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Characterizing the Influence of Gap Junction Communication on Cell Volume Dynamics

Bona Mu

Buffalo State College, mub01@mail.buffalostate.edu

Advisor

Derek L. Beahm

First Reader

Derek L. Beahm

Second Reader

I. Martha Skerrett

Third Reader

Gregory J. Wadsworth

Department Chair

I. Martha Skerrett

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Abstract of Thesis

Characterizing the Influence of Gap Junction Communication on Cell Volume Dynamics

Cell volume changes are associated with both normal and disease processes, and can serve as a signal for altering cell activities. Most cell types in a multicellular organism express gap junction channels that allow for the direct transfer of ions, metabolites, and other small molecules between neighboring cells. Little is known about the potential role of gap junctions in modulating cell volume dynamics. The present study characterizes what happens to the swelling rates of fast swelling and slow swelling cells when they are coupled together by gap junctions. The initial hypothesis was that water transport through gap junctions from a fast swelling cell would increase the swelling rate of the slow swelling cell. I first verified endogenous gap junction communication in CHO cell lines stably transfected to express either the water channel AQP4 to create a fast swelling cell type or CD81 membrane protein to create a normal slow swelling cell type. Relative volume changes associated with exposure to a 30% hypo-osmotic shock were measured in CHO-AQP4 and CHO-CD81 homogeneous populations and in mixed populations. Swelling curves were fit to a single exponential function for homogeneous populations or a double exponential for mixed populations. Average time constants (τ) of these fits were compared between the different groups, with CHO-AQP $\tau=9.7$ s whereas CHO-CD81 $\tau=40.6$ s. The fast and slow τ of mixed populations were 7.4 sec and 34.2 sec, respectively. There was a trend for both cell types to reach their new volumes faster when cocultured. Attempts to block gap junctions with a non-specific inhibitor, carbenoxolone, generated mixed results. Models to explain the data, as well as the advantages and disadvantages of the experimental strategy, are discussed to help direct future studies on the role of gap junctions in cell volume dynamics.

State University of New York
College at Buffalo
Department of Biology

Characterizing the Influence of Gap Junction Communication on Cell Volume Dynamics

A Thesis in
Biology
by

Bona Mu

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Approved by:

Derek L. Beahm, Ph.D.
Assistant Professor of Biology
Chairperson of the Thesis Committee/Thesis Adviser

I. Martha Skerrett, Ph.D.
Chair and Associate Professor of Biology

Kevin J. Miller, Ed.D.
Interim Dean of the Graduate School

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Introduction

Cell Volume and Volume Regulation

Cell size is relevant to many basic processes in cell physiology. It is a critical aspect of cell health and function. Volume changes are associated with both normal and disease processes. Cell volume alters during cell growth, differentiation, hypertrophy, apoptosis and migration, which in turn affects cell metabolism (Wehner et al., 2003). The simplest consequence of volume change is a change in the cytosolic concentration of biologically active macromolecules, small molecules such as metabolites and signaling molecules, and ions. For example, an increase in cell size due to cell growth dilutes transcriptional inhibitor and activates cell proliferation as demonstrated in a recent work on budding yeast size (Schmoller et al., 2015).

Cell volume is determined by the intracellular and extracellular concentrations of osmotically active components. The plasma membrane is highly permeable to water relative to the ions, small polar molecules and proteins that make up the bulk of biological solutions. An osmotic potential across the plasma membrane is established when there is a difference between the concentrations of membrane impermeable components on either side of the membrane. This osmotic pressure drives water across the membrane, resulting in a change in cell volume. Cell swelling occurs when either the osmolarity of the extracellular solution is reduced or the osmolarity of the intracellular cytosol is increased. Cell shrinkage occurs under opposite conditions. In general, the $\text{Na}^+\text{-K}^+\text{-ATPase}$ plays a key role in generating and maintaining the steady-state cell volume. This pump forces out additional osmolytes in the form of Na^+ to offset the osmotic pressure that is generated by excess impermeable negatively charged macromolecules in the

cytosol (Nylander-Koski et al., 2005). In the absence of this pump, as is the case of energy depletion, a cell will swell and eventually burst as positive ions enter to balance the excess negative charge presented by the cytosolic proteins which in turn results in water entry by simple diffusion across the membrane.

There are always challenges to cell volume constancy. Intracellular and extracellular osmotic changes can be responsible for cell volume change. Those changes may be caused by physiological changes in the water or sodium content of blood, altered metabolic processes, energy depletion, hormones etc. Although most cells are in a relatively constant isosmotic microenvironment in mammals, some cell types such as liver cells, blood cells, gastrointestinal cells and kidney cells face greater challenge in maintaining cell volume homeostasis. Given the importance of cell volume, its constancy is usually maintained through homeostatic mechanisms involving changes in membrane flux of ions and osmotically active small molecules. When shifted away from steady-state, acute cell swelling leads to the activation of regulatory volume decrease (RVD) involving KCl efflux. In RVD, the efflux of K^+ and Cl^- by cotransport using K^+/Cl^- symporters or swelling activated K^+ and Cl^- transporters, forces water to follow their path so the cells shrink back towards normal volume in hypotonic conditions (Hoffmann and Simonsen, 1989). Volume-regulated anion channels (VRACs) are involved in the efflux of Cl^- and organic osmolytes such as taurine and amino acids (Jentsch, 2016). Recent works have identified VRAC component as LRRC8 proteins complex (Qiu et al., 2014; Voss et al., 2014). Intracellular free Ca^{2+} increases due to cell swelling may also open Ca^{2+} activated Cl^- and K^+ channels (Hazama and Okada, 1988; Hazama and Okada, 1990; Jentsch et al., 2002; Okada et al., 1990). These channels and transporters act to reduce cell volume under hypotonic conditions. Interestingly, the loss of

osmolytes (including organic osmolytes) during RVD cannot be quickly recovered upon return to isotonic media so cells will initially shrink in the originally isotonic media and show reduced volume compared to normal volume until the natural osmolyte concentration is restored in the cytoplasm.

Acute cell shrinkage upon exposure to hypertonic solutions is safeguarded by a regulatory volume increase (RVI) mechanism that often consists of Na^+ and Cl^- influx (Okada et al., 2001). Na^+/H^+ exchangers and $\text{Cl}^-/\text{HCO}_3^-$ anion exchangers, and $\text{Na}^+/\text{K}^+/\text{2Cl}^-$ cotransporters are involved in the process. Both RVD and RVI tend to return the cell's volume to a specific setpoint in the continued presence of the non-isotonic condition. These mechanisms, however, can in turn impact other cell physiological processes. Hence the importance of understanding the molecular mechanisms involved in volume regulation.

Gap Junction Structure, Function, and Regulation

In multicellular organisms, it is essential for cells to coordinate with each other. The cells of most solid tissues communicate with each other through gap junction channels. Gap junction channels form plaques with a few to thousands of units and are composed of connexins in chordates and innexins in prechordates (Skerrett and Williams, 2016). Combining X-ray crystallography, electron microscope image analysis and chemical measurements, a structural model was constructed (Caspar et al., 1977; Makowski et al., 1977). Gap junctions (GJs) are intercellular channels composed of two connexons (hemichannels) that are docked head-to-head across a narrow ~ 2 nm extracellular gap between cells in contact, which is likely how the term gap junction was coined. In vertebrates, the hemichannel provided by each cell is a hexamer of connexin (Cx)

proteins. Human connexins are encoded by 21 genes (Söhl and Willecke, 2003) and are commonly named using an estimated molecular weight nomenclature system (Kumar and Gilula, 1996). Connexins have four α helical transmembrane domains and two extracellular loops that are highly conserved among the family. Connexin intracellular cytoplasmic N-terminus is conserved while more variations occur at the cytoplasmic loop and the intracellular C-terminus within connexin family. The loop and the C- terminal region contain sites that are prone to post translational regulations such as phosphorylation. The genomes of most vertebrate species contain multiple distinct connexin genes and a given cell type usually expresses more than one connexin. Two other families of gap junction proteins are pannexins that form unopposed channels and their homologous counterparts expressed in invertebrates called innexins (Baranova et al., 2004; Goodenough et al., 1996). Interestingly, innexins and connexins have no sequence homology.

Gap junction channels are basically aqueous filled pores that directly connect the cytosolic compartments of cells, allowing chemical communication through simple diffusion. Gap junction plaques are clusters of a few to thousands of individual channels, allowing the formation of large functional cell networks such as the astrocyte syncytium. The width of the pore averages 20Å and fluorescent dye transfer tests have shown that hydrophilic molecules up to 1 KDa in size can permeate the channel. Given their ability to exchange ions, metabolites, and small signaling molecules between cells, gap junctions serve a variety of functional roles within a cell population. The ability to share ions is the basis for electrical signal propagation and synchronization, allowing the fast conduction of electrical impulses through cardiovascular and neural tissues (Bennett and Zukin, 2004; Rohr, 2004). The ability to share larger molecules is the basis for metabolic cooperation, wherein cells can share metabolites and precursors including simple carbohydrates,

amino acids, and nucleotides (Goldberg et al., 1999). Gap junction channels also allow the passage of signaling molecules and secondary molecules cAMP (Lawrence et al., 1978), inositol 1,4,5-triphosphate (IP3) (Saez et al., 1989), polyamines, siRNA (Valiunas et al., 2005), ATP and microRNAs (Katakowski et al., 2010). Cationic and anionic probes testing GJ permeability reveals that permeability is dependent on the connexin composition of GJ, and provide selectivity over solute size and charge (Kanaporis et al., 2011).

Because of the significance of gap junctions in the normal cell physiology (embryogenesis, neurogenesis, cell growth, cell differentiation, cell migration, cell synchronization, metabolic coordination, etc.), disruption of its function causes pathologies. Connexin mutations disrupt tissue homeostasis and lead to many known human hereditary diseases, such as deafness, skin disease or cataractogenesis (Gerido and White, 2004). Connexin deletion can cause embryonic lethality in Cx26, Cx43 and Cx45 knockout mice (Gabriel et al., 1998; Kumai et al., 2000; Reaume et al., 1995). Homozygous Cx46 deletion produces cataracts (Gong et al., 1997) whereas Cx50 knockout mice have smaller lenses and display irregular and diffuse nuclear opacity (White et al., 1998). Cx32 mutation in myelinating Schwann cells in humans cause an inherited disease of peripheral neuropathy (Bergoffen et al., 1993). Cardiac failures are often associated with an increase in Cx40 expression, a decrease of Cx43 (Dupont et al., 2001; Wang and Gerdes, 1999) and an increase in Cx45 expression (Yamada et al., 2003).

Gap Junction channels, like other types of membrane channels, transition between an open and closed state in a process referred to as gating. Understanding these gating mechanisms is important in understanding the possible ways that volume changes can affect gap junction function. Gap junction channel gating is sensitive to voltage and the chemical environment of the cytoplasm.

Gap junctions close with increasing trans-junctional voltage that occurs when the plasma membrane voltages of the two coupled cells are different (Spray et al., 1979). Chemical gating involves intracellular molecules and ions, such as Ca^{2+} and H^+ , to initiate a slow gating of the gap junction channel. High concentration of Ca^{2+} in the intracellular space can close channels but different types of GJs have different sensibilities to calcium concentration. A $\text{Ca}^{2+}/\text{CaM}$ -dependent regulatory mechanism is present in α -subfamily connexins where calmodulin binding sites located at the carboxyl tail assists calcium in the closing of channels (Xu et al., 2012). Calmodulin binding site located in Cx50 intracellular loop also regulates GJ closing (Chen et al., 2011). Previous reports have shown that hemichannels are regulated by extracellular calcium and have a low open probability at ~ 1.8 mM (Lopez et al., 2016). Extracellular calcium levels above 1 mM reduce hemichannel open probability (Sáez et al., 2005). The calcium sensing motifs are said to reside in the pore lumen, with the binding of calcium creating a positive electrostatic barrier blocking ionic conduction (Bennett et al., 2016).

Intracellular acidic pH also closes gap junctions in a mechanism involving carboxyl tail interacting with cytoplasmic loop in a particle-receptor mechanism during Cx43 gating by H^+ (Duffy et al., 2002; Ek-Vitorin et al., 1996; Kreusch et al., 1998). In addition to voltage and chemical gating mechanisms, gap junction conduction and permeability can be modulated by post translational modifications, usually targeting the cytoplasmic tail region of the connexin protein. Phosphorylation was shown to alter unitary conductance state in human Cx43 (Moreno et al., 1994). Phosphorylation of Cx43 tail has also been shown to affect its life cycle and likely triggers gap junction degradation (Thévenin et al., 2013).

Studies of Gap Junctions and Cell Volume and/or Water Transport

Because gap junctions are aqueous channels permeable to the ions and small molecules that make up the majority of the osmolytes in the cytoplasm, it is predicted to be difficult to establish an osmotic gradient across the channel. Molecules and proteins that are impermeable to gap junctions represent only 5-10% of the total intracellular osmolytes and changes in their concentration could possibly establish a small osmotic pressure to drive water transport through the channel. Also, it is possible for cells to alter their concentrations of gap junction permeable ions and small molecules by regulating different types and levels of ion pumps and transporters. And even in the absence of an osmotic gradient, hydrostatic pressures could act as a driving force for water transport through gap junctions. A fluid circulation model that explains the transparency of the lens in the absence of vasculature has been supported by experiments testing fluid movement, lens intracellular hydrostatic pressure and water-protein concentration ratio (Candia et al., 2012; Gao et al., 2011; Vaghefi et al., 2011). The model predicts that the current flow measured in lens is due to sodium flow in the extracellular space entering cells at the center of the lens and redirected out to the equator through gap junctions. Water follows this current flow, creating an internal circulation system for nutrient delivery and waste removal (Mathias et al., 2007; Mathias et al., 1997).

The role of gap junctions in volume dynamics is currently unexplored. Some studies describe the consequences of cell volume changes on gap junctions but rarely provide insight on the mechanism of change. Cell shrinkage has been associated with a loss of gap junctions as examined by electron microscopy (Barr et al., 1968; Barr et al., 1965), and by measuring junctional membrane permeability (Loewenstein et al., 1967). The loss of junctions is most probably due to

them being ripped out of the intercellular junction area as the cells shrink away from each other. Cell swelling, on the other hand, has been associated with either an enhancement of gap junctions (Scemes and Spray, 1998) or an inhibition of gap junctions (De Mello, 2010; Kimelberg and Kettenmann, 1990; Ngezahayo and Kolb, 1990; Zhao and Santos-Sacchi, 1998). The enhancement could arise simply from increased membrane contact area allowing for more gap junction channel formation from the pool of hemichannels residing in the plasma membranes of the cells, but this remains to be proven. Alternatively, enhanced junctional conductance could arise from cytosolic changes that occur upon swelling. The increase of junctional currents in astrocytes seen after exposure to hypotonic shock may be due to Ca^{2+} signaling that up-regulates the expression of connexins and increases the number of gap junctions (Scemes and Spray, 1998). But, increased intracellular calcium levels has also been shown to inhibit gap junctional currents through the activation of a calmodulin pathway (Light et al., 2003). Again, the few gap junction studies related to cell volume focus on what happens to gap junctions upon cell swelling or shrinkage, whereas no studies discuss the influence of gap junctions on cell volume changes in neighboring cells.

Studies have shown seemingly contradictory results regarding the potential role of gap junction or their component hemichannels in volume buffering. Water permeability testing of *Xenopus* oocytes expressing Cx30 and Cx43 hemichannels subjected to a 20 mOsm hypertonic shock showed no significant difference in shrinking rate compared to non-injected oocytes, and much milder shrinking compared to AQP expressing oocytes (Hansen et al., 2013). In pyramidal neurons that naturally lack aquaporin channels, knock out of pannexin 1 did not reduce spreading depolarization-induced dendritic swelling (Sword et al., 2016). Although these two studies are about hemichannels and pannexins, the permeability similarities between gap junctions and

hemichannels (connexins or pannexins) makes us wonder whether or not an osmotic gradient can exist across the channel to drive water flow. However, brain blood interface abundant Cx30 and Cx43 gap junction number and dye coupling increase in AQP KO mice, which may explain the observed limited effect of the AQP deletion on brain water and ion homeostasis (Katoozi et al., 2017; Strohschein et al., 2011). If true, this finding hints at a possible water permeability function for gap junction channels.

This project seeks to address the question of how gap junctions might affect the dynamics of cell volume changes, especially in cell populations that are heterogeneous in terms of membrane water flux (Figure 1). Given that the rate and extent of volume change can trigger other cellular events, it is important to know if and how gap junction communication affects these changes. The hypothesis is that water or solute transport through gap junctions will alter the swelling rates of coupled cells in a population where the cells have a different membrane permeability to water. Differences in membrane permeability were generated by using a mix of gap junction competent Chinese Hamster Ovary (CHO) cells expressing either a water channel, AQP4, or a control membrane protein, CD81. Aquaporins are molecular water channels that facilitate rapid and selective movement of water in osmotic gradients (Agre et al., 1993). Upon exposure to hypotonic solutions, these two cell types will swell at different rates and thereby create a transient non-isotonic condition between the cytoplasmic compartments of the two cells. How cells respond to this situation in the presence and absence of gap junctions is explored. The specific goals of this study were to 1) verify the existence of gap junction communication within and between the two different stably transfected CHO cell lines used in this study, and 2) quantify

swelling rates in homogeneous and mixed cell populations in the absence and presence of gap junction blockers.

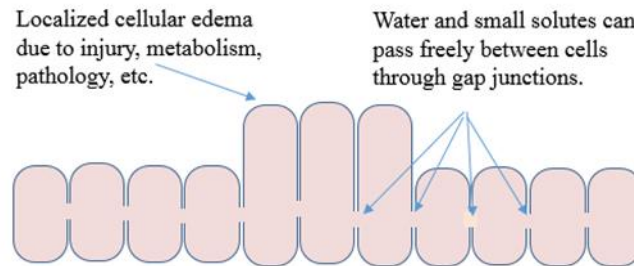


Figure 1: Schematic representation showing localized cell swelling in a tissue. The question being explored is whether gap junction communication between neighboring cells can affect the rate or extent of volume changes.

Methods

1. Cell lines and cell culture

This study uses a CHO (Chinese Hamster Ovary) cell line stably transfected with human AQP4 to generate a rapid swelling cell type and a CHO cell line stably transfected with a control membrane protein, human CD81, to generate a normal swelling cell type. These cell lines were kindly provided by Aeromics LLC (Cleveland, OH). Cells were cultured in canted neck 25 cm² culture flasks T-25 (Corning Inc., Corning, NY) at 37 degrees Celsius and 5% CO₂, in 5-6 ml of media consisting of Ham's F12 nutrient mixture (Gibco, Life technologies, Grand Island, NY), 10% Fetal bovine serum (Gibco, Grand Island, NY), 1% of 10,000 Units/ml penicillin streptomycin (Gibco, Grand Island, NY), and Hygromycin B at 500µg/ml (Invitrogen, Carlsbad, CA). Cells were passed

to new culture flasks when they reached near confluency, which occurred in 3-4 days. Passing cells involved removing old media and washing the cells in a calcium-free media or PBS (BupH phosphate buffered saline packs, Thermo Fisher Scientific Inc., Rockford, IL) and then incubating the cells for several minutes in 1ml of TrypLE dissociation buffer (Gibco, Grand Island, NY). Cell detachment was verified with light microscopy. Cells were collected in a sterile Eppendorf tube and centrifuged for 4 minutes at 600 rcf in a tabletop microcentrifuge. The supernatant was aspirated off and the cells were resuspended in 1 ml of fresh media. New T-25 culture flasks were seeded with 50 μ l of this 1 ml resuspension.

For cell volume measurements, sterile glass coverslips in 35mm petri dishes were seeded with various volumes of the resuspended cells to generate different cell densities. This protocol evolved over the course of the project to optimize heterogeneity in mixed cell populations. The final protocol used 300 μ l out of 1ml cell suspensions to seed the coverslip followed by a 20-minute incubation time to allow cells to attach to the coverslip and then replacing the media containing unattached cells with fresh media. Mixed cell populations were generated by combining suspended cells in different ratios to get the 300 μ l of cell suspension used to seed a coverslip. This way, cells reached confluency faster and generated more contacts between the different cell types rather than generating large colonies of a single cell type.

2. Parachute assay for gap junction communication

Gap junction communication was assayed using variations of the “preloading” assay (Goldberg et al., 1995) and the “parachute assay” (Ziambaras et al., 1998). One cell population is labelled with a membrane resident lipophilic dye and a different population is labelled with a

cytosolic gap junction permeable dye and then the two populations are mixed together and assayed for transfer of the gap junction permeable dye into the membrane labelled cells. In my work, 5 μ M DiI from Vybrant™ DiI Cell-Labeling Solution (Molecular Probes, Eugene, OR) was used to label adherent recipient cells that were plated on a glass bottom dish suitable for confocal imaging. DiI is a red fluorescent membrane resident dye. Donor cells were labelled in suspension with 5 μ M BCECF-AM (Molecular Probes, Eugene, OR) which is a membrane permeant dye that then becomes trapped in the cell cytosol after non-specific esterases cleave the AM ester groups. BCECF is green fluorescent. After thorough washing of both recipient and donor cells, the donor cells are added to the dish which contains the DiI labeled recipient cells. Cells were imaged at different times after mixing with an Fluoview FV10i (Olympus, Center Valley, PA) confocal fluorescence microscope using the red filter set for DiI and the green filter set for BCECF. Green fluorescence in red DiI labeled cells indicates the presence of functioning gap junction channels. In some experiments, 100 μ M of the gap junction blocker carbenoxolone (Sigma Aldrich, St. Louis, MO) was added to the culture dish containing the freshly mixed cells.

3. Measuring cell volume dynamics

Measurement of relative volume changes resulting from osmotic challenges will be achieved by a microfluidic cell volume sensor that uses impedance measurements to determine the resistivity of a small volume of solution bathing the cells (Ateya et al., 2005). As cells swell from a hypotonic challenge, they occupy a greater volume within a microfluidic chamber which reduces the available volume for external current flow and this is measured as an increase in chamber resistance to a constant current (see Figure 2). This technique provides for fast solution

exchange times of less than 1 sec and real-time measurement of relative volume changes in substrate-attached cells.

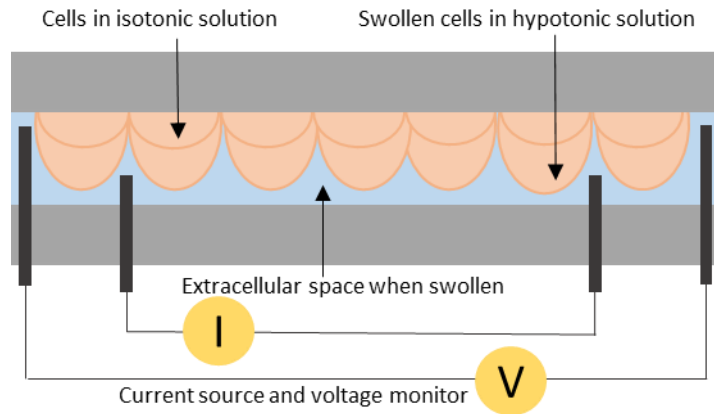


Figure 2: Principles of volume detection in a cell volume cytometer. A glass coverslip with adherent cells is used to create the ceiling of a microfluidic chamber with a 20 μm height dimension. A pair of electrodes in the chamber output a constant current. A different pair of electrodes senses the voltage across the length of the chamber and outputs the resistance of the chamber. Not shown are ports for solution entry and exit. Changes in cell volume are directly related to changes in chamber resistance.

Cells are grown to >80% confluence on sterile 22x22 mm glass coverslips placed in 35mm petri dishes to be ready for testing using the cell volume sensor instrument CVC-7000 created by SUNY Buffalo and used in various cell volume related studies (Ateya et al., 2005; Heo et al., 2008a; Heo et al., 2008b; Hua et al., 2010; Kowalsky et al., 2010). Coverslips are washed in isotonic solution for 2 minutes and then clamped on the microfluidic chamber to create a sealed environment. Solutions are perfused through inlets, flush and/or outlet ports located in the sealed chamber at 1.0 psi. Isotonic solution was perfused for 5 minutes providing a stable baseline of resistance. For solution exchanges, a new solution was perfused for 30 secs through a flush port in the chamber prior to reaching the cells to prevent mixing that occurs in solution exchanges. Then

the new solution was directed over the cells at its full composition. Cells were exposed to various osmotic shocks for various durations. For the drug treatment groups, cells were perfused with a 100 μ M carbenoxolone (CBX) in isotonic solution for 10 minutes prior to exposing them to the drug in hypotonic solution.

Stock solutions were prepared to have roughly the same salt concentrations. A 50% hypotonic stock solution was prepared at pH 7.4 with the following composition: NaCl 60mM, KCl 5mM, MgCl₂ 2mM, CaCl₂ 1mM, Hepes 10mM. An isotonic solution was prepared the same way but with the addition of 160mM sucrose. The addition of sucrose reduces the electrical conductivity of the solution because it interferes with ion mobility. This would result in a bulk shift in the impedance signal when changing solutions. To minimize this bulk shift, small amounts of NaCl were added to the isotonic solution until its conductivity matched that of the hypotonic solution (1113-1119 μ S/cm at room temperature), as measured by a conductivity meter. Osmolarities of the final solutions were measured using a freezing-point osmometer. The two solutions could then be mixed at different ratios to generate matching conductivity solutions of different osmolarities. For most experiments, the osmolarity of the isotonic solution ranged between 310-320 mOsm whereas the 30% hypoosmotic solution ranged between 213-215 mOsm.

4. Swelling curve fitting and data analysis

Swelling curves were fit to a single exponential for homogeneous cell population or double exponential for mixed cell populations using a Levenberg-Marquardt Algorithm (LMA). Chamber resistance (R), which was a direct measure of total volume of the cell population, was modelled by the following equation for the mixed cell populations: $R=R_0-R_1\exp(-t/\tau_f)-R_2\exp(-t/\tau_s)$, where

the fast time constant (τ_f) and slow time constant (τ_s) characterize swelling of the CHO-AQP cells and CHO-CD81 cells, respectively, and where R1 and R2 reflects the relative contribution of those cell types to total volume change. A linear component was added to accommodate a drifting baseline observed in some of the experiments. Time constants for the different experimental groups were averaged over multiple trial and presented as mean and standard deviation. Student t-test was used to determine statistical significance between groups.

Results

1. Assaying gap junction communication in CHO cell lines

Parachute (also known as “preloading”) assays were used to verify gap junction communication in the CHO-AQP and CHO-CD81 cell lines. In a parachute assay, adherent recipient cells are labelled with a membrane dye (DiI) that fluoresces red and is confined to the labelled cell. Then donor cells in suspension are loaded with a gap junction permeant dye that fluoresces green (BCECF). The donor cells are then added to the culture dish containing the adherent recipient cells and cultured for several hours to allow for gap junction formation. Fluorescence microscopy is used to determine if the green fluorescent dye from the donor cells is transferred into the recipient cells labelled with red fluorescent membrane dye. Such transfer is a positive indication of functional gap junction communication. This assay is considered more qualitative than quantitative, and simply demonstrates the existence or absence of gap junction communication without reference to the number of channels.

Time was spent trying different variations of the assay and optimizing the protocol. Variations included labelling both donor and recipient cells while they were in suspension versus labelling a sub-confluent adherent cell population with one dye and labeling a suspended population in the other dye. Labelling cells in suspension required repeated centrifugation and resuspension steps to remove excess dye so that it could not label the other cell population when the cells were mixed together. Labelling both cell populations in suspension required longer times to detect gap junction coupling after mixing and plating on glass bottom dishes (data not shown). Also, it was harder to wash away excess DiI compared to excess BCECF when cells were in suspension (data not shown), so the final protocol involved staining adherent cells with DiI and parachuting suspended cells loaded with BCECF.

Extended staining times with DiI appeared to alter cell morphology as seen under normal phase light microscopy, and so I determined the minimum staining time required for adherent cells. Figure 3 shows the results from staining adherent CHO-CD81 cells with 100 μ l of 200 times diluted Vybrant DiI cell-labeling solution in normal growth media for only 5, 10, or 15 minutes. These short staining times maintained cell morphology and the results indicated that even a 5-min staining time was sufficient to uniformly stain the whole population, with longer times not resulting in qualitatively better staining. Loading suspended cells in 10 μ M BCECF was sufficient to produce very strong cytoplasmic signals.

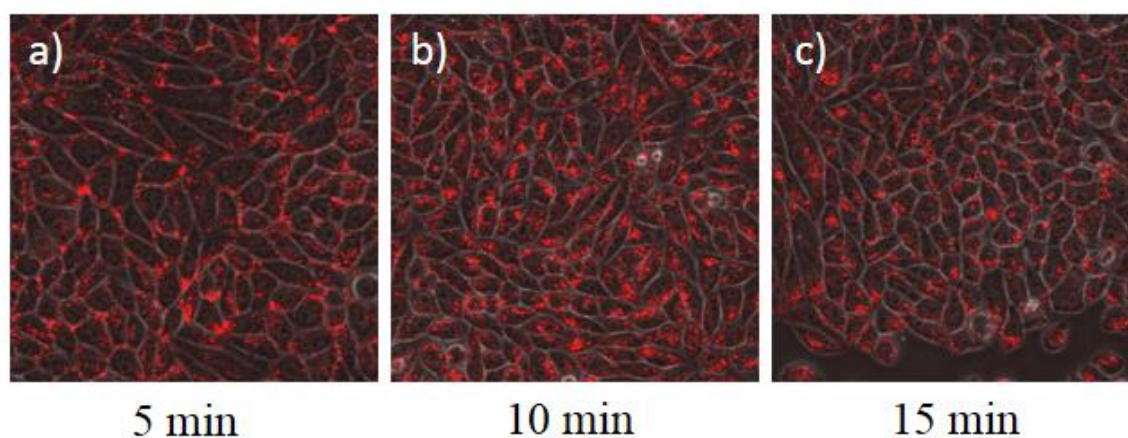


Figure 3: CHO-CD81 cells stained with lipophilic dye DiI for different times. a), b) and c) are confocal images taken 5 mins, 10 mins and 15 mins after Vybrant DiI staining of CHO-CD81 cells grown on glass bottom dishes, respectively.

Parachute assays performed at different times over the course of my thesis work confirmed existence of gap junction communication for the different cell lines. Examples of positive coupling detected at different times for cell mixes are shown in Figures 4-5. These examples show coupling between CHO-AQP and CHO-CD81 cells, which is the critical experimental group. Homogeneous populations of CHO-AQP or CHO-CD81 also showed positive coupling (data not shown). As shown in Figure 4, dye transfer through gap junctions could be seen in as little as 15 minutes after adding donor cells, even before they had a chance to adhere and spread on the glass coverslip. After one day of co-culture, as shown in Figure 5, there is dye found in more DiI labelled recipient cells and note the reduced intensity of the BCECF labelled cells which I believe is due to a loss of dye to more neighboring cells and due to cell division, which dilutes the dye. Although positive gap junction coupling was detected, it was not seen between every neighboring cell pair. This finding is consistent with previously published reports suggesting that although CHO cells express Cx43, gap junction coupling is weaker and less extensive than in other cell types (Musil et al., 2000b).

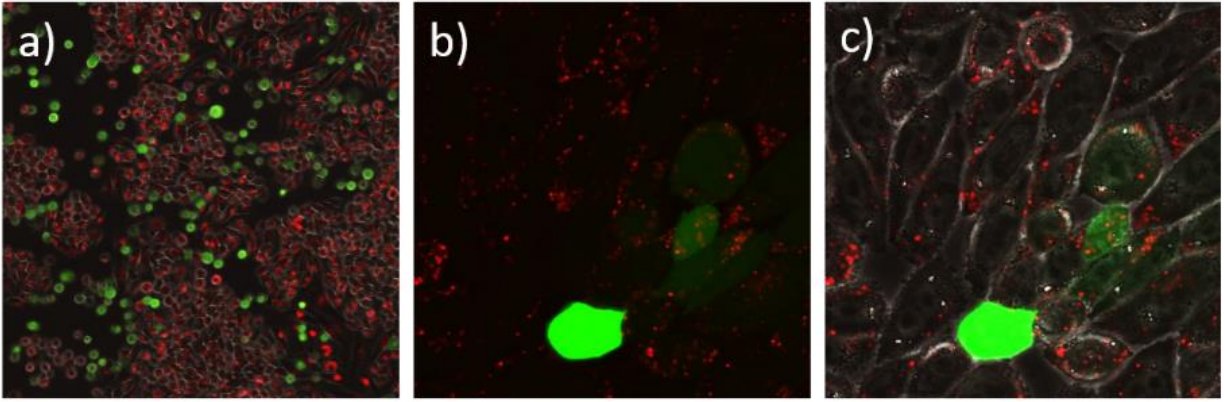


Figure 4: Parachute assay of dye transfer, post 15 min. The panels show images of BCECF loaded CHO-AQP cells that were dropped onto adherent DiI stained CHO-CD81 cells at 10x magnification (a) or 120x magnification (b,c). Panel (c) includes a phase contrast image of panel (b). Note green fluorescence from a brightly labelled donor cell to neighboring DiI cells.

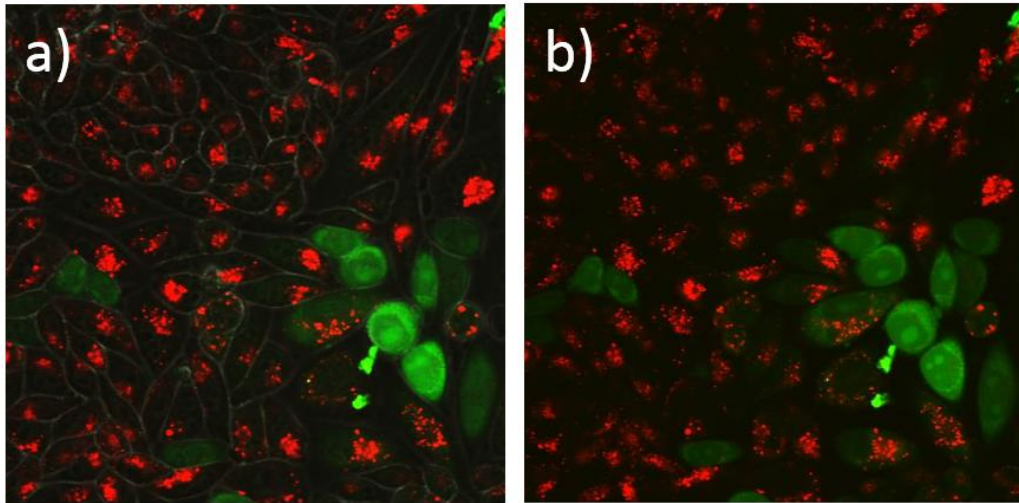


Figure 5: Parachute assay of dye transfer, post 1 day. Dye spread from BCECF loaded CHO-AQP donor cells to DiI labelled CHO-CD81 recipient cells 1 day after cell mixing. Images collected at 60x magnification with (a) and without (b) phase contrast.

During the thesis work, I observed changes in cell morphology such that cells showed a large number of membrane blebs. These blebs may have been due to culture conditions or passage number of the cell lines or cell cycle stage. The cell cultures with blebs were still used for swelling

assays and so I wanted to determine if they still formed gap junctions despite the blebs. Figure 6 shows the transfer of dye from blebbing cells to recipient cells, suggesting that the blebbing morphology did not interfere with the cells ability to form gap junctions with neighboring cells.

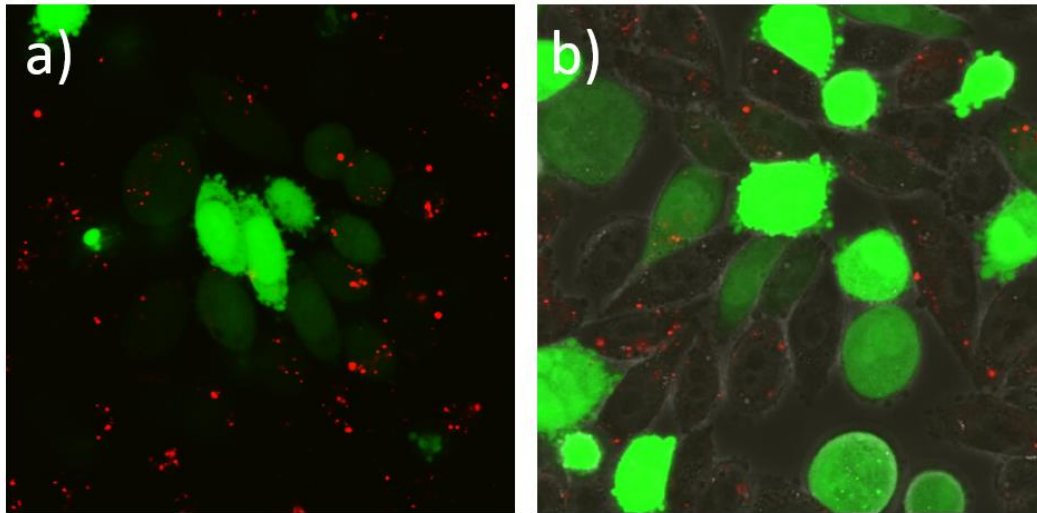


Figure 6: Parachute assay of dye transfer at high magnification, post 3 hrs. Parachute assay results observed at 120x magnification 3hrs after adding BCECF loaded CHO-AQP cells to adherent DiI stained CHO-CD81 cells. Green fluorescence shown spreading from donor cells to acceptor cells. a) and b) are different fields of view and with b) including a phase contrast image layer.

2. Confirm efficacy of GJ blockers to be used in main experiments.

To directly demonstrate the effect of gap junctions on volume dynamics, swelling experiments were to be performed on the same cell population in the presence of the endogenous gap junction coupling and after blocking gap junction communication. Unfortunately, there are no known blockers that target gap junctions specifically. However, there are molecules known to reduce gap junction coupling but they may also affect other cell processes. One of the more popular blockers is CBX. I used the parachute assay to test the ability of CBX to block gap junctions. Cells did not do well in long term incubation with carbenoxolone (1 day) so images were collected at 2-

3 hours after cell mixing in the absence or presence 100 μ M CBX. Figure 7 is several fields of view of cells cultured in absence of the drug, showing typical dye transport to neighboring cells whereas Figure 8 is two representative fields of view for cells that were co-cultured in presence of CBX and show no dye transfer. In some fields, there may be transfer to a single cell in the entire field, suggesting that carbenoxolone is not 100% effective in eliminating gap junction communication.

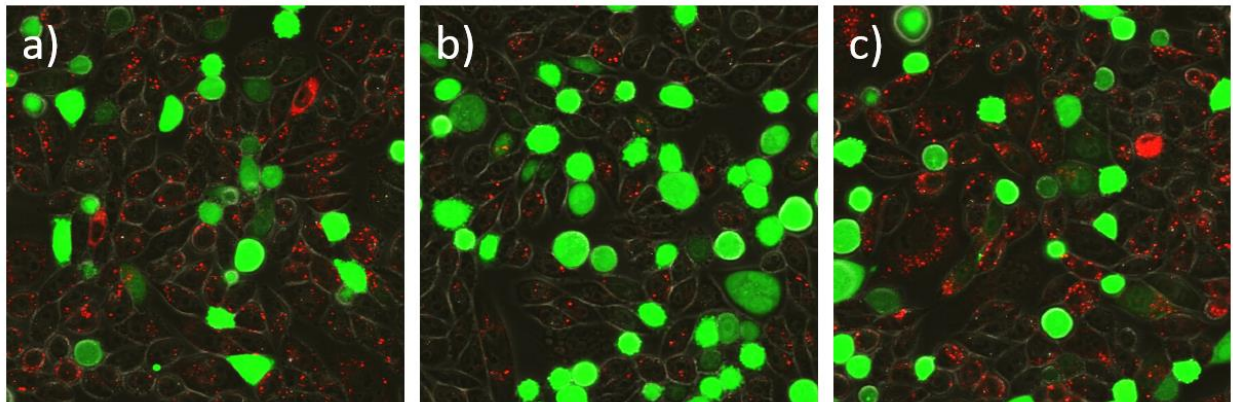


Figure 7: Parachute assay without CBX. Parachute assay results observed at 60x magnification 3 hrs after adding BCECF loaded CHO-AQP cells to adherent DiI labelled CHO-CD81 cells in the absence of CBX. a), b), and c) are different fields and include a phase contrast image overlay.

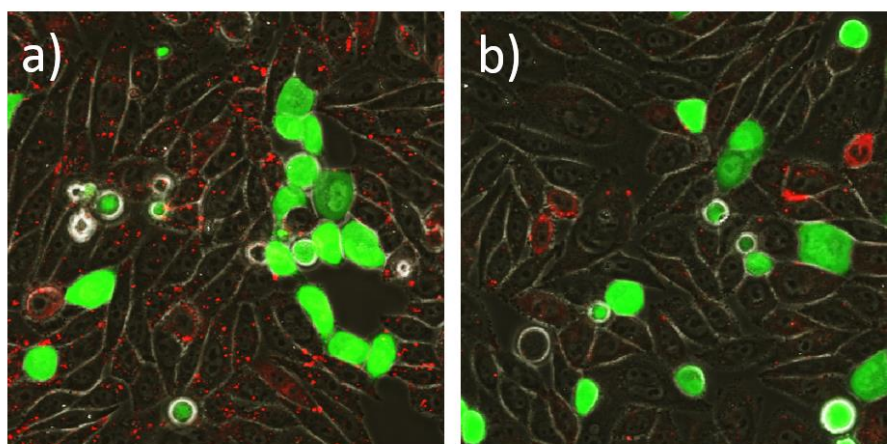


Figure 8: Parachute assay in presence of gap junction blocker, CBX. Parachute assay results observed at 60x magnification 3 hrs after adding BCECF loaded CHO-AQP cells to adherent DiI labelled CHO-CD81 cells in the presence of 100 μ M CBX. a) and b) are different fields of view and include a phase contrast image overlay in experiments performed on the same cell populations as those in Figure 7.

3. Cell Volume Dynamics

Impedance techniques on a microfluidic platform were used to measure relative changes in the volume of a cell population in real time. The swelling of human embryonic kidney (HEK) cells transfected with Aquaporin 4 was documented using the same type of real time sensor that is used in my studies (Heo et al., 2008a). I monitored swelling in CHO-AQP4 and CHO-CD81 populations separately, and then in a mixed cell population. Large or prolonged osmotic challenges, as well as high ambient room temperature were seen to induce a regulated volume decrease (RVD), as shown in Figure 9. Alternatively, this reduced volume could indicate a loss in cell number during the perfusion step, but this seems less likely because subsequent swelling episodes resulted in similar signal amplitudes. RVD would complicate the mathematical fitting of the data and alter the intracellular osmolytes such that repeated swelling episodes (as needed for the gap junction blocker

studies) would produce different swelling curves. Future studies will examine the onset and extent of RVD in gap junction coupled cells, but this was not the aim of my thesis.

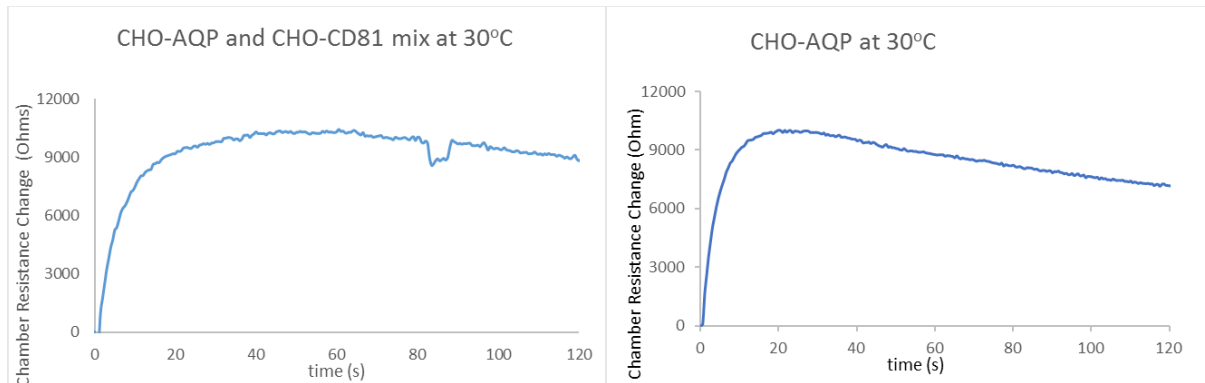


Figure 9: RVD observed at elevated temperatures with 30% osmotic challenge. Regulatory Volume Decrease (RVD) or cell loss observed in CHO cell populations during a 30% hypoosmotic challenge when the room temperature was an unusual 29-30°C. This volume reduction was not normally seen at normal room temperatures of 20-22°C.

To avoid RVD, a 15-30% osmotic challenge limited to 2-3 minutes was found to be sufficient to generate reproducible swelling curves with a strong signal-to-noise ratio for good fitting. An example of a typical experiment is shown in Figure 10, where a CHO-AQP cell population was subjected to repeated hypo-osmotic challenges of 15% or 30%. The top panel shows the timeline of the entire experiment, and the bottom panels show swelling curves for each osmotic challenge along with the data fit to a single exponential function (red line) and the resulting time constant. The bulk shifts in resistance seen immediately prior to and at the end of a swelling curve in the timeline panel reflects changes in chamber pressure as solutions are re-routed through a flush port during solution exchanges. The data also shows the total volume change is greater with greater osmotic challenges, as would be predicted. This data and many other examples show that 30% osmotic challenge for 2-3 minute rarely results in RVD and generates swelling curves that

are reproducible in the same cell population. Additional experiments (data not shown) demonstrated reproducible swelling curves for different solution flow rates, suggesting that shear stress was not a factor during routine experiments.

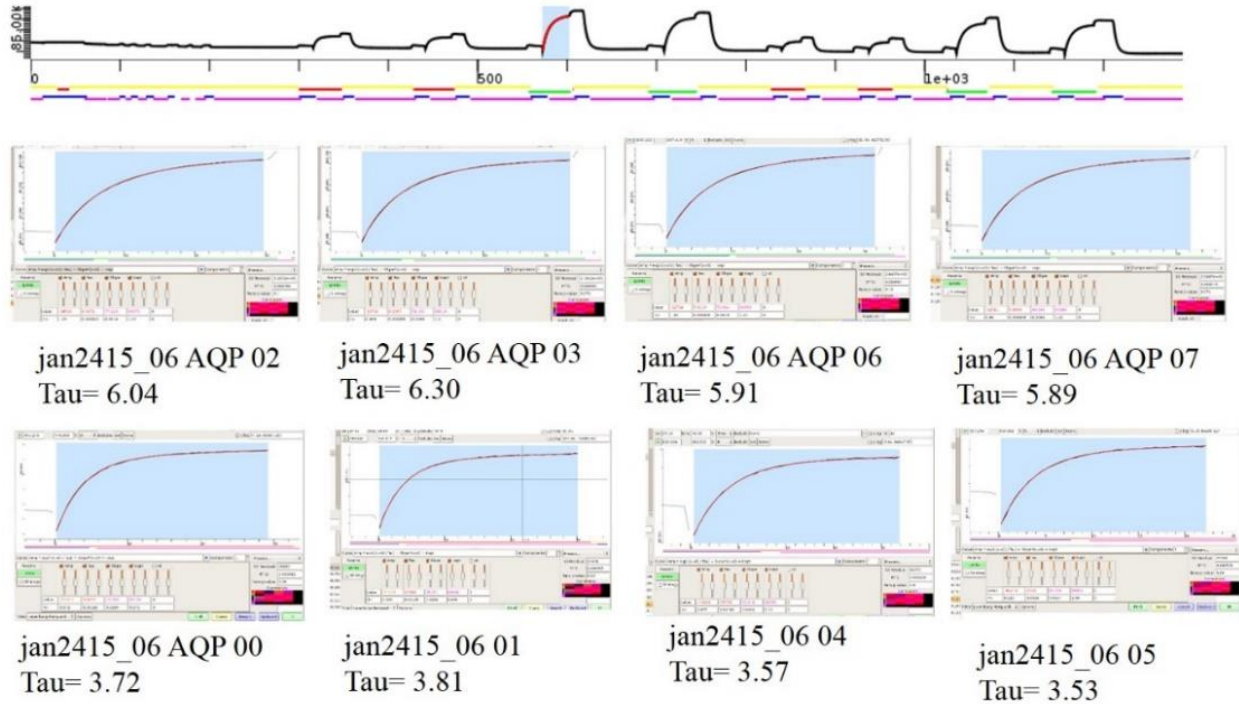


Figure 10: Reproducible swelling kinetics with repeated exposures to osmotic challenges. Swellings time constants are reproducible in well-behaved cell populations exposed to 15-30% hypoosmotic challenges. Top panel shows timeline of experiment. Individual panels show exponential fits to the swelling curves generated by exposure to 15% osmotic challenge (first, second, fifth and sixth swelling curves all displayed on top row of individual panels) or 30% osmotic challenge (third, fourth, seventh, and eighth swelling curves, all displayed on bottom row of individual panels).

Experiments were performed on over 100 different coverslips during my thesis work. Many of these did not generate meaningful data, due to instrument malfunction or clogging of the cell chamber midway during perfusions. Other data were discarded when the room temperature was recorded as exceeding 24°C or if extensive RVD was observed. The remaining data is presented

in Figure 11 and derived from experiments on 27 different CHO-AQP cultures, 16 different CHO-CD81 cultures and 50 different mixed cocultures where cells were mixed in 1:1 to 1:3 ratio. Both the fast and slow components of swelling in the mixed populations were faster than the time constants observed in homogeneous populations of CHO-AQP or CHO-CD81, showing that the mixed cell population as a whole reaches its new steady-state volume faster than the slowest swelling cell type, CHO-CD81. Models to possibly explain these findings are discussed later.

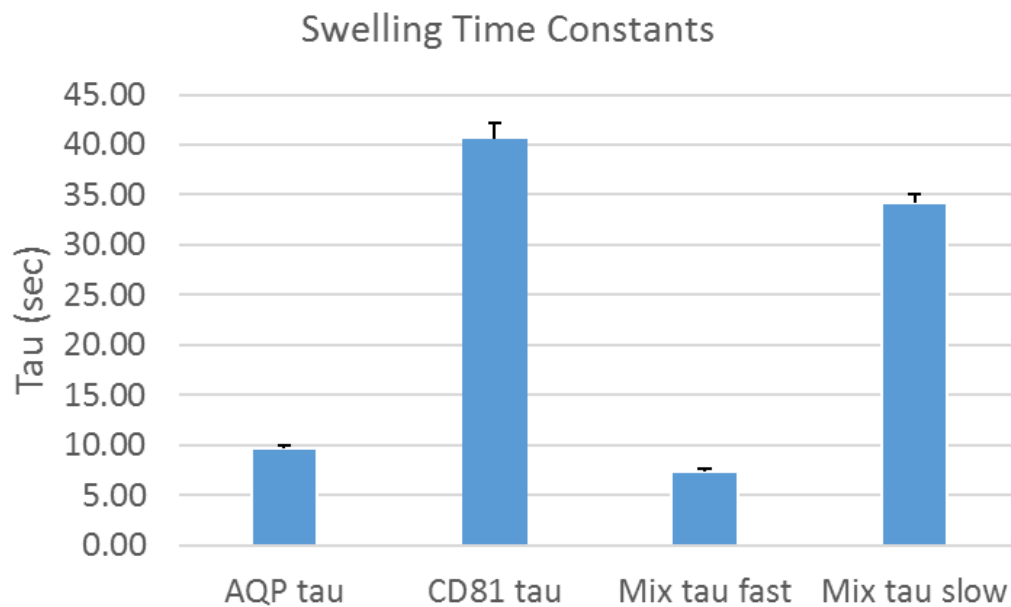


Figure 11: Average swelling time constants for different cell populations exposed to a 30% osmotic challenge. Data expressed as Mean and SEM from n=27 CHO-AQP cultures, n=16 CHO-CD81 cultures, and n=50 Mix cocultures. Single-tail Student t-tests yielded $p = 0.0011$ for AQP tau vs Mix tau fast, and $p = 0.0378$ for CD81 tau vs Mix tau slow.

Cell volume experiments in the presence of gap junction blockers:

I proceeded to determine if gap junction blockers would alter the swelling behavior in mixed populations of CHO-AQP and CHO-CD81 cocultures. Because swelling curves are reproducible

for any one culture, the effects of the blocker can be looked at in the same cell population. The cells were subjected to two rounds of 30% hypoosmotic challenge followed by 10 minutes of perfusion in 100 μ M CBX in isotonic solution. After allowing time for the blocker to work two rounds of 30% hypoosmotic challenge were conducted in the presence of the drug. The second swelling curve in the absence of the drug and the first swelling curve in the presence of the drug were fit to double exponential functions to determine the fast and slow time constants. Data from individual experiments is shown in Figure 12 to highlight the fact that cells responded differently to the drug during different experimental runs. When averaged, no statistical difference between pre- and post-drug data were seen. If gap junction communication sped up the swelling process as shown in the previous figure, then I would expect that blocking gap junctions would slow it down back to same levels seen in homogeneous populations of CHO-AQP or CHO-CD81. This was the case in trials 10 and 11, but not in most cases. This protocol was optimized over time, and some of the earlier runs included data where cells were incubated in the drug without continuous perfusion which may have adversely affected the cells.

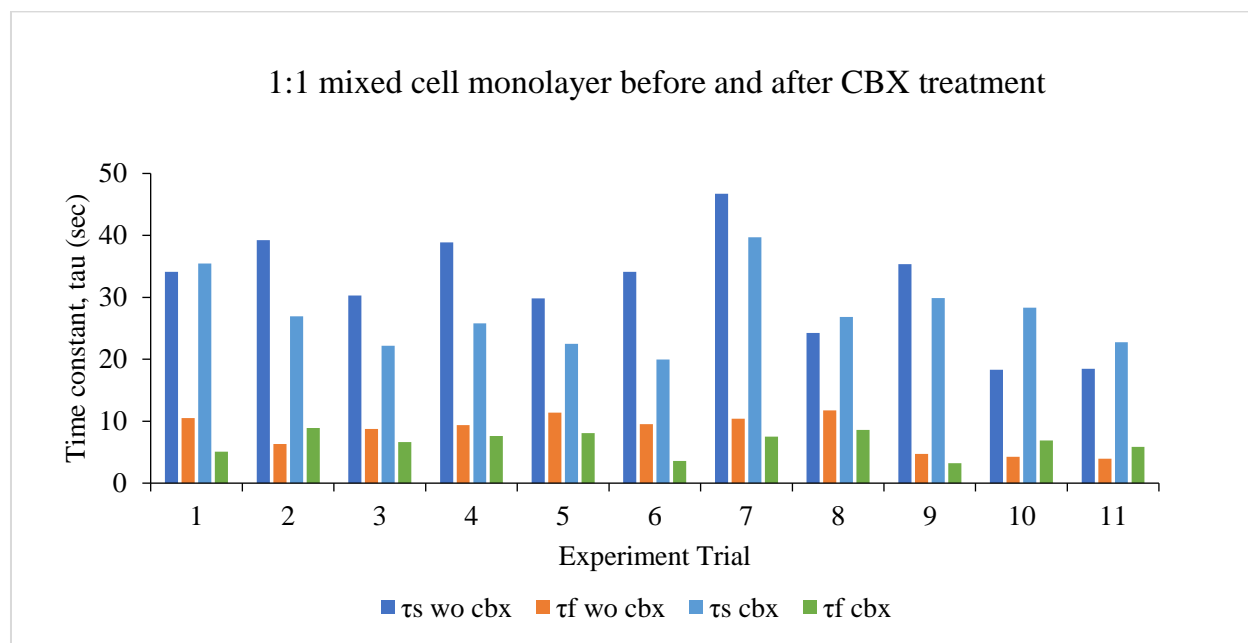


Figure 12: Swelling time constants before and during CBX treatment. Fast and slow time constants characterizing the swelling of 11 different CHO-AQP + CHO-CD81 mixed cocultures before and during exposure to 100 μ M carbenoxolone.

Discussion

Cell volume is a critical aspect of normal and pathological cell physiology and changes in volume can trigger other cell events. It is not clear if the rate of change or extent of change serves as a signal. The consequence of gap junction communication on cell volume dynamics is relatively unexplored. Gap junctions could possibly provide buffering capacity against localized changes in hydrostatic pressure or volume by redistributing the pressure and volume throughout the syncytium of cells. Alternatively, localized volume changes could be exaggerated by the continuous gap junction-mediated supply of additional solutes to the swelling region by cells outside of the affected region. The question is “what happens to the volumes of gap junction coupled cells when conditions cause their intracellular environments to differ in osmolarity?” Given that gap junctions are permeable to both water and the ions and small molecules that make

up most of the cytosol's osmolarity, what will transfer most between the cells through gap junctions– water or solutes? Understanding this simple question will help enable us to predict the influence of gap junction communication on volume dynamics of cell populations within a tissue or organ.

Our experimental strategy involved generating a difference in the intracellular osmolarity in gap junction coupled cells, and characterizing the resulting volume changes in the cells in the presence and absence of functional gap junction channels. Due to limitations in equipment and techniques, I conducted this study in cell populations rather than isolated cell pairs. The osmolarity difference between cytosolic compartments was generated by co-culturing gap junction competent cells that have very different membrane permeability to water and exposing that population to a hypotonic challenge.

The CHO cell line has been shown by several groups to express endogenous Cx43 gap junction channels that result in medium levels of cell-cell communication as assayed by fluorescent dye transfer (Musil et al., 2000a). To obtain differences in water permeability, I used two different stably transfected CHO cell lines: CHO-AQP4 expressing an aquaporin water channel (AQP4) and CHO-CD81 expressing a 26-KDa plasma membrane cell surface protein (CD81) serving as a control. AQP4 water transporting protein was initially found in lung alveolus (Hasegawa et al., 1994), and is expressed in the brain (Borgnia et al., 1999).

The expression of these membrane proteins could affect the synthesis and trafficking of endogenous Cx43 protein and/or the assembly of functional gap junction channels. It was therefore important to verify that Cx43 mediated cell-cell communication remains intact in these cell lines

and between cells of the two different cell lines. Results from parachute assays confirmed the existence of gap junction communication in and between these cell types. Furthermore, the assay was used to demonstrate that carbenoxolone dramatically reduced gap junction communication, as determined by a reduction in the incidence of dye transfer between donor to recipient cells. But these assays also demonstrated that gap junction communication was not as extensive or abundant as seen in other cell types or cells transfected to express connexins. Variations in the extent of coupling between different experiments could account for some of the variation in the data. Using cell lines with more robust gap junction communication would benefit this line of research.

The major finding of this work was a trend for the co-cultured cells to reach a new steady-state volume faster upon exposure to a hypotonic challenge compared to cells from a homogeneous population of either type. While the trend was statistically significant with $p < 0.05$, it was not very significant. Many factors could have played a role in the variation in time constants observed between experiments performed at different times during my thesis work. Although the experimental design allowed for direct comparisons of time constants between the three experimental groups (CHO-AQO, CHO-CD81, CHO-AQP+CHO-CD81) for any given trial, this was not how things worked out. In some trials, I performed more swelling assays on one or two of the experimental groups rather than all three. This was based on quality of cell monolayers and on available time during the day and on assuming the cells would behave consistently so that I could average all data across multiple trials. Unfortunately, while there was consistency of time constants within a single trial, the time constants could vary greatly between trials, especially for the CHO-CD81 cells. This variation was linked to room temperature, quality of monolayer and mounting, and cell passage number. There was a trend for CHO-CD81 cells to swell faster at high passage

numbers and this could reflect poor health and a more permeable plasma membrane. Future studies should ensure that swelling experiments should be performed on all three experimental groups for best comparison and experiments should use only cells limited to a certain passage number.

Other factors could introduce variability between different trials, including the distribution of cell cycle stages, the presence or absence of membrane blebs, and/or the extent to which regulated volume changes affected swelling rates. Cells respond to hypotonic challenges differently at different stages in the cell cycle (Chen et al., 2002; Shen et al., 2000). In my experiments, cells were not synchronized and because contact inhibition is due to mechanical interaction and constraint, a delay between cell-cell contact and the onset of proliferation inhibition is present (Puliafito et al., 2012). There is considerable variation in initial cell volumes in such a population, most likely as a result of cells being in different stages of the cell cycle (Byun et al., 2015). In other words, growing the CHO cells to confluency did not arrest the cells in G1 phase. Hence, some of the variation in time constants seen across different experiments might be due to a difference in the proportion of cell cycle stages present in each population. All things considered, it might be important for future experiments to use synchronized cells.

Membrane blebs were observed on many but not all occasions when culturing CHO cells, both in the plastic T-25 flasks and on glass coverslips. Blebbing occurs temporarily at confined locations where the attachment between the membrane and the underlying cytoskeleton are ruptured (Albrecht-Buehler, 1982; Cunningham, 1995). Researchers have found that in confluent CHO cells in culture, blebs and microvilli become prominent during a specific state of the cell cycle (G2 phase), likely preparing for cell round up during mitosis (Porter et al., 1973). Such membrane bleb formation is also found in mitotic Hela cells and trypsinized cells (Moes et al.,

2011). Cells with many blebs creates complications in our interpretation of swelling assay results, since those local protrusions are indicative of hydrostatic pressure induced morphology change.

Volume regulatory decrease

CHO cells do not show RVD response because they do not produce TRPV4 (Becker et al., 2005). Some broad-spectrum Cl⁻ channel blockers such as NPPB and DIDS, as well as selective inhibitors DCPIB and NS3728 are inhibitors of regulatory volume decrease by blocking VRAC (Hoffmann et al., 2009). As mentioned before, CBX can also block VRAC. CBX blocking potency for VRAC is at a similar level to gap junction blocking in astroglia but the process is independent of connexins (Benfenati et al., 2009). These two pieces of information are valuable for future experiments because it is important in follow up experiments to control RVD during cell swelling.

Qualitative models

When exposed to a hypotonic gradient, water enters a cell at a rate that is proportional to the size of the osmotic gradient, the total membrane area, and the permeability constant of the membrane. Water continues to enter the cell, increasing the volume of the cell, until the cytosol is isotonic with the external media. While cells with a higher water permeability swell faster, they do not swell larger compared to cells that have a lower water permeability because final volume change is determined only by the size of the initial osmotic gradient. An interesting situation arises in the case of gap junction coupled cells, because the higher water permeable cell will develop a

cytosol that is hypotonic relative to the cytosol of the slower swelling cell for a limited amount of time until both cells reach isotonicity with both the external media and with each other.

While we work on a mathematical model of cell volume to account for transport properties of gap junctions, we can consider two qualitative models to explain how gap junctions might be speeding up the volume changes in cocultured cells of different water permeability. These models represent the extreme cases of where only water or only solutes are transported between the cells and assumes similar membrane areas and initial cell volume across all cells.

Model 1: Only water is transported through gap junctions

Upon exposure to a hypotonic solution, both CHO-AQP and CHO-CD81 cells begin to swell. As CHO-AQP cells swell much faster, their cytosolic compartments become more hypotonic relative to those of the CHO-CD81 cells. Water moves into the CHO-81 cells, effectively contributing to the water influx across the plasma membrane and resulting in a faster swelling rate for the CHO-CD81 cells. This would suggest that more water needs to come into the CHO-AQP cells across its membrane to accommodate the loss to the CHO-CD81 cells and would possibly slow down the time it takes for the CHO-AQP cell to reach isotonicity with the external media. If there are no solute fluxes and only water is transported through gap junctions, then both cell types would eventually swell to the same volume dictated by the requirement that their cytosols reach isotonicity with the external media.

Model 2: Only solutes are transported through gap junctions.

Upon exposure to a hypotonic solution, both CHO-AQP and CHO-CD81 cells begin to swell. As CHO-AQP cells swell much faster, cytosolic compartments become more hypotonic relative to those of the CHO-CD81 cells. Cytosolic solutes are then at a higher concentration in the CHO-CD81 cells relative to the CHO-AQP cells and begin to diffuse down their concentration gradient through gap junctions into the CHO-AQP cells. The reduced levels of solutes in the CHO-CD81 cells reduces the amount of water that needs to enter the cell across the membrane to satisfy the condition of matching tonicity with the external media. Hence, the CHO-CD81 cells do not swell as much as they normally would in absence of gap junctions with the CHO-AQP cells and the time to reach a new steady-state volume is shorter. Equally, the solutes added to the CHO-AQP cell would cause it to swell to a larger extent than normal to reach isotonicity with the external media.

These models have very different predictions for the relative final cell volumes of CHO-AQP and CHO-CD81 cells in the case of water versus solute transport through gap junctions being responsible for the faster time to new steady-state volumes upon hypotonic challenges. Distinguishing between the possible models will require observation of volume changes at the single cell level, and the lab is currently working on these studies.

Interestingly, both models would predict that the CHO-AQP cells would reach their final volume slower when communicating with the CHO-CD81 cells, whereas the time constants for these cells were also faster than they were in homogeneous populations of only CHO-AQP cells. One explanation is that the fast time constants are not as reliable due to the finite time of solution exchange, even if that time is 1-2 seconds. Alternatively, it could be due to CHO-AQP cells having two components to its swelling profile, one being a fast component due to the water channels and

the other being the slower component from transport through gap junctions with CHO-CD81 cells. This slower component could be absorbed into the slow component of the double exponential fit that is usually attributed to the CHO-CD81 cells, leading to errors in estimating fast component.

Bias may be given to the second model where solutes are transported from CHO-CD81 cells to CHO-AQP cells because it is predicted that it is very hard to generate an osmotic gradient across a gap junction channel given that the channel is permeable to most cytosolic solutes including ions. In the absence of an osmotic gradient, hydrostatic pressure could drive water flow, but cells are not thought of as tolerating large hydrostatic pressures. It is difficult to model the cell pair volume dynamics without the aid of mathematical tools. More information needs to be gathered and integrated into a model, including to what extent the small but finite difference in gap junction impermeable proteins and macromolecules could contribute to establishing an osmotic gradient and how much hydrostatic pressure can a cell handle.

Volume dynamics is no longer seen as a simple osmosis issue. The modern description of cytoplasmic fluid properties is a poroelastic and viscoelastic one (Mahadevan et al., 2013; Sachs and Sivaselvan, 2015). Cell swelling dynamics is not only determined by its boundaries with the extracellular environment, the semi-permeable plasma membrane, but also the underlying cellular cortex and the cytoskeletal network that provides support and changes to the membrane. When cells swell, they become softer and more deformable, with diminished cellular elasticity (Moeendarbary et al., 2013). On the other hand, researchers have found that osmotic stress is internal to the cell and not confined to the cell cortex. Exposure to distilled water does not always lyse cells (Guo et al., 2014) and cells do not get stiffer with hypotonic swelling (Spagnoli et al., 2008), suggesting a role of the cytoskeleton in the regulation of cell volume. Giant unilamellar

vesicles composed of basic lipids and cholesterol to artificially mimic primitive cells are devoid of cytoskeletal structure or channel protein. Under hypotonic conditions, they exhibit pulsatile properties, going through cycles in which lytic tension leads to rupture of the cell membrane which then reseals and gives rise to another round of swelling (Chabanon et al., 2017; Ogłęcka et al., 2014). The dissimilarity between artificial osmotic swelling response and cell swelling response is another line of evidence suggesting that the crosslinked cytoskeletal meshwork and various membrane protein channels have dramatic effects on osmotic swelling behavior.

Carbenoxolone as a gap junction blocker

The experimental platform allowed for the measurement of swelling rates in the absence and presence of gap junction blockers within the same cell population. It is hypothesized that the lack of extracellular space between gap junction channels and their large pore size are the main reasons natural inhibitors that mediate gap junction activity are not found (Srinivas et al., 2001). Most of the known gap junction inhibitors have limited gap junction specificity. Gap junction blockers (GJBs) include triarylmethanes (TRAMs), fenamates, long chain n-alkyl alcohols (heptanol and octanol), glycyrrhetnic acid and its derivatives (carbenoxolone), fatty acid and fatty acid amides (anandamide and oleamide), quinines and derivatives (quinidine and mefloquine), 2-aminophenoxyborate (2-APB) and derivatives, and more (Bodendiek et al., 2012; Harks et al., 2003; Harks et al., 2001; Juszczak and Swiergiel, 2009). Open probability of gap junction channels can also be altered by mimetic peptides (Gap26 and Gap27) containing sequences from the extracellular loop (Boitano and Evans, 2000).

Carbenoxolone (CBX) is best known in cellular physiology as a modestly potent, reasonably effective, water-soluble blocker of gap junctions. CBX can block both swelling activated anion channels VRACs and gap junctions. CBX was also reported to block Ca^{2+} channels, pannexin channels and P2X7 acceptors at concentrations similar to or lower than those that block connexin channels. Inhibition of gap junction channel currents require application of high doses of carbenoxolone (200 μM) for 10-15 min, to reach steady-state. CBX inhibits junctional conductance up to 80% in a concentration-dependent manner, and complete blockade of gap junction channels is not observed even with concentrations up to 200 μM .

In this study, the results using CBX were not clear. Often the drug resulted in either no effect or increased swelling rates, but a few good examples showing a reduced swelling rate. If gap junctions were increasing the swelling rates of CHO-AQP and CHO-CD81 cells in co-culture, then I expected to see that the rates would decrease back to control homogeneous culture levels after exposing the population to CBX. These experiments were carried out on cultures of high passage number, and it could be that such cultures showed little gap junction communication in the first place. Again, the problem with interpreting the data comes from not having enough data on the homogeneous populations within the same experimental run. CBX had either no effect or slightly reduced the time constant in these homogeneous populations assayed at different days, so it could be that the data from the mixed populations reflects the normal action of CBX to slightly increase swelling rates independent of gap junctions. CBX does affect other channels and transporters at the concentration used in my experiments, so maybe these interactions are responsible for the observations. CBX did behave as expected in a few runs, but clearly more experiments with better controls are needed to understand the effects of CBX. Future experiments

should include the use of other gap junction blockers, such as oleamide, glycerritinic acid, and octanol-1 (Rozenal et al., 2001). These drugs have different modes of action and can be used to separate out any nonspecific effect a particular drug is having on volume dynamics.

In summary, a new strategy to look at the effects of gap junctions on cell volume dynamics is developed here and a general trend for gap junction coupled cells to reach a new volume faster when exposed to a hypotonic challenge is demonstrated. Gap junction mediated transport of water and/or solutes between cells that normally swell at different rates will affect volume dynamics. While we are confident in this trend, additional work needs to be carried out to strengthen a model explaining how gap junctions affect cell volume changes. Improvements in the experimental design were identified in this discussion, and include the use of cell lines with more robust gap junction communication, better cell culture control with restrictions on passage number, ensuring data is collected from all three experimental groups for any given culture time, and the use of multiple gap junction blockers to eliminate non-specific effects. These studies represent an initial attempt to understand the unexplored roles that gap junctions have in cell volume dynamics.

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