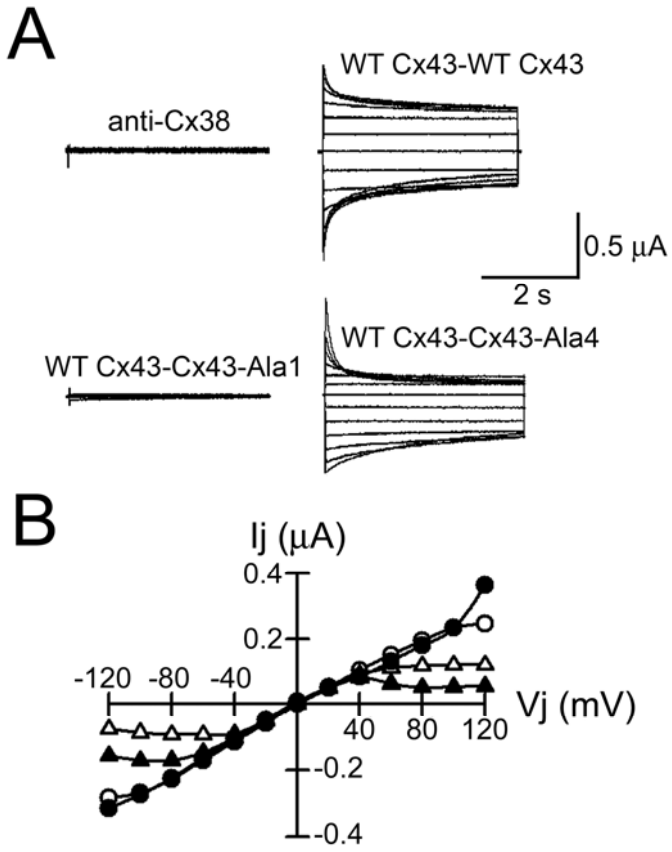

Figure 1.5: Gap-junctional currents in paired oocytes.

A. Typical gap-junctional currents. Oocytes injected with anti-Cx38 antisense oligonucleotide (anti-Cx38), or expressing WT Cx43 or poly-Ala mutants, were paired, and the junctional currents (Ij) were measured upon 4-s transjunctional voltage (Vj) steps between -120 and 120 mV, applied in 20-mV increments from a holding voltage of -60 mV. Four sets of Ij records are shown, which correspond to anti-Cx38-anti-Cx38, WT Cx43-WT Cx43, WT Cx43-Ala1 and WT Cx43-Ala4 oocyte pairs. The recordings are representative of 6-10 experiments *per* condition, obtained using the dual-electrode voltage-clamp technique. B. Current-voltage relationship of the WT Cx43-WT Cx43 and WT Cx43-Ala4 records in panel A. The circles and triangles correspond to the WT Cx43-WT Cx43 and WT Cx43-Ala4 records, respectively. Open symbols, instantaneous currents; filled symbols, currents measured at the end of the voltage pulse (4 s).

Although there was some asymmetry in the time dependency of the junctional currents elicited by transjunctional voltage pulses in the WT Cx43-Ala4 gap-junctional channels (Fig. 1.5, WT Cx43-Ala4 trace and I-V curve), the voltage dependency of the current was generally conserved (slow inactivation at large junctional voltages). The magnitude of the gap-junctional currents cannot be easily quantified because of the time-dependent formation of the channels. However, WT Cx43/Cx43-Ala4 channels either form less efficiently or have lower conductance and/or open probability than WT Cx43 channels because the current amplitude 2 h after pairing was smaller, and after 6 h (trace shown in Fig. 1.5A) was similar, to that of the WT Cx43 channels just 2 h after pairing (Fig. 1.5A, right panels). Fig. 1.5A (bottom left) also shows the absence of gap-junctional currents between an oocyte expressing WT Cx43 and another one expressing Cx43-Ala1 after overnight pairing. Junctional currents were also absent between oocyte pairs expressing one WT Cx43 and the other one Cx43-Ala2 (n = 6) or Cx43-Ala3 (n = 6), even after overnight pairing (not shown). These electrophysiology experiments were performed by Dr. Yongyue Chen in Dr. Luis Reuss' laboratory.

DISCUSSION

Cx43 poly-Ala mutants can form hemichannels whose permeability to large hydrophilic solutes is increased by lowering [Ca^{2+}] and PKC inhibition, and at least one of the complete helix mutants (Cx43-Ala4) can form gap-junctional channels. The docking of hemichannels to form the gap-junctional channels depends on the structure of the two extracellular connexin loops, which are believed to form interdigitated sheets that insulate the channel from the extracellular solution (Harris, 2001). Gap-junctional channel formation by docking of connexons of different connexin composition

(heterotypic channels) occurs only with certain isoforms. i.e., is highly selective (Harris, 2001). A change in the conformation of the extracellular loops, preventing hemichannel docking, is likely to exist in the poly-Ala mutants due to alterations in the packing of the transmembrane helices. Changes in helix packing of the poly-Ala mutants are likely because small residues such as Ala favor closer helix packing in membrane proteins (Eilers *et al*., 2000, Adamian *et al*., 2001, Curran *et al*., 2003) and the substitution of native polar residues can affect helix-helix interaction (Eilers *et al*., 2000 and Curran *et al*., 2003).

The fact that all Cx43-Ala mutants form functional hemichannels suggests that none of the helices contains polar residues needed for the formation of a large pore permeable to hydrophilic solutes. Even though the basic functions of Cx43 are maintained in most poly-Ala mutants, variations in the primary sequence of the helices may tune these functions by alterations in helix packing and/or electrostatics, resulting in functional changes, e.g., pore size and substrate selectivity in the examples studied.

The replacement of complete helices ("helix-scanning mutagenesis"), introduced here to identify those helices whose primary sequence is essential for function, could prove very useful for studies of other membrane proteins. It is significantly less labor intensive than Cys- and Ala-scanning mutagenesis (Cunningham *et al*., 1989 and Frillingos *et al*., 1998), and can be used as a primary helix-screening tool, followed by the traditional individual substitutions of residues in the poly-Ala-replacement mutants that display functional changes. It is also possible that complete helix replacement may simplify the rational engineering of simple, stable proteins for specific applications, such as their use in sensing devices.

The most widely accepted hemichannel model, the C^{α} model of Fleishman *et al*., (2004) is based on the notion that phylogenetically conserved native (especially

hydrophilic residues) are more likely in pore-forming helices of channels because they are essential for function of proteins that form a hydrophilic pore across the membrane. However, none of the transmembrane helix residues is essential for the expression, oligomerization, function and regulation of Cx43 hemichannels. These results indicate that Cx43 tolerates transmembrane helix mutations well, even multiple simultaneous mutations, and that it is not warranted to conclude that conserved residues point to pore-forming helices. The observations presented in this chapter indicate that experimental studies, as opposed to sequence analysis, are needed to confirm or disprove the hemichannel model of Fleishman *et al*. (2004). In the next chapter, I present data using a modified SCAM to attempt experimental identification of pore-lining residues in transmembrane a helices of Cx43 hemichannels.